

PRACA  
DOKTORSKA

**INSTYTUT CHEMII ORGANICZNEJ  
POLSKIEJ AKADEMII NAUK**



**Selektywna funkcjonalizacja witaminy B<sub>12</sub> w pozycji  
*mezo* i w obrębie pętli nukleotyduowej**

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## 1. Spis publikacji wchodzących w skład rozprawy doktorskiej

### Publikacje przeglądowe:

1. **A. J. Wierzba**, S. Hassan, D. Gryko, *Asian J. Org. Chem.* **2018**, 10.1002/ajoc.201800579, praca zaakceptowana  
*Synthetic Approaches Toward Vitamin B<sub>12</sub> Conjugates*

### Publikacje oryginalne:

1. **A. J. Wierzba**, M. Wojciechowska, J. Trylska, D. Gryko, *Bioconjugate Chem.* **2016**, 27, 189–197  
*Vitamin B<sub>12</sub> Suitably Tailored for Disulfide-Based Conjugation*
2. M. Równicki, M. Wojciechowska, **A. J. Wierzba**, J. Czarnecki, D. Bartosik, D. Gryko, J. Trylska, *Sci. Rep.* **2017**, 7, 7644  
*Vitamin B<sub>12</sub> as a Carrier of Peptide Nucleic Acid (PNA) into Bacterial Cells*
3. T. Pieńko, **A. J. Wierzba**, M. Wojciechowska, D. Gryko, J. Trylska, *J. Phys. Chem. B* **2017**, 121, 2968–2979  
*Conformational Dynamics of Cyanocobalamin and Its Conjugates with Peptide Nucleic Acids*
4. **A. J. Wierzba**, A. Wincenciuk, M. Karczewski, V. I. Vullev, D. Gryko, *Chem. Eur. J.* **2018**, 24, 10344–10356  
*meso-Modified Cobalamins: Synthesis, Structure, and Properties*
5. E. Braselmann, **A. J. Wierzba**, J. T. Polaski, M. Chromiński, Z. E. Holmes, S. Hung, D. Batan, J. R. Wheeler, R. Parker, R. Jimenez, D. Gryko, R. T. Batey, A. E. Palmer, *Nat. Chem. Bio.* **2018**, 14, 964–971  
*A Multicolor Riboswitch-Based Platform for Imaging of RNA in Live Mammalian Cells*
6. **A. J. Wierzba**, K. Maximova, A. Wincenciuk, M. Równicki, M. Wojciechowska, E. Nexø, J. Trylska, D. Gryko, *Chem. Eur. J.* **2018**, 10.1002/chem.201804304  
*Does a Conjugation Site Affect Transport of Vitamin B<sub>12</sub>-PNA Conjugates into Bacterial Cells?*

## 2. Spis wystąpień konferencyjnych

Wyniki przedstawione w niniejszej pracy zostały zaprezentowane na konferencjach:

1. Tenth International Conference on Porphyrins and Phthalocyanines ICPP-10; Monachium, Niemcy 2018:  
*Synthetic approaches toward vitamin B<sub>12</sub> conjugates*  
**prezentacja ustna**
2. German-Polish-Baltic Conference on Organic Chemistry; Hamburg, Niemcy 2018:  
*Vitamin B<sub>12</sub> as a drug delivery agent - a chemical point of view*  
**prezentacja ustna**
3. Międzynarodowy Kongres Młodych Chemików YoungChem2017; Lublin 2017:  
*Vitamin B<sub>12</sub> as a drug delivery agent - a chemical point of view*  
**prezentacja ustna**
4. International Symposium on Synthesis and Catalysis ISySyCat; Evora, Portugalia 2017:  
*Vitamin B<sub>12</sub> as a drug delivery agent - a chemical point of view*  
**prezentacja ustna**
5. Warszawskie Seminarium Doktorantów Chemików–ChemSession'17; Warszawa 2017:  
*Selective modifications of cobalamin at the meso position*  
**prezentacja posterowa**
6. Ninth International Conference on Porphyrins and Phthalocyanines ICPP-9; Nankin, Chiny 2016:  
*Conjugable vitamin B<sub>12</sub> derivatives*  
**prezentacja posterowa**
7. IX Kopernikańskie Seminarium Doktoranckie; Toruń 2015:  
*Synteza pochodnej witaminy B<sub>12</sub> z aktywnym ugrupowaniem disiarczkowym*  
**prezentacja posterowa uhonorowana nagrodą**



### 3. Spis publikacji niewchodzących w skład rozprawy doktorskiej

Publikacje oryginalne:

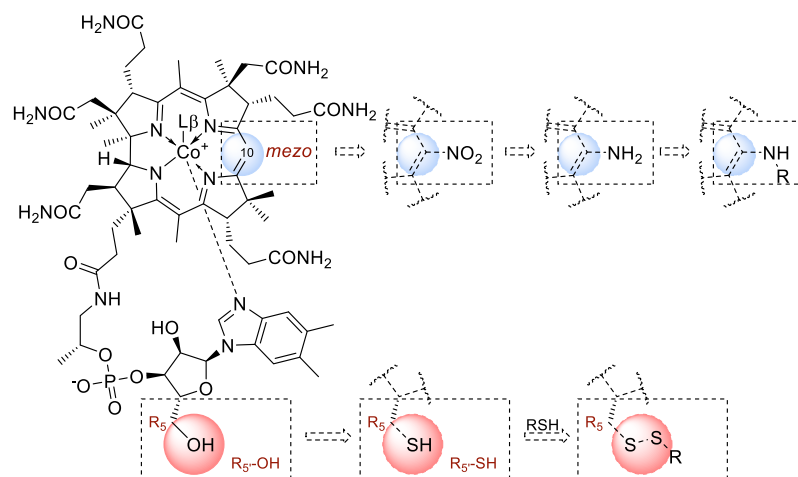
1. R. Kowalczyk, **A. J. Wierzba**, P. J. Boratyński, J. Bąkiewicz, *Tetrahedron* **2014**, 70, 5834-5842  
*Enantioselective Conjugate Addition of Aliphatic Thiols to Divergently Activated Electron Poor Alkenes and Dienes*
2. R. Kowalczyk, P. J. Boratyński, **A. J. Wierzba**, J. Bąkiewicz, *RSC Advances* **2015**, 5, 66681-66686  
*Site and stereoselectivity in sulfa-Michael addition to equivocally activated conjugated dienes*

## 4. Przewodnik po rozprawie doktorskiej

### 4.1 Założenia i cel pracy

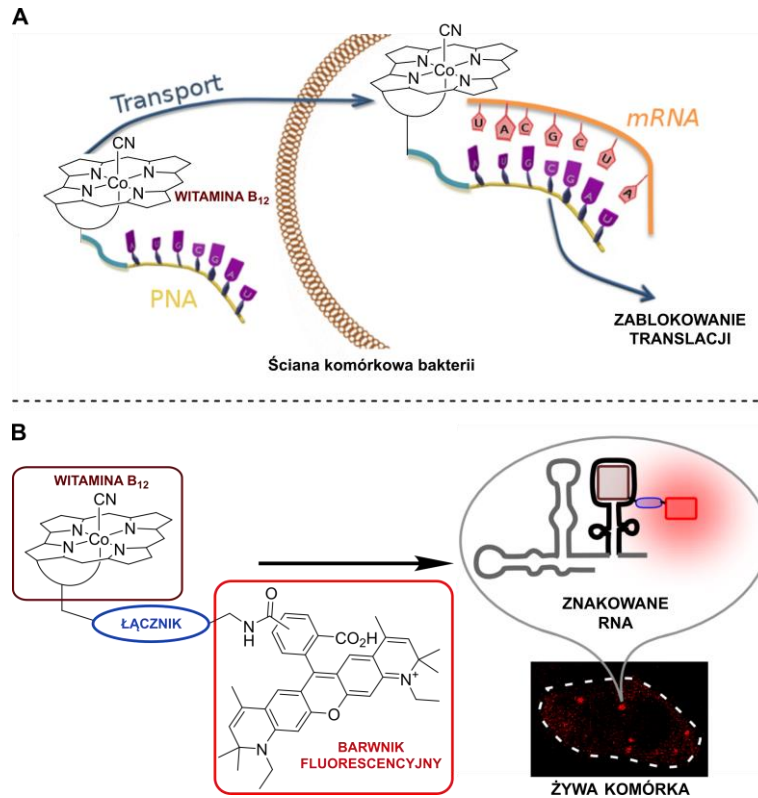
Dostarczenie aktywnej substancji do miejsca jej działania jest często bardzo trudnym zadaniem. Związki naturalne posiadające zdolność penetrowania komórek są atrakcyjnymi kandydatami do transportowania substancji biologicznie czynnych, np. leków, które w swej niezmodyfikowanej postaci nie mogą dostać się do wnętrza komórek. Związkiem, który spełnia powyższe kryteria i jest równocześnie niezbędnym składnikiem odżywczym dla wszystkich ssaków i niektórych bakterii jest witamina B<sub>12</sub> (kobalamina, Rysunek 1).<sup>[1,2]</sup>

Wykorzystanie witaminy B<sub>12</sub> jako nośnika wymaga odpowiedniej modyfikacji jej struktury, która umożliwi selektywne przyłączenie transportowanego związku. Z uwagi na duży stopień złożoności struktury kobalaminy, nie jest to zadanie łatwe. **Celem mojej pracy było opracowanie syntezy nowych pochodnych witaminy B<sub>12</sub>, posiadających w swej strukturze reaktywne ugrupowania pozwalające na łączenie witaminy B<sub>12</sub> ze związkami o znaczeniu biologicznym w pozycji R<sub>5</sub> w sposób odwracalny.** Jako motyw rozłączalny wybrałam mostek disiarczkowy (Rysunek 1). Ponadto, **za cel postawiłam sobie również opracowanie metodologii funkcjonalizacji kobalaminy w pozycji mezo (C10), która do tej pory nie była rozpatrywana w kontekście tworzenia koniugatów** (Rysunek 1). Poza utworzeniem nowego narzędzia syntetycznego, opracowanie metodologii funkcjonalizacji kobalaminy w tej pozycji umożliwi głębsze zbadanie wpływu rodzaju podstawnika w pozycji C10 na właściwości strukturalne, fotofizyczne czy elektrochemiczne kobalaminy.



Rysunek 1. Wybrane pozycje modyfikacji w strukturze witaminy B<sub>12</sub>.

Obecnie istniejące oraz nowo opracowane metodologie postanowiłam wykorzystać do przygotowania koniugatów witaminy B<sub>12</sub> z syntetycznymi oligonukleotydami, a konkretnie peptydowym kwasem nukleinowym (ang. *peptide nucleic acid*, PNA, Rysunek 2, **A**)<sup>[3]</sup> oraz barwnikami fluorescencyjnymi (Rysunek 2, **B**).

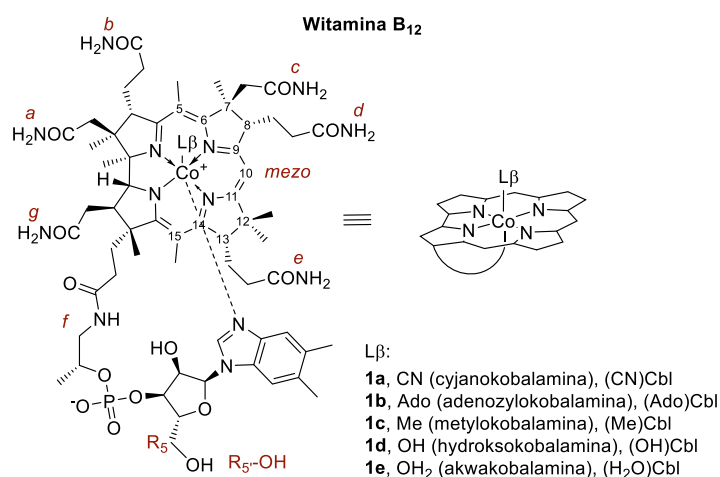


**Rysunek 2.** Witamina B<sub>12</sub> jako transporter oligonukleotydów PNA (A). Witamina B<sub>12</sub> jako część koniugatu służącego do znakowania RNA (B).

Zastosowanie krótkich (do kilkunastu merów), modyfikowanych oligonukleotydów zaburzających ekspresję genów bakterii poprzez komplementarne łączenie się z bakteryjnym RNA, co w rezultacie blokuje produkcję białek bakteryjnych i w efekcie hamuje wzrost bakterii, może być obiecującą alternatywą dla obecnie nadużywanych antybiotyków.<sup>[4,5]</sup> **Oligonukleotydy nie posiadają zdolności penetrowania komórek bakterii, dlatego też celem pracy było zweryfikowanie czy witamina B<sub>12</sub> może być efektywnym transporterem dla oligomerów PNA (Rysunek 2, A).** Nie było bowiem doniesień na temat wykorzystania kobalaminy jako transportera związków do komórek bakterii. Natomiast, **połączenie witaminy B<sub>12</sub> z barwnikami fluorescencyjnymi miało na celu zweryfikowanie czy otrzymane koniugaty mogą posłużyć do śledzenia fragmentów mRNA oraz krótkich, niekodujących fragmentów RNA w żywych komórkach ssaków.** Witamina B<sub>12</sub> jest ligandem dla przełączników RNA (ryboprzełączników, ang. *riboswitches*)<sup>[6,7]</sup> oraz posiada zdolność wygaszania fluorescencji połączonych z nią barwników.<sup>[8]</sup> W związku z tym, odpowiednio zaprojektowane połączenie kobalaminy z barwnikiem może potencjalnie posłużyć do znakowania RNA przy założeniu, że wygaszona przez oddziaływanie barwnika z koryną fluorescencja będzie obserwowana po związaniu kobalaminy przez ryboprzełącznik (Rysunek 2, B).

## 4.2 Wstęp literaturowy

Witamina B<sub>12</sub> (kobalamina, **1**, Rysunek 3) jest składnikiem niezbędnym do prawidłowego funkcjonowania ssaków, jak również niektórych bakterii.<sup>[9]</sup> W komórkach eukariotycznych przekształcana jest ona do metaloorganicznych kofaktorów: adenozylokobalaminy (**1b**) lub metylokobalaminy (**1c**), które biorą udział w procesach związanych z metabolizmem aminokwasów oraz syntezą nukleotydów.<sup>[10]</sup> Witamina B<sub>12</sub> jest kluczowym elementem dla właściwego funkcjonowania układu pokarmowego, nerwowego oraz krwionośnego.



**Rysunek 3.** Struktura witaminy B<sub>12</sub> oraz jej uproszczona forma.

Jako substancja egzogenna kobalamina transportowana jest w organizmach ssaków przez złożony i selektywny system trzech białek transportujących: haptokoryny (ang. *haptocorrin*, HC), czynnika wewnętrznego (ang. *intrinsic factor*, IF) oraz transkobalaminy (ang. *transcobalamin*, TC).<sup>[11]</sup> Fakt ten czyni witaminę B<sub>12</sub> atrakcyjnym kandydatem do dostarczania związków do wnętrza komórek. W istocie, istnieją doniesienia na temat przykładów wykorzystania witaminy B<sub>12</sub> jako efektywnego nośnika leków przeciwnowotworowych,<sup>[12,13]</sup> peptydowych,<sup>[14,15]</sup> barwników fluorescencyjnych<sup>[16–18]</sup> czy znaczników radioizotopowych<sup>[19,20]</sup> do komórek eukariotycznych. Jednak utworzenie takich połączeń wymaga uprzedniej modyfikacji struktury witaminy B<sub>12</sub> w taki sposób, aby posiadała ona grupę funkcyjną o określonej reaktywności (umożliwiająca przyłączanie związków) oraz była wciąż rozpoznawana przez białka transportujące kobalaminę. Struktura witaminy B<sub>12</sub> oferuje szereg pozycji, które można poddać modyfikacji lub bezpośredniej funkcjonalizacji, jednak nie wszystkie są jednakowo dostępne syntetycznie.<sup>[21]</sup>

Witamina B<sub>12</sub> (Rysunek 3) składa się z centralnego jonu kobaltu na trzecim stopniu utlenienia skompleksowanego wewnątrz makrocyklicznego pierścienia koryny, który posiada szereg podstawników metylowych, acet- (*a*, *c*, *g*) oraz propionamidowych (*b*, *d*, *e*, *f*). Ligandem aksjalnym α dla jonu kobaltu jest dimetylobenzimidazol, natomiast ligand β, różni się w zależności od formy kobalaminy (L<sub>β</sub> = CN, dla cyjanokobalaminy **1a**). Dimetylobenzimidazol jest częścią nukleotydu,

w skład którego wchodzi pierścień rybozy oraz grupa fosforanowa, która łącząc się z grupą propionamidową *f* zamyka tzw. pętlę nukleotydową kobalaminy.

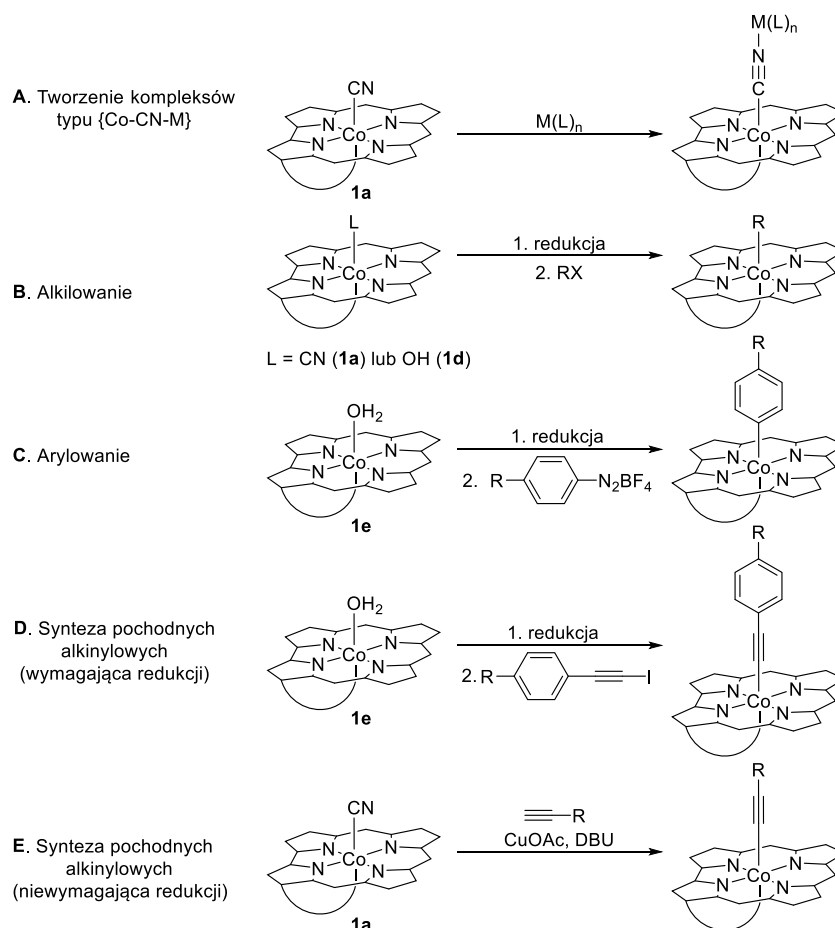
W przypadku komórek eukariotycznych udowodniono, że przyłączanie związków w niektórych pozycjach negatywnie wpływa na proces rozpoznania kobalaminy przez jej białka transportujące.<sup>[22]</sup> Dzieje się tak w przypadku modyfikowania pozycji *c* oraz w mniejszym stopniu pozycji *d*, podczas gdy funkcjonalizacja w pozycji *b* tylko w nieznacznym stopniu zaburza transport kobalaminy. Wpływ modyfikacji w pozycjach *a* i *g* nie został do tej pory opisany, natomiast przyłączanie związków w pozycji *e*, na centralnym jonie kobaltu lub w pozycji  $R_5$ , prowadzi do pochodnych witaminy  $B_{12}$ , które są rozpoznawane w równym stopniu co jej niezmodyfikowany wariant. Z tego względu, właśnie te pozycje były najczęściej wybierane jako miejsca koniugacji. Niezwykle ważne jest zapewnienie, aby w docelowym koniugacie żaden z elementów nie blokował działania drugiego, tj. zmodyfikowana witamina musi być nadal rozpoznawana przez serię białek zaangażowanych w jej pobieranie, a cząsteczka leku/barwnika musi oddziaływać ze swoim receptorem, w celu wywołania pożądanego efektu terapeutycznego/analizacyjnego. Dlatego też, poza doбором odpowiedniej pozycji, przyłączenie docelowego leku/barwnika często poprzedza się wprowadzeniem łącznika o określonej długości i charakterze (hydrofilowe, hydrofobowe, rozłączalne bądź nie), który oddziela od siebie te dwa elementy.

Z uwagi na duży stopień złożoności cząsteczki, modyfikacje struktury witaminy  $B_{12}$  nie należą do zadań łatwych. Niemniej, wysiłki naukowców doprowadziły do opracowania efektywnych podejść syntetycznych umożliwiających tworzenie koniugatów z kobalaminą. Poniżej podsumowałam te, które prowadzą do pochodnych ze związkami przyłączonymi na centralnym jonie kobaltu, w obrębie pierścienia makrocyclicznego oraz w pozycji  $R_5$  pierścienia rybozy.

Cyjankowy ligand kobalaminy **1a** może tworzyć heterojądrowe kompleksy o ogólnym motywie  $\{Co-CN-M\}$ , w których kompleks metalu koordynuje do liganda CN poprzez atom azotu (Schemat 1, A).<sup>[13]</sup> Takie kompleksy można uważać za proleki, ponieważ oczekuje się, że uwalnianie aksjalnego liganda w komórce będzie miało miejsce po konwersji witaminy  $B_{12}$  do jej kofaktorów. Wśród przykładów koniugatów z witaminą  $B_{12}$  otrzymanych tą metodą można wymienić kompleksy z pochodnymi *cis*- lub *trans*platyny,<sup>[23,24]</sup> czy też pochodne z kompleksami renu posiadające w swej strukturze ligandy karbonylowe jako źródło terapeutycznego CO.<sup>[25,26]</sup>

Redukcja witaminy  $B_{12}$  prowadzi do jej form z jonem kobaltu na II i I stopniu utlenienia posiadających odpowiednio charakter rodnikowy oraz nukleofilowy.<sup>[27]</sup> Reakcja zredukowanej witaminy (**1a**, **1d** lub **1e**) z elektrofilami prowadzi kolejno do jej alkilowych (Schemat 1, B),<sup>[28]</sup> arylowych (Schemat 1, C)<sup>[29]</sup> bądź alkinylowych pochodnych (Schemat 1, D).<sup>[30]</sup> Opracowano również bardziej wydajną i efektywną metodę syntezy pochodnych alkinylowych, która nie wymaga zastosowania warunków redukujących (Schemat 1, E).<sup>[31]</sup> Należy zaznaczyć, że wiązanie  $Co-C_{sp^3}$  w alkilowych pochodnych jest słabe ( $\sim 30$  kcal/mol) i ulega rozerwaniu zarówno pod wpływem światła, jak i podwyższonej temperatury. Właściwość ta może jednak okazać się korzystna

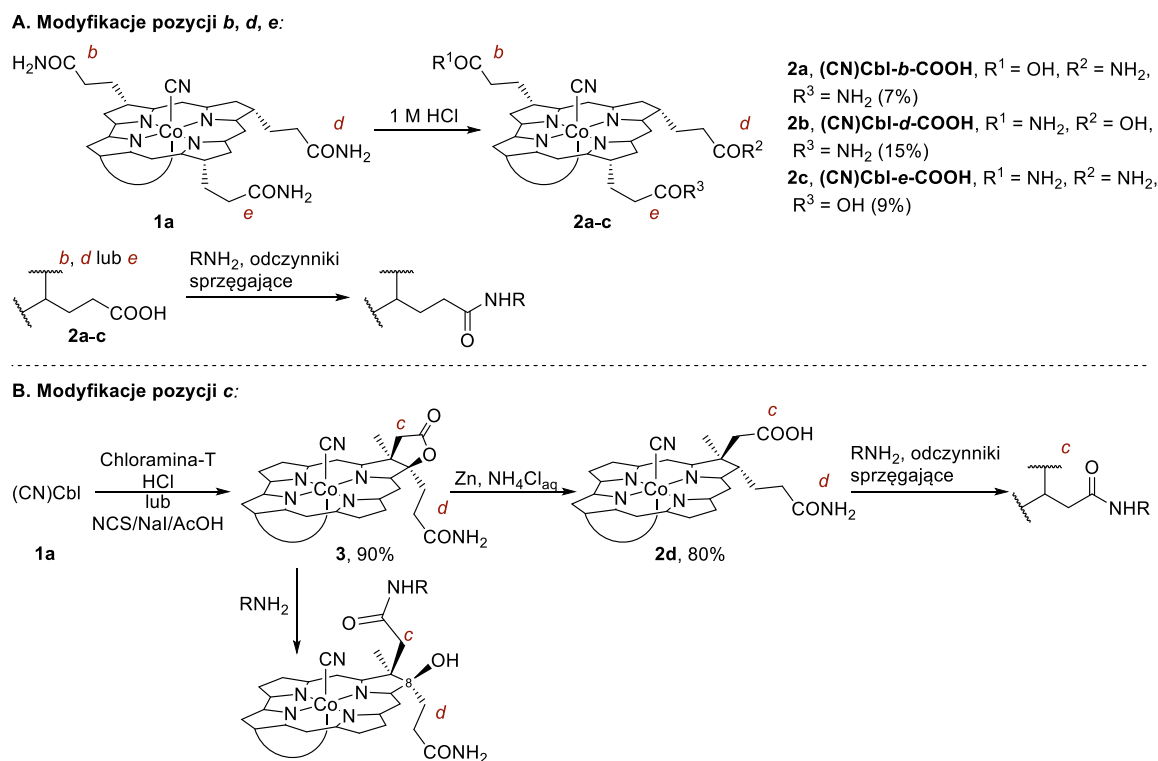
w przypadku przygotowywania tzw. rozłączalnych koniugatów witaminy – przyłączony związek może zostać wówczas uwolniony poprzez naświetlenie. Takie podejście wykorzystano do przygotowania koniugatów z lekami przeciwnowotworowymi lub barwnikami fluorescencyjnymi.<sup>[17]</sup> Problem niestabilności opisywanych koniugatów rozwiązano wprowadzając podstawnik alkinyłowy (Schemat 1, **D i E**), gdyż utworzone wiązanie Co-C<sub>sp</sub> jest odporne zarówno na termo- jak i fotolizę.<sup>[31]</sup>



**Schemat 1.** Modyfikacje witaminy B<sub>12</sub> na jonie kobaltu (M = metal, L = ligand, R = lek, barwnik, łącznik, itp.).

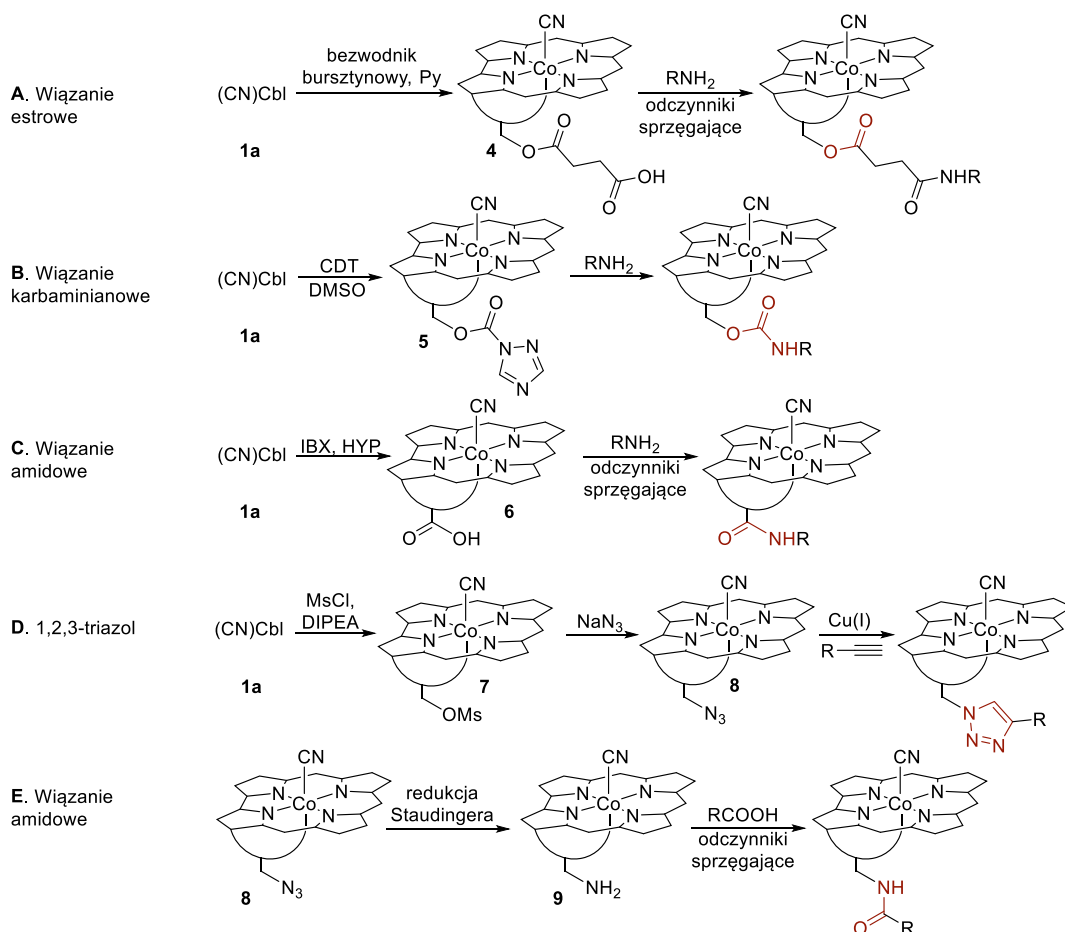
Modyfikacja łańcuchów bocznych *b*, *d* i *e* wymaga kwasowej hydrolizy kobalaminy **1a**, która prowadzi między innymi do mieszaniny kwasów karboksylowych **2a-c** (Schemat 2, **A**) powstających z wydajnościami odpowiednio 7%, 15% i 9%.<sup>[32]</sup> Kwas karboksylowy **2a-c** można kolejno poddać reakcji z aminami w obecności odczynników sprzęgających lub po uprzedniej aktywacji NHS (*N*-hydroksysukcynoimidem). Tę metodę wykorzystano w celu przyłączania do kobalaminy m.in. związków chelatujących metale<sup>[33,34]</sup> lub leków hormonalnych.<sup>[35,36]</sup> Z uwagi na brak negatywnego wpływu modyfikacji w pozycji *e* na rozpoznanie kobalaminy przez jej białka transportujące, właśnie ta pozycja była najczęściej wykorzystywana do tworzenia koniugatów w obrębie pierścienia makrocyklicznego. Mimo to, poważnym ograniczeniem pozostaje niska wydajność pierwszego etapu hydrolizy oraz żmudny proces oczyszczania kwasów monokarboksylowych **2a-c**. Natomiast, modyfikacje w pozycji *c* można przeprowadzić selektywnie na drodze bromo- lub chlorolaktonizacji<sup>[37,38]</sup> i następczego otwarcia laktonu **3** aminami<sup>[39]</sup>

lub na drodze jego redukcji do odpowiedniego kwasu monokarboksyłowego **2d**,<sup>[40]</sup> który kolejno można poddać reakcji sprzęgania (Schemat 2, **B**). Z uwagi na negatywny wpływ modyfikacji w tej pozycji na transport kobalaminy nie jest ona szeroko stosowana.



Charakter oraz strategiczne położenie grupy R<sub>5</sub>-OH rybozy sprawia, że jest ona obecnie jedną z najczęściej wykorzystywanych w kontekście tworzenia koniugatów z witaminą B<sub>12</sub>. Wykazano, że modyfikacje w tej pozycji nie mają negatywnego wpływu na proces rozpoznawania kobalaminy przez białka transportujące obecne w organizmach ssaków.<sup>[22]</sup> Pierwszą próbą modyfikacji tej pozycji była estryfikacja bezwodnikiem kwasu bursztynowego, która w zależności od ilości użytego elektrofila, prowadziła do estryfikacji jednej (pierwszorzędowej) lub obu grup hydroksylowych w obrębie rybozy (Schemat 3, **A**).<sup>[41]</sup> Powstające estry są jednak niestabilne i ulegają hydrolizie nawet w łagodnych warunkach. Kolejną i obecnie najczęściej wykorzystywaną metodą funkcjonalizacji grupy R<sub>5</sub>-OH jest reakcja tworzenia wiązania karbaminianowego na drodze aktywacji grupy hydroksylowej CDT (1,1'-karbonyl-di-(1,2,4-triazol)) i następczej reakcji z aminami (Schemat 3, **B**).<sup>[42]</sup> Ponadto, grupa R<sub>5</sub>-OH może zostać utleniona do kwasu karboksylowego **6**, który reaguje z aminami w obecności odczynników sprzęgających, jednak etap utleniania zachodzi z niską wydajnością około 30% (Schemat 3, **C**).<sup>[43]</sup> Interesującym podejściem jest przekształcenie grupy hydroksylowej w grupę azydkową na drodze mesylowania i następczego podstawienia azydkiem sodu (Schemat 3, **D**).<sup>[44]</sup> Witaminowy azydek **8** okazał się bardzo dobrym partnerem w katalizowanej jonami miedzi(I) reakcji 1,3-dipolarnej cykloaddycji azydków do terminalnych alkinów

(ang. *copper(I)-catalyzed azide-alkyne cycladdition*, CuAAC)<sup>[45,46]</sup> oraz w wariacie niewymagającym użycia miedziowego katalizatora (ang. *strain-promoted azide-alkyne cycladdition*, SPAAC).<sup>[47]</sup> Azydek **8** może także zostać zredukowany odpowiednią fosfiną do aminy **9** w reakcji Staudingera (Schemat 3, **E**). Amina **9** reaguje z kwasami karboksylowymi w obecności odczynników sprzęgających lub z aktywnymi estrami NHS wybranych kwasów karboksylowych, dając amidowe pochodne.<sup>[48]</sup> W pozycji R<sub>5</sub> kobalaminy przyłączano leki,<sup>[49]</sup> barwniki fluorescencyjne,<sup>[50]</sup> związki chelatujące metale<sup>[51]</sup> oraz kompleksy metali.<sup>[52]</sup>



**Schemat 3.** Modyfikacje witaminy B<sub>12</sub> w pozycji R<sub>5</sub> (R = lek, barwnik, łącznik, itp.).

Poza wymienionymi przykładami opracowano również metody prowadzące do pochodnych kobalaminy ze związkami przyłączonymi jednocześnie na jonie kobaltu, jak i w pozycji R<sub>5</sub>. Aby to osiągnąć stosuje się reakcje tworzenia kompleksów między ligandem cyjankowym a kompleksami metali (Schemat 1, **A**),<sup>[53]</sup> reakcje alkilowania jonu kobaltu (Schemat 1, **B**)<sup>[54]</sup> lub tworzenia pochodnych alkinylowych na kobaltcie (Schemat 1, **E**)<sup>[55]</sup> razem z funkcjonalizacją pozycji R<sub>5</sub> za pomocą wiązania karbaminianowego (Schemat 3, **B**) lub sekwencji reakcji prowadzącej do grupy azydkowej (Schemat 3, **D**).<sup>[56]</sup> Należy jednak pamiętać o odpowiedniej kolejności przeprowadzanych modyfikacji. Istnieją również doniesienia na temat funkcjonalizacji grupy



fosforanowej, nie były one jednak szeroko stosowane z uwagi na ograniczoną stabilność otrzymanych pochodnych.<sup>[57]</sup>

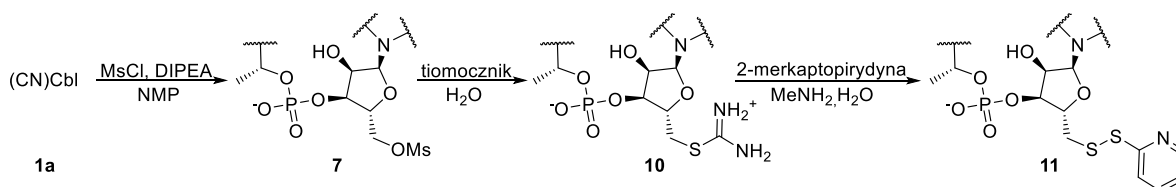
Syntetyczne podejścia prowadzące do koniugatów z witaminą B<sub>12</sub> zostały opisane w przeglądzie:

**A. J. Wierzba**, S. Hassan, D. Gryko, *Asian J. Org. Chem.* **2018**, 10.1002/ajoc.201800579

*Synthetic Approaches Toward Vitamin B<sub>12</sub> Conjugates*

### 4.3 Modyfikacje witaminy B<sub>12</sub> w obrębie pętli nukleotydowej

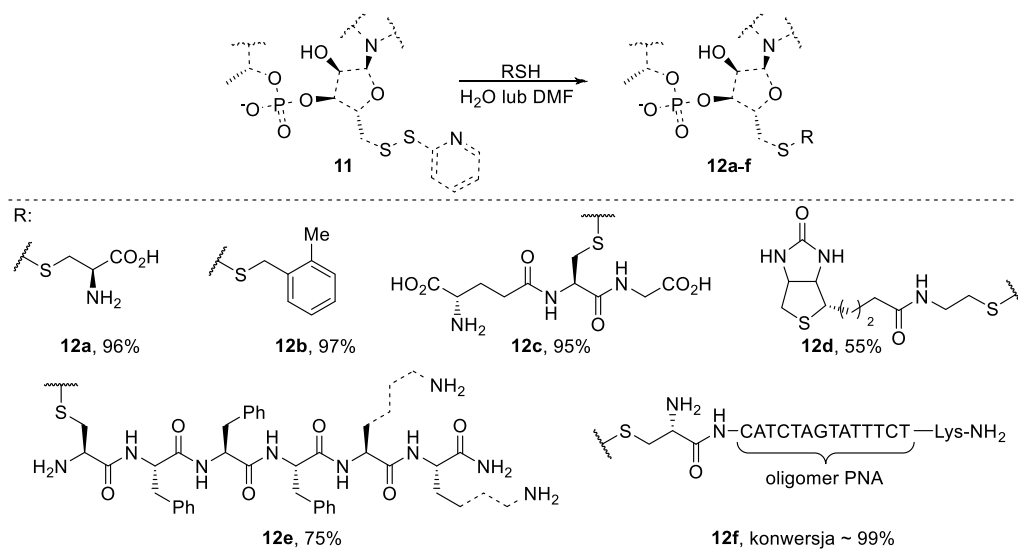
Modyfikacje struktury kobalaminy stanowią wyzwanie i często wymagają czasu, cierpliwości oraz żmudnych procesów optymalizacyjnych. Grupa R<sub>5</sub>-OH jest atrakcyjną pozycją do potencjalnych modyfikacji i opisane w literaturze podejścia syntetyczne obejmują możliwość funkcjonalizacji tej grupy na drodze estryfikacji, tworzenia wiązania karbaminianowego, utleniania i następczego sprzęgania z aminami oraz substytucji prowadzącej do wprowadzenia w pozycji R<sub>5</sub> grupy azydkowej, która może być wykorzystana w katalizowanej jonami miedzi(I) 1,3-dipolarnej cykloaddycji azydków do alkinów (Schemat 3). Poza estryfikacją, która prowadzi do pochodnych charakteryzujących się ograniczoną stabilnością, zastosowanie wyżej wymienionych metod skutkuje otrzymaniem stabilnych koniugatów. Z drugiej strony, atrakcyjnym dopełnieniem opisanych podejść syntetycznych, w mojej ocenie, byłaby możliwość tworzenia koniugatów, których elementy połączone są wiązaniami rozłączalnymi w określonych warunkach. Zaproponowane podejście eliminuje występowanie niekorzystnych oddziaływań między nośnikiem a przyłączanym związkiem i dodatkowo pozwala na uwolnienie tego związku pod wpływem określonego czynnika. W związku z tym, postanowiłam opracować syntezę pochodnej kobalaminy, która reagowałaby ze związkami posiadającymi grupę tiolową poprzez utworzenie rozłączalnego wiązania disiarczkowego. W celu wprowadzenia grupy tiolowej w pozycji R<sub>5</sub>, zaprojektowałam sekwencję reakcji obejmującą odpowiednio mesylowanie pierwszorzędowej grupy hydroksylowej, podstawienie grupy mesylowej tiomocznikiem oraz następczą zasadową hydrolizę w obecności 2-merkaptopirydyny (Schemat 4). Trójetapowa synteza prowadziła do otrzymania disiarczku **11**, który wydzieliłam z łączną wydajnością 60%.<sup>[58]</sup>



**Schemat 4.** Sekwencja reakcji prowadząca do związku **11**.

Etap podstawienia grupy mesylowej zoptymalizowałam biorąc pod uwagę rodzaj nukleofila oraz jego ilość, czas reakcji, temperaturę, rodzaj rozpuszczalnika, a także ilości reagentów. Podobnie zoptymalizowałam etap zasadowej hydrolizy poprzez dobór odpowiedniej zasady, czasu reakcji, a także ilości reagentów. Jedynie metyloamina prowadziła do selektywnej hydrolizy soli tiouroniowej **10**, w wyniku której obserwowałam powstanie pochodnej witaminy z grupą tiolową (SH) w pozycji

R<sub>5</sub>, której wydzielenie było jednak niemożliwe z uwagi na fakt tworzenia się dimerycznego produktu (disiarczku witaminy B<sub>12</sub>). Dlatego też, dodatek 2-merkaptopirydyny na etapie hydrolizy okazał się idealnym rozwiązaniem i w efekcie selektywnie otrzymałam pochodną **11**. Wprowadzone ugrupowanie –SSPy charakteryzuje się selektywną reaktywnością względem tioli, a reakcja tworzenia disiarczków z jego udziałem nie wymaga dodatkowych odczynników i jako produkt uboczny generuje jedynie tautomer 2-merkaptopirydyny, który może być łatwo usunięty ze środowiska reakcji. Pochodną **11** poddałam reakcji odpowiednio z: tiolami, cysteiną oraz krótkimi peptydami zawierającymi cysteinę, otrzymując disiarczki z bardzo dobrymi wydajnościami (Schemat 5). Ponadto, związek **11** reagował również ilościowo z bardziej złożonymi strukturami, tj. peptydowym kwasem nukleinowym (pochodna **12f**).



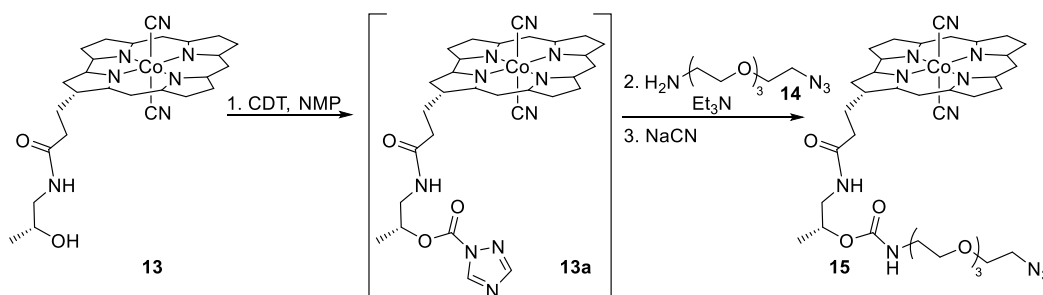
Schemat 5. Synteza disiarczków **12a-f**.

Opisana reakcja tworzenia disiarczków jest wysoce selektywna i organiczna ją jedynie dobór rozpuszczalnika (warunkowany rozpuszczalnością substratu **11**), który musi mieć charakter polarny protyczny lub aprotyczny. Nowo otrzymane pochodne witaminy B<sub>12</sub> **10**, **11** oraz **12a-f** poddałam pełnej charakterystyce metodami spektroskopii NMR, spektrometrii mas, analizy elementarnej oraz wysokosprawnej chromatografii cieczowej w układzie faz odwróconych (RP HPLC). Dodatkowo, pochodną **11** poddałam krystalizacji, co umożliwiło potwierdzenie jej struktury metodą rentgenowskiej analizy strukturalnej. Wykazałam również, że połączenie disiarczkowe w docelowych koniugatach może być selektywnie rozłączone w obecności glutationu (tiolu obecnego w komórkach eukariotycznych).

Opisana procedura funkcjonalizacji została opublikowana w artykule:

A. J. Wierzba, M. Wojciechowska, J. Trylska, D. Gryko, *Bioconjugate Chem.* **2016**, 27, 189–197  
*Vitamin B<sub>12</sub> Suitably Tailored for Disulfide-Based Conjugation*

Niektóre bakterie, w przeciwieństwie do ssaków posiadają zdolność rozpoznawania tzw. niepełnych korynoidów (ang. *incomplete corrinoids*).<sup>[18,59]</sup> Fakt ten otwiera nowe możliwości w kontekście tworzenia narzędzi, które pozwolą na dostarczanie związków do komórek bakterii w sposób selektywny. Do niepełnych korynoidów zalicza się kobinamid **13** – pochodna witaminy B<sub>12</sub> pozbawiona fragmentu nukleotydowego (Schemat 6). Związek ten można stosunkowo łatwo otrzymać poddając cyjanokobalaminę działaniu promieniowania mikrofalowego w obecności NaCN.<sup>[60]</sup> Do tej pory nie były znane możliwości jego bezpośredniej funkcjonalizacji, dlatego też w toku prowadzonych przeze mnie badań postanowiłam podjąć próbę podstawienia drugorzędowej grupy hydroksylowej będącej częścią łańcucha proponamidowego *f*. Podejścia obejmujące mesyłowanie lub tosyłowanie tej grupy okazały się nieskuteczne, w związku z tym postanowiłam aktywować ją za pomocą CDT (1,1'-karbonyl-di-(1,2,4-triazolu)), a następnie bez wydzielania produktu pośredniego, przeprowadziłam reakcję z aminą **14** posiadającą terminalną grupę azydkową. Zaproponowane podejście prowadziło do oczekiwanego produktu **15**, który wydzieliłam metodą preparatywnej wysokosprawnej chromatografii cieczowej w układzie faz odwróconych w formie mieszaniny produktów różniących się rodzajem ligandów aksjalnych (form  $\alpha$ -akwa,  $\beta$ -cyjano oraz  $\alpha$ -cyjano,  $\beta$ -akwa) z wydajnością 84%.<sup>[61]</sup> Dodatek NaCN prowadził do otrzymania kompleksu dicyjano **15**.



Schemat 6. Synteza pochodnej kobinamidu **15**.

Otrzymany związek scharakteryzowałam metodą spektroskopii NMR, spektrometrii mas, analizy elementarnej oraz wysokosprawnej chromatografii cieczowej w układzie faz odwróconych. Opracowane przeze mnie podejście pozwala na wprowadzenie do struktury kobinamidu związków z reaktywnymi grupami funkcyjnymi (w przypadku pochodnej **15** jest to grupa azydkowa) i tym samym otwiera możliwość wykorzystania tej pochodnej jako potencjalnie selektywnego transportera związków do komórek bakterii.

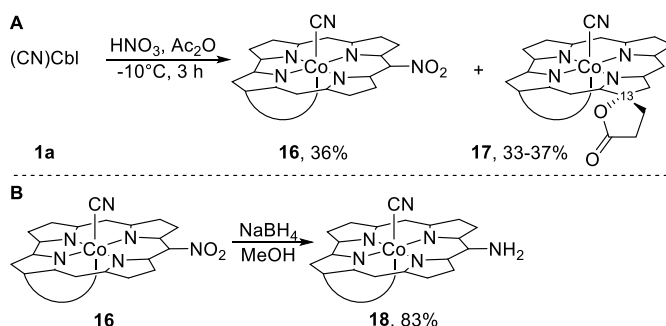
Opisane podejście syntetyczne jest częścią artykułu:

**A. J. Wierzba**, K. Maximova, A. Wincenciuk, M. Równicki, M. Wojciechowska, E. Nexø, J. Trylska, D. Gryko, *Chem. Eur. J.* **2018**, 10.1002/chem.201804304

*Does a Conjugation Site Affect Transport of Vitamin B<sub>12</sub>-PNA Conjugates into Bacterial Cells?*

#### 4.4 Modyfikacje witaminy B<sub>12</sub> w pozycji *mezo*

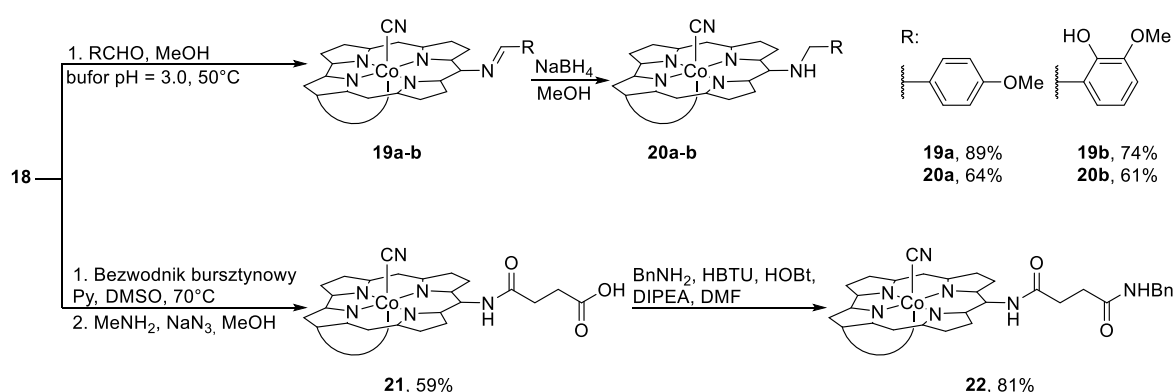
Pozycją w obrębie struktury kobalaminy, która do tej pory nie była rozpatrywana w kontekście tworzenia koniugatów jest pozycja *mezo* (C10), będąca częścią pierścienia korynowego. Funkcjonalizacja kobalaminy w tej pozycji pozwoliłaby nie tylko na stworzenie nowego narzędzia syntetycznego, ale również umożliwiłaby zbadanie wpływu podstawników w tej pozycji na całą strukturę kobalaminy. Można się bowiem spodziewać, że podstawniki o różnym charakterze elektronowym w pozycji *mezo* będą wpływały na cały charakter liganda ekwatorialnego (koryny), co z kolei może zmieniać charakter jonu centralnego (tzw. efekt cis).<sup>[62,63]</sup> W literaturze istnieją jedynie doniesienia na temat kobalaminy z podstawnikami Cl,<sup>[64]</sup> Br<sup>[65]</sup> oraz NO<sup>[66]</sup> w pozycji *mezo*. W związku z tym, postanowiłam rozszerzyć spektrum *mezo*-modyfikowanych kobalamin o pochodną z podstawnikiem NO<sub>2</sub> (Schemat 7, A), która może zostać poddana następczym przekształceniom. W tym celu przetestowałam serię różnych odczynników nitrujących, jednak najbardziej efektywnym okazała się mieszanina HNO<sub>3</sub> (65%) i bezwodnika octowego (1:1 v/v).<sup>[67]</sup> Kluczową rolę w tej reakcji pełniła temperatura i dopiero obniżenie jej do -10°C prowadziło do otrzymania produktu **16** z zadowalającą wydajnością 36%. Drugim głównym produktem powstającym w reakcji nitrowania był, nieopisany dotąd w literaturze, *e*-lakton **17** o odwróconej konfiguracji na atomie węgla C13 (Schemat 7, A). Strukturę związku **17** określiłam w oparciu o widma dwuwymiarowe NMR. Ponadto, krystalizacja produktu jego hydrolizy i następcza rentgenowska analiza strukturalna otrzymanego monokryształu pozwoliły mi na pośrednie potwierdzenie stereochemii. Niezależnie od zastosowanych warunków nie udało mi się uniknąć procesu tworzenia *e*-laktonu **17**. Jednak nie należy tego traktować za wadę opracowanej metodologii, ponieważ reakcja nitrowania prowadzi do dwóch pochodnych witaminy, które można poddać dalszym modyfikacjom. Redukcja *e*-laktonu prowadzi bowiem do *e*-kwasu o odwróconej konfiguracji na atomie węgla C13, który może być poddany następczym reakcjom sprzęgania, co stanowi alternatywną metodę modyfikacji w pozycji *e* w stosunku do mało wydajnej i nieselektywnej hydrolizy kobalaminy **1a** opisaney w podrozdziale 4.2.



Schemat 7. Nitrowanie cyjanokobalaminy oraz redukcja *mezo*-nitrokobalaminy **16** do aminy **18**.

Redukcja nitro-pochodnej **16** (o barwie czerwonej) borowodorkiem sodu prowadziła selektywnie do aminy **18** o barwie ciemnogrnatowej (Schemat 7, B), którą wydzieliłam z wydajnością 83%.

Przeprowadziłam próby reakcji aminy **18** z aldehydami i optymalizację warunków tej reakcji obejmującą ilości reagentów, czas reakcji, temperaturę oraz rozpuszczalnik (Schemat 8). W konsekwencji otrzymałam iminy **19a-b** z bardzo dobrymi wydajnościami. Zsyntezowane iminy poddałam kolejno redukcji do amin drugorzędowych **20a** oraz **20b**. Próby przeprowadzenia reakcji aminy **18** z kwasami karboksylowymi w obecności odczynników sprzęgających nie powiodły się, prawdopodobnie z uwagi na niedostateczną nukleofilowość aminy **18**. Jednak, stosując odpowiednio duży nadmiar bezwodnika bursztynowego otrzymałam *mezo*-amid **21**. Co więcej, terminalną grupę karboksylową w związku **21** poddałam następczej reakcji z aminami w obecności odczynników sprzęgających, co umożliwiło dalszą modyfikację podstawnika *mezo* kobalaminy (Schemat 8, pochodna **22**).



Schemat 8. Funkcjonalizacja witaminy B<sub>12</sub> w pozycji *mezo*.

Zinterpretowałam serię widm dwuwymiarowych NMR otrzymanych *mezo*-pochodnych **16**, **18** oraz **22** i porównałam je z serią widm dla niemodyfikowanej kobalaminy **1a**. Ponadto, otrzymałam kryształy dla pochodnych **16** oraz **22**, które zostały poddane rentgenowskiej analizie strukturalnej. Porównałam również widma absorpcji związków **1a**, **16**, **18**, **19a**, **20a** oraz **22**, w których pasma absorpcji nitrowanej kobalaminy **16** wykazują wyraźne przesunięcie hipsochromowe, podczas gdy dla aminy **18** obserwuje się przesunięcie batochromowe, co jest zgodne z charakterem obu podstawników i widoczne jest w barwie obu pochodnych (**16** – czerwona, **18** – ciemnogrnatowa). W związku z tym, że zarówno biologiczne, jak i katalityczne właściwości witaminy B<sub>12</sub> związane są z właściwościami utleniająco-redukującymi centralnego jonu kobaltu, określiłam również potencjały redukcji kobaltu metodą woltamperometrii cyklicznej. Nitro-pochodna **16** charakteryzowała się najwyższym potencjałem redukcji (-0.69 V), podczas gdy wynik dla *mezo*-aminy **18** (-0.96 mV) był bardzo zbliżony do wartości otrzymanej dla kobalaminy **1a** (-1.01 V).

Opracowana metodologia pozwoliła, nie tylko na funkcjonalizację kobalaminy w pozycji *mezo*, ale również na określenie wpływu charakteru podstawnika *mezo* na właściwości strukturalne, fotofizyczne i elektrochemiczne witaminy B<sub>12</sub>, co w konsekwencji umożliwiło głębsze zrozumienie wpływu liganda ekwatorialnego zarówno na jon centralny, jak i strukturę całej cząsteczki.

Wyniki przedstawione w tym podrozdziale zostały opublikowane w artykule:

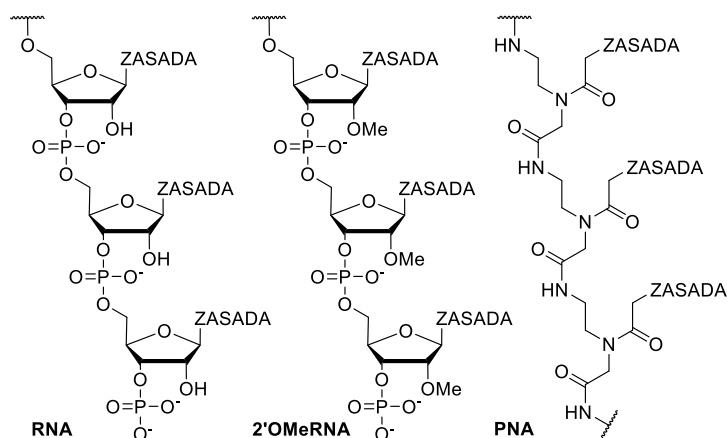
A. J. Wierzba, A. Wincenciuk, M. Karczewski, V. I. Vullev, D. Gryko, *Chem. Eur. J.* **2018**, *24*, 10344–10356

*meso-Modified Cobalamins: Synthesis, Structure, and Properties*

#### 4.5 Synteza połączeń witaminy B<sub>12</sub> z peptydowymi kwasami nukleinowymi

Rosnąca oporność bakterii względem obecnie stosowanych terapii antybiotykowych skłania naukowców do opracowywania nowych podejść umożliwiających walkę z infekcjami bakteryjnymi. Jednym z nich jest wykorzystanie antysensownych nukleotydów, które posiadają zdolność łączenia się z komplementarnymi fragmentami mRNA i w efekcie powodują zahamowanie procesu translacji.<sup>[4,5]</sup> Odpowiednie zaprojektowanie sekwencji takiego nukleotydu umożliwia zatem zahamowanie kluczowych procesów związanych z biosyntezą białek w komórce bakterii.

W związku z tym, że naturalne oligonukleotydy charakteryzują się ograniczoną trwałością, stosuje się ich syntetyczne analogi wykazujące większą stabilność, wśród których możemy wyróżnić peptydowe kwasy nukleinowe (PNA), zawierające w swej strukturze fragmenty *N*-(2-aminoetylo)glicyny (Rysunek 4).<sup>[3]</sup> Ponadto, oligomery PNA łączą się z pojedynczymi komplementarnymi niemi DNA lub RNA silniej niż te z komplementarnymi łańcuchami DNA.



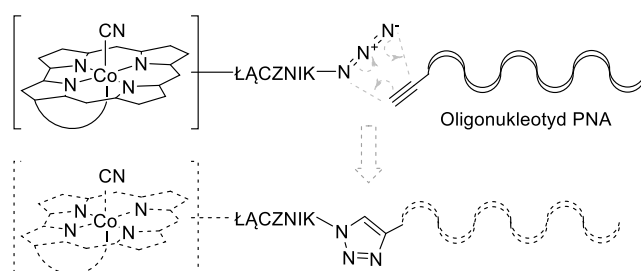
**Rysunek 4.** Struktury RNA, 2'OMeRNA oraz PNA. ZASADA: adenina, guanina, cytozyna, tymina lub uracyl.

Głównym czynnikiem ograniczającym zastosowanie modyfikowanych oligonukleotydów jako potencjalnych środków przeciwbakteryjnych jest ich brak zdolności wnikania do komórek bakteryjnych. Dlatego też, poszukuje się skutecznych i nieinwazyjnych metod ich dostarczenia. Obecnie stosowane podejścia opierają się o wykorzystanie połączeń PNA z peptydami transportującymi, np. z peptydem (KFF)<sub>3</sub>K, jednak te metody wciąż mają wiele ograniczeń.<sup>[68]</sup>

Niektóre bakterie, podobnie jak komórki eukariotyczne, wykształciły drogę dla transportu kobalaminy angażującą serię białek transportujących. W przypadku bakterii *E. coli* są to m.in. białka BtuB, TonB, BtuF oraz BtuCD.<sup>[10]</sup> W związku z tym, można przypuszczać, że analogicznie jak w komórkach

eukariotycznych, witamina B<sub>12</sub> może okazać się skutecznym transporterem związków do komórek bakterii.

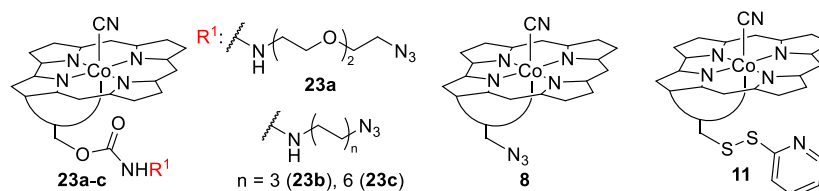
Jednym z celów mojej pracy było zaadaptowanie nowo opracowanych oraz opisanych już w literaturze metod do syntezy koniugatów witaminy B<sub>12</sub> z modyfikowanymi oligonukleotydami, a konkretnie peptydowym kwasem nukleinowym (PNA). Sekwencja PNA była zaprojektowana w taki sposób, aby łączyła się komplementarnie z określonym fragmentem bakteryjnego mRNA, prowadząc do zahamowania procesu translacji. Dotychczas nie było doniesień na temat wykorzystania kobalaminy jako nośnika związków do komórek bakterii, dlatego też w pierwszym etapie badań należało zweryfikować, czy witamina B<sub>12</sub> będzie efektywnym transporterem modyfikowanych oligonukleotydów. Nie było również wiadomo, które z pozycji w witaminie B<sub>12</sub> mogą być wykorzystane do tworzenia koniugatów z oligonukleotydami bez negatywnego wpływu na rozpoznanie przez białka transportujące bakterii. Biorąc pod uwagę wszystkie te aspekty opracowałam syntezę serii pochodnych witaminy, które kolejno wykorzystane zostały do utworzenia koniugatów z odpowiednio sfunkcjonalizowanym monomerem PNA w katalizowanej jonami miedzi(I) 1,3-dipolarnej cykloaddycji azydków do alkinów (Rysunek 5). Oligomer PNA przyłączany do witaminy posiadał terminalną grupę alkinową, wprowadzoną do struktury na ostatnim etapie syntezy na podłożu stałym. W związku z tym, modyfikacja kobalaminy wymagała wprowadzenia do jej struktury funkcji azydkowej.



**Rysunek 5.** Schematyczna reprezentacja metodologii stosowanej do otrzymywania koniugatów witaminy B<sub>12</sub>-PNA.

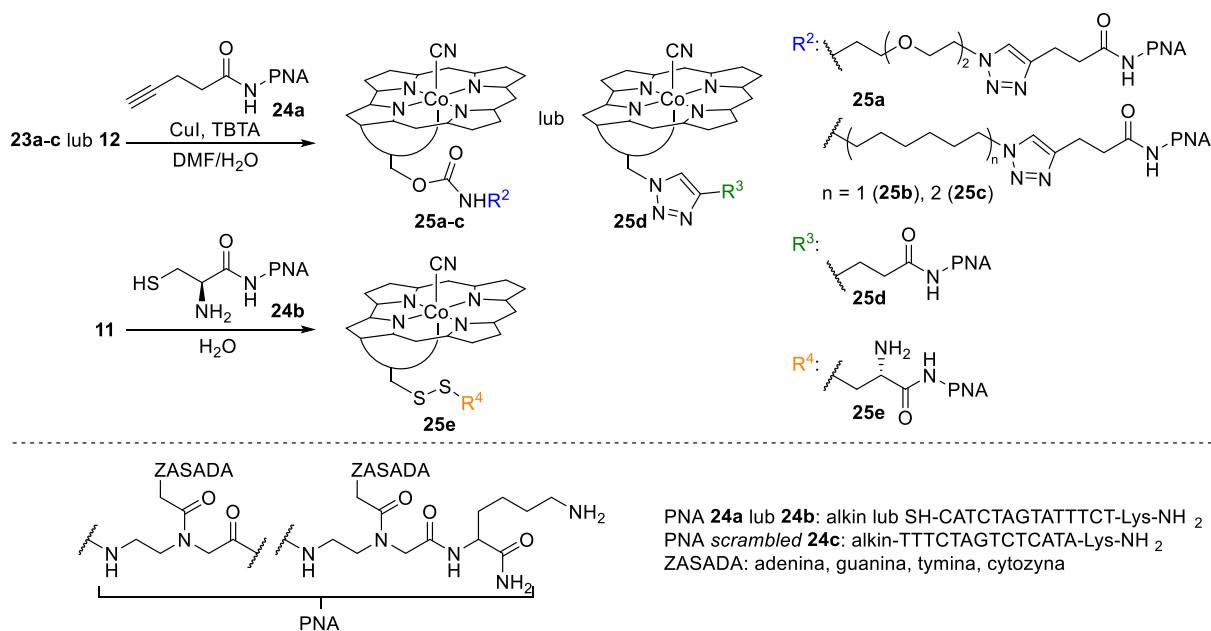
Pozycja R<sub>5</sub> w obrębie rybozy została wybrana jako modelowe miejsce modyfikacji, ponieważ założono, że podobnie jak w przypadku komórek eukariotycznych, jej modyfikacja nie będzie w znacznym stopniu zaburzała procesu rozpoznania koniugatu. Wprowadzenie łącznika między transporterem a dostarczonym związkiem często korzystnie wpływa na proces transportu z uwagi na odpowiednie odseparowanie ograniczające wzajemne oddziaływanie związków. Dlatego, w celu zweryfikowania wpływu obecności łącznika na transport, przygotowałam serię pochodnych kobalaminy z łącznikami o różnej długości i odmiennym charakterze (hydrofilowe **23a**, hydrofobowe **23b-c**, Rysunek 6) oraz taką, która pozwalała na bezpośrednie przyłączenie oligomeru (**8**). Co więcej, otrzymałam również pochodną **11**, która umożliwia utworzenie koniugatu z odpowiednio sfunkcjonalizowanym łańcuchem PNA za pomocą rozłączalnego w warunkach redukujących wiązania disiarczkowego (Rysunek 6).<sup>[69]</sup> Serię pochodnych kobalaminy z łącznikami w pozycji R<sub>5</sub> otrzymałam wykorzystując podejście opracowane przez McEvana i współpracowników polegające na aktywacji

grupy hydroksylowej CDT i następczej reakcji z aminą (w tym przypadku aminą posiadającą terminalną grupę azydkową), prowadzące do utworzenia wiązania karbaminianowego (**23a-c**).<sup>[42]</sup> Pochodną witaminy z grupą azydkową w pozycji R<sub>5</sub> (**8**) przygotowałam korzystając z procedury opracowanej w zespole Gryko obejmującej odpowiednio mesyłowanie grupy hydroksylowej oraz podstawienie azydkiem sodu.<sup>[44]</sup> Pochodną **11** zsyntezowałam wykorzystując podejście opisane w podrozdziale 4.3.<sup>[69]</sup>



Rysunek 6. Pochodne witaminy B<sub>12</sub> wykorzystywane do syntezy koniugatów z PNA

Synteza oligomerów PNA oraz docelowych koniugatów została przeprowadzona we współpracy z Centrum Nowych Technologii Uniwersytetu Warszawskiego. Koniugaty witamina B<sub>12</sub>-PNA **25a-d** zostały otrzymane w reakcji 1,3-dipolarnej cykloaddykcji katalizowanej za pomocą CuI w obecności liganda TBTA (tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)metylo]amina), natomiast koniugat **25e** otrzymano na drodze tworzenia wiązania disiarczkowego między oligomerem PNA zawierającym terminalną cysteinę (**24b**) oraz pochodną witaminy **11** (Schemat 9). Oba opracowane podejścia pozwoliły na otrzymanie zaprojektowanych związków.



Schemat 9. Synteza koniugatów witaminy B<sub>12</sub> i PNA.

Sekwencja przyłączanego PNA (licząca 14 zasad, zakończona lizyną w celu poprawy rozpuszczalności, **24a** lub **24b**, Schemat 9) została zaprojektowana w taki sposób, aby hamowała ekspresję genu odpowiedzialnego za syntezę białka czerwonej fluorescencji (ang. *red fluorescent protein*, RFP) obecnego w odpowiednio zmodyfikowanych szczepach *Escherichia coli*



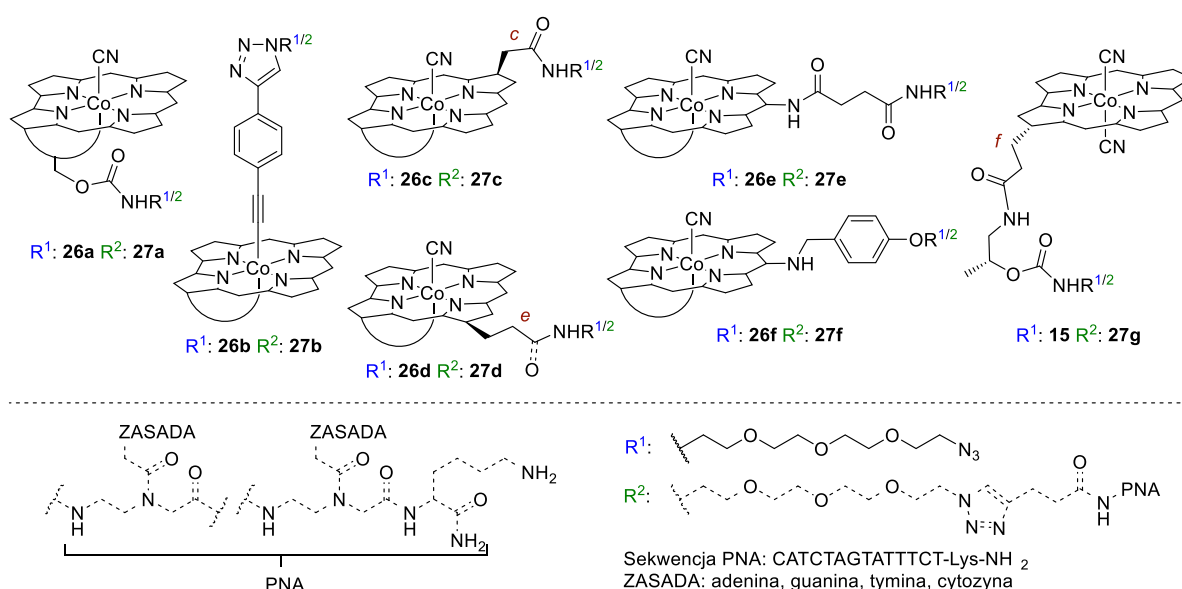
oraz *Salmonella* Typhimurium.<sup>[70]</sup> Jako kontrolę przygotowano koniugat witaminy B<sub>12</sub> z sekwencją PNA, która była dobrana w taki sposób, aby nie zaburzała żadnego z procesów zachodzących w bakterii (PNA scrambled, **24c**). Po efektywnym wprowadzeniu koniugatów z PNA **24a-b** do komórek bakterii oczekiwany był spadek intensywności fluorescencji RFP, podczas gdy koniugat z sekwencją PNA scrambled **24c** nie powinien negatywnie wpływać na fluorescencję. W istocie, właśnie taki efekt obserwowano. Najbardziej efektywne wygaszenie fluorescencji miało miejsce w przypadku zastosowania koniugatu **25c** z najdłuższym alkielowym łącznikiem między witaminą B<sub>12</sub> a PNA. Przeprowadzone badania dowiodły, że witamina B<sub>12</sub> może być efektywnym transporterem dla syntetycznych oligonukleotydów przyłączonych do kobalaminy w pozycji R<sub>5</sub>.

Wynik przedstawione powyżej zostały opisane w artykule:

M. Równicki, M. Wojciechowska, **A. J. Wierzba**, J. Czarnecki, D. Bartosik, D. Gryko, J. Trylska, *Sci. Rep.* **2017**, 7, 7644

#### Vitamin B<sub>12</sub> as a Carrier of Peptide Nucleic Acid (PNA) into Bacterial Cells

Podobnie jak w przypadku komórek eukariotycznych, spodziewałam się zależności między miejscem przyłączenia związku do witaminy, a efektywnością transportu. Dlatego też w kolejnym etapie badań opracowałam syntezę pochodnych witaminy z łącznikiem przyłączonym do kobalaminy w różnych pozycjach. Dobór miejsca modyfikacji determinowany był łatwością i dostępnością metod syntetycznych. Łącznik na bazie glikolu polietylenowego, posiadający terminalną grupę azydkową (R<sup>1</sup>), wprowadziłam kolejno na jonie kobaltu, w pozycjach R<sub>5</sub>, c, e, mezo, a także przyłączyłam go do tzw. niepełnego korynoidu – kobinamidu **13** (pochodne **26a-f** oraz **15**, Rysunek 7).



**Rysunek 7.** Koniugaty witaminy B<sub>12</sub> z PNA przyłączonym w różnych miejscach w cząsteczce.

W celu otrzymania pochodnej modyfikowanej w obrębie jonu kobaltu zaprojektowałam sekwencję reakcji z wykorzystaniem metodologii opracowanej w zespole Gryko (metoda modyfikacji została

opisana w podrozdziale 4.2, Schemat 1, E),<sup>[31]</sup> polegającej na wprowadzeniu 1,4-dietynylobenzenu jako liganda  $\beta$  oraz następczej reakcji CuAAC z odpowiednim diazydkiem prowadzącej do pochodnej **26b**. Pochodną **26c** z łącznikiem w pozycji *c* otrzymałam wykorzystując *c*-lakton **3** jako wyjściowy substrat, który po redukcji do *c*-kwasu **2d**<sup>[40]</sup> poddałam reakcji z amino-azydkiem (analogiczne podejście syntetyczne zostało opisane w podrozdziale 4.2, Schemat 2, B). Otrzymany w reakcji nitrowania *e*-lakton **17** okazał się użyteczną pochodną do modyfikacji pozycji *e* na drodze redukcji laktonu do kwasu, a następnie reakcji sprzęgania z aminą. W efekcie otrzymałam pochodną **26d**. Pochodne **26c** oraz **26d** z łącznikiem przyłączonym w pozycji *mezo* otrzymałam wykorzystując autorskie podejście opisane w podrozdziale 4.4,<sup>[67]</sup> natomiast synteza modyfikowanego, niepełnego korynoidu **15** została opisana w podrozdziale 4.3.<sup>[61]</sup> Wszystkie otrzymane pochodne oczyściłam metodą chromatografii cieczowej w układzie faz odwróconych oraz scharakteryzowałam m.in. metodą spektroskopii NMR oraz spektrometrii mas.

Synteza docelowych koniugatów **27a-g** została przeprowadzona we współpracy z Centrum Nowych Technologii Uniwersytetu Warszawskiego. Otrzymane koniugaty B<sub>12</sub>-PNA **27a-g** zostały poddane analogicznym badaniom biologicznym, jak uprzednio opisane związki z PNA przyłączonym w pozycji R<sub>5</sub>.<sup>[61]</sup> Uzyskane wyniki wykazały, że modyfikacje w obrębie pierścienia korynowego (pozycja *c*, *e* i *mezo*) wpływają niekorzystnie na transport oligonukleotydów do komórek bakterii *E. coli*, podczas gdy pochodna modyfikowana na kobalcie **27b** transportuje PNA, przy czym efektywność wygaszania fluorescencji jest większa przy mniejszych stężeniach koniugatu. Bardzo interesującym wynikiem był ten uzyskany dla niepełnego korynoidu **27g**, ponieważ przy zapewnieniu odpowiednio dużego stężenia koniugatu, wygaszenie fluorescencji było niemal tak efektywne jak dla pochodnych modyfikowanych w pozycji R<sub>5</sub> (**27a**). Rezultat ten potwierdza, że bakterie mają zdolność transportowania pochodnych kobalaminy nieposiadających pętli nukleotydowej.

Spośród wszystkich testowanych wariantów pochodne modyfikowane w pozycji R<sub>5</sub>, najefektywniej wygaszały fluorescencję, co wskazuje, że podobnie jak dla komórek eukariotycznych, modyfikacje w tej pozycji w najmniejszym stopniu zakłócają transport kobalaminy do komórek bakterii.

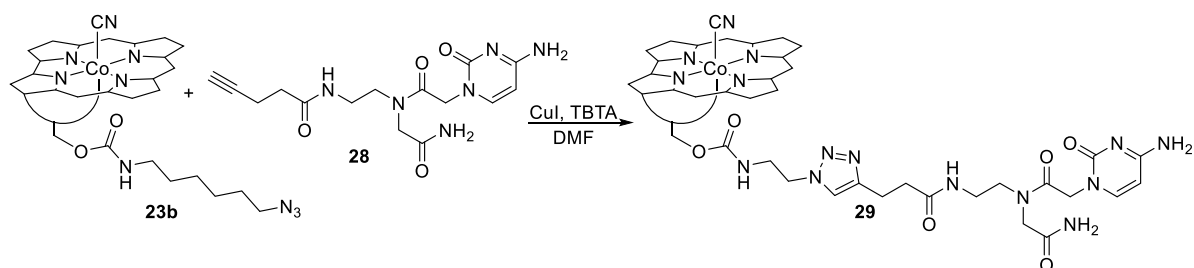
Wyniki przedstawione powyżej zostały opisane w artykule:

A. J. Wierzba, K. Maximova, A. Wincenciuk, M. Równicki, M. Wojciechowska, E. Nexø, J. Trylska, D. Gryko, *Chem. Eur. J.* **2018**, 10.1002/chem.201804304

*Does a Conjugation Site Affect Transport of Vitamin B<sub>12</sub>-PNA Conjugates into Bacterial Cells?*

W ramach współpracy z Centrum Nowych Technologii wykonane zostały również symulacje dynamiki molekularnej dla 14-merowego łańcucha PNA (CATCTAGTATTTCT-Lys-NH<sub>2</sub>), koniugatu witaminy B<sub>12</sub> z tym oligomerem (**25b**) oraz dla koniugatu z pojedynczym monomerem (pierwszym w sekwencji- monomerem C, koniugat **29**, Schemat 10).<sup>[71]</sup> Wykonane symulacje miały na celu określenie wzajemnego oddziaływania dwóch elementów koniugatu i zweryfikowanie czy są one korzystne w kontekście oddziaływania PNA z docelowym fragmentem mRNA. W celu sprawdzenia

czy wyniki uzyskane z symulacji są zbieżne z danymi uzyskanymi eksperymentalnie zaprojektowałam syntezę koniugatu z monomerem C (**29**), dla którego przeprowadzona została symulacja. Wykorzystując syntezę na podłożu stałym przygotowałam odpowiednio zmodyfikowany monomer **28** z terminalną grupą alkinową, który kolejno poddałam reakcji z kobalaminą **23b** w obecności układu katalitycznego CuI/TBTA, otrzymując pochodną **29** (Schemat 10). Podjęłam szereg prób mających na celu krystalizację pochodnej **29**, jednak okazały się nieskuteczne. Aby określić oddziaływania między fragmentami koniugatu (witaminą B<sub>12</sub> a monomerem C), poddałam analizie serię widm dwuwymiarowych tej pochodnej, w tym widm <sup>1</sup>H-<sup>1</sup>H ROESY oraz <sup>1</sup>H-<sup>15</sup>N HSQC. Po zestawieniu wyników uzyskanych z widm NMR z oddziaływaniami obecnymi w konfiguracjach cząsteczek uzyskanych z trajektorii symulacji dynamiki molekularnej wykazałam, że oddziaływania obecne na widmie były obserwowane również za pomocą symulacji.



Schemat 10. Synteza połączenia witaminy B<sub>12</sub> z monomerem PNA.

Wyniki przedstawione powyżej zostały opisane w artykule:

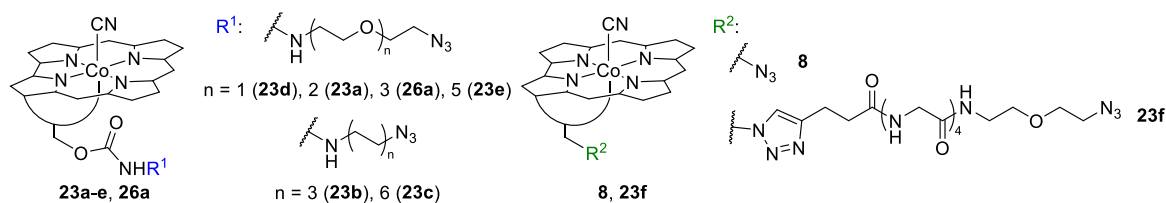
T. Pieńko, A. J. Wierzba, M. Wojciechowska, D. Gryko, J. Trylska, *J. Phys. Chem. B* **2017**, *121*, 2968–2979

*Conformational Dynamics of Cyanocobalamin and Its Conjugates with Peptide Nucleic Acids*

#### 4.6 Synteza połączeń witaminy B<sub>12</sub> z barwnikami fluorescencyjnymi

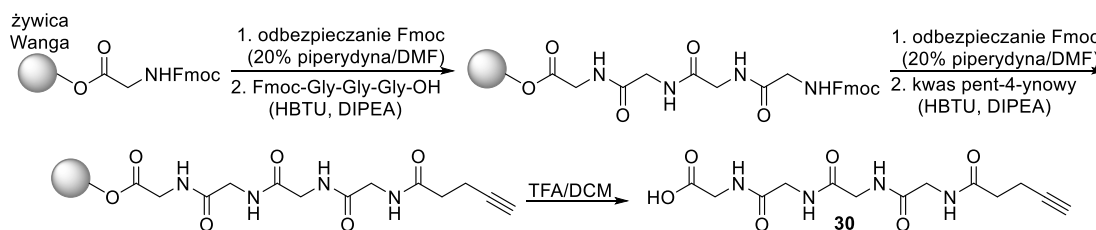
Przełączniki RNA (ryboprzełączniki, ang. *riboswitches*) regulują ekspresję genów na poziomie transkrypcji i translacji poprzez wiązanie niskocząsteczkowych metabolitów, do których zalicza się również witamina B<sub>12</sub>.<sup>[6,7]</sup> Założeniem tej części pracy było wykorzystanie naturalnej zdolności witaminy B<sub>12</sub> (będącej częścią koniugatu z barwnikiem fluorescencyjnym) do wiązania się z RNA w celu wywołania odpowiedniej odpowiedzi spektralnej, która pozwoli na zobrazowanie danego fragmentu RNA. W tym celu zaprojektowałam serię sond fluorescencyjnych będących połączeniem witaminy B<sub>12</sub> i barwnika. Pozycja R<sub>5'</sub> została wybrana jako miejsce modyfikacji, a katalizowana jonami miedzi(I) 1,3-dipolarna cykloaddycja azydków do alkinów posłużyła mi jako metoda łączenia odpowiednio sfunkcjonalizowanej kobalaminy z barwnikiem fluorescencyjnym. W związku z tym, że oddziaływanie barwnika z koryną prowadzi do wygaszenia jego fluorescencji, kluczowy jest dobór rodzaju połączenia między składowymi koniugatu.<sup>[8]</sup> W tym celu zsyntezowałam serię witaminowych pochodnych z terminalną grupą azydkową przyłączoną do kobalaminy bezpośrednio (pochodna **8**)

lub za pomocą łączników o różnej długości i odmiennym charakterze: hydrofilowe **27a-c** oraz **28f**, hydrofobowe **27d-e**, dodatkowo zsyntezowałam również łącznik peptydowy **27g** (Rysunek 8).



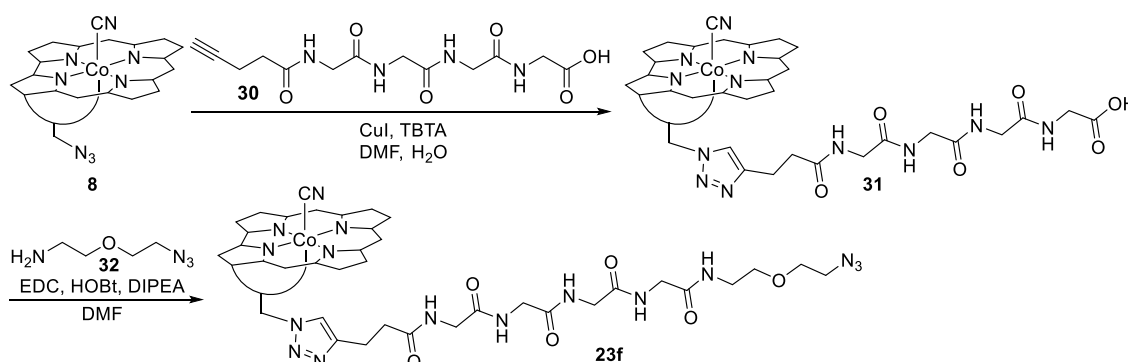
**Rysunek 8.** Pochodne witaminy B<sub>12</sub> z terminalną grupą azydkową.

Pochodne **23a-e** oraz **26a** otrzymałam według wcześniej opracowanych metod (opisanych w podrozdziale 4.5). Natomiast syntezę zaprojektowanego peptydowego łącznika w pochodnej **23f** rozpocząłam od sekwencji reakcji przeprowadzonych na podłożu stałym, w wyniku czego otrzymałam związek bifunkcyjny **30** z grupami odpowiednio karboksylową i alkinową (Schemat 11).



**Schemat 11.** Synteza łącznika **30**.

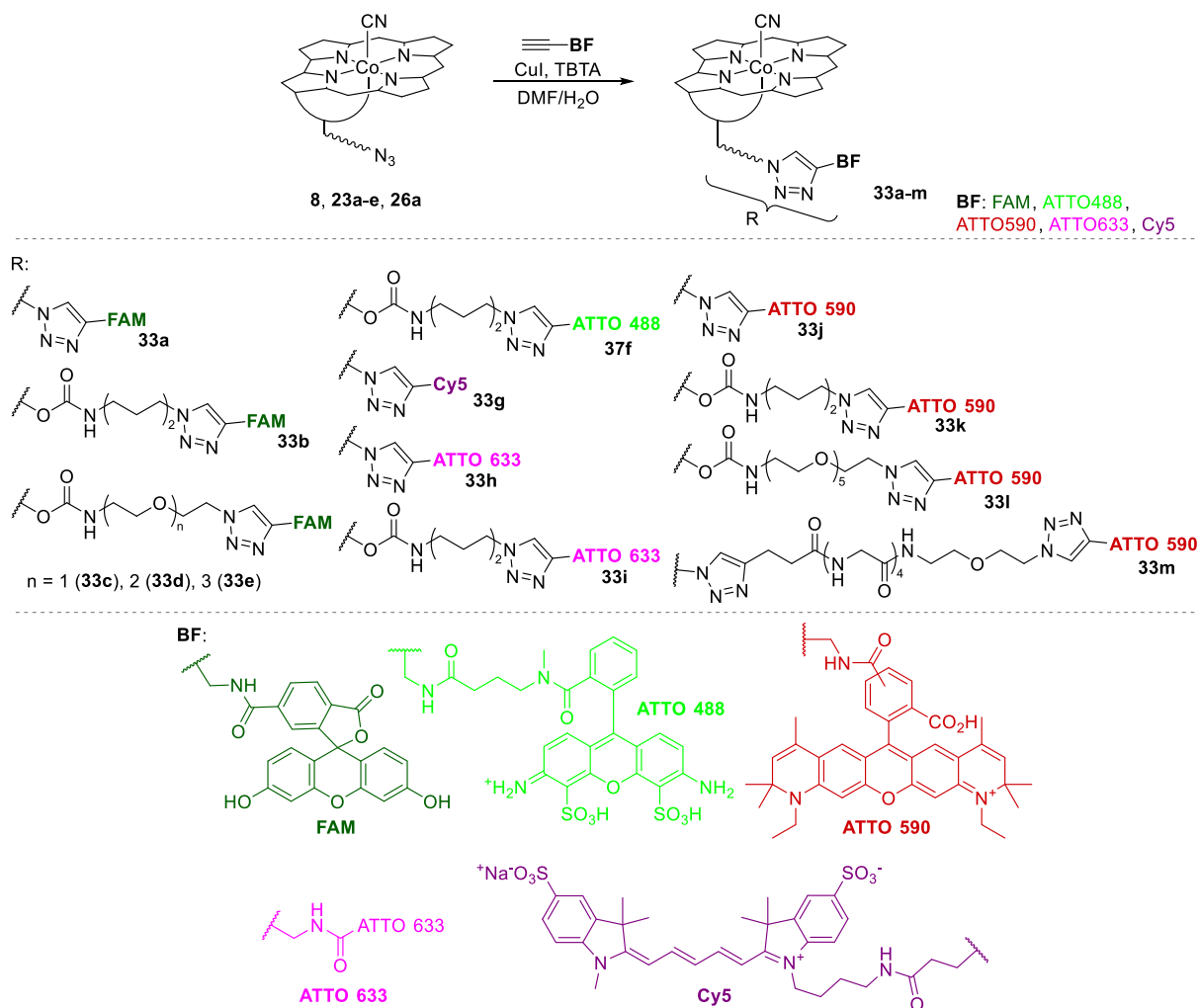
Pochodną **30** poddałam kolejno reakcji z azydkiem **8** otrzymując kwas **31** (Schemat 12). Ostatnim etapem przygotowania związku **23f** była reakcja amino-azydku **32** z kwasem **31** w obecności EDC (chlorowodorek *N*-etylo-*N'*-[(3-dimetyloamino)propylo]karbodiimidu) jako odczynnika sprzęgającego (Schemat 12).



**Schemat 12.** Synteza azydku **23f**.

Etap przygotowania docelowych koniugatów przeprowadziłam wykorzystując pochodne kobalaminy **8**, **23a-e**, **26a** oraz handlowo dostępne barwniki fluorescencyjne absorbujące w zakresie od 490 nm do ok. 650 nm, posiadające terminalną grupę alkinową (Schemat 13). Wykorzystując układ katalityczny CuI/TBTA zsyntezowałam trzynaście sond molekularnych **33a-m**, które zostały poddane

badaniom biologicznym w grupie A. Palmer na University of Colorado Boulder (Schemat 13). Spośród testowanych koniugatów, pochodne **33g** oraz **33i** wykazywały najkorzystniejsze właściwości fotofizyczne po związaniu z RNA, ponadto opracowana metodologia okazała się być porównywalnie dobra, a w niektórych analizowanych przypadkach nawet bardziej skuteczna niż obecnie stosowane podejścia do obrazowania RNA w żywych komórkach ssaków.<sup>[72]</sup>



**Schemat 13.** Synteza połączeń witaminy B<sub>12</sub> z barwnikami fluorescencyjnymi (BF). Struktura barwnika ATTO633 nie jest dostępna.

Wyniki syntetyczne przedstawione w tym podrozdziale są częścią artykułu:

E. Braselmann, **A. J. Wierzba**, J. T. Polaski, M. Chromiński, Z. E. Holmes, S. Hung, D. Batan, J. R. Wheeler, R. Parker, R. Jimenez, D. Gryko, R. T. Batey, A. E. Palmer, *Nat. Chem. Bio.* **2018**, *14*, 964–971

*A Multicolor Riboswitch-Based Platform for Imaging of RNA in Live Mammalian Cells*

#### 4.7 Podsumowanie

Badania przeprowadzone przeze mnie doprowadziły do opracowania metod syntezy nowych pochodnych witaminy B<sub>12</sub> zmodyfikowanych w obrębie pętli nukleotydowej oraz w pozycji *mezo*. Ponadto, zaprojektowane podejścia wykorzystałam do otrzymania koniugatów z modyfikowanymi oligonukleotydami oraz barwnikami fluorescencyjnymi. Za największe osiągnięcia uważam:

- a) opracowanie syntezy pochodnej kobalaminy **11**, pozwalającej na selektywne i bezpośrednie przyłączanie tioli w pozycji R<sub>5'</sub> w obrębie rybozy za pomocą rozłączalnego w warunkach redukujących wiązania disiarczkowego
- b) opracowanie metodologii funkcjonalizacji kobalaminy **1a** w pozycji *mezo* oraz szczegółowe zbadanie wpływu właściwości elektronowych podstawnika w tej pozycji na właściwości strukturalne, fotofizyczne i elektrochemiczne kobalaminy **1a**
- c) opracowanie metodologii funkcjonalizacji kobinamidu **13** na drodze utworzenia wiązania karbaminianowego
- d) opracowanie metodologii syntezy koniugatów witaminy B<sub>12</sub> **1a** z peptydowym kwasem nukleinowym w pozycjach R<sub>5'</sub>, *c*, *e*, *mezo* oraz na kobalcie, a także wykazanie po raz pierwszy, że witamina B<sub>12</sub> może być efektywnym transporterem tych związków do komórek bakterii, co otwiera możliwości projektowania nowych systemów przeciwbakteryjnych
- e) opracowanie metodologii syntezy koniugatów witaminy B<sub>12</sub> **1a** z barwnikami fluorescencyjnymi w pozycji R<sub>5'</sub>, które stanowią użyteczne narzędzie do obrazowania fragmentów mRNA oraz krótkich, niekodujących fragmentów RNA w żywych komórkach ssaków.

Badania przedstawione w niniejszej rozprawie stanowią zwartą całość i jasno demonstrują użyteczność pochodnych kobalaminy jako substratów do tworzenia koniugatów, które mogą być dostarczane do komórek eukariotycznych jak również do komórek bakterii. Opracowane przeze mnie metodologie mogą stanowić podstawę dalszych badań w dziedzinie wykorzystania witaminy B<sub>12</sub> jako transportera.

## 4.8 Literatura

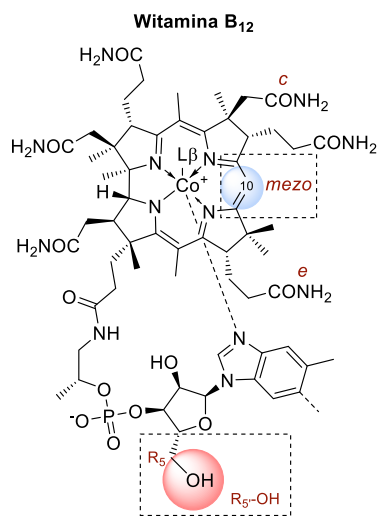
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## 5. Streszczenie w języku polskim



nie należy do zadań łatwych.

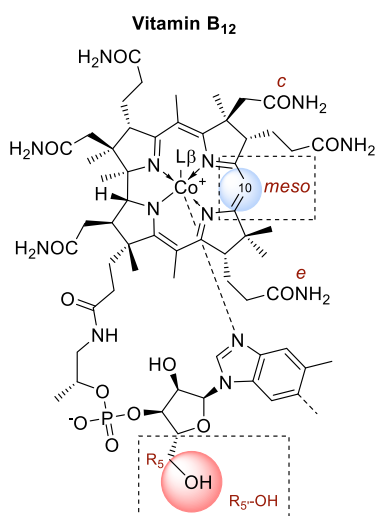
Celem moich badań była synteza nowych pochodnych witaminy B<sub>12</sub> umożliwiających przyłączanie do niej związków w pozycji R<sub>5</sub> w sposób odwracalny, a także opracowanie metodologii funkcjonalizacji kobalaminy w pozycji *mezo* (C10). Istniejące oraz nowo opracowane metodologie postanowiłam wykorzystać do przygotowania koniugatów witaminy B<sub>12</sub> z syntetycznymi oligonukleotydami, a konkretnie – peptydowym kwasem nukleinowym (ang. *peptide nucleic acid*, PNA) oraz barwnikami fluorescencyjnymi.

W pierwszym etapie badań opracowałam syntezę pochodnej kobalaminy reaktywnej względem tioli oraz wykazałam, że utworzone połączenie disiarczkowe między witaminą a przyłączanym związkiem może być redukowane w obecności glutationu (tiolu obecnego w komórkach eukariotycznych). Kolejno, zmodyfikowałam strukturę kobalaminy w pozycji *mezo* w reakcji nitrowania. W wyniku redukcji grupy nitrowej w pozycji C10 otrzymałam *mezo*-aminę, którą następnie funkcjonalizowałam w reakcjach z aldehydami oraz bezwodnikami kwasów. Ponadto, zbadałam wpływ charakteru elektronowego podstawników w pozycji C10 na strukturę oraz właściwości fotofizyczne i elektrochemiczne kobalaminy.

W kolejnym kroku opracowałam metodologie łączenia witaminy B<sub>12</sub> z modyfikowanymi oligonukleotydami PNA w pozycjach R<sub>5</sub>, *c*, *e*, *mezo* oraz na kobalcie, wykorzystując w tym celu łączniki o różnej długości i odmiennym charakterze. Opracowałam również metodę przyłączania do kobalaminy barwników fluorescencyjnych w pozycji R<sub>5</sub> z zastosowaniem katalizowanej jonami miedzi(I) reakcji 1,3-dipolarnej cykloaddycji azydków do terminalnych alkinów (CuAAC).

Przeprowadzone badania wykazały, że witamina B<sub>12</sub> może być efektywnym transporterem modyfikowanych oligonukleotydów do komórek bakterii oraz służyć jako element sondy molekularnej wykorzystywanej do obrazowania fragmentów mRNA oraz krótkich, niekodujących fragmentów w żywych komórkach ssaków.

## 6. Streszczenie w języku angielskim/ Abstract in English



Vitamin B<sub>12</sub> (cobalamin) is an essential nutrient for mammals and certain bacteria. As an exogenous compound, cobalamin reaches mammalian cells via a system of transport proteins and this fact makes it an attractive candidate for the delivery of cargoes into cells. Development of conjugates requires suitably designed building blocks and vitamin B<sub>12</sub> possesses a number functional groups susceptible to chemical modifications, however complex nature of cobalamin makes them extremely challenging.

The goal of my studies was to design an efficient methodology allowing for the preparation of cobalamin conjugates in a reversible manner at the R<sub>5</sub>' position. Moreover, I decided to functionalize cobalamin at the *meso* position – which has not been considered so far for the synthesis of conjugates. I have decided to use newly developed and already established methodologies to prepare vitamin B<sub>12</sub> conjugates with synthetic oligonucleotides, namely peptide nucleic acid (PNA) and fluorescent dyes.

At the first stage of my research, I have developed a three-step synthesis leading to vitamin B<sub>12</sub> derivative highly reactive toward thiols and proved that disulfide bond formed during preparation of conjugates with this molecule, reduces in the presence of glutathione (a thiol present in eukaryotic cells). Functionalizations at the *meso* position involved nitration and subsequent reduction leading to *meso*-aminocobalamin. The obtained compound can be successfully reacted with aldehydes and acid anhydrides. In addition, I investigated the effect of the electronic properties of C10 substituents on the structural, photophysical and electrochemical properties of cobalamin.

Another part of my research focused on the development of methodologies leading to vitamin B<sub>12</sub> conjugates with synthetic oligonucleotides (PNA) appended at the R<sub>5</sub>', *c*, *e*, *meso* position and cobalt, with the use of spacers of various character and length. I have also developed a method for attaching cobalamin to fluorescent dyes at the R<sub>5</sub>' position using copper(I)-catalyzed 1,3-dipolar cycloaddition of azides to terminal alkynes (CuAAC).

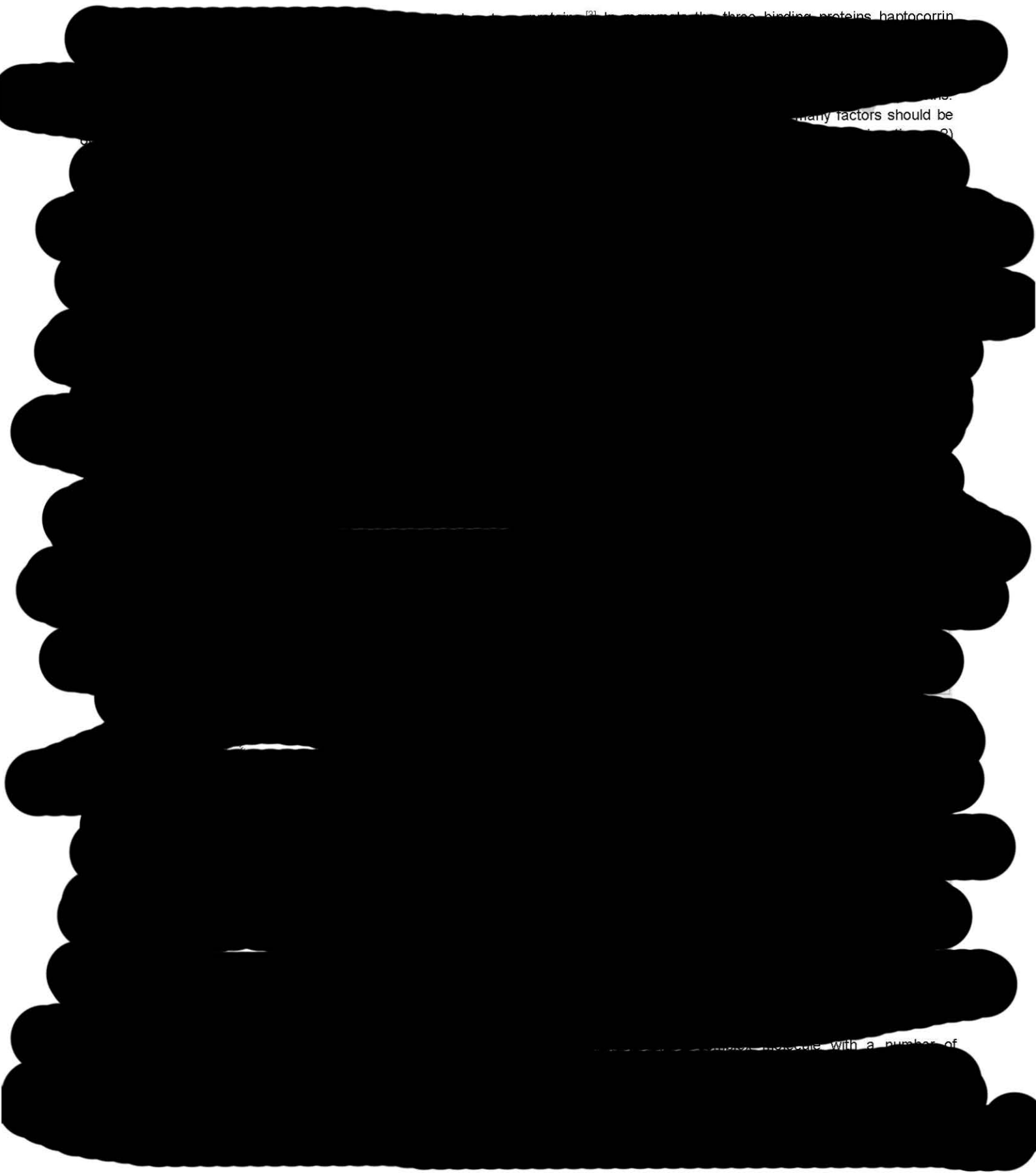
My studies demonstrate that vitamin B<sub>12</sub> can serve as an efficient carrier for synthetic oligonucleotides (PNA) into bacterial cells, which opens new routes for developing antibacterial agents. Additionally, cobalamin conjugates with fluorescent dyes can be applied as molecular probes to track mRNA and small noncoding RNA in live mammalian cells.

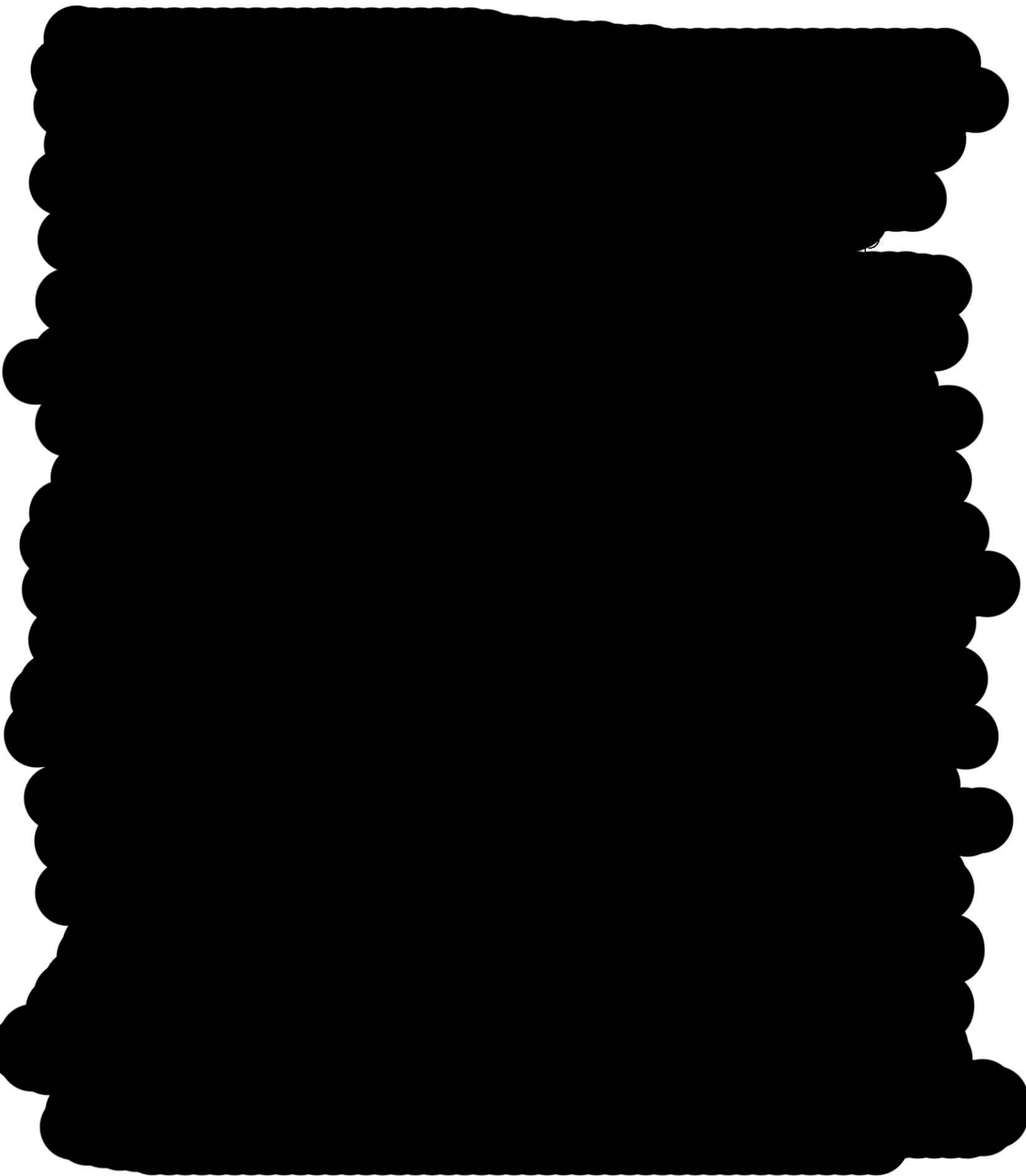
## **7. Publikacje przeglądowe**



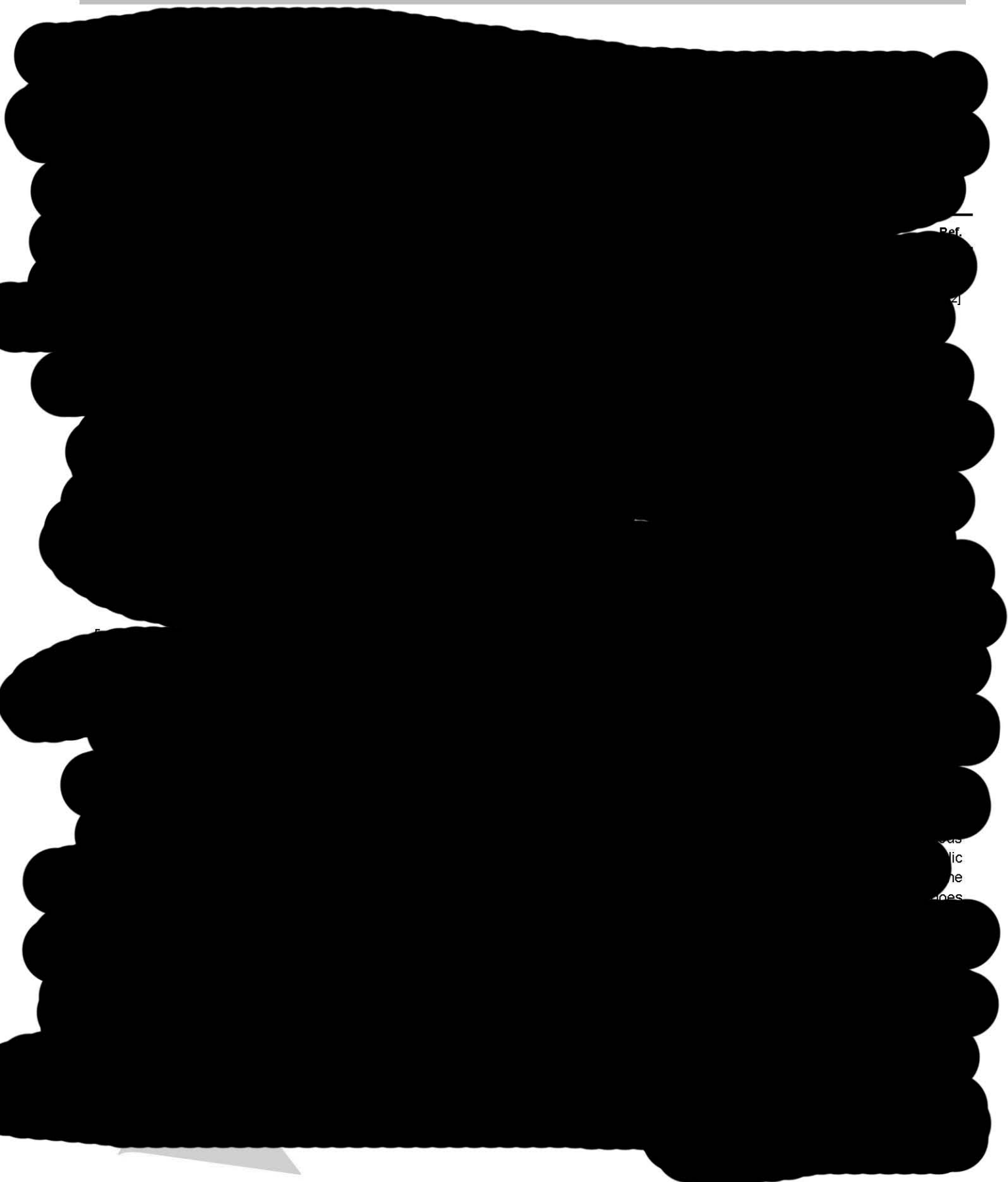
## **Synthetic approaches toward vitamin B<sub>12</sub> conjugates**

Aleksandra J. Wierzba, Sidra Hassan and Dorota





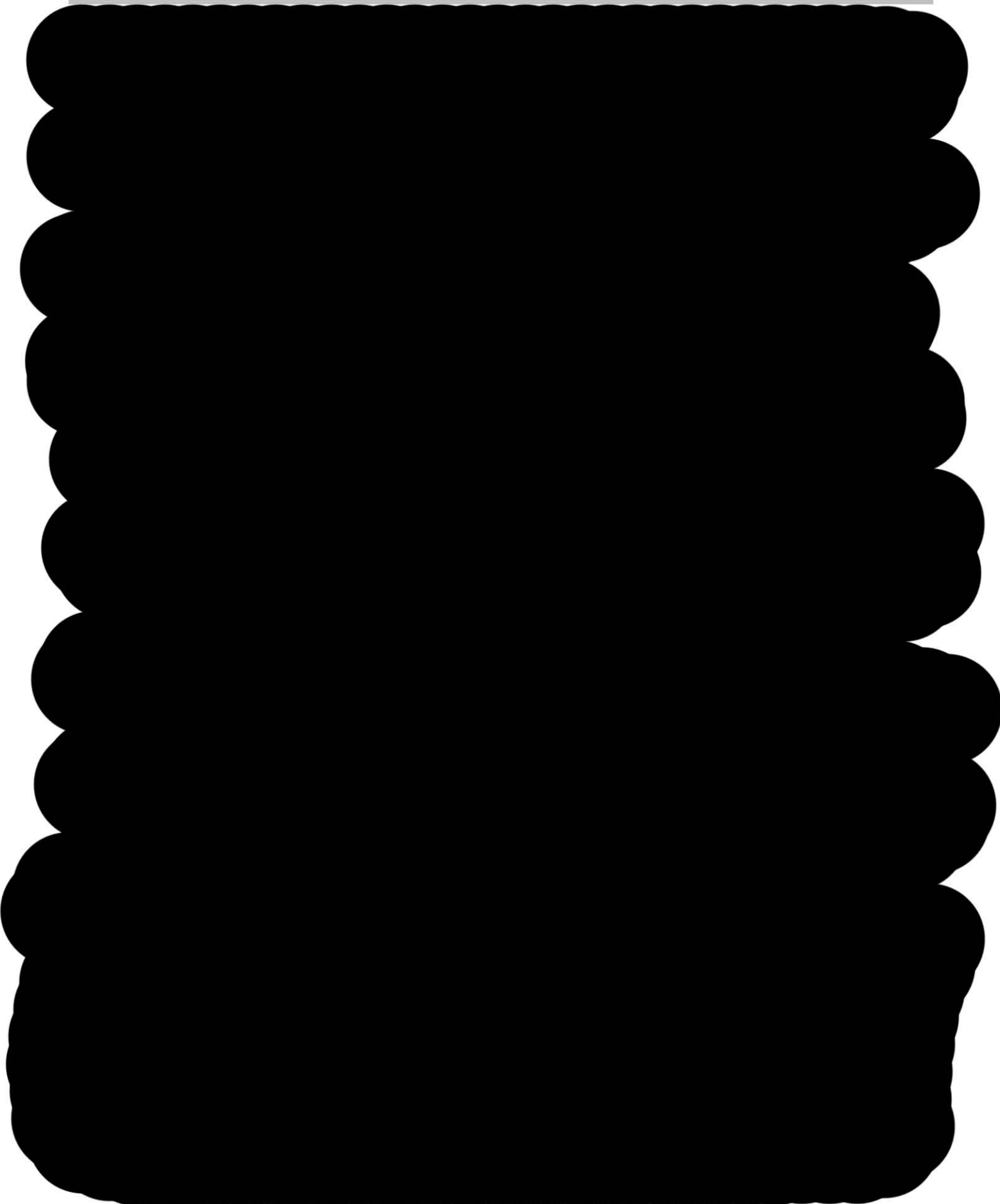


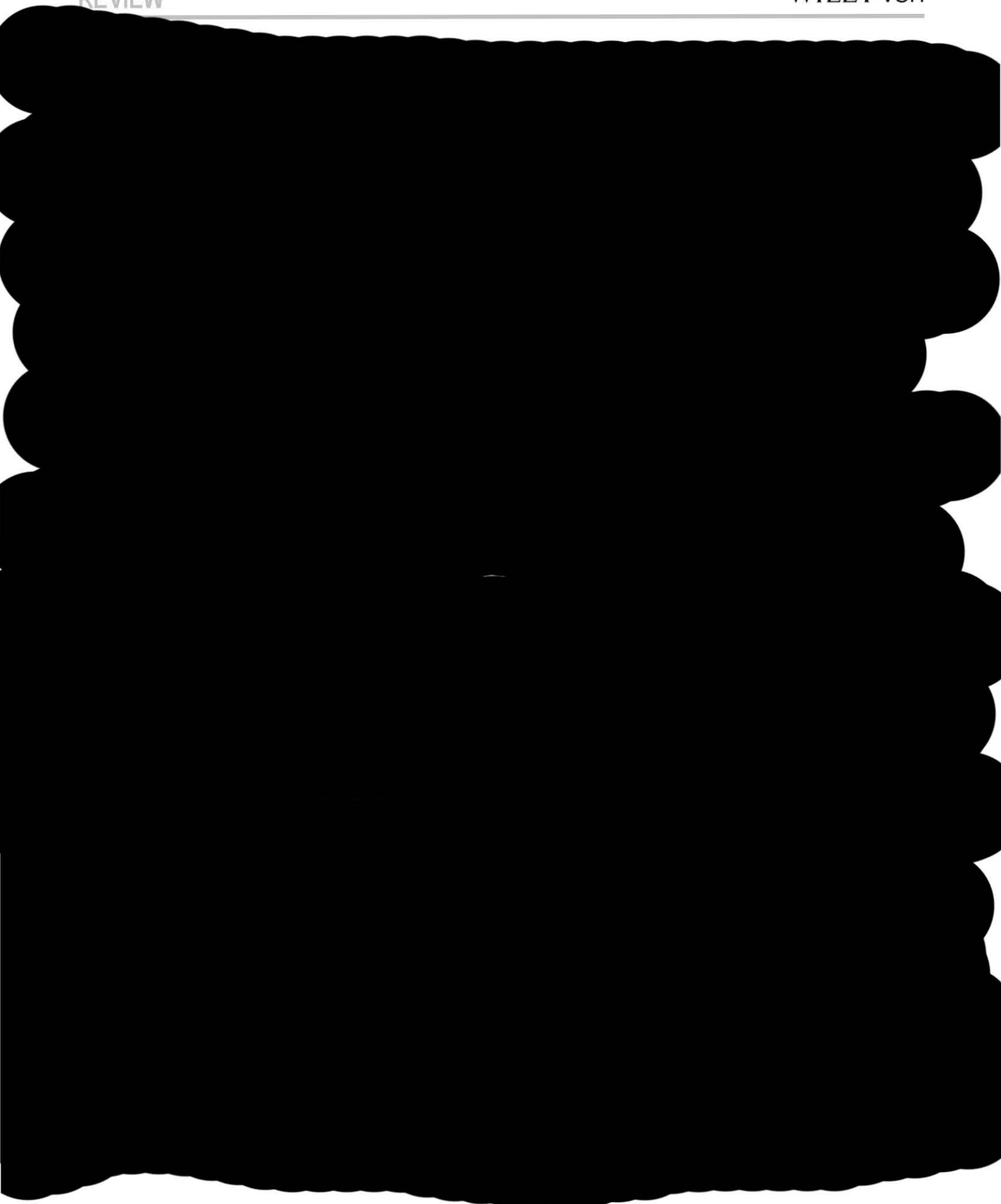


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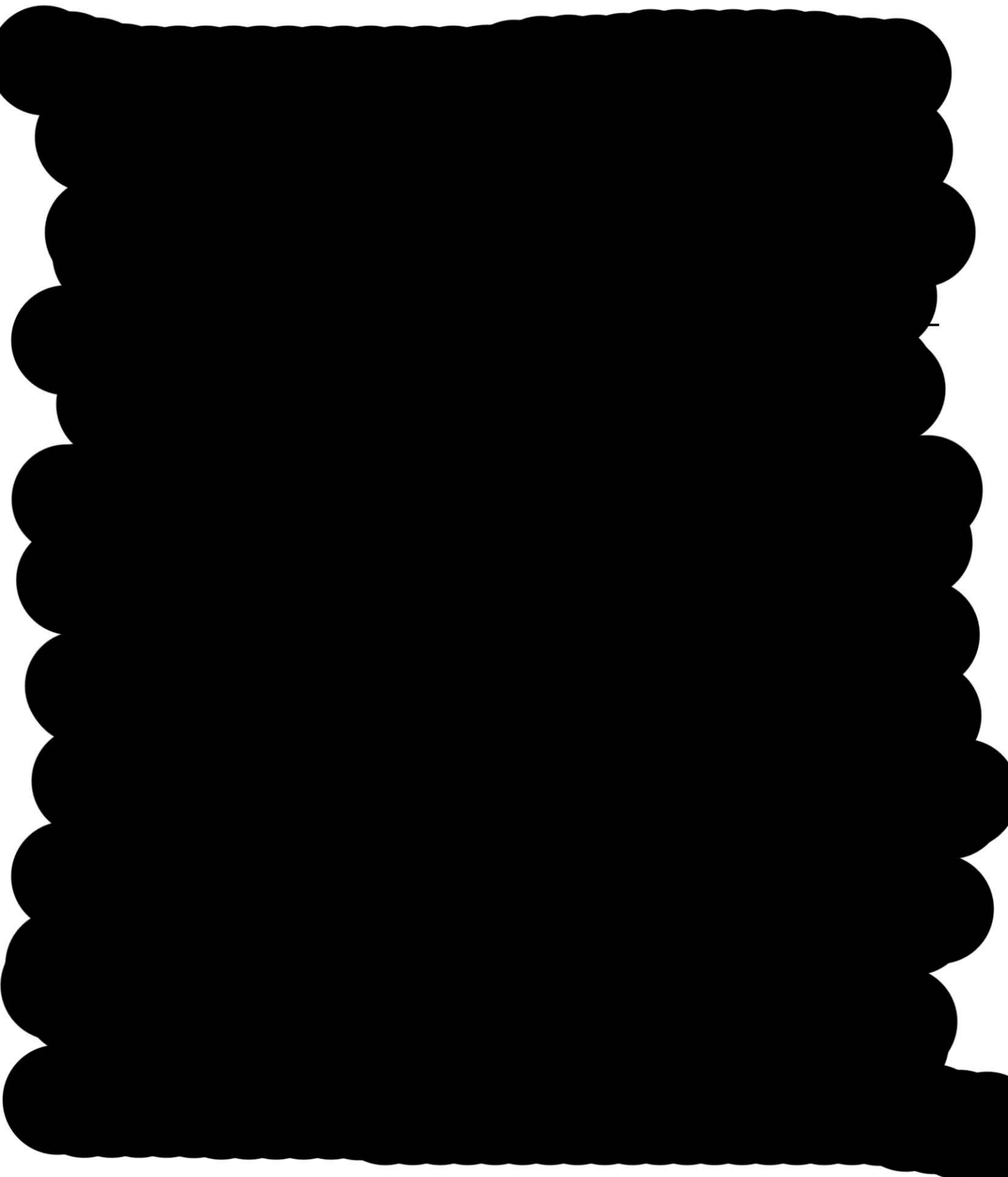
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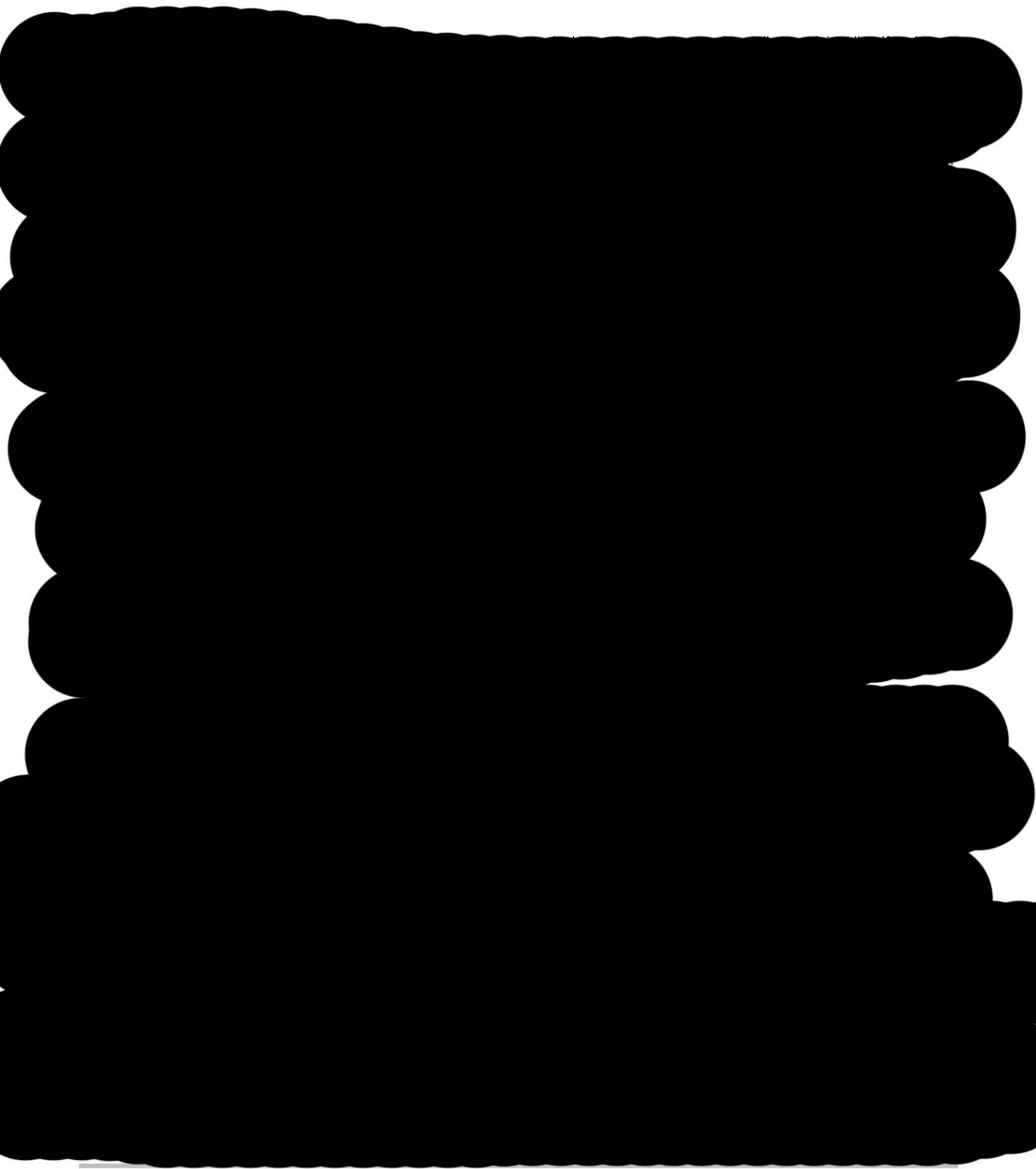




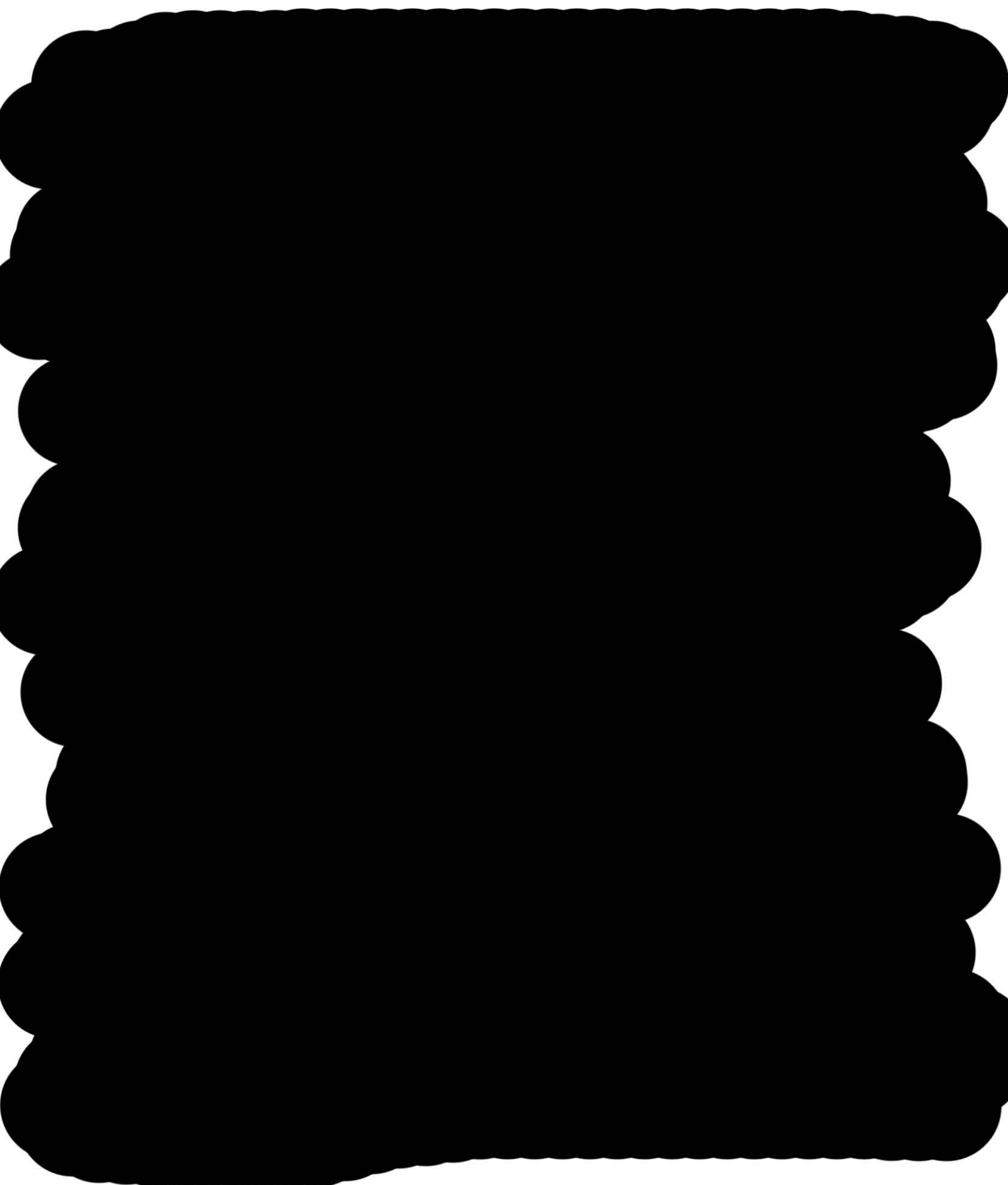


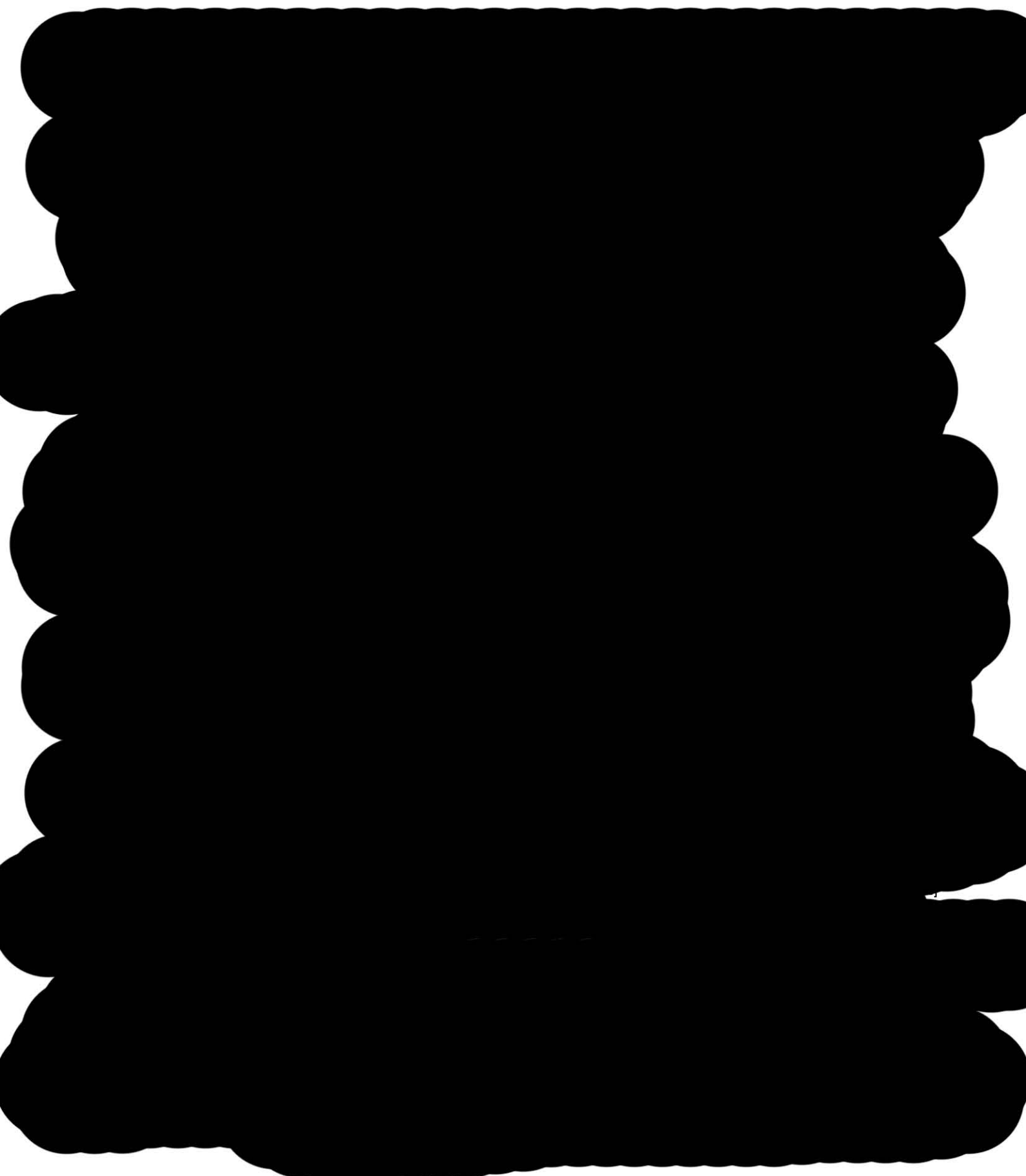








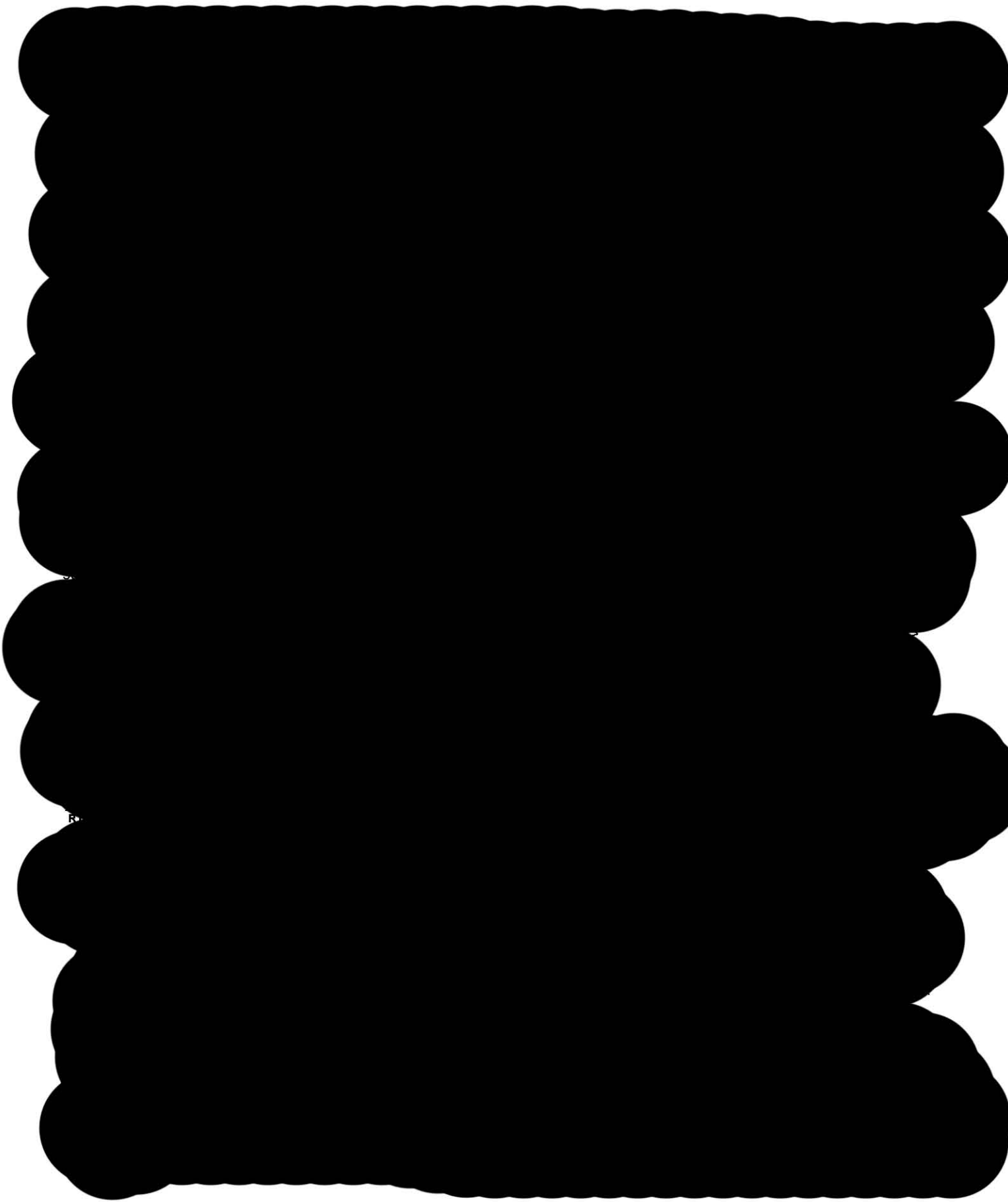




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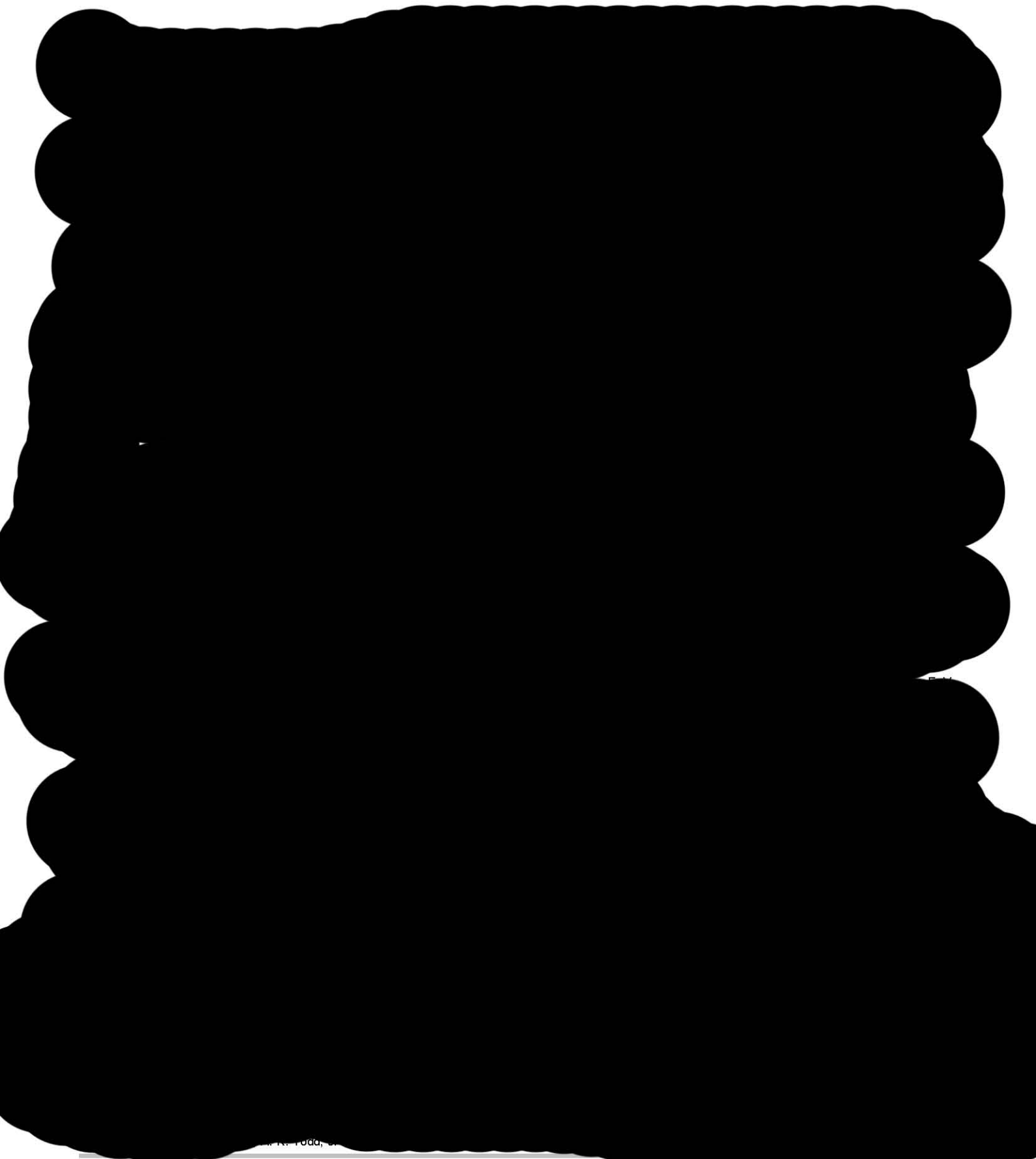
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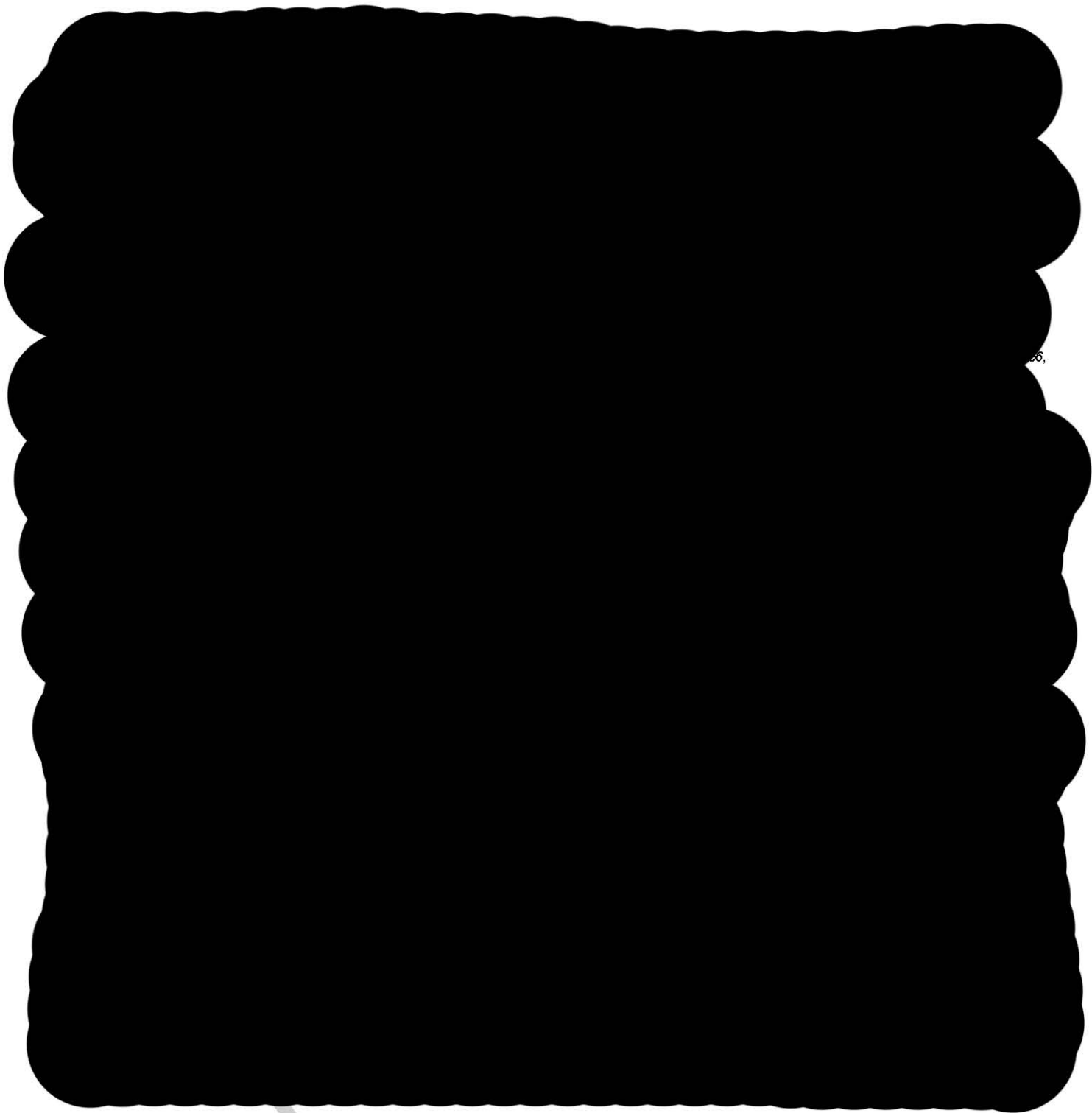




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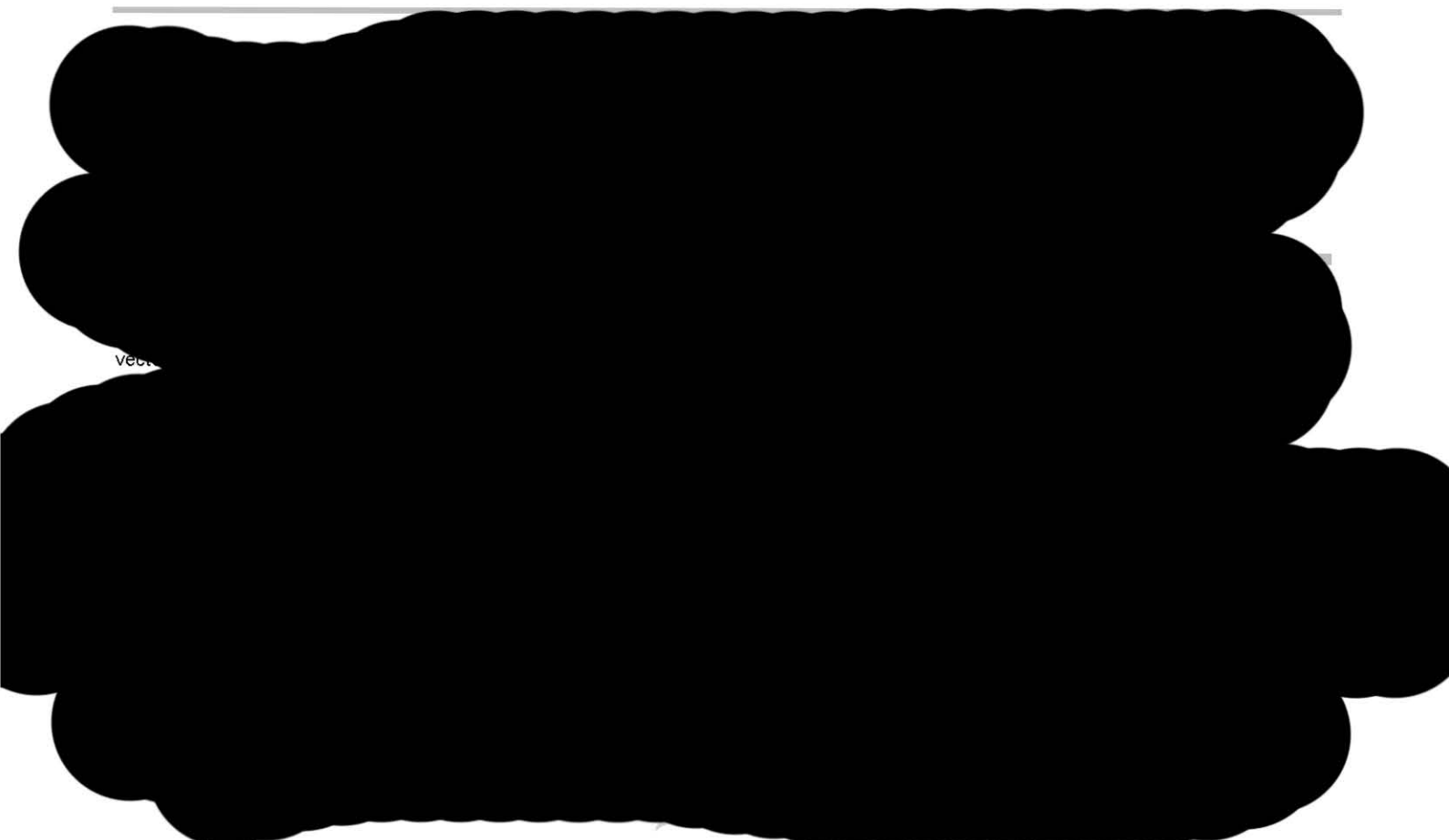






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[http://ww2.icho.edu.pl/Gryko\\_group/index.html](http://ww2.icho.edu.pl/Gryko_group/index.html)

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From: **AsianJOC** <[em@editorialmanager.com](mailto:em@editorialmanager.com)>  
Date: Wed, Oct 31, 2018 at 1:41 PM  
Subject: Decision on your paper ajoc.201800579 for Asian Journal of Organic Chemistry  
To: Dorota Gryko <[dgryko@gmail.com](mailto:dgryko@gmail.com)>

Title: Synthetic approaches toward vitamin B12 conjugates

DOI: 10.1002/ajoc.201800579

for citing the article before publication in an issue, please use this DOI number.

---

Dear Professor Gryko,

Thank you for your submission of 02 Oct 2018. We are pleased to inform you that the above-mentioned paper has been accepted for publication in Asian Journal of Organic Chemistry.

For the next steps, please:

1) revise your paper according to the reviewer/editor comments; see the end of this email. Please highlight the changes made during revision by giving the text a yellow background. As Supporting Information does not undergo editing, please do not mark up or highlight changes to the Supporting Information file, but list any changes in the "Respond to Reviewers" box. IMPORTANT: For faster processing, please ensure chemical structures are embedded in the manuscript file in ChemDraw format and all other images are embedded as high-resolution .jpeg, .tif, or similar formats as per the [Checklist](#)

2) check that you have included biographical sketches (maximum length 560 characters) in the text file, as well as portrait-quality color photographs of the authors and an 18x18 cm frontispiece image.

3) go to <https://asianjoc.editorialmanager.com/> and upload your revision by clicking on "Submissions Needing Revision" in the "Revisions" section, then choosing "Revise submission" from the "Action Links" menu. Your username is Your username is: dgryko. If you have forgotten your password, please use the "Send Access Credentials" option given on the login screen.

4) insert a point-by-point response to the reviewer comments in the "Response to Reviewers" box, including a list of changes made and a rebuttal to any comments with which you disagree.

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Please submit your revised version within one week of receiving this email.

Yours sincerely,

Dr. Dinesh Talwar  
Associate Editor  
Asian Journal of Organic Chemistry

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#### REVIEWER REPORT

##### EVALUATION:

##### Reviewer's Responses to Questions

1. Please rate the importance of the field under review

Reviewer #1: Very important

Reviewer #2: Important

Reviewer #3: Very important

---

**2. Please rate the citation of previous publications**

Reviewer #1: Good balanced overview

Reviewer #2: Good balanced overview

Reviewer #3: Good balanced overview

---

**3. Please rate the length of the manuscript**

Reviewer #1: Concise

Reviewer #2: Concise

Reviewer #3: Concise

---

**4. Please rate the critical selection of presented data**

Reviewer #1: Good balanced overview

Reviewer #2: Good balanced overview

Reviewer #3: Good balanced overview

---

**5. Please rate the instructive presentation of the content**

Reviewer #1: Good logical approach

Reviewer #2: Good logical approach

Reviewer #3: Good logical approach

---

**6. Have other overviews been published recently which would make this one obsolete?**

Reviewer #1: No

Reviewer #2: No

Reviewer #3: No

**COMMENTS TO AUTHOR:**

Reviewer #1: In the last 15 years, numerous groups developed bioconjugates of Cbls for medicinal and biological applications. In these systems they took advantage of the sophisticated delivery mechanism of B12 into cells involving three transport proteins. The Gryko group contributed significantly to this area and developed various efficient strategies for B12 conjugation that are used by different groups worldwide. The present review by Gryko and coworkers represents the most detailed description of "synthetic approaches toward vitamin B12 conjugates" so far. Sites of potential modifications, synthetic methodologies and applications are thoroughly discussed. It will give the expert but also the freshman an useful overview of the area. It is nicely written and the tables give a good overview.

I suggest only some minor corrections:

- Compound 28a has been drawn wrongly. The two nitrogens are missing.
- The format of metal-ligand bonds is not consistent in the drawings.
- H<sub>2</sub>OCbl (1e) is positively charged.
- Alberto's overview should be mentioned The Porphyrin Handbook; Kadish, K. M., Smith, K. M., Guilard, R., Eds.; World Scientific, 2012; (25), 83-130.

Reviewer #2: This article is a truly excellent review of the most recent synthetic approaches for the preparation of

vitamin B12 conjugates. The authors have covered both the synthetic methodologies and the applications of the ensuing conjugates in a comprehensive manner and I am certain that researchers involved in the field will greatly appreciate the effort of the authors in compiling this review.

I fully support the publication of this article and it is, thus, my recommendation to the editorial office of Asian Journal of Organic Chemistry to accept this work.

I have very much appreciated the attention devoted to the literary style of the document and I could not identify any mistakes. Overall in the review article of Wierzba et al.: the chosen subject, scopes, objectives and chemistry are clearly defined and well contextualized. The subject is presented in a systematic, logical and well structured manner. All relevant literature has been cited.

Reviewer #3: An excellent and comprehensive review. Well thought out and pictorially described. Will serve as a go to reference for persons in the field and an excellent introduction to those entering it. Dr. Grkyo is the leader of this field and is ideally placed to write such a relevant review.

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## **8. Publikacje oryginalne**






## Vitamin B<sub>12</sub> Suitably Tailored for Disulfide-Based Conjugation

Aleksandra Wierzba,<sup>†</sup> Monika Wojciechowska,<sup>‡</sup> Joanna Trylska,<sup>\*,‡</sup> and Dorota Gryko<sup>\*,†</sup>

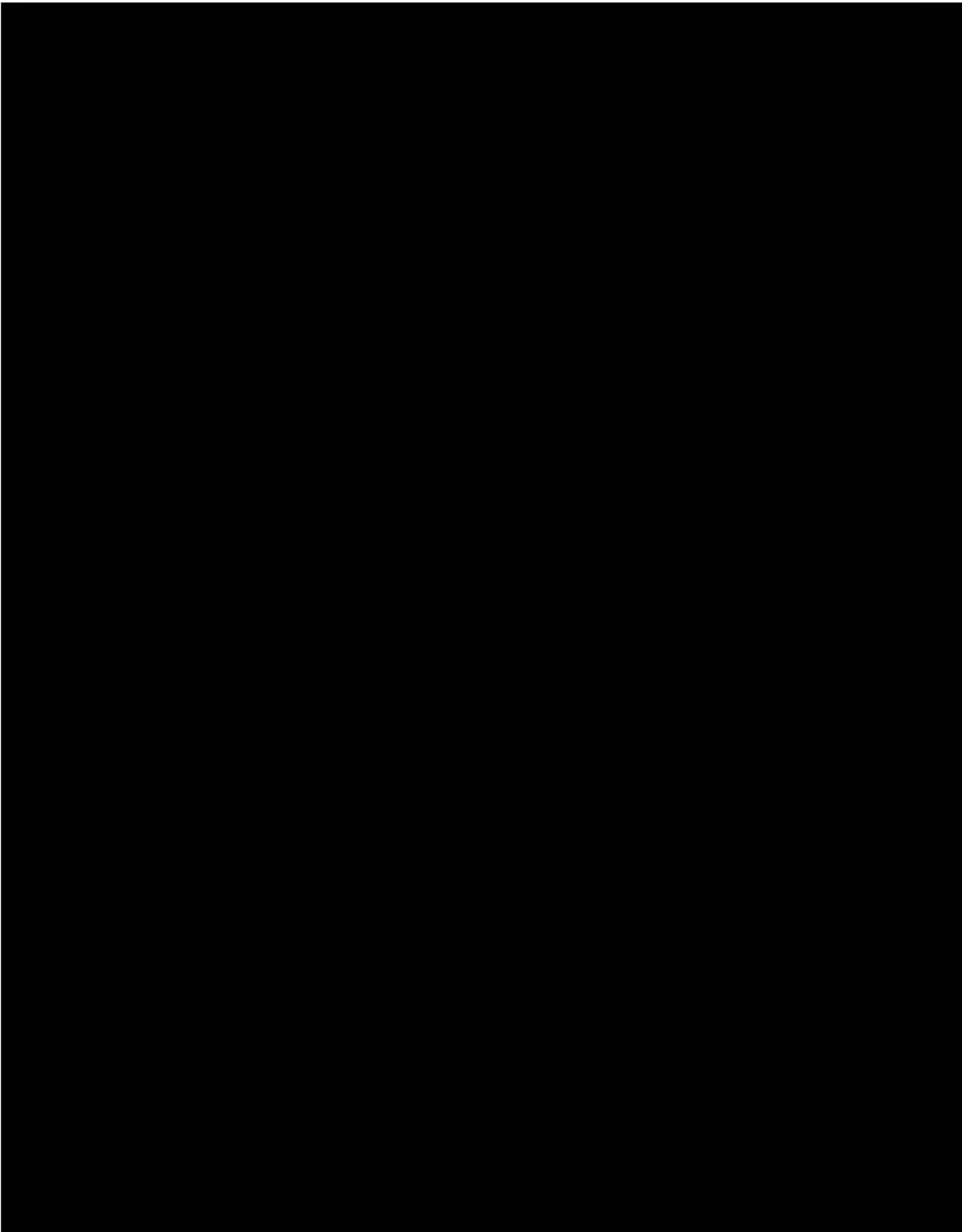
<sup>†</sup>Institute of Organic Chemistry, Polish Academy of Sciences, M. Kasprzaka 44/52, 01-224 Warsaw, Poland

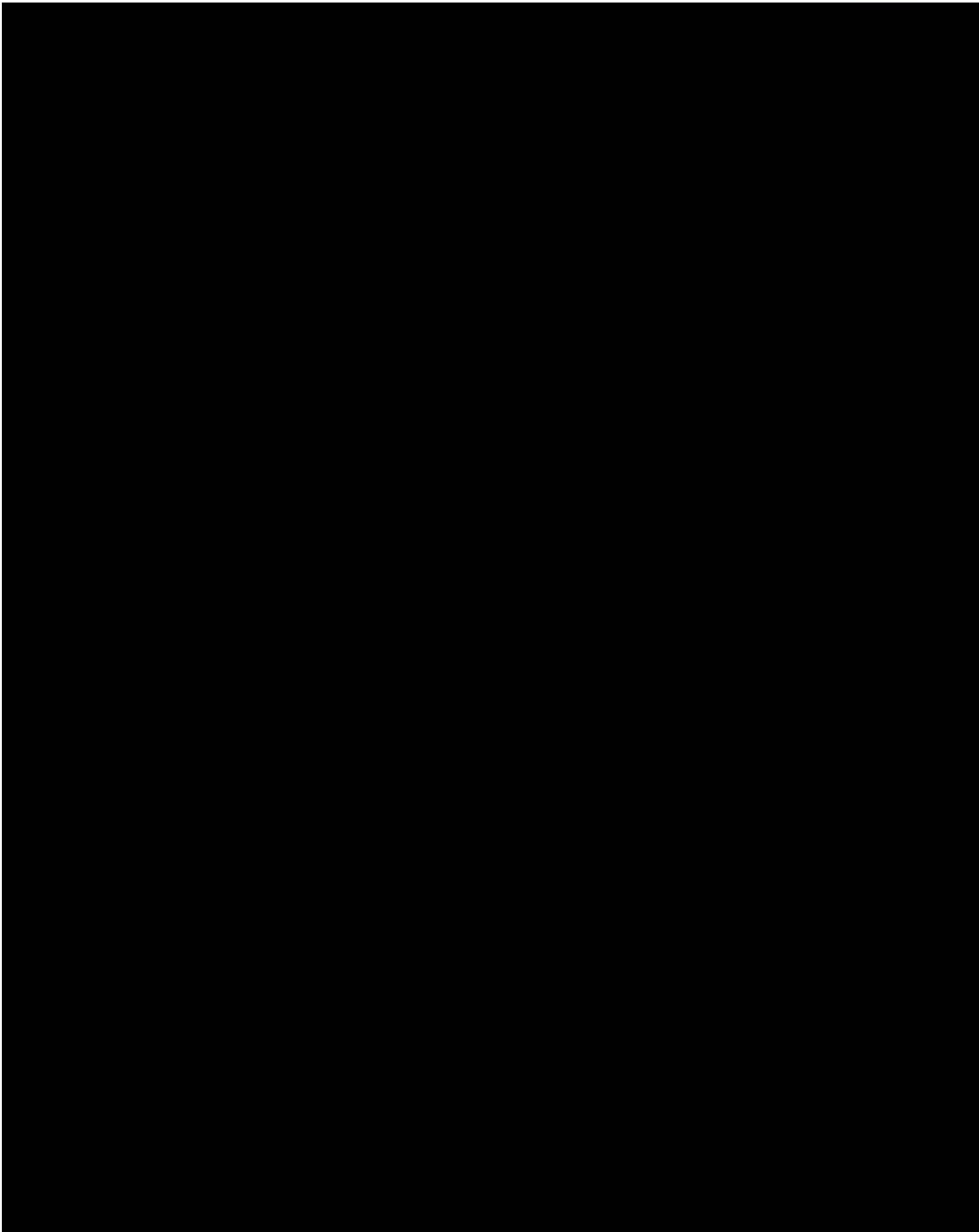
<sup>‡</sup>Centre of New Technologies, University of Warsaw, S. Banacha 2c, 02-097 Warsaw, Poland

 Supporting Information

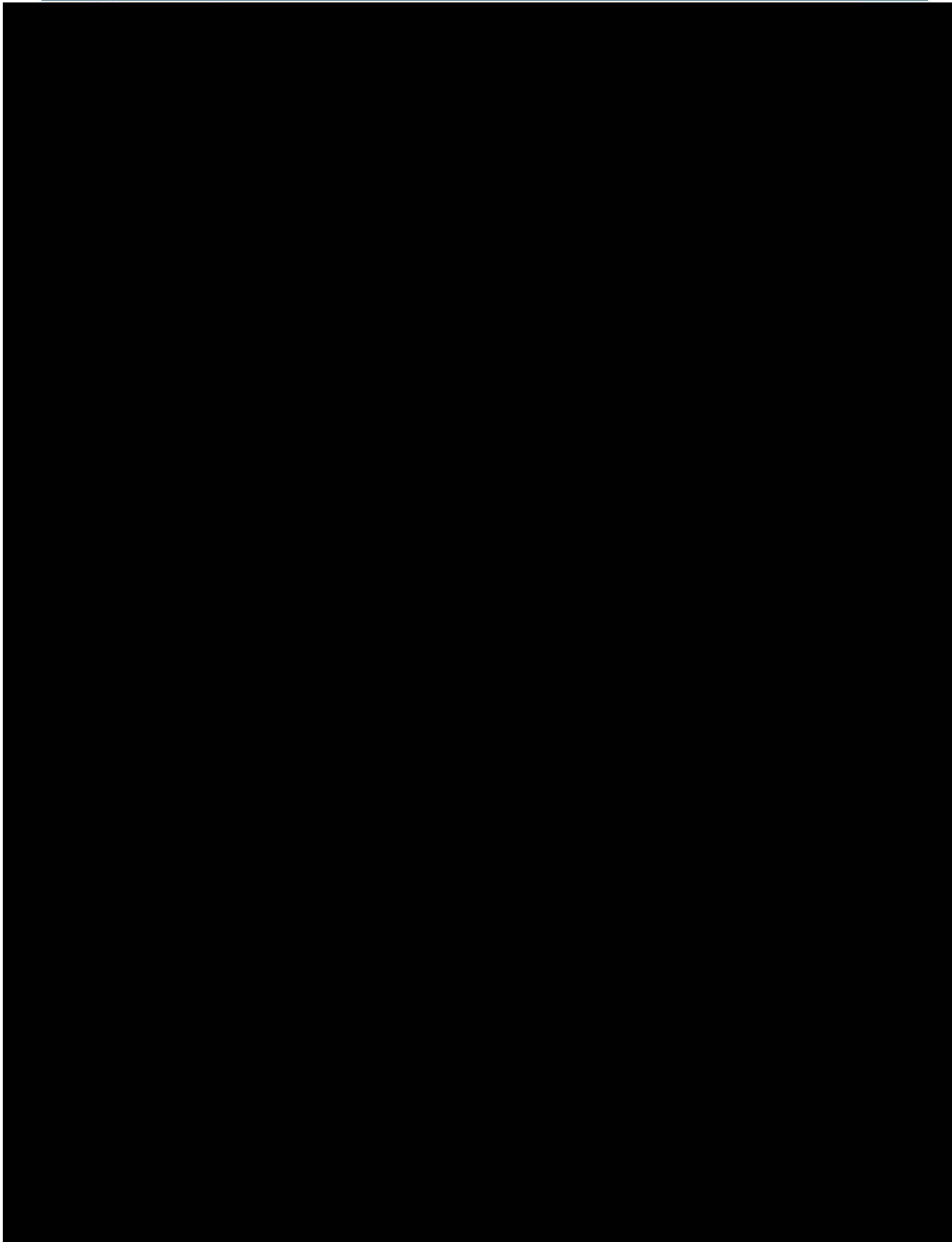


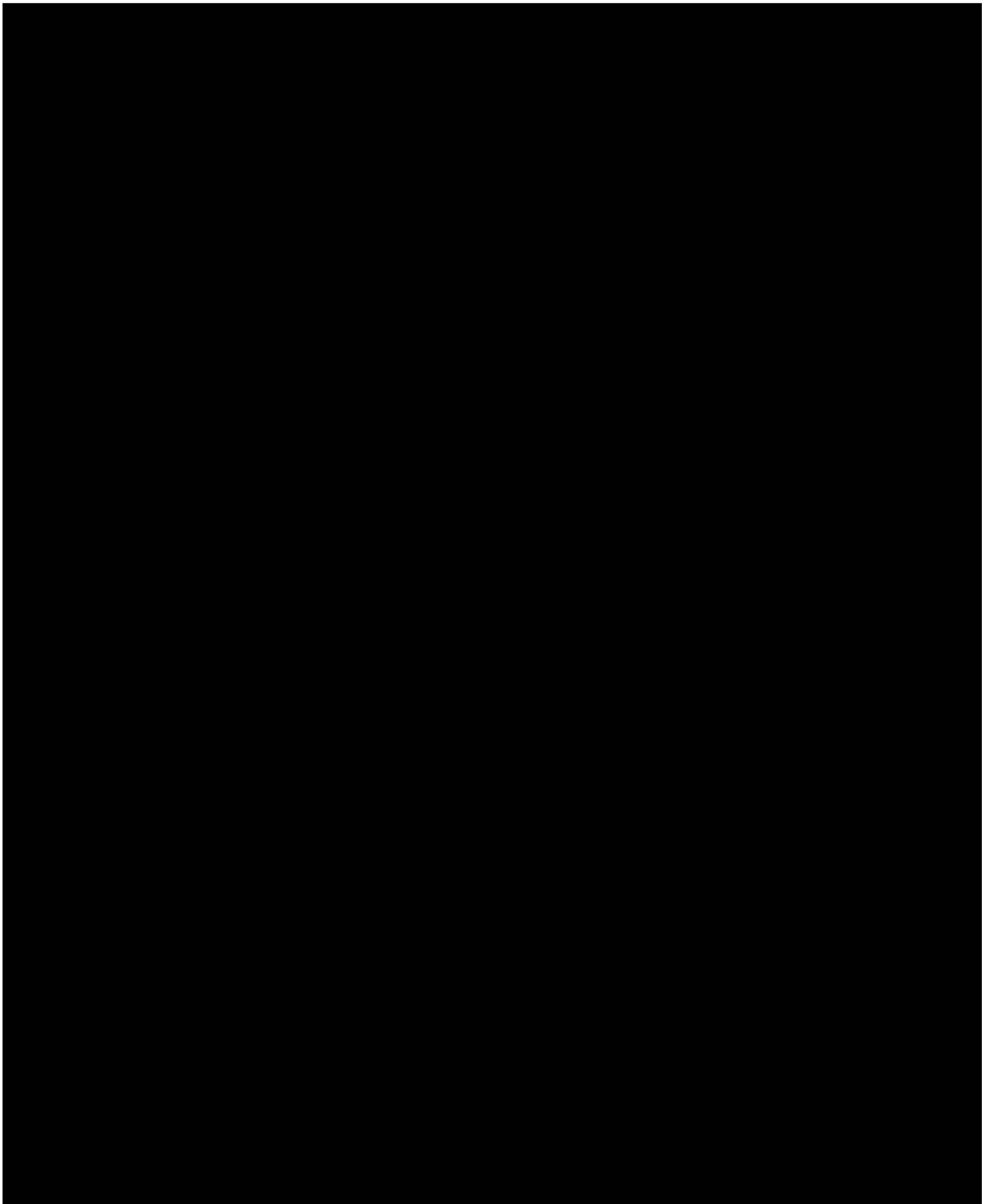




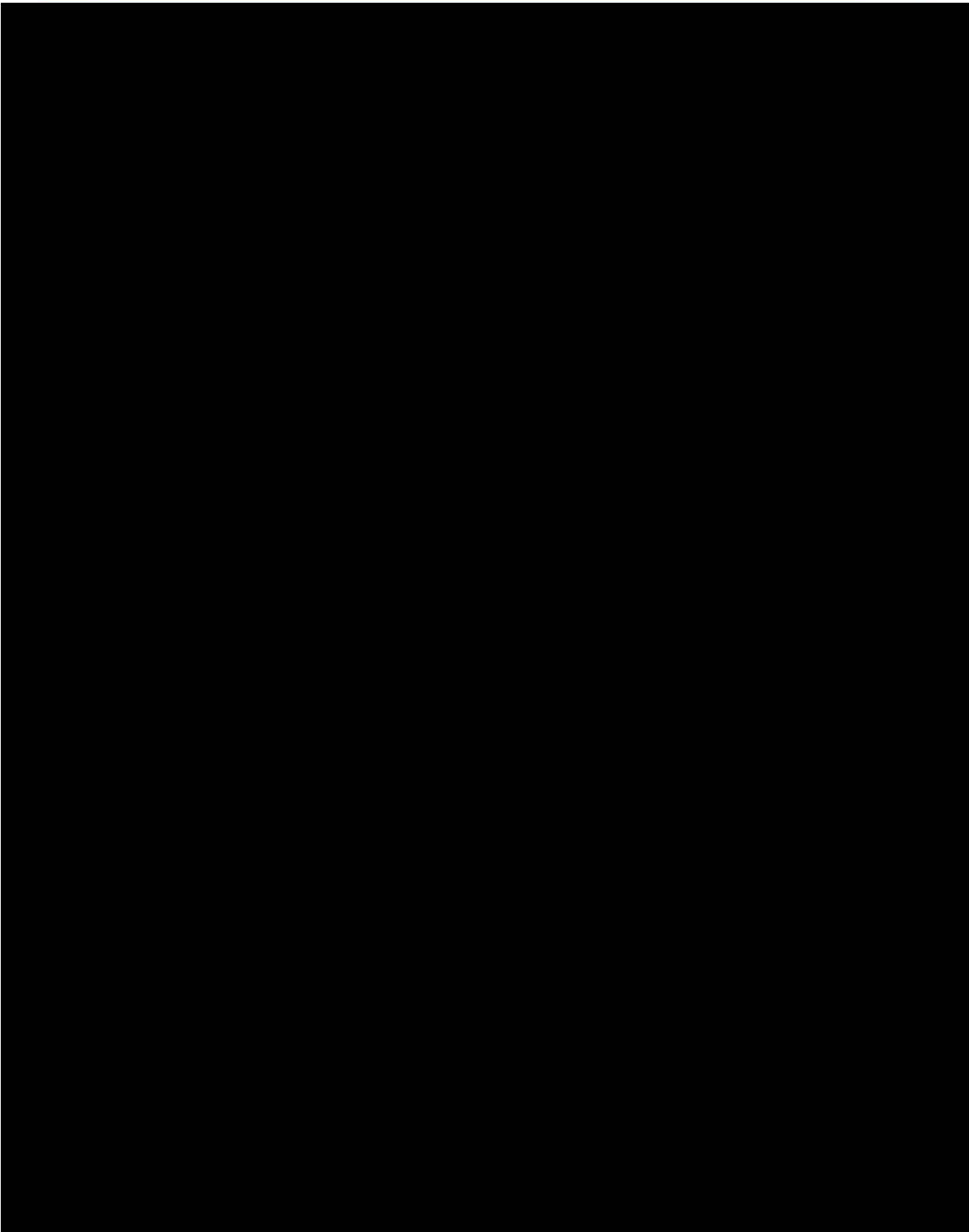














**Supporting information  
for**

**Vitamin B<sub>12</sub> Suitably Tailored for Disulfide-Based Conjugation**

Aleksandra Wierzba,<sup>†</sup> Monika Wojciechowska,<sup>‡</sup> Joanna Trylska,<sup>\*,‡</sup> Dorota Gryko<sup>\*,†</sup>

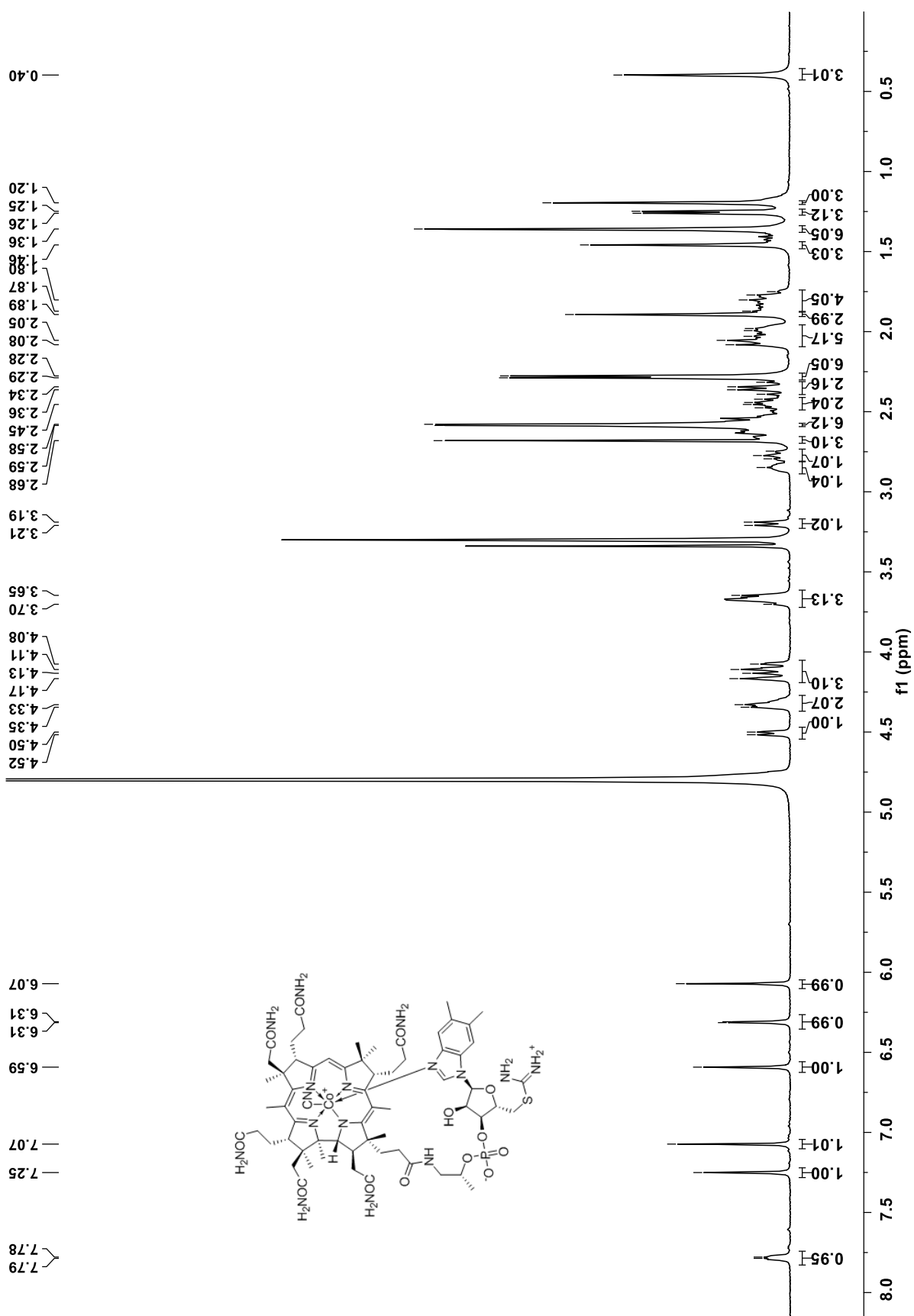
<sup>†</sup>Institute of Organic Chemistry Polish Academy of Sciences, M. Kasprzaka 44/52, 01-224 Warsaw, Poland

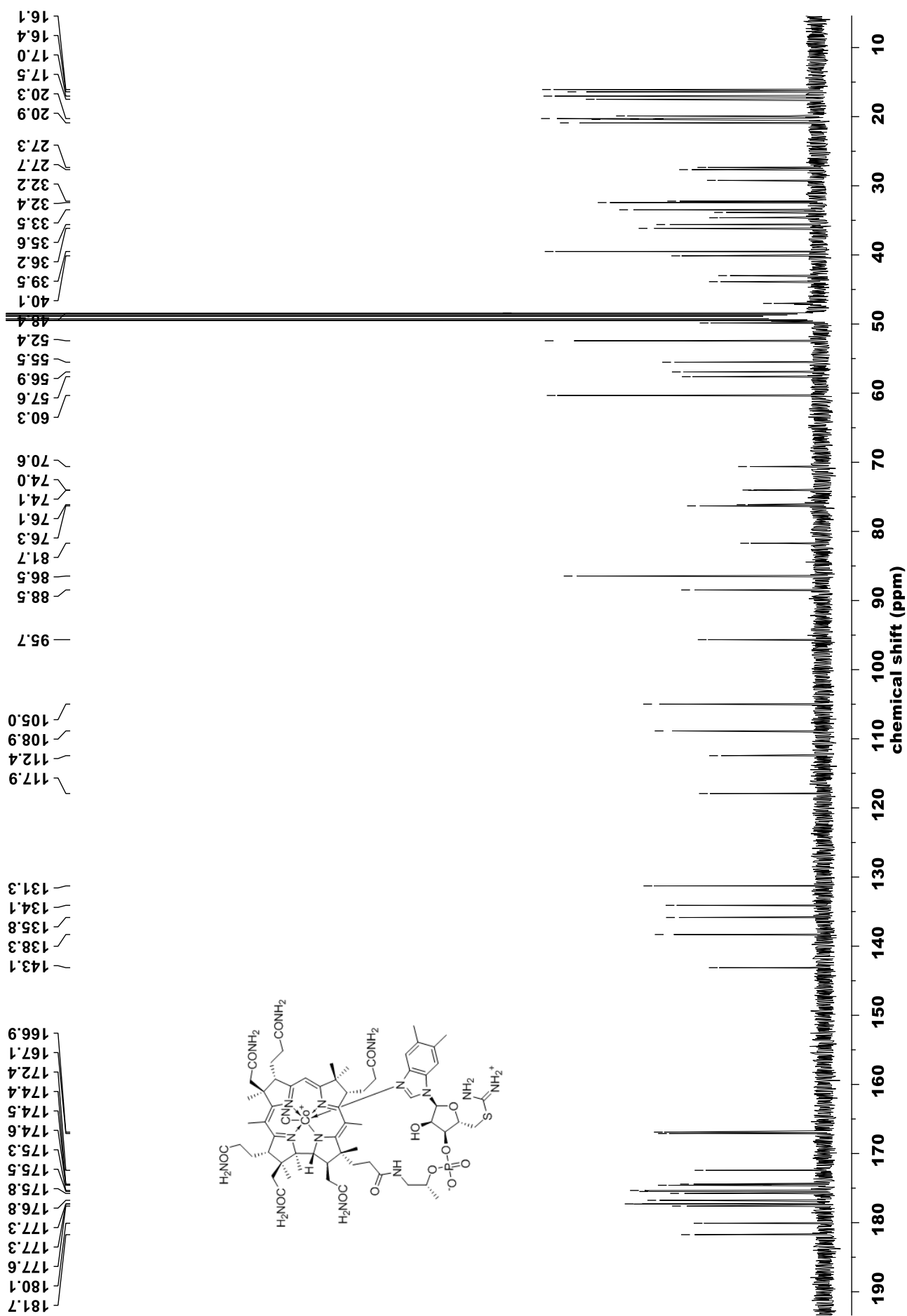
<sup>‡</sup>Centre of New technologies University of Warsaw, S. Banacha 2c, 02-097 Warsaw, Poland

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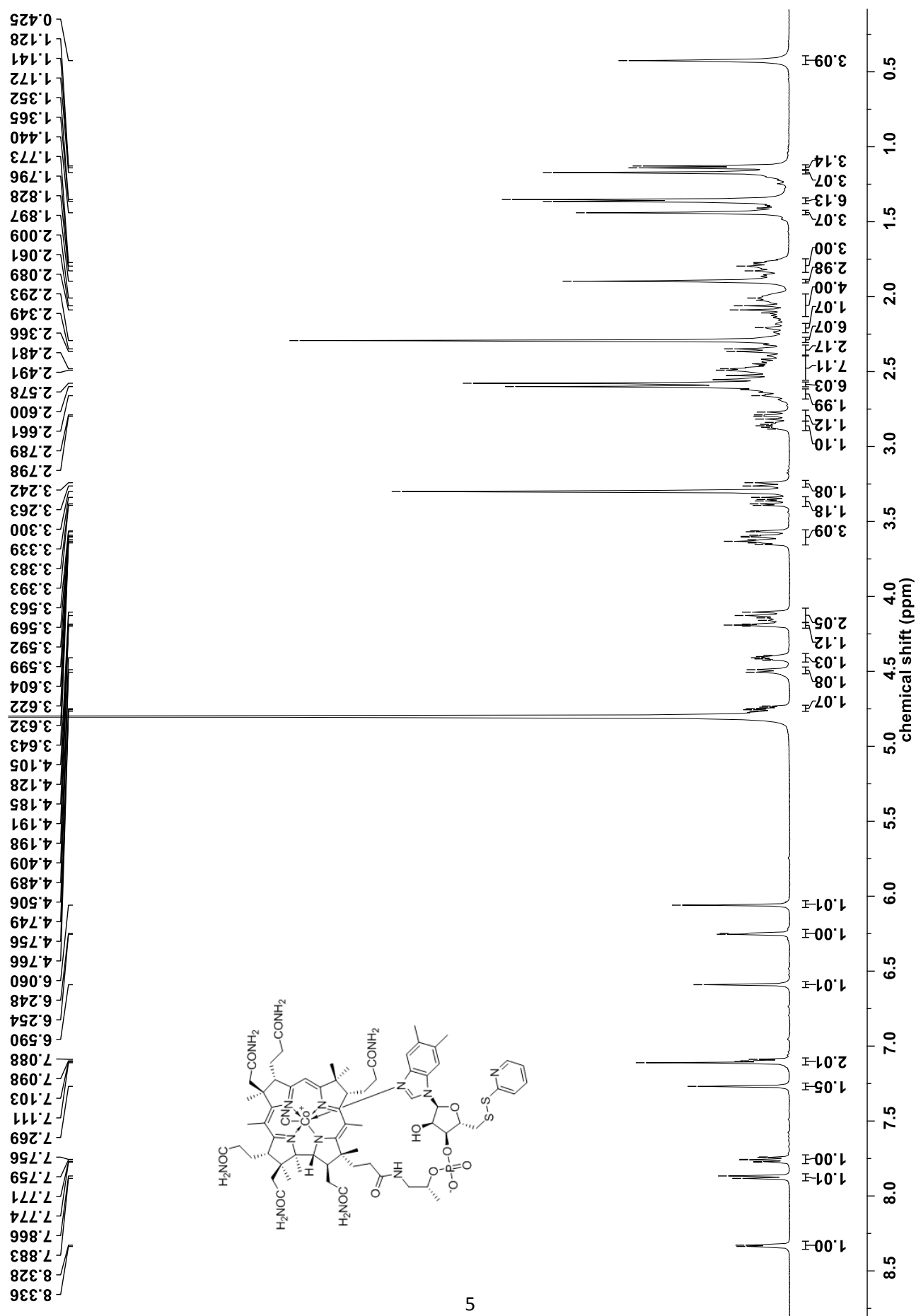
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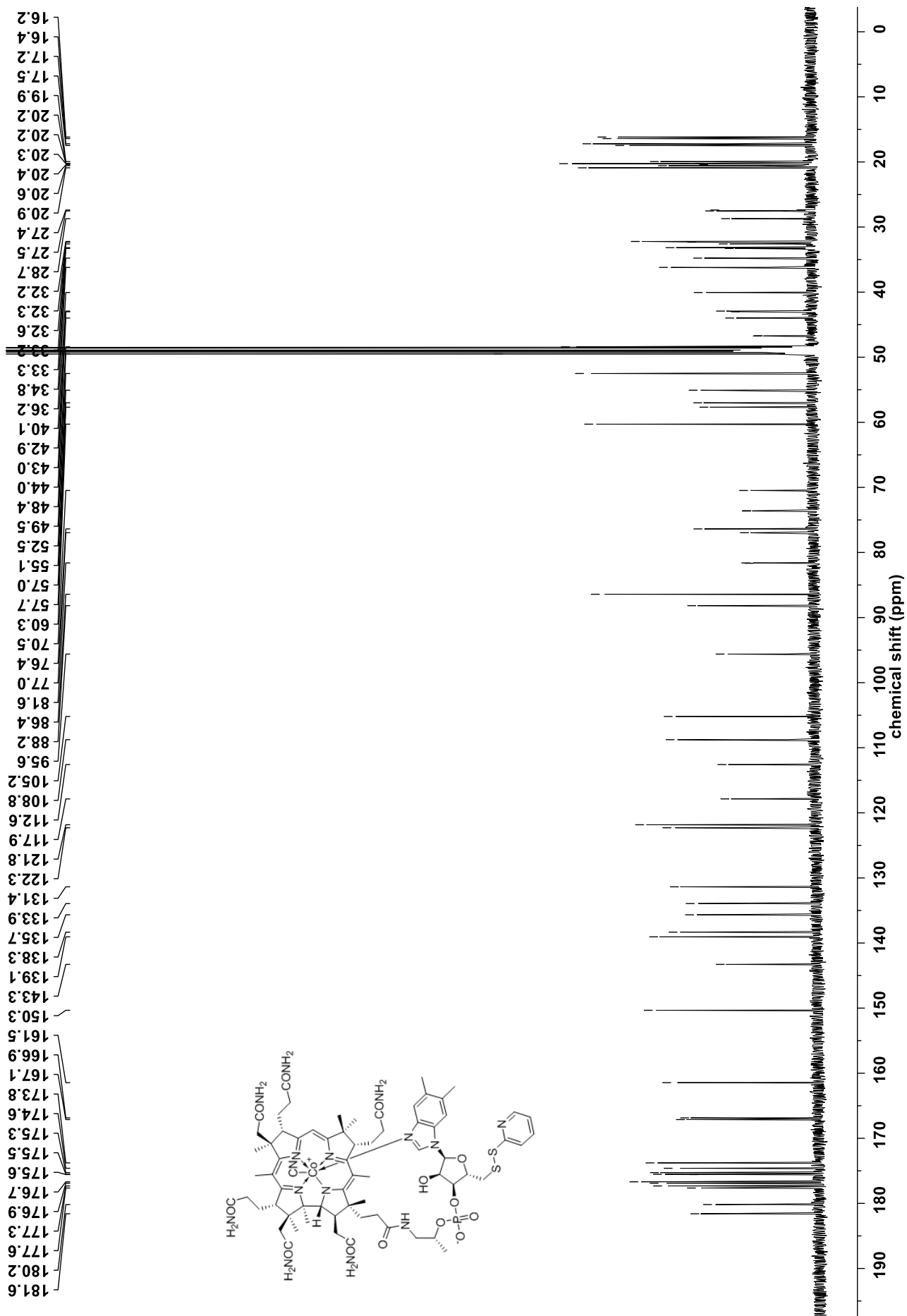
1. Copy of NMR spectra of compound **3** recorded in CD<sub>3</sub>





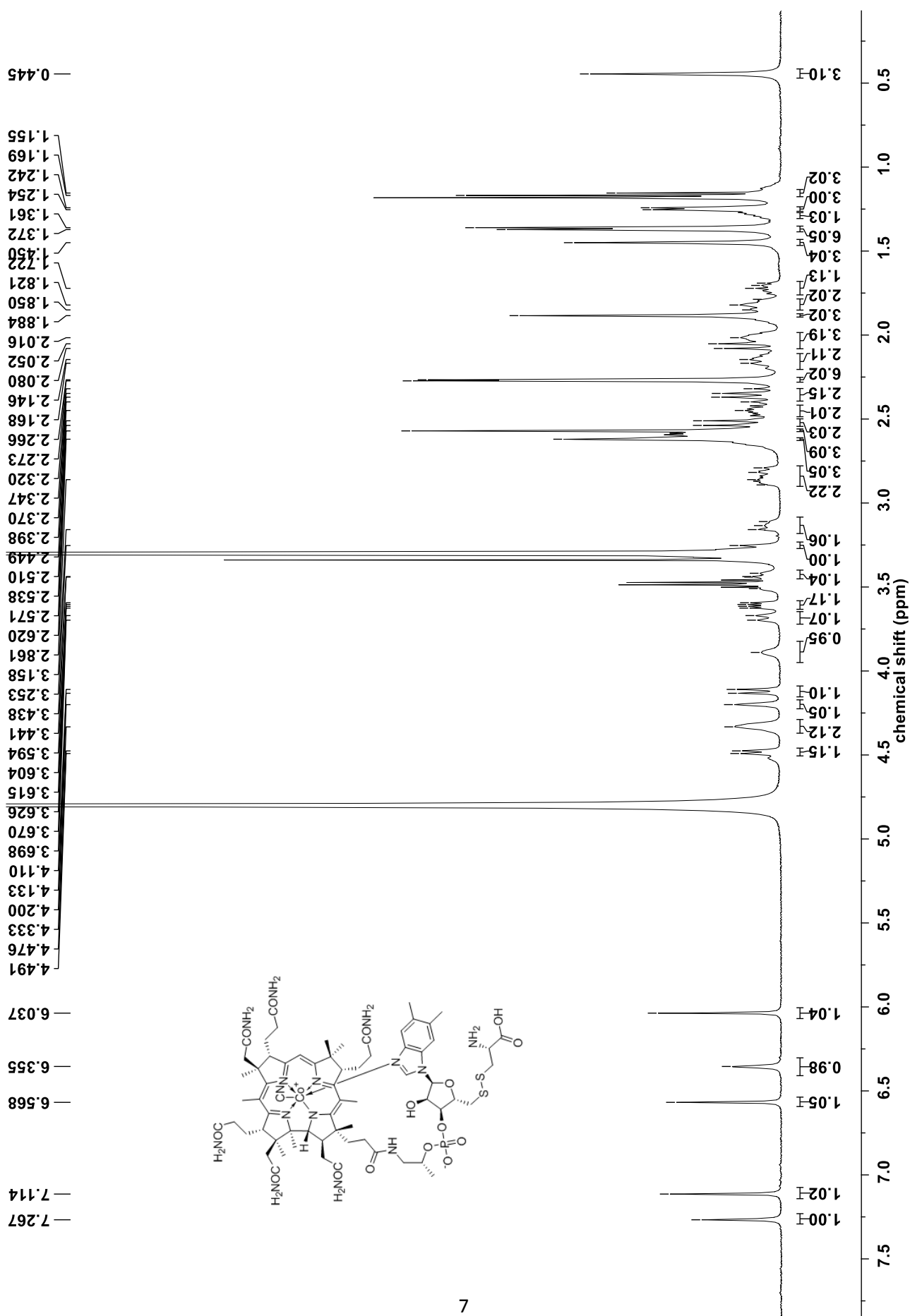
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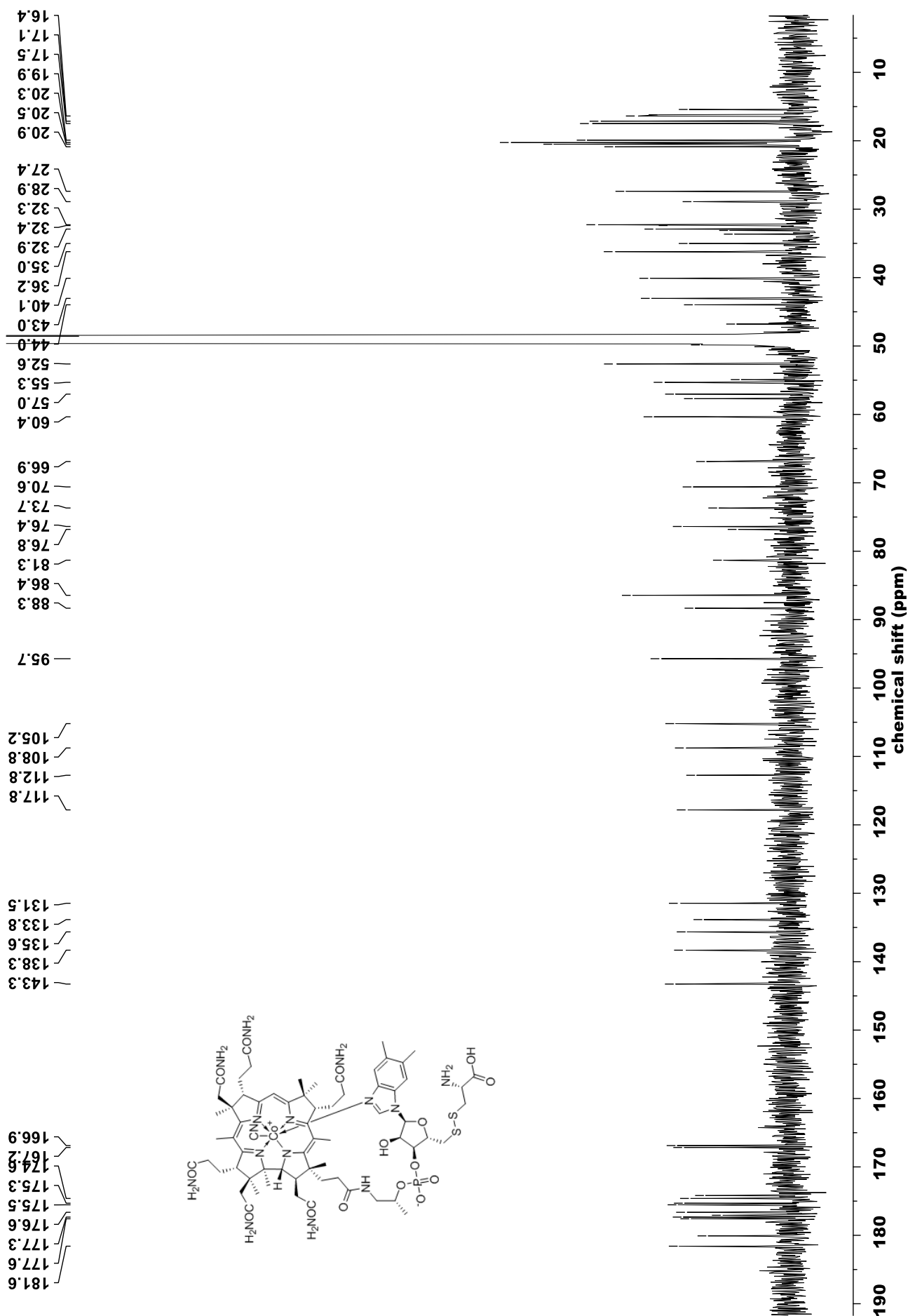




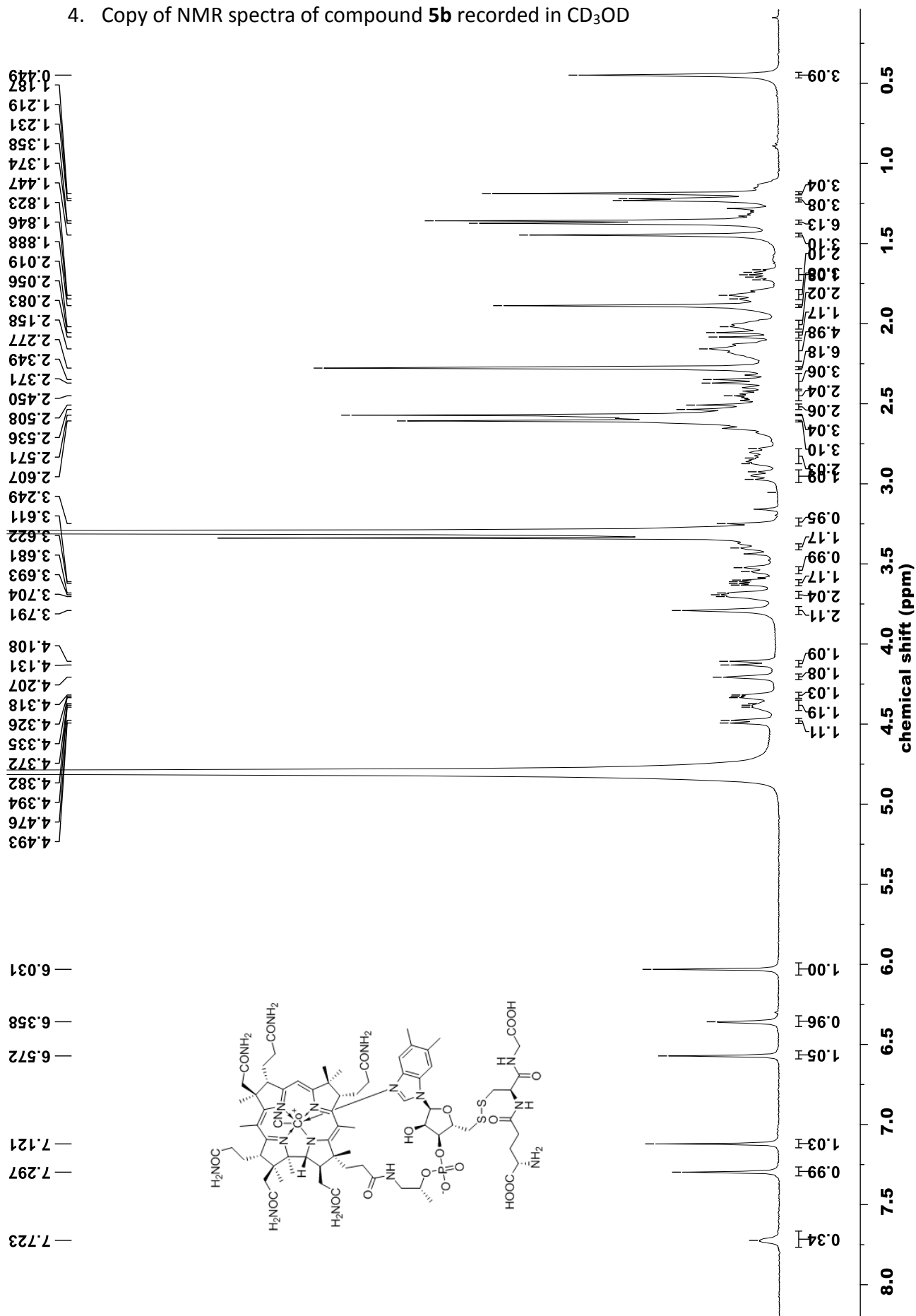


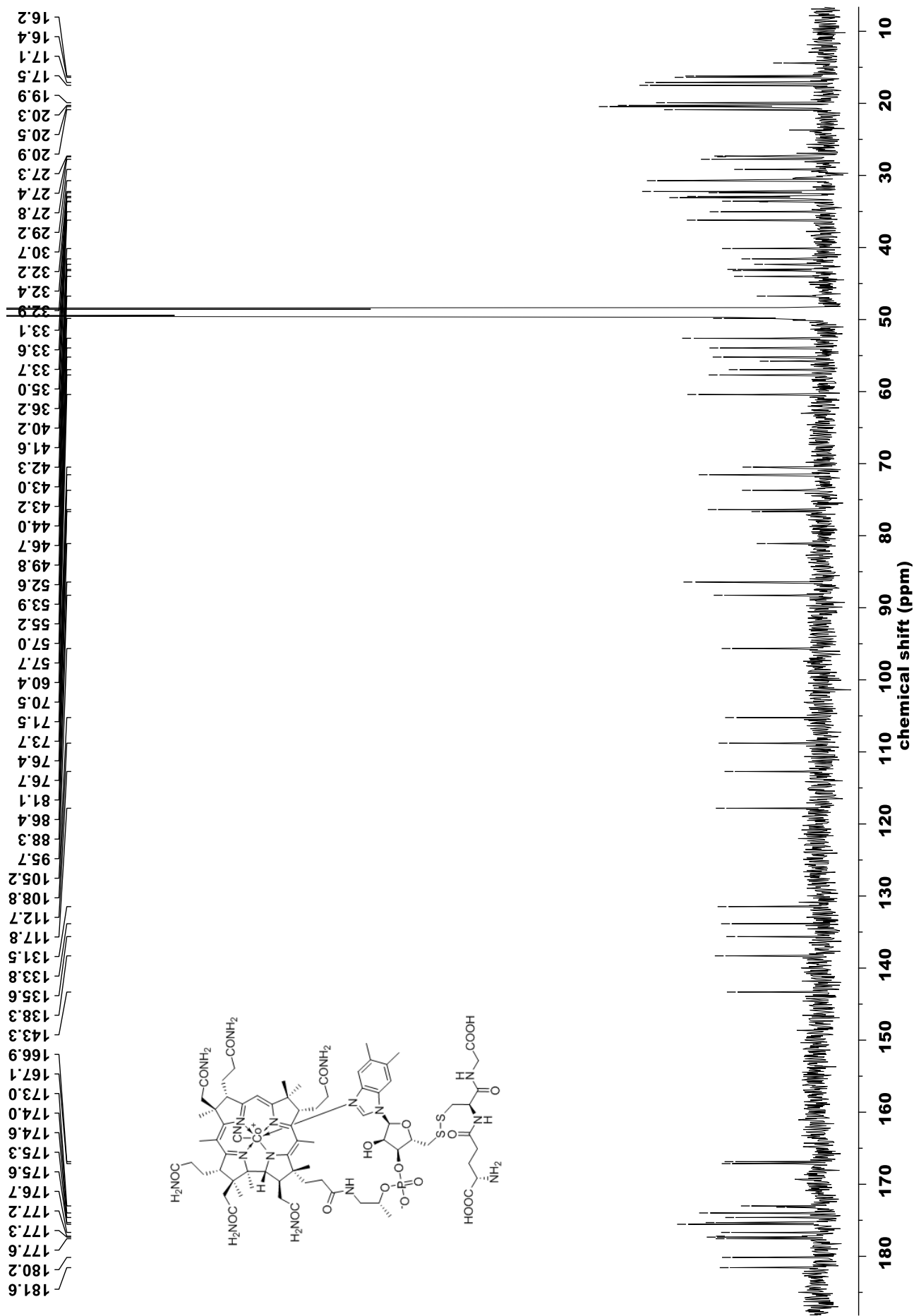
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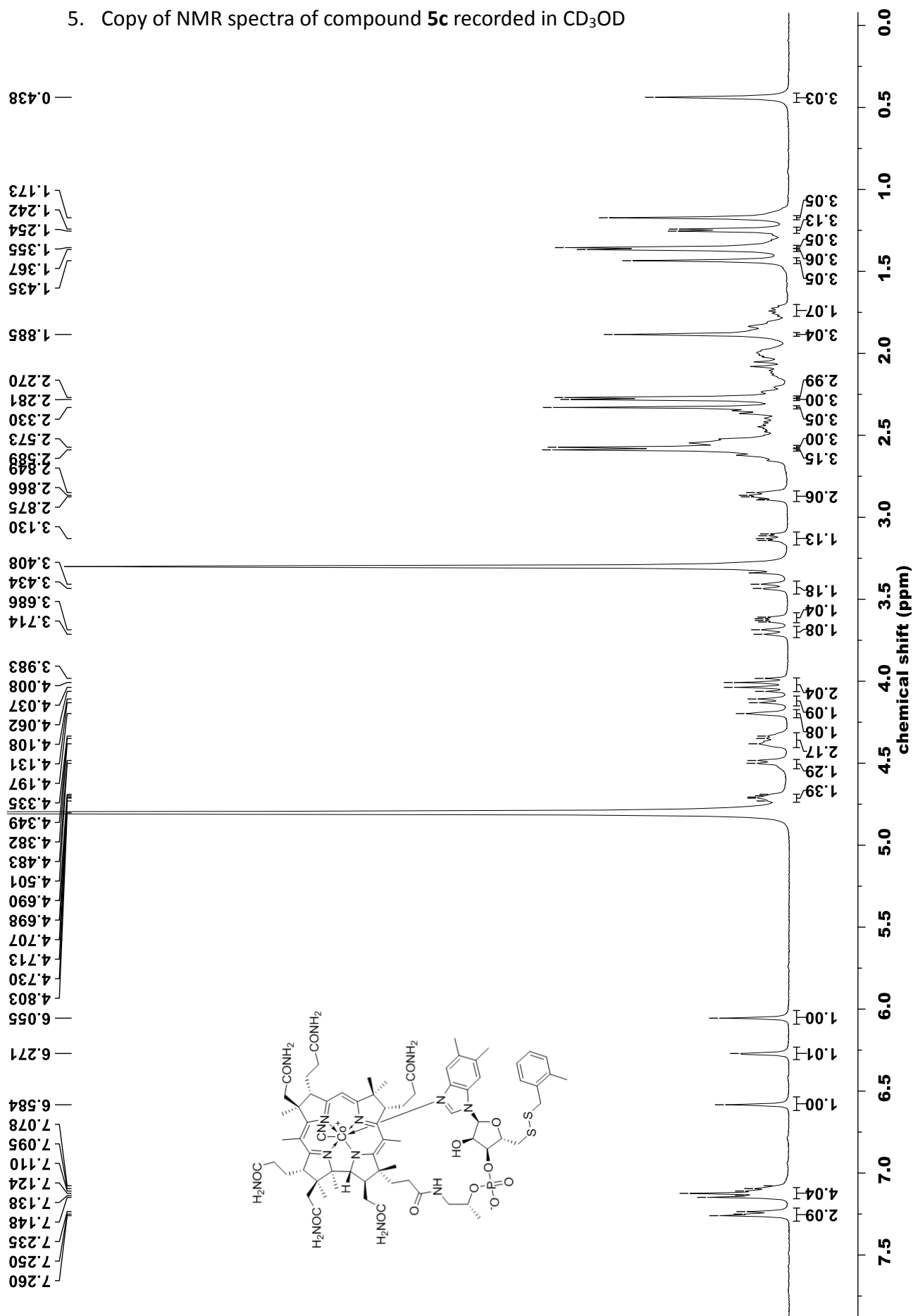


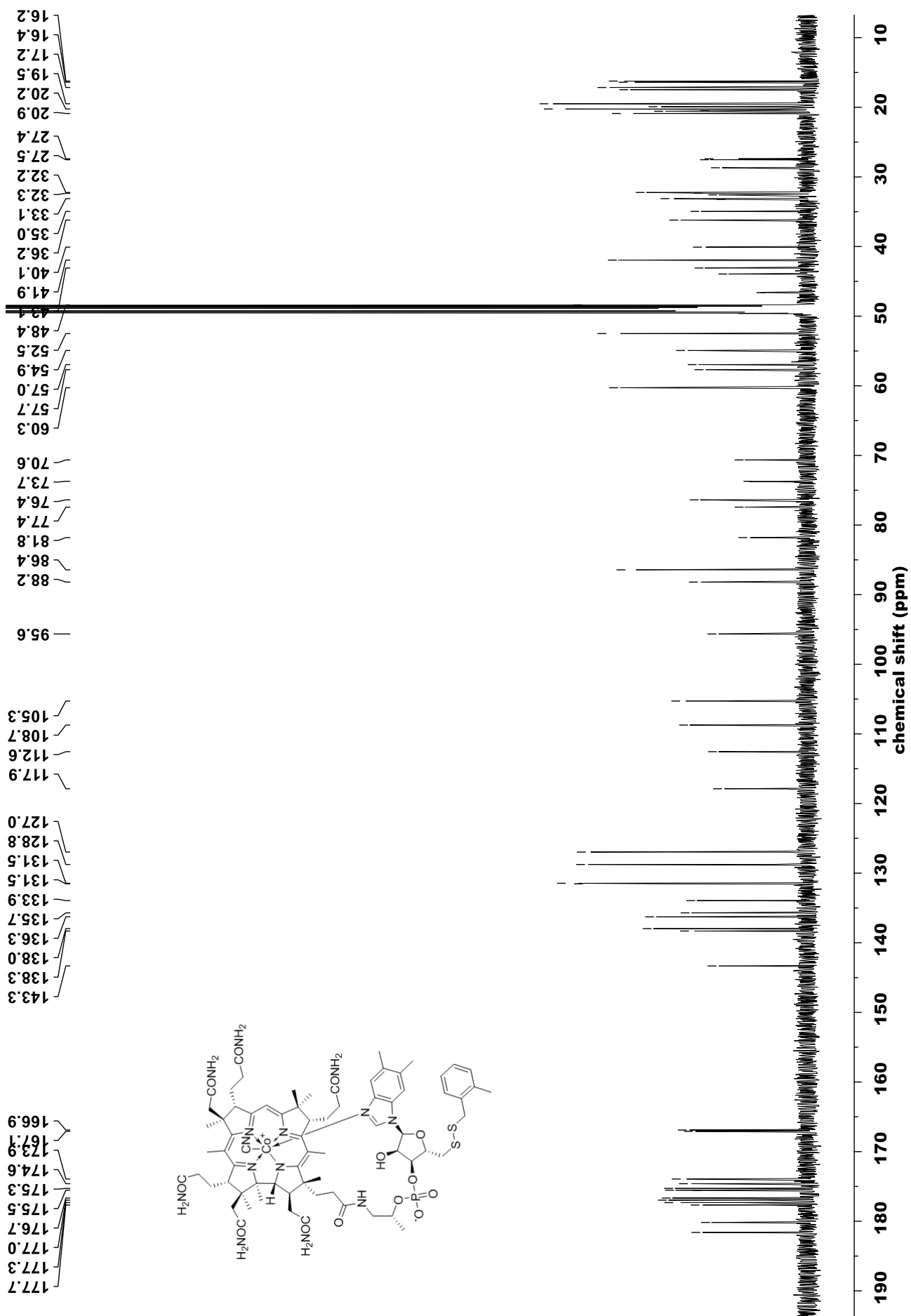
4. Copy of NMR spectra of compound **5b** recorded in CD<sub>3</sub>OD



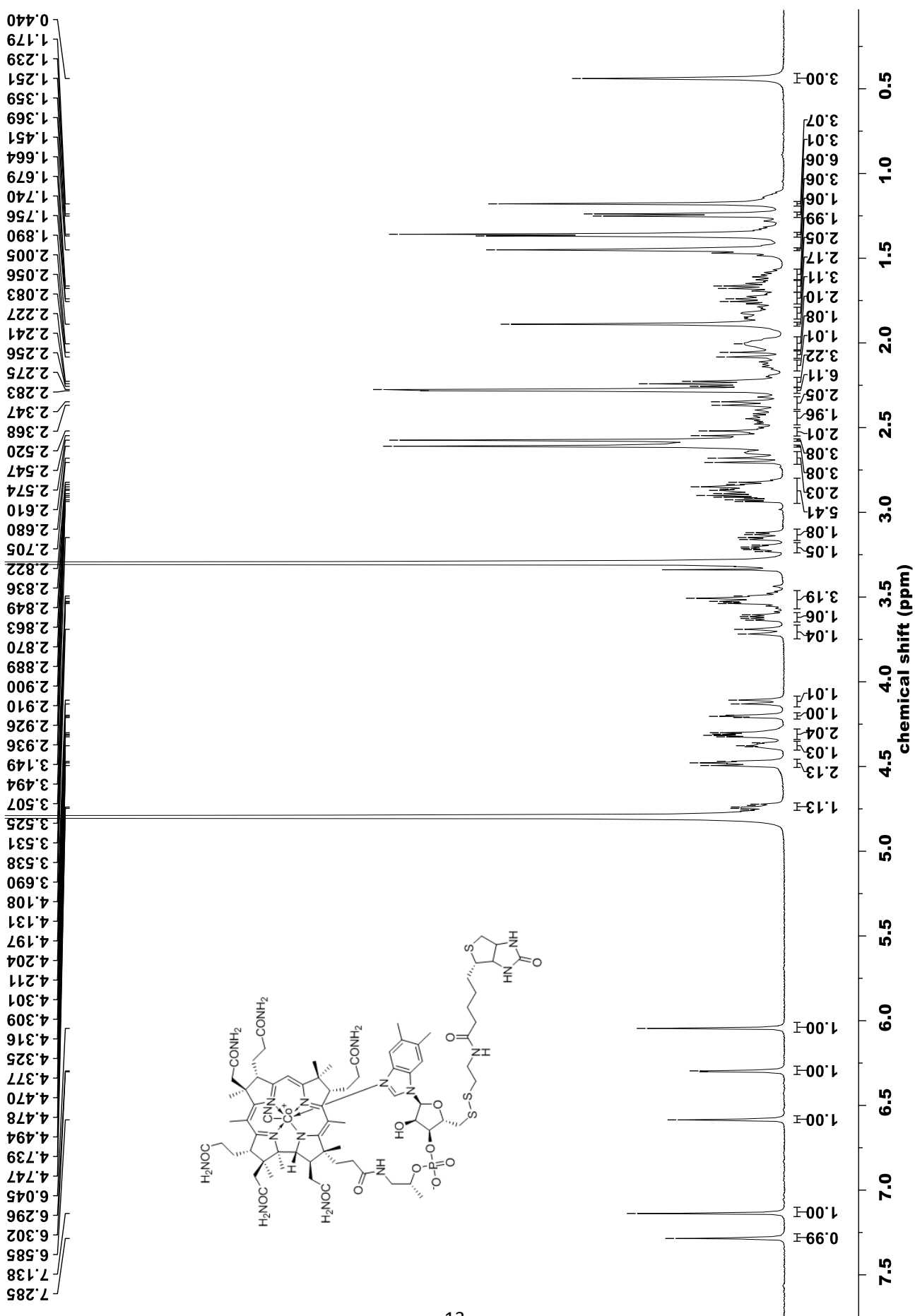


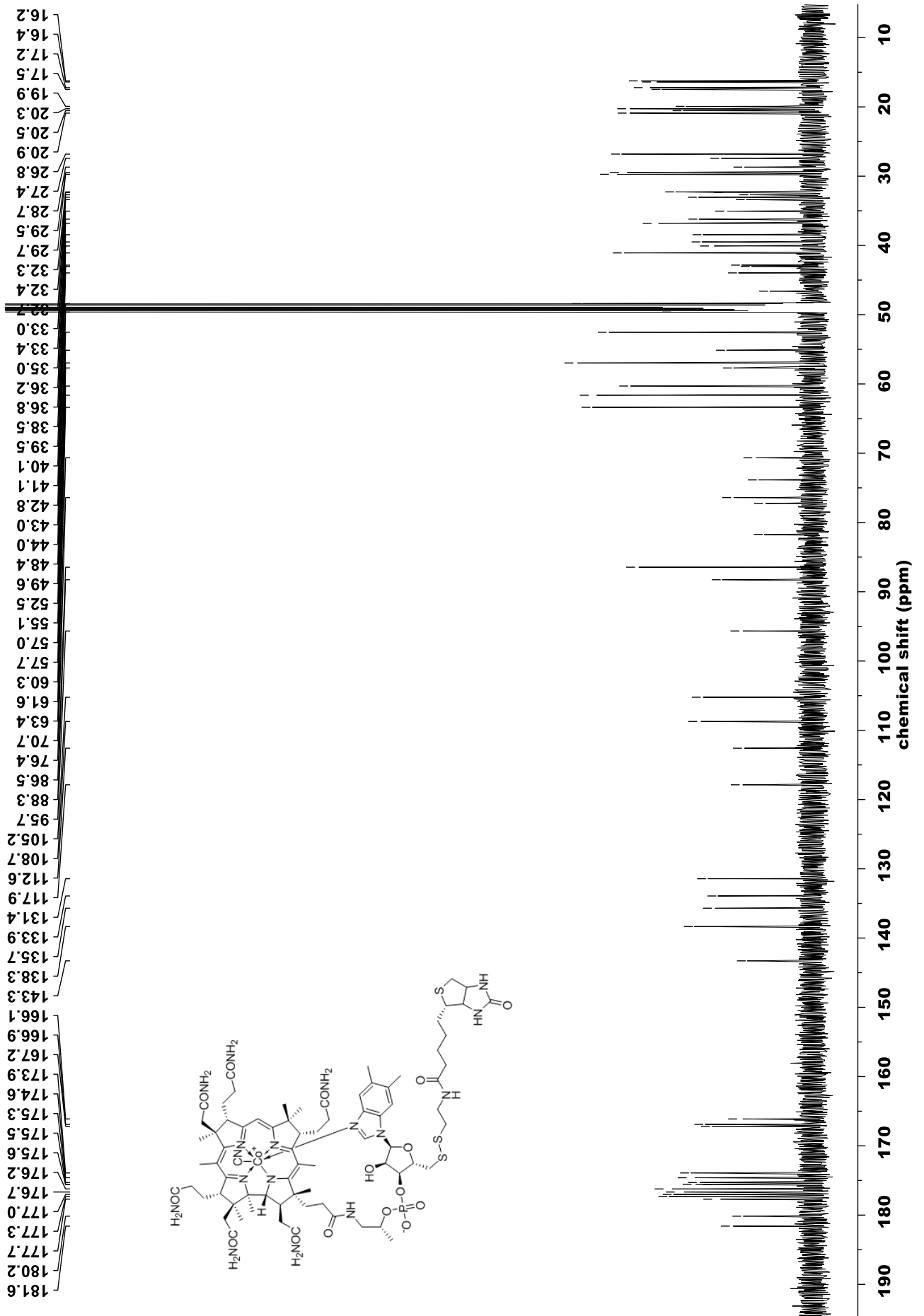
5. Copy of NMR spectra of compound **5c** recorded in CD<sub>3</sub>OD





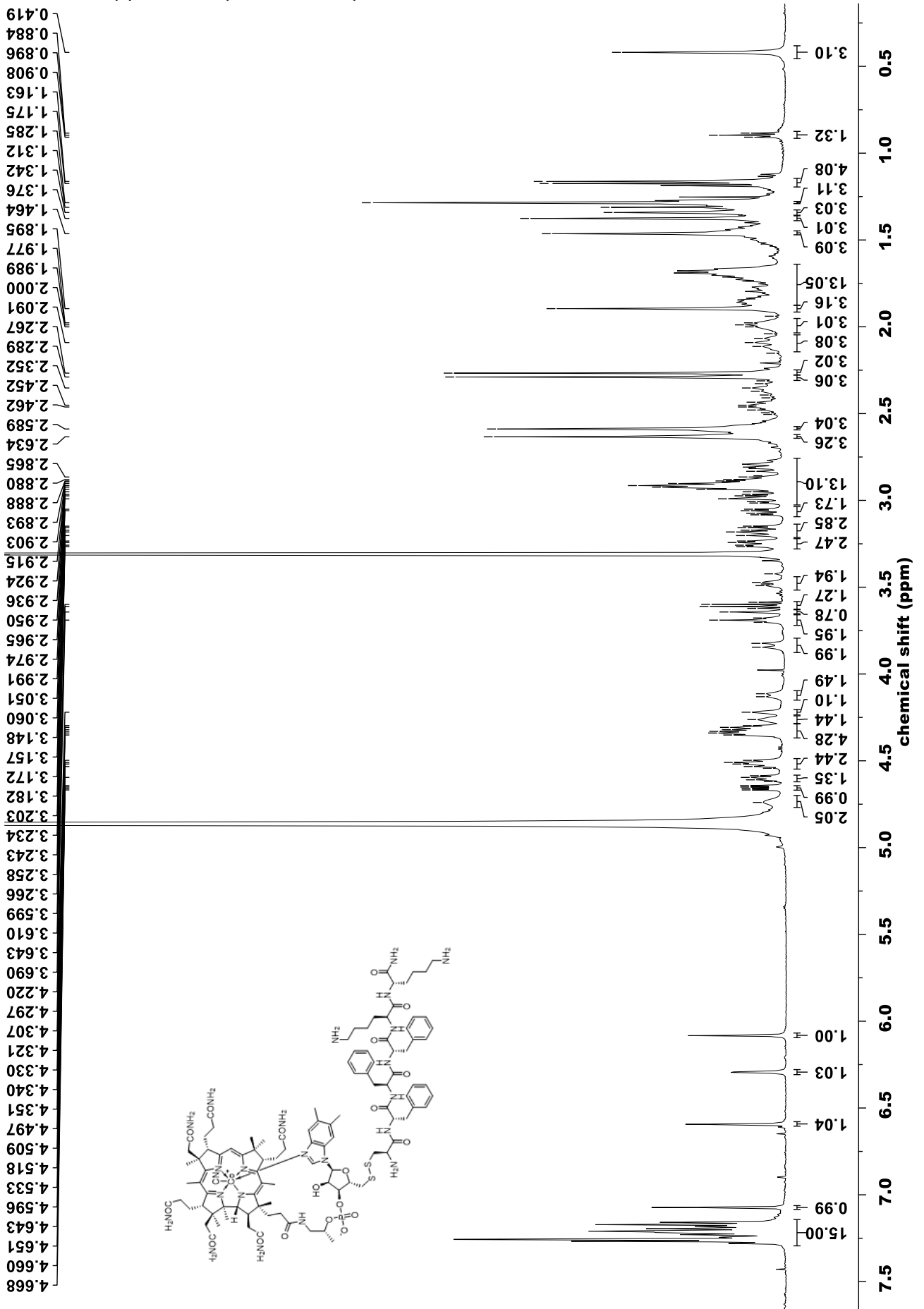
6. Copy of NMR spectra of compound **5** recorded in CD<sub>3</sub>OD

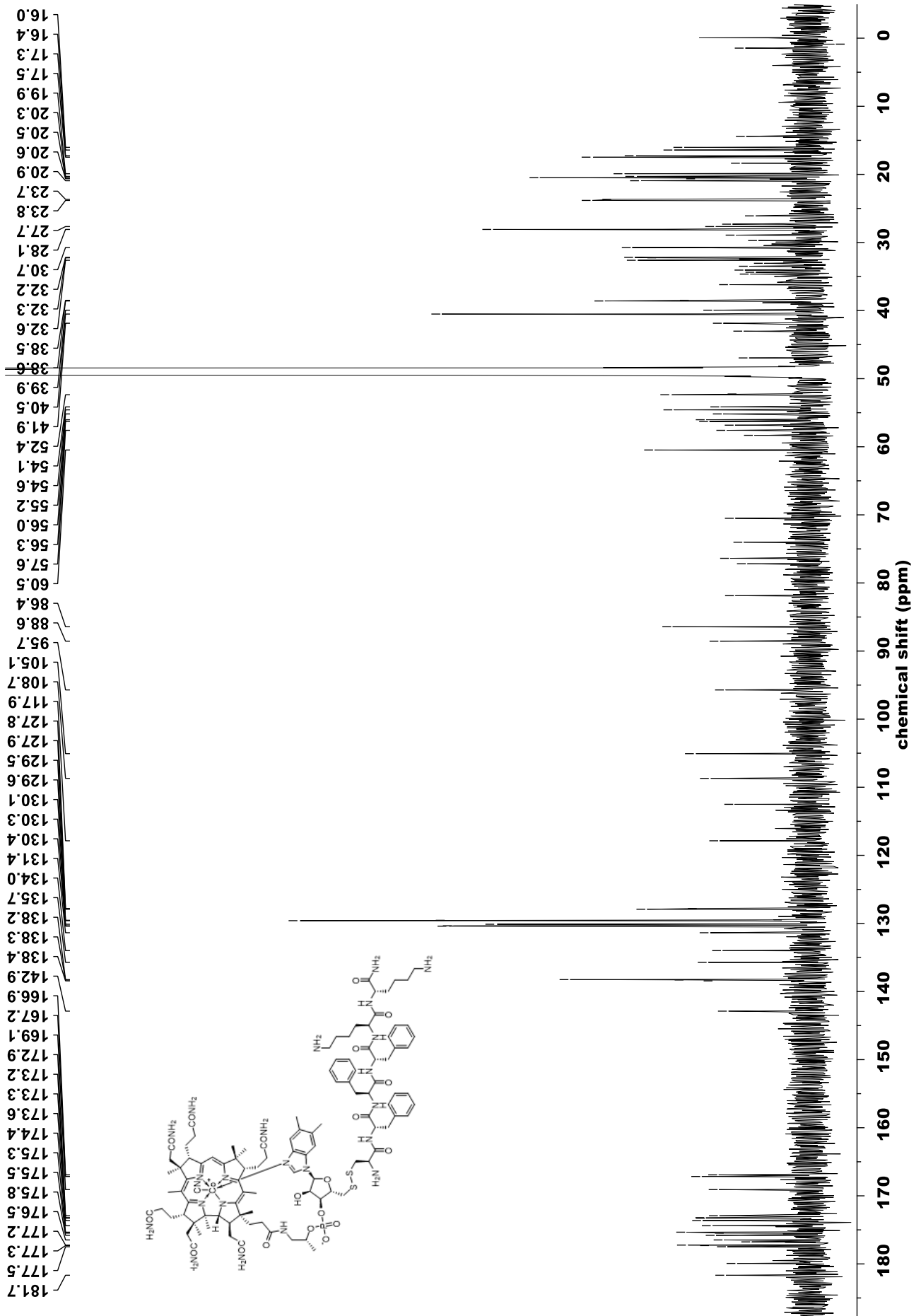






7. Copy of NMR spectra of compound **5e** recorded in CD<sub>3</sub>OD

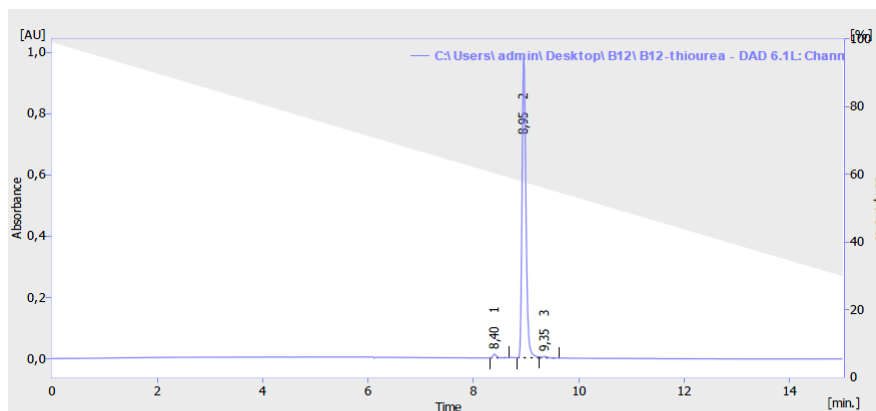
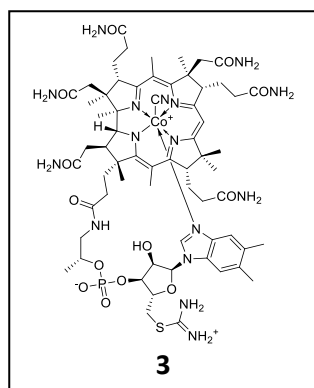




## 8. Copies of HPLC chromatograms

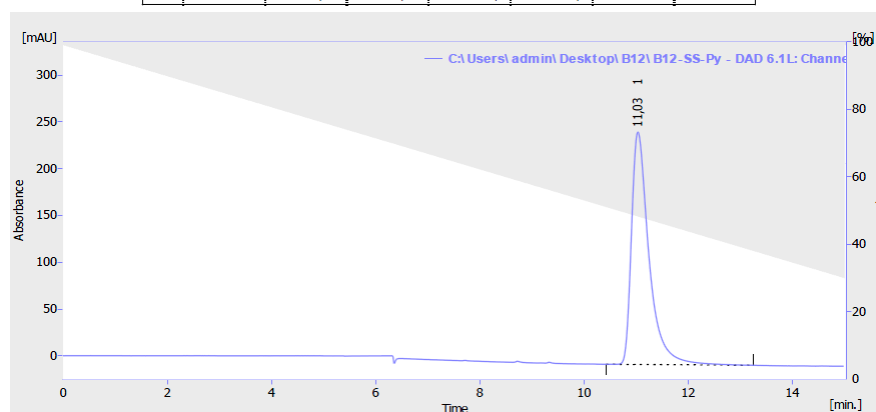
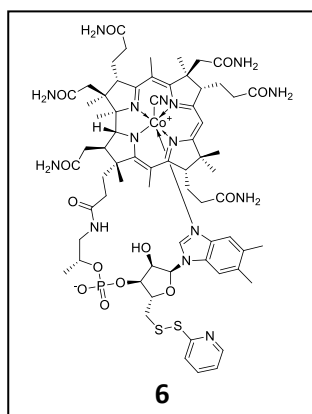
### HPLC Method 1.

Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN[%]	λ [nm]
Initial	99	1	361
15	30	70	



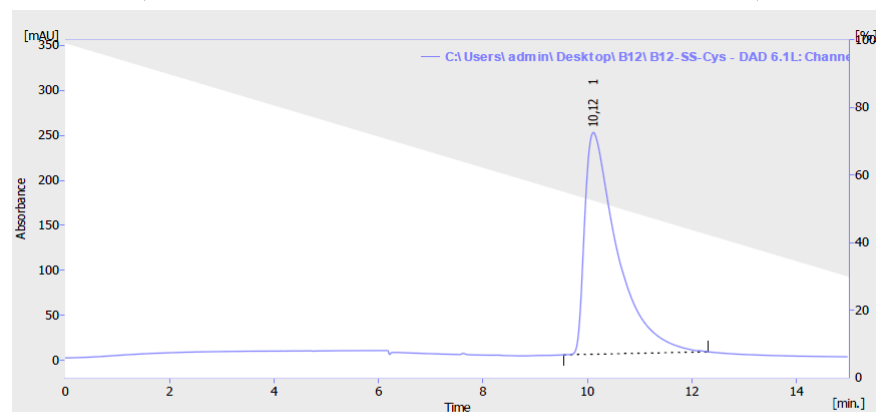
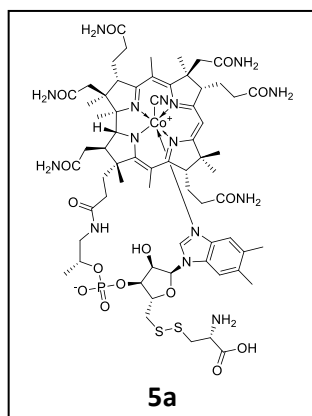
Result Table (Uncal - C:\Users\admin\Desktop\B12\B12-thiourea - DAD 6.1L: Channel 2)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	8,400	56,764	12,321	1,1	1,2	0,08	984
2	8,950	5051,591	979,539	98,2	98,3	0,08	998
3	9,350	35,897	4,739	0,7	0,5	0,10	356
Total		5144,252	996,599	100,0	100,0		



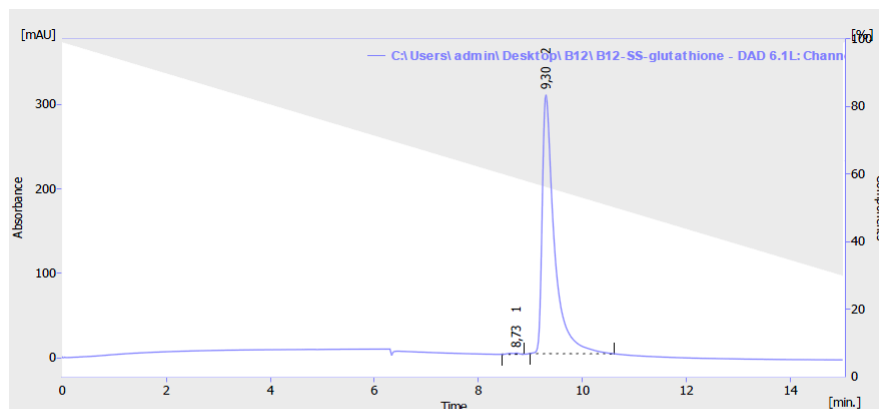
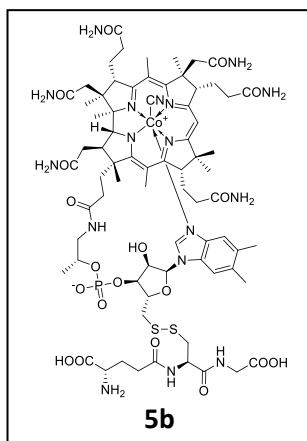
Result Table (Uncal - C:\Users\admin\Desktop\B12\B12-SS-Py - DAD 6.1L: Channel 2)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	11,033	5824,450	248,546	100,0	100,0	0,35	779
Total		5824,450	248,546	100,0	100,0		



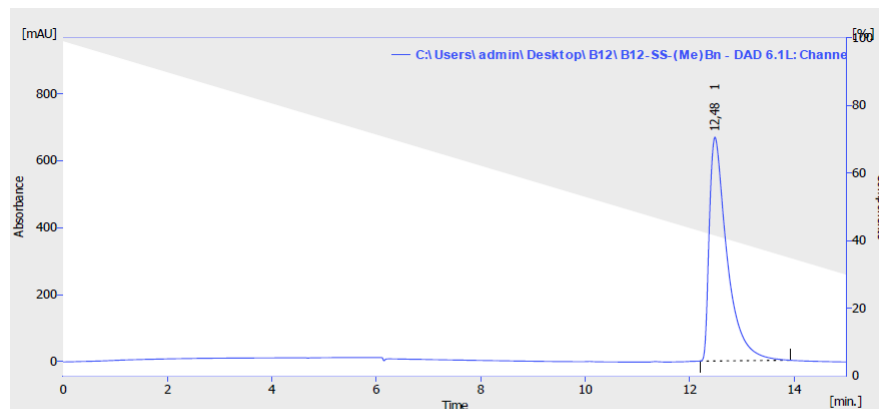
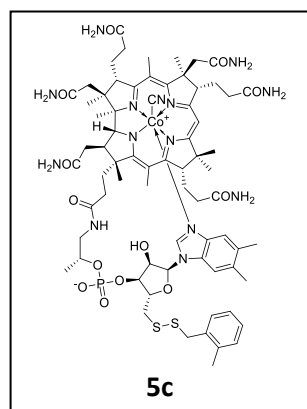
Result Table (Uncal - C:\Users\admin\Desktop\B12\B12-SS-Cys - DAD 6.1L: Channel 2)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	10,117	10699,143	246,159	100,0	100,0	0,63	828
Total		10699,143	246,159	100,0	100,0		



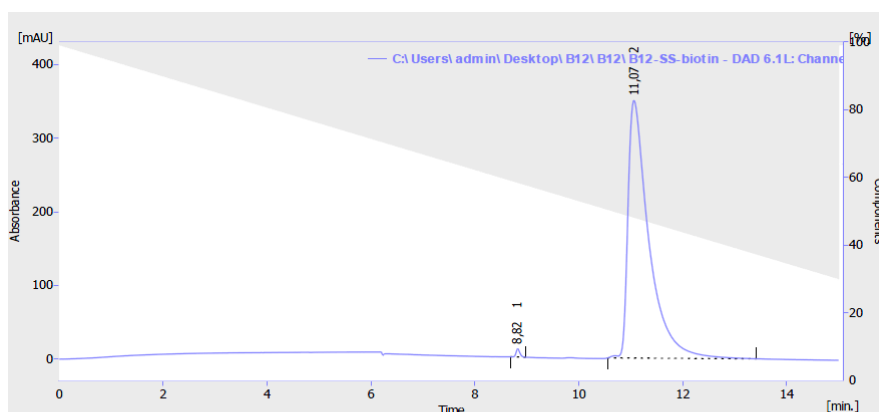
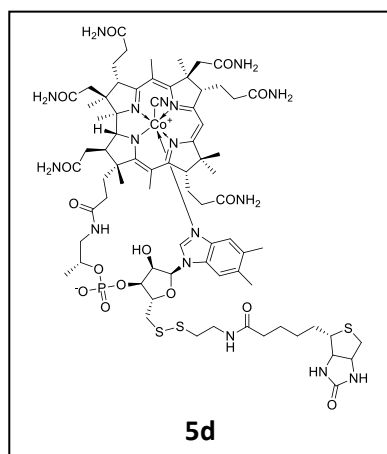
Result Table (Uncal - C:\Users\admin\Desktop\B12\B12-SS-glutathione - DAD 6.1L: Channel 2)

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	8,733	16,601	1,405	0,3	0,5	1000
2	9,300	4998,699	306,863	99,7	99,5	708
Total		5015,295	308,268	100,0	100,0	



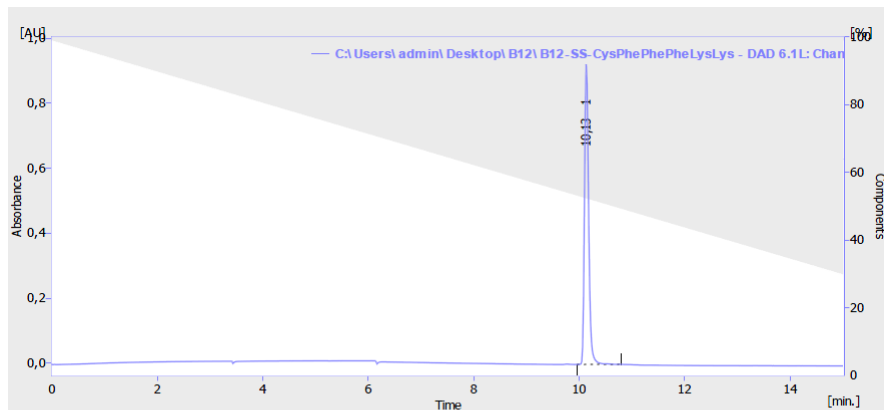
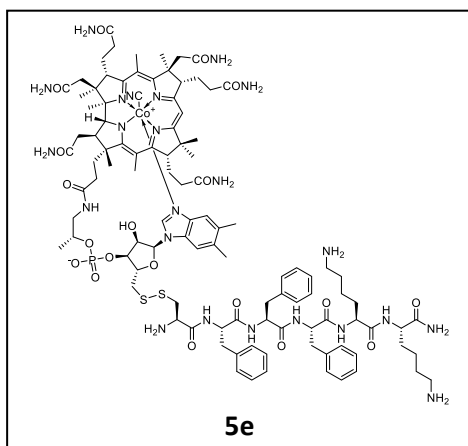
Result Table (Uncal - C:\Users\admin\Desktop\B12\B12-SS-(Me)Bn - DAD 6.1L: Channel 2)

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	12,483	15827,590	669,379	100,0	100,0	0,37
Total		15827,590	669,379	100,0	100,0	



Result Table (Uncal - C:\Users\admin\Desktop\B12\B12-SS-biotin - DAD 6.1L: Channel 2)

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	8,817	55,932	10,746	0,6	3,0	0,10
2	11,067	9791,746	349,556	99,4	97,0	843
Total		9847,678	360,302	100,0	100,0	

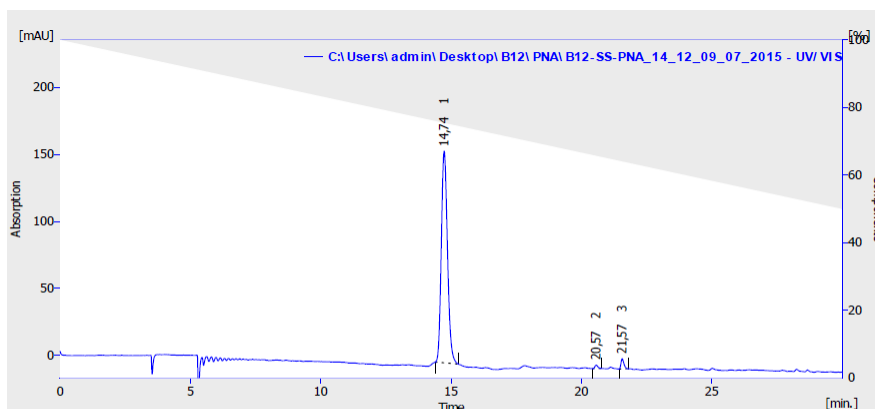
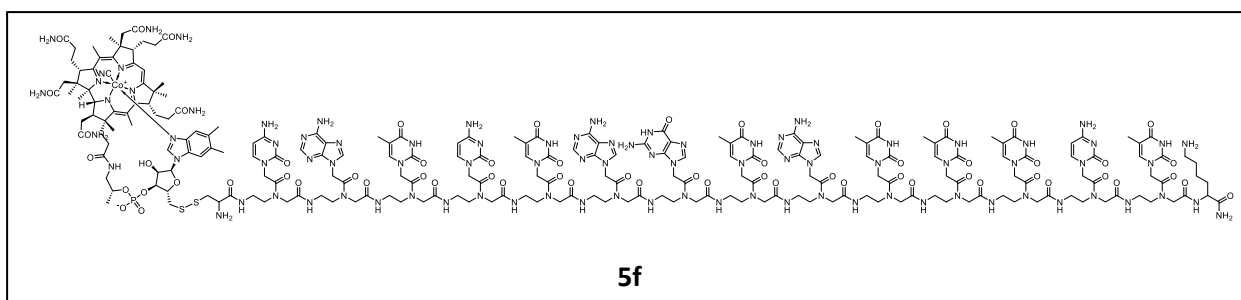


Result Table (Uncal - C:\Users\admin\Desktop\B12\B12-SS-CysPhePheLys - DAD 6.1L: Channel 2)

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	5044,155	923,409	100,0	100,0	0,10	334
Total	5044,155	923,409	100,0	100,0		

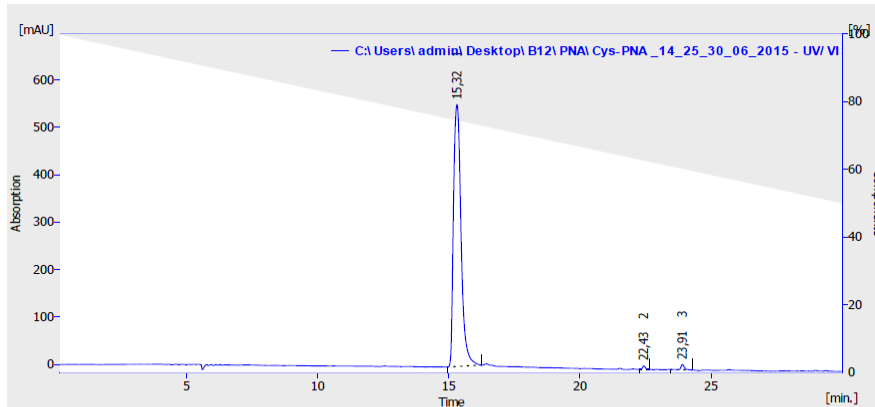
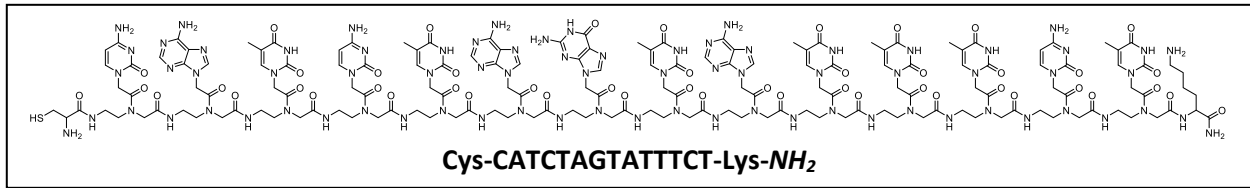
### HPLC Method 2.

Time [min]	H <sub>2</sub> O+1%TFA [%]	MeCN+1%TFA[%]	λ [nm]
Initial	100	0	267
30	50	50	



Result Table (Uncal - C:\Users\admin\Desktop\B12\PNA\B12-SS-PNA\_14\_12\_09\_07\_2015 - UV/VIS)

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	2605,396	158,256	97,1	93,9	0,26	865
2	20,555	2,589	0,8	1,5	0,13	833
3	58,387	7,669	2,2	4,6	0,13	894
Total	2684,337	168,514	100,0	100,0		

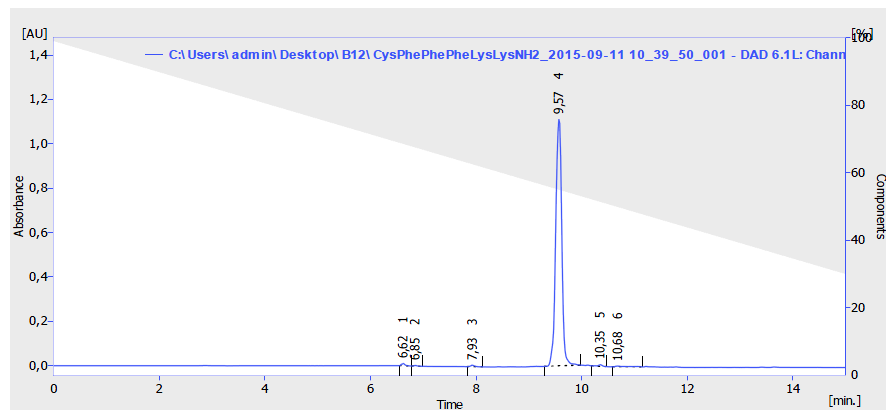
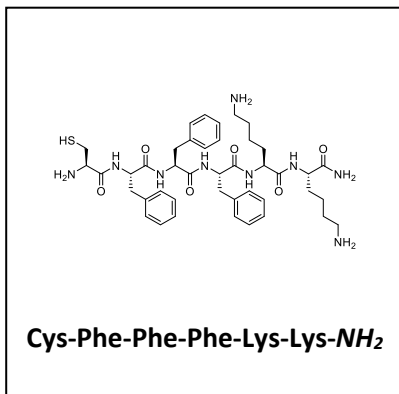


*Result Table (Uncal - C:\Users\admin\Desktop\B12\PNA\Cys-PNA\_14\_25\_30\_06\_2015 - UV/VIS)*

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity	
1	15,317	11032,977	552,056	98,6	96,8	0,32	909
2	22,433	52,124	7,188	0,5	1,3	0,12	0
3	23,908	100,597	10,995	0,9	1,9	0,14	396
Total		11185,698	570,239	100,0	100,0		

**HPLC Method 3.**

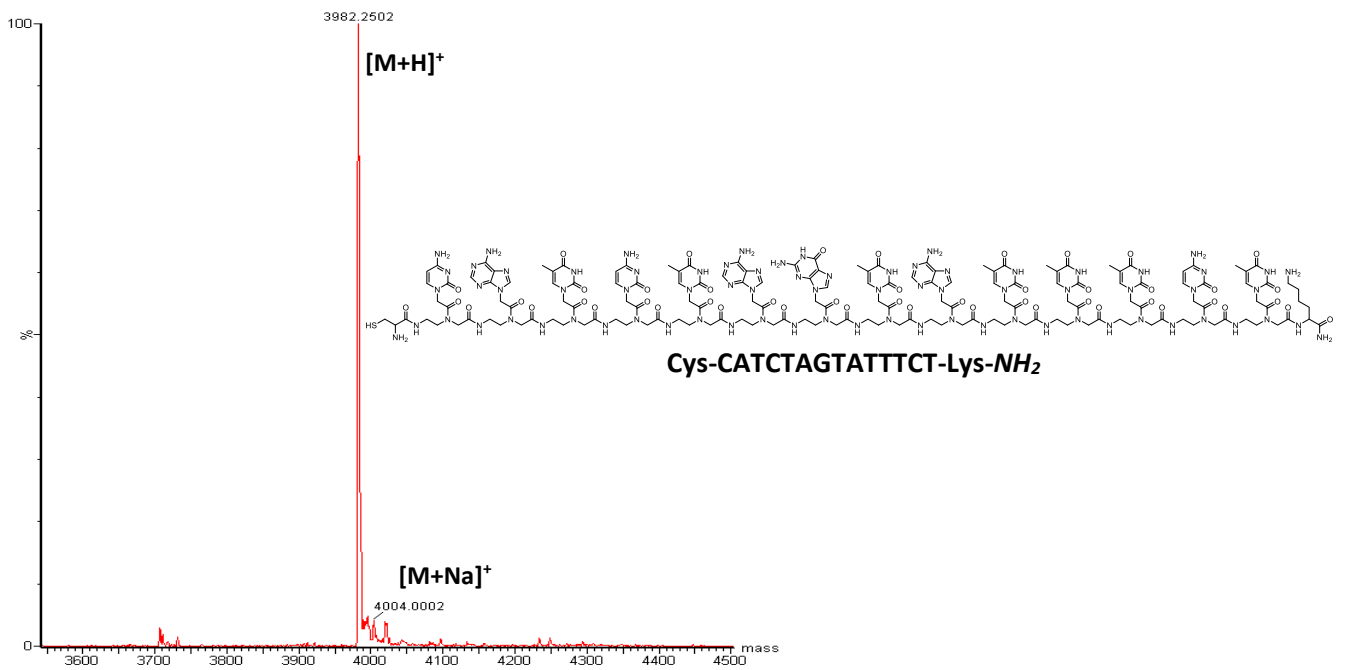
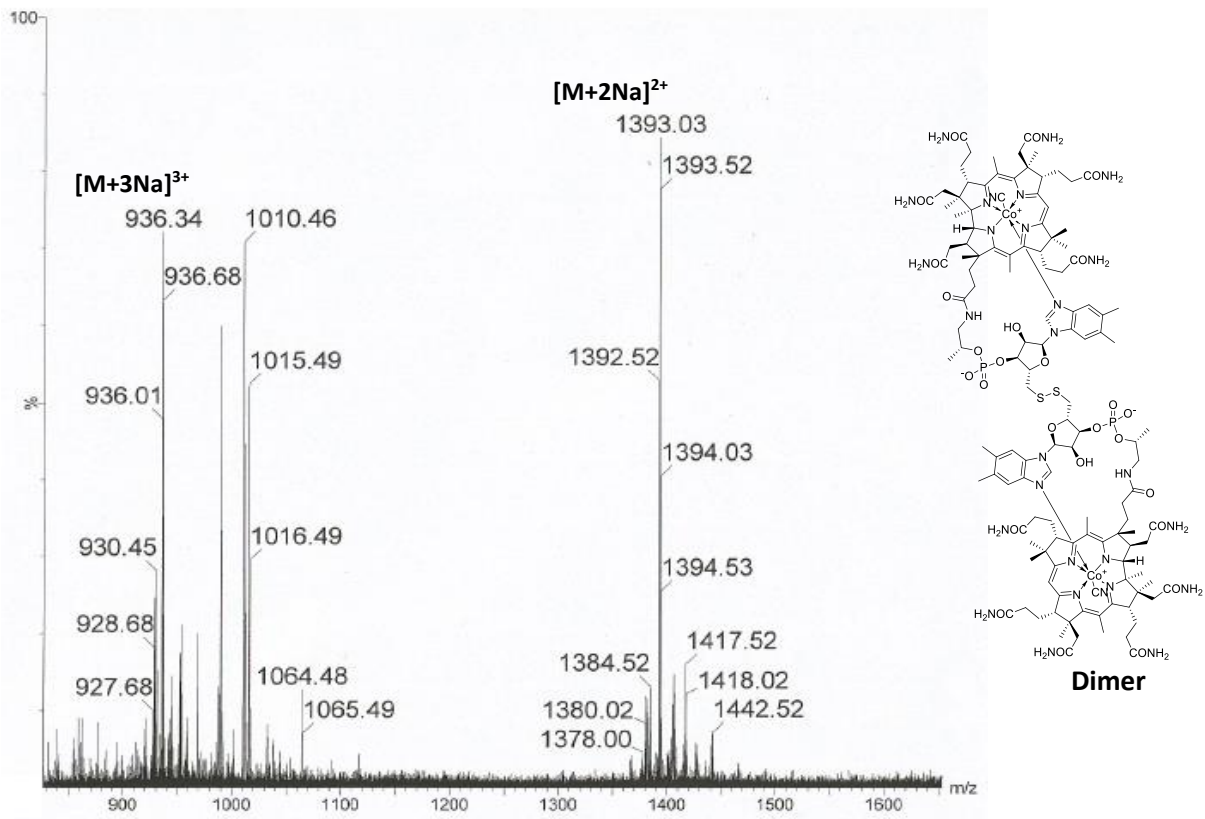
Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN[%]	λ [nm]
Initial	99	1	254
15	30	70	

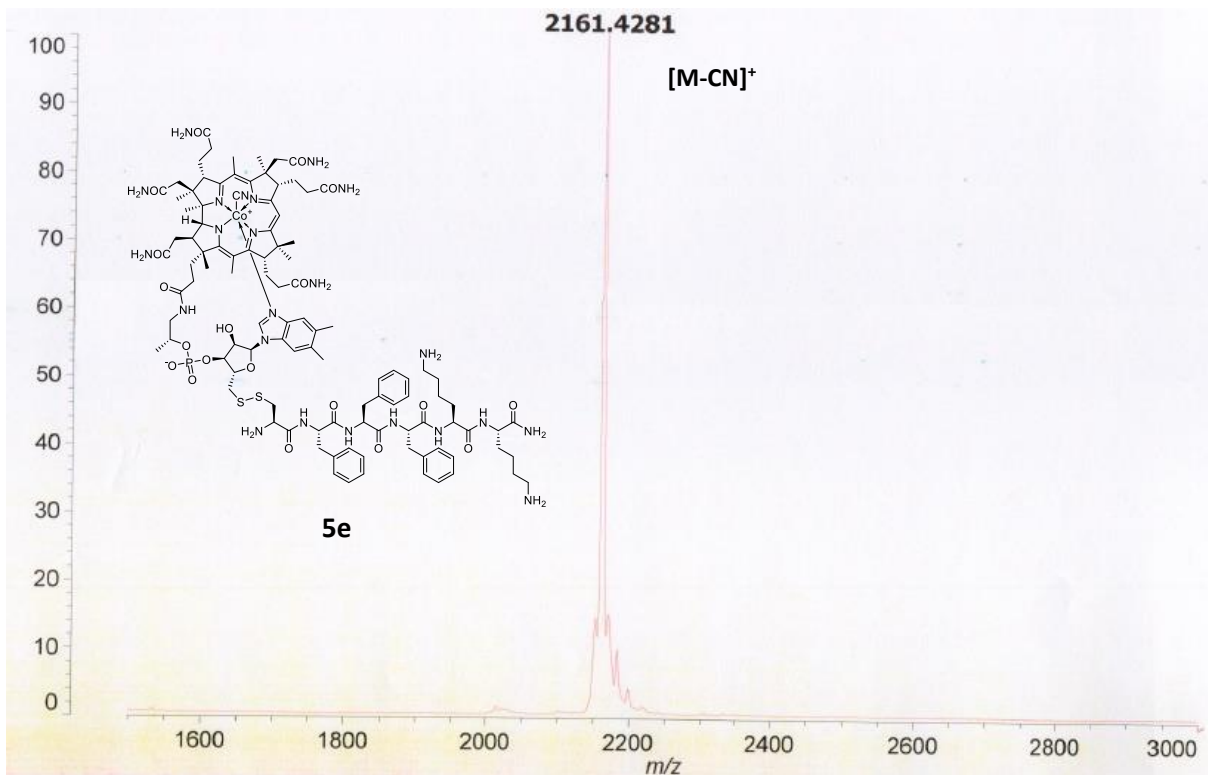
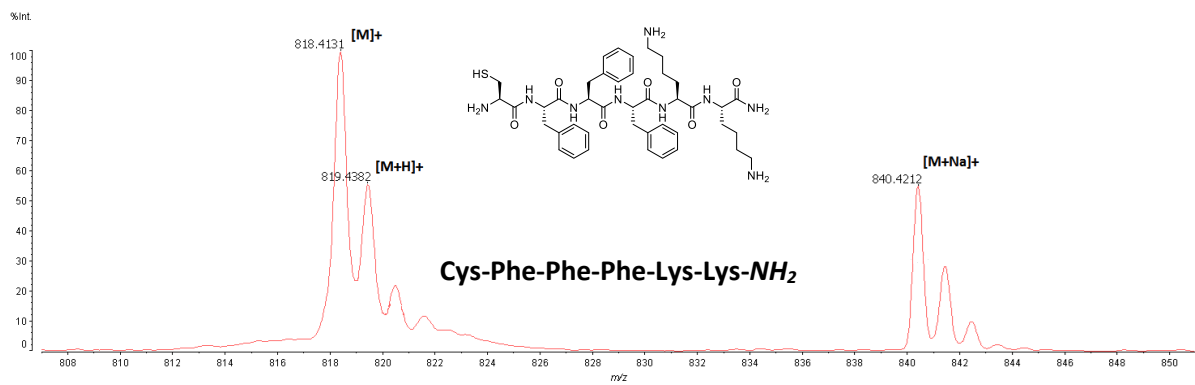
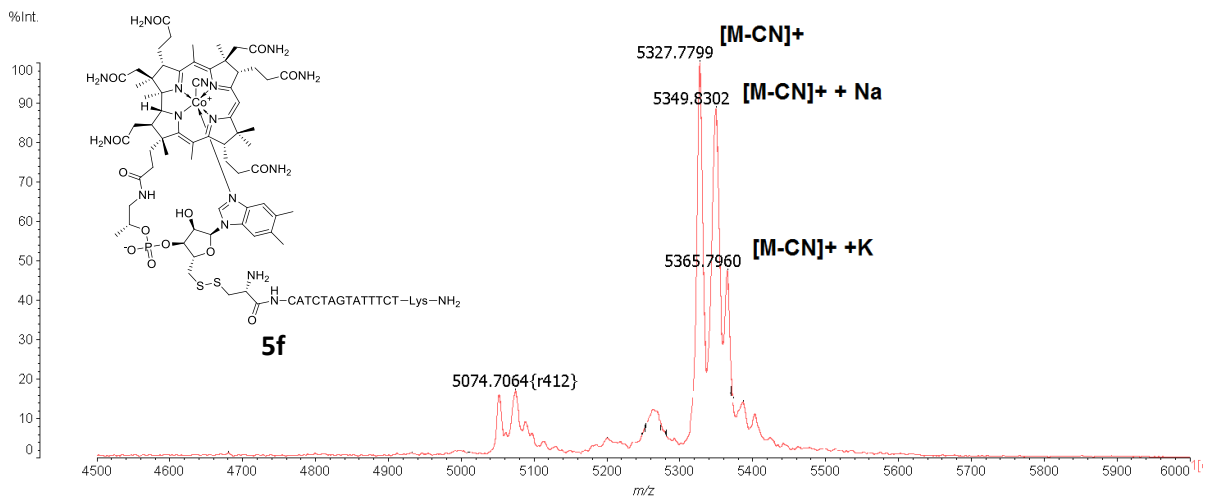


*Result Table (Uncal - C:\Users\admin\Desktop\B12\CysPhePhePheLysLysNH2\_2015-09-11 10\_39\_50\_001 - DAD 6.1L: Channel 1)*

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity	
1	6,617	45,223	10,048	0,5	0,9	0,08	966
2	6,850	12,282	2,794	0,1	0,2	0,07	981
3	7,833	34,303	6,241	0,4	0,5	0,10	964
4	9,567	8866,819	1112,686	98,0	97,4	0,13	614
5	10,350	36,934	7,036	0,4	0,6	0,08	856
6	10,683	53,053	3,280	0,6	0,3	0,13	837
Total		9048,614	1142,084	100,0	100,0		

9. Copies of MS spectra







10. X-ray structure of B<sub>12</sub>-pyridyl disulfide (B<sub>12</sub>-5'-SSPy) 6

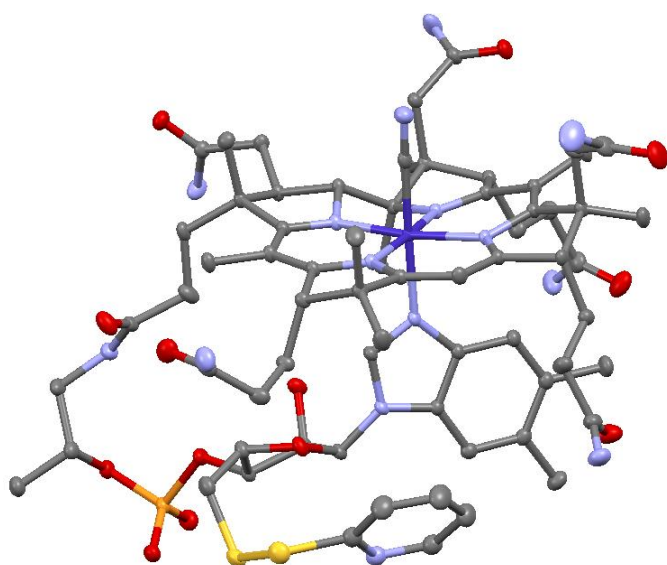


Figure 1.

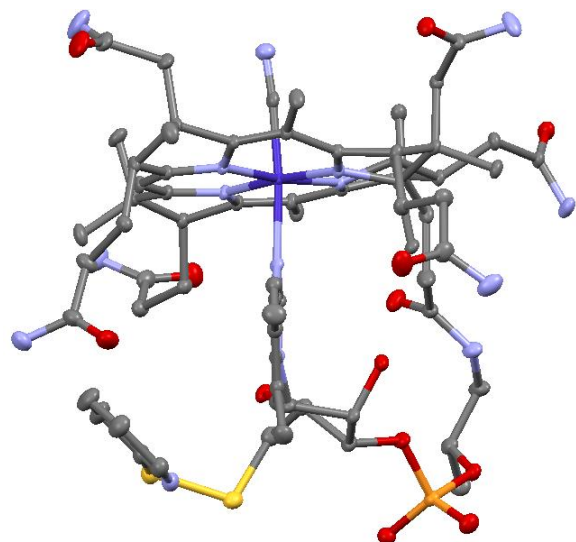


Figure 2.

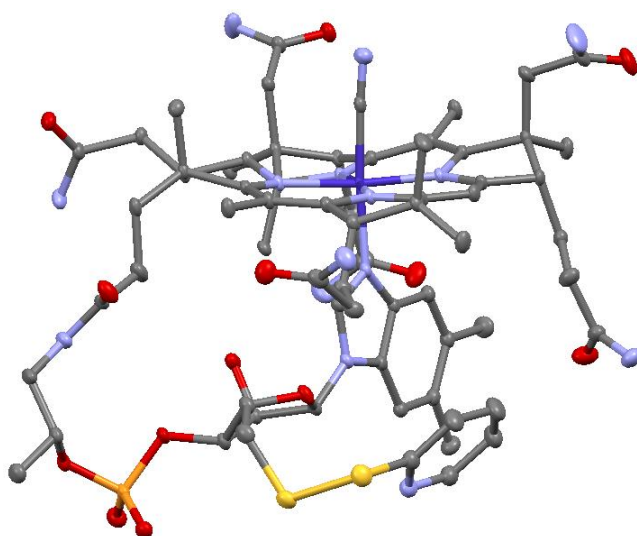
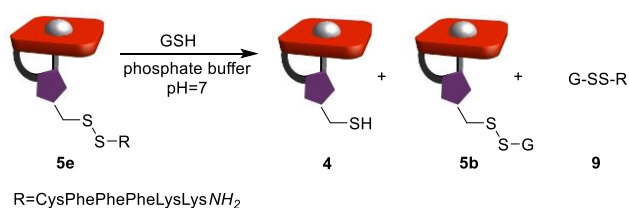


Figure 3.

## 11. Reduction of disulfides **5e** and **5c** with GSH

**Scheme 1.** Reduction of disulfide **5e**.



**Table 1.** Reduction of disulfide **5e**,  $c_{\text{GSH}} = 1 \text{ mM}$ .<sup>a</sup>

Entry	Time [h]	Yield <sup>b</sup> <b>4</b> [%]	Yield <sup>b</sup> <b>5b</b> [%]	Conv. <sup>b</sup> <b>5e</b> [%]
1	1	17	31	52
2	2	21	45	66
3	3	21	51	72
4	4	18	57	75
5	5	11	64	75
6	6	7	68	75
7	24	0	75	75

<sup>a</sup> General conditions: substrate **5e** (1  $\mu\text{mol}$ ), GSH (10 equiv.), phosphate buffer pH = 7 (10 mL,  $c = 50 \text{ mM}$ ), rt. <sup>b</sup> Based on HPLC analyses.

**Table 2.** Reduction of disulfide **5e**,  $c_{\text{GSH}} = 2.5 \text{ mM}$ .<sup>a</sup>

Entry	Time [h]	Yield <sup>b</sup> <b>4</b> [%]	Yield <sup>b</sup> <b>5b</b> [%]	Conv. <sup>b</sup> <b>5e</b> [%]
1	1	29	46	75
2	2	31	55	86
3	3	26	60	86
4	4	20	65	85
5	5	10	72	82
6	6	4	76	80
7	24	0	81	81

<sup>a</sup> General conditions: substrate **5e** (1  $\mu\text{mol}$ ), GSH (10 equiv.), phosphate buffer pH = 7 (4 mL,  $c = 50 \text{ mM}$ ), rt. <sup>b</sup> Based on HPLC analyses.

**Table 3.** Reduction of disulfide **5e**,  $c_{\text{GSH}} = 5 \text{ mM}$ .<sup>a</sup>

Entry	Time [h]	Yield <sup>b</sup> <b>4</b> [%]	Yield <sup>b</sup> <b>5b</b> [%]	Conv. <sup>b</sup> <b>5e</b> [%]
1	1	53	41	94
2	2	56	39	95
3	3	51	43	94
4	4	44	48	92
5	5	33	59	92
6	6	22	68	90
7	24	0	90	90

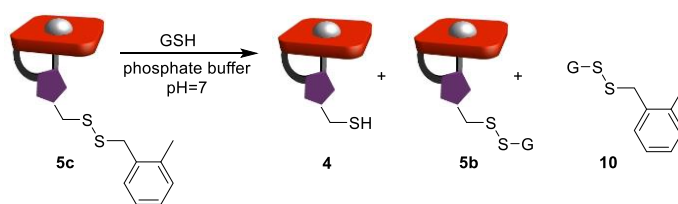
<sup>a</sup> General conditions: substrate **5e** (1  $\mu\text{mol}$ ), GSH (10 equiv.), phosphate buffer pH = 7 (2 mL,  $c = 50 \text{ mM}$ ), rt. <sup>b</sup> Based on HPLC analyses.

**Table 4.** Reduction of disulfide **5e**,  $c_{\text{GSH}} = 10 \text{ mM}$ .<sup>a</sup>

Entry	Time [h]	Yield <sup>b</sup> <b>4</b> [%]	Yield <sup>b</sup> <b>5b</b> [%]	Conv. <sup>b</sup> <b>5e</b> [%]
1	1	55	39	94
2	2	54	39	93
3	3	46	48	94
4	4	39	54	93
5	5	26	67	93
6	6	16	76	92
7	24	0	92	92

<sup>a</sup> General conditions: substrate **5e** (1  $\mu\text{mol}$ ), GSH (10 equiv.), phosphate buffer pH = 7 (1 mL,  $c = 50 \text{ mM}$ ), rt. <sup>b</sup> Based on HPLC analyses.

**Scheme 2. Reduction of disulfide **5c**.**



**Table 5. Reduction of disulfide **5c**,  $c_{\text{GSH}} = 1 \text{ mM}$ .<sup>a</sup>**

Entry	Time [h]	Yield <sup>b</sup> <b>4</b> [%]	Yield <sup>b</sup> <b>5b</b> [%]	Conv. <sup>b</sup> <b>5c</b> [%]
1	1	7	3	10
2	2	12	5	17
3	4	18	7	25
4	6	21	10	31
5	24	21	24	45
6	48	8	42	50

<sup>a</sup> General conditions: substrate **5c** (1  $\mu\text{mol}$ ), GSH (10 equiv.), phosphate buffer pH = 7 (10 mL,  $c = 50 \text{ mM}$ ), rt. <sup>b</sup> Based on HPLC analyses.

**Table 6. Reduction of disulfide **5c**,  $c_{\text{GSH}} = 5 \text{ mM}$ .<sup>a</sup>**

Entry	Time [h]	Yield <sup>b</sup> <b>4</b> [%]	Yield <sup>b</sup> <b>5b</b> [%]	Conv. <sup>b</sup> <b>5c</b> [%]
1	1	23	10	33
2	2	37	7	44
3	4	50	12	62
4	6	51	28	79
5	24	21	68	89
6	48	0	95	95

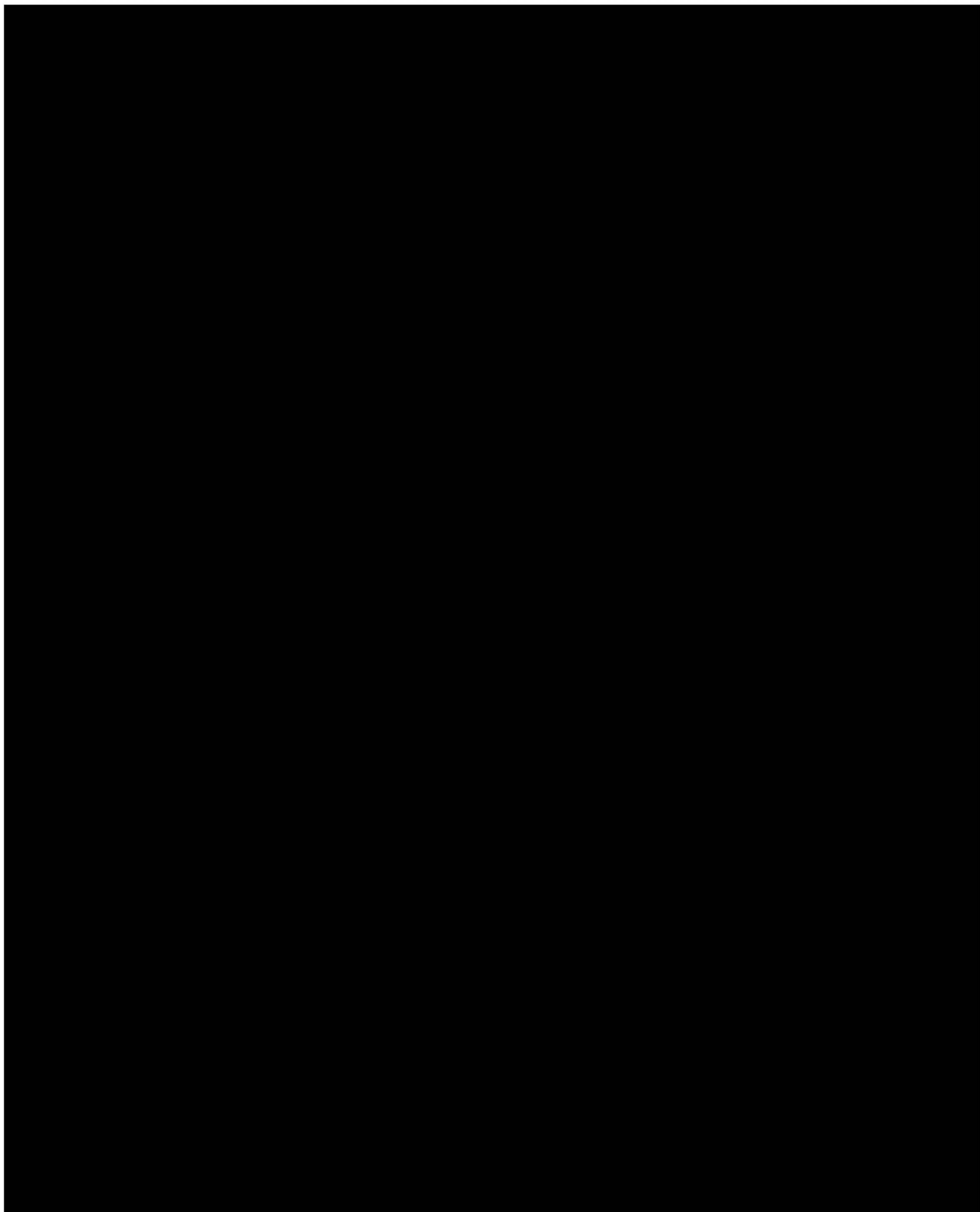
<sup>a</sup> General conditions: substrate **5c** (1  $\mu\text{mol}$ ), GSH (10 equiv.), phosphate buffer pH = 7 (2 mL,  $c = 50 \text{ mM}$ ), rt. <sup>b</sup> Based on HPLC analyses.

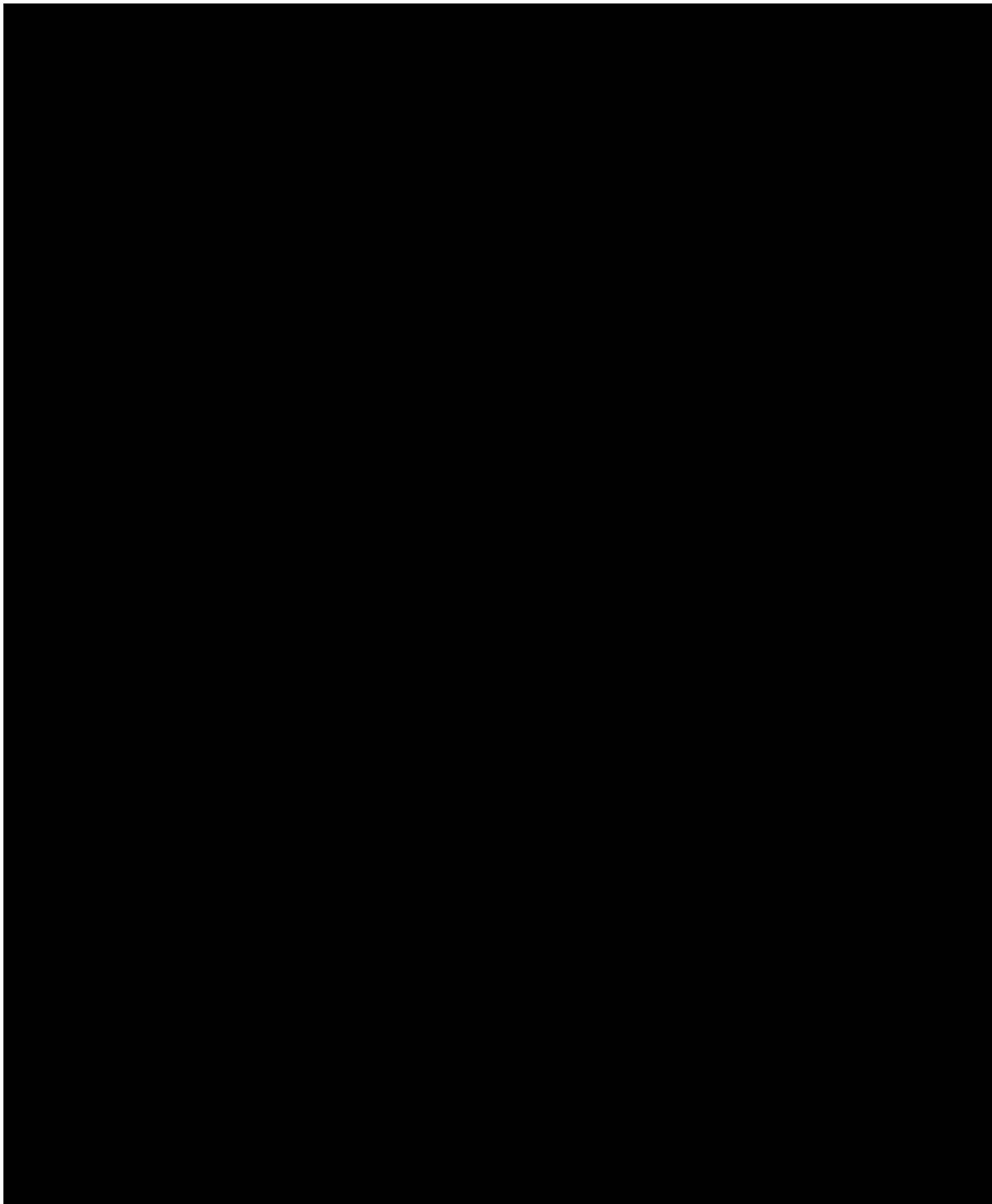
■ Natural Products

## *meso*-Modified Cobalamins: Synthesis, Structure, and Properties

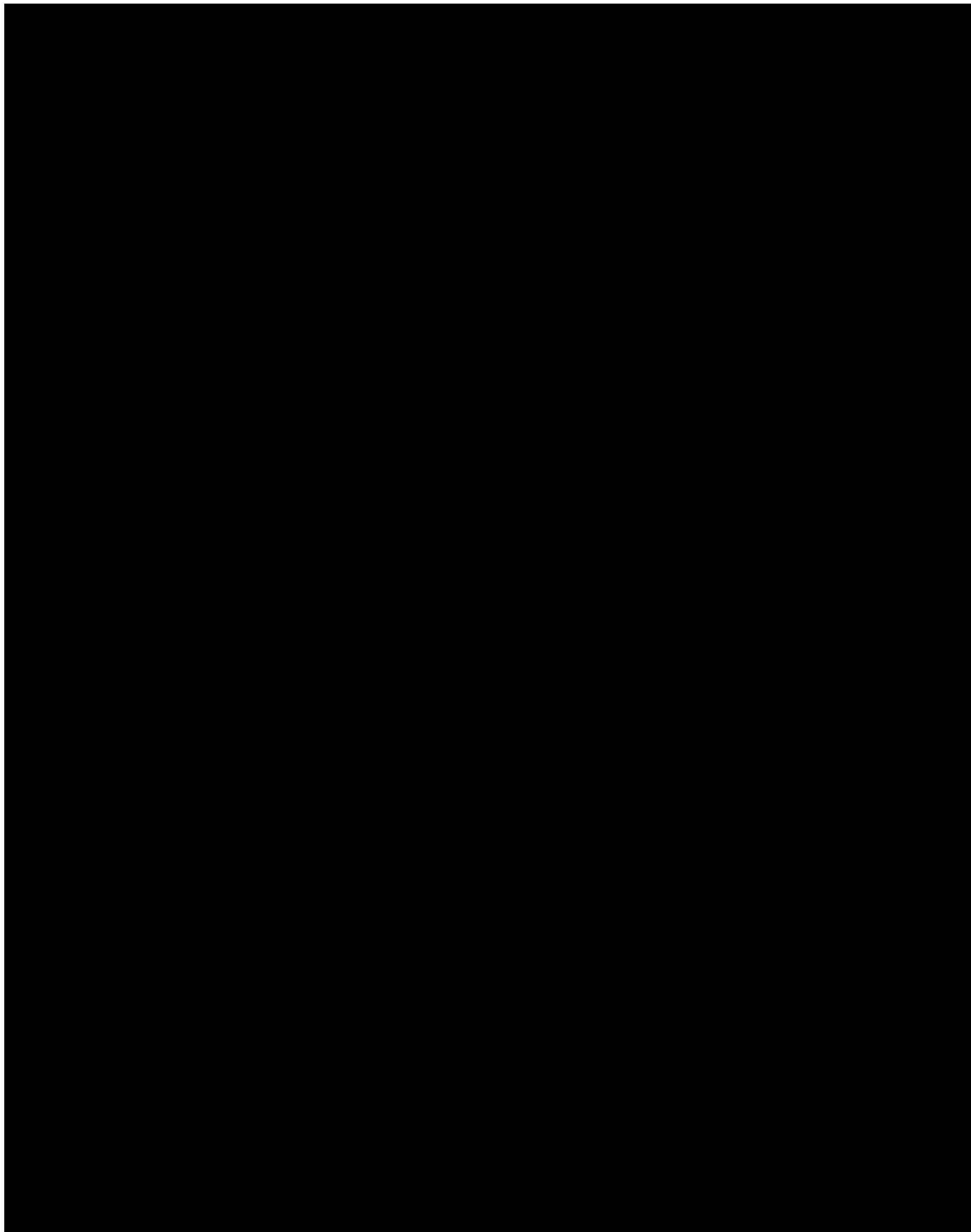
Aleksandra J. Wierzba,<sup>[a]</sup> Aleksandra Wincenciuk,<sup>[a, b]</sup> Maksymilian Karczewski,<sup>[a]</sup>  
Valentine I. Vullev,<sup>\*[c]</sup> and Dorota Gryko<sup>\*[a]</sup>

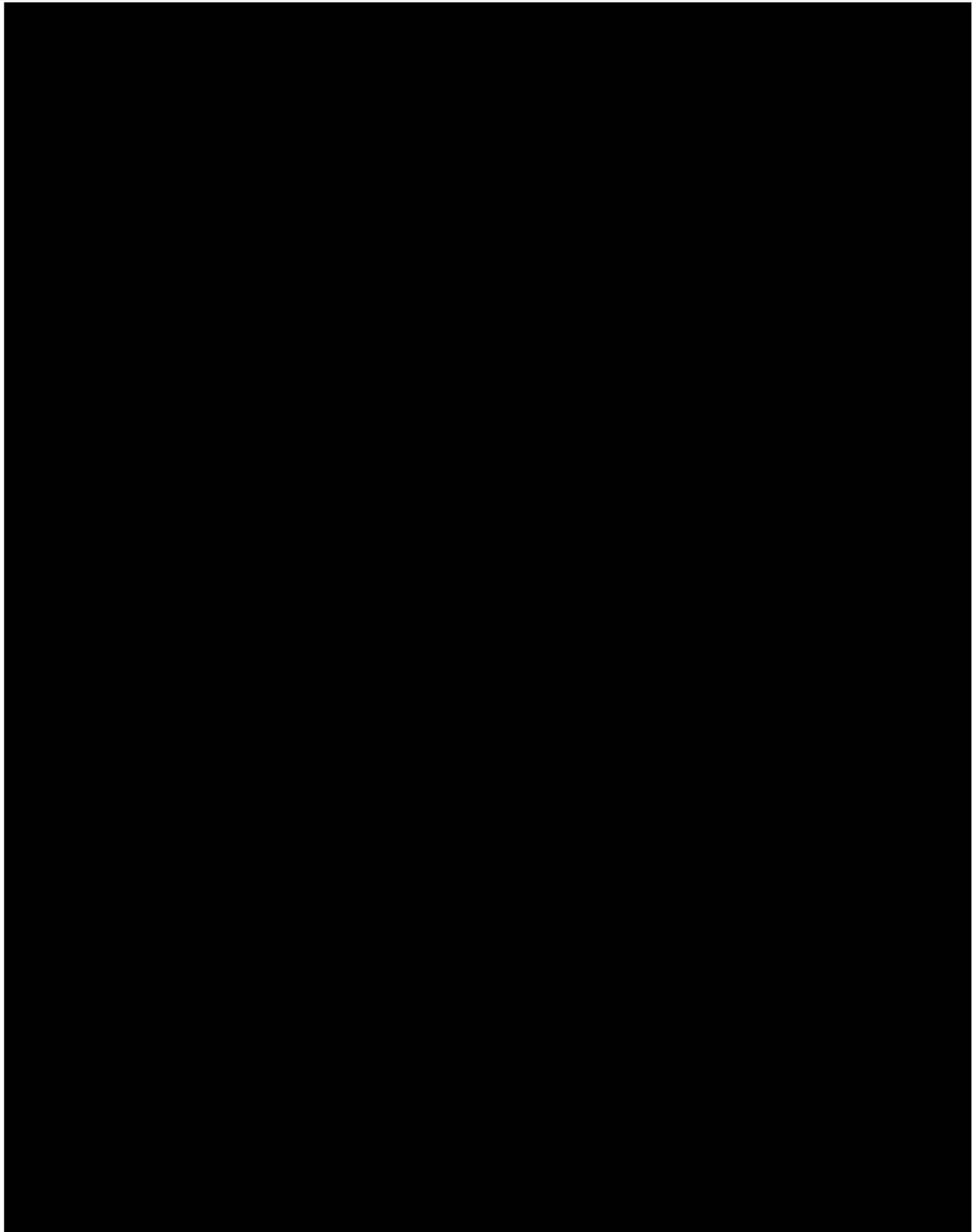


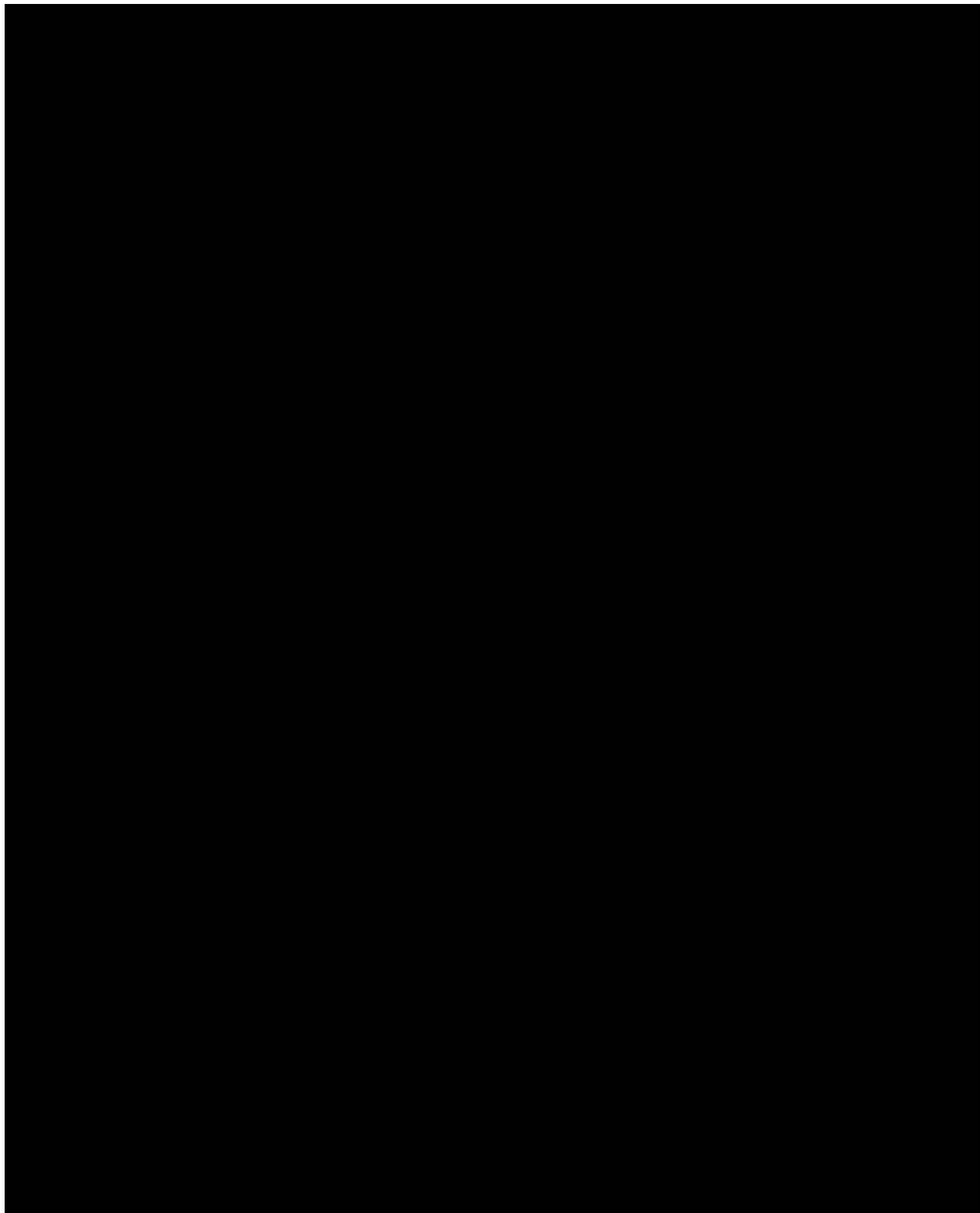


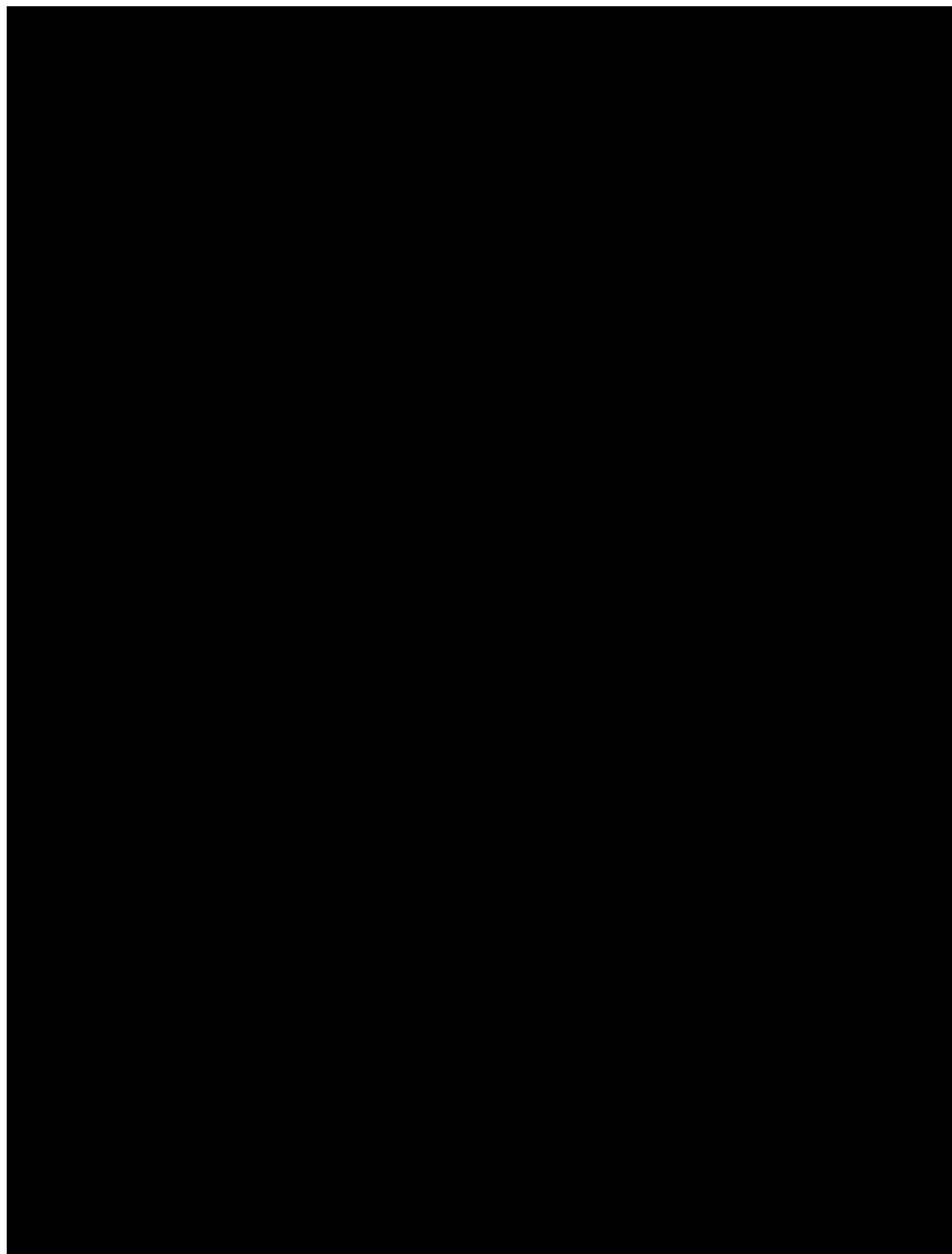


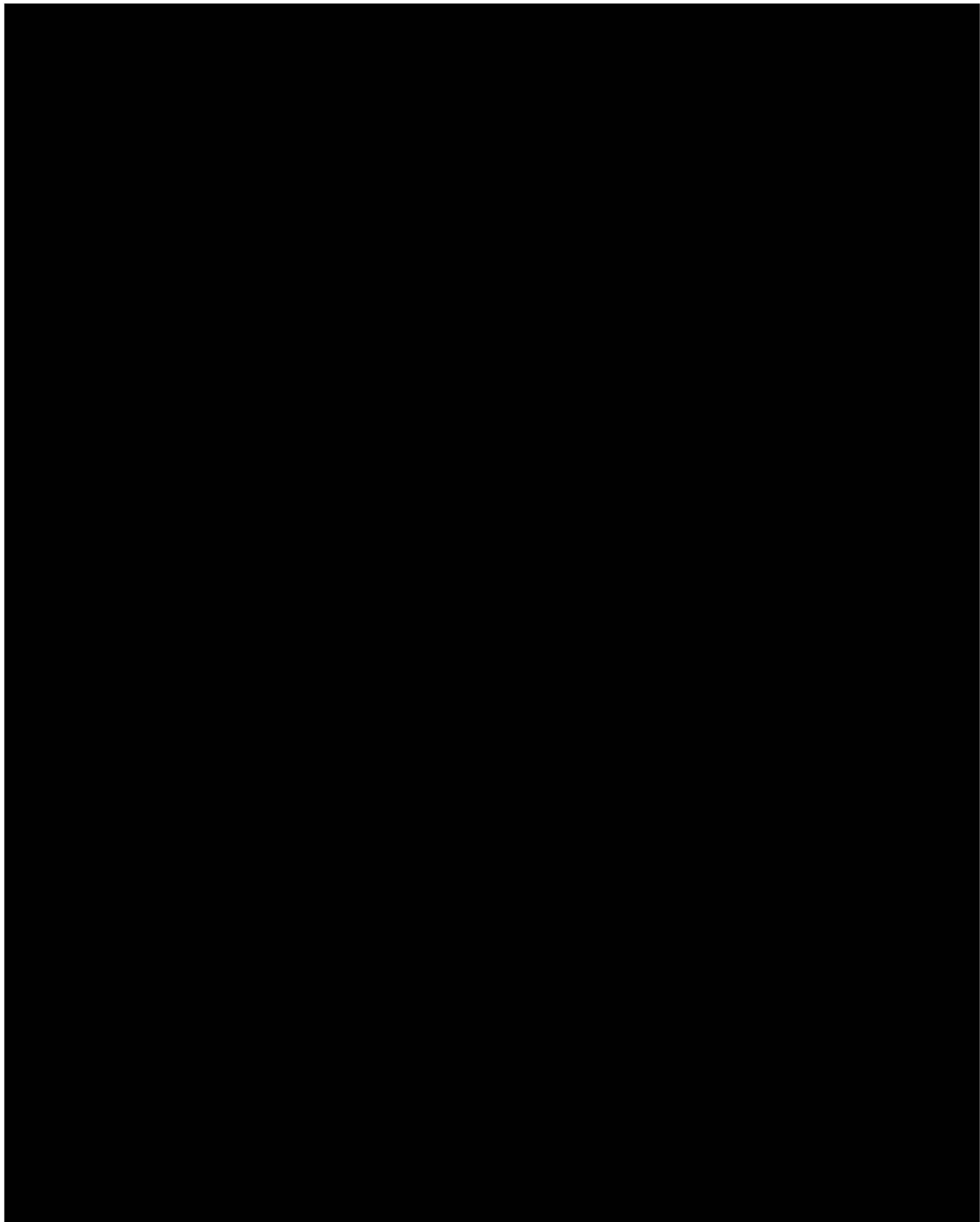


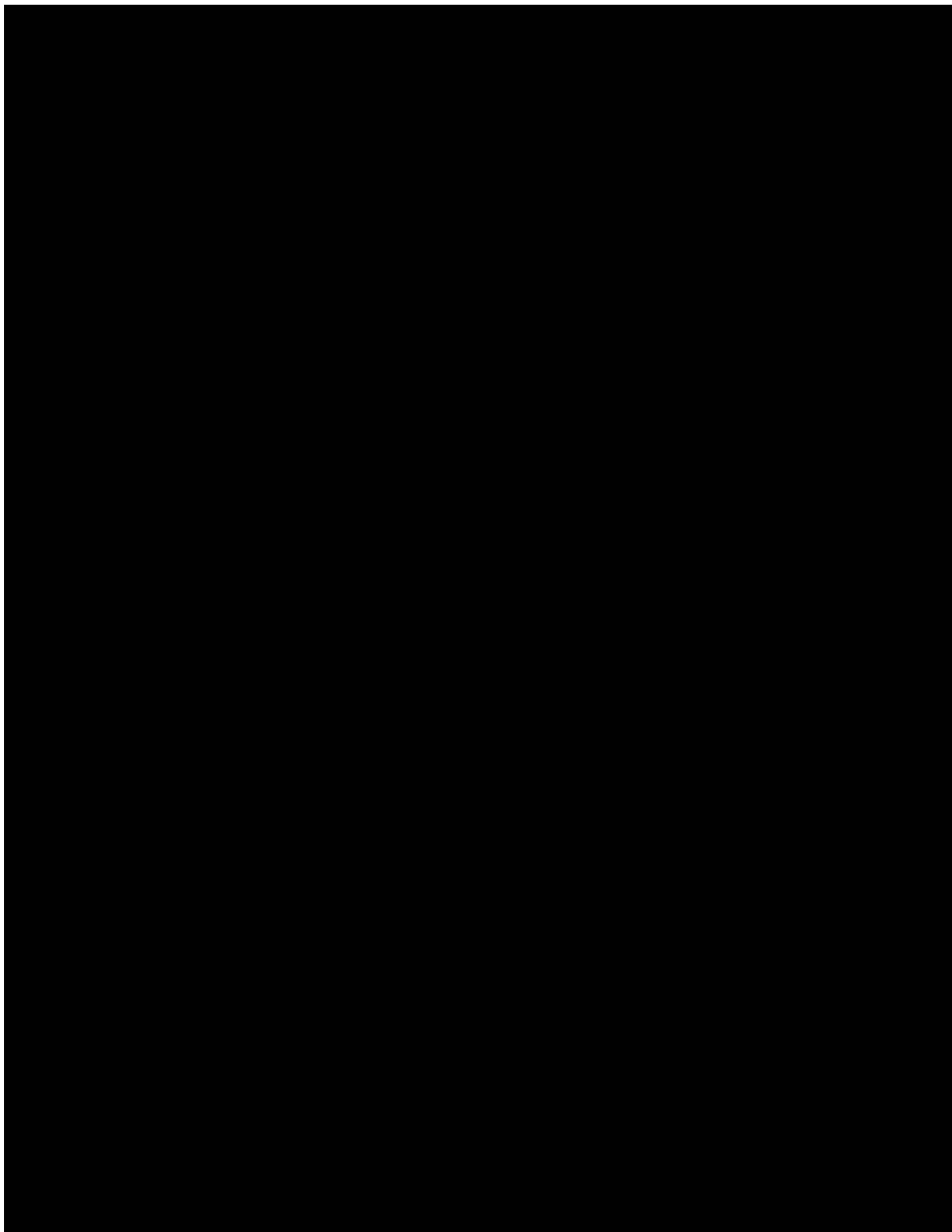


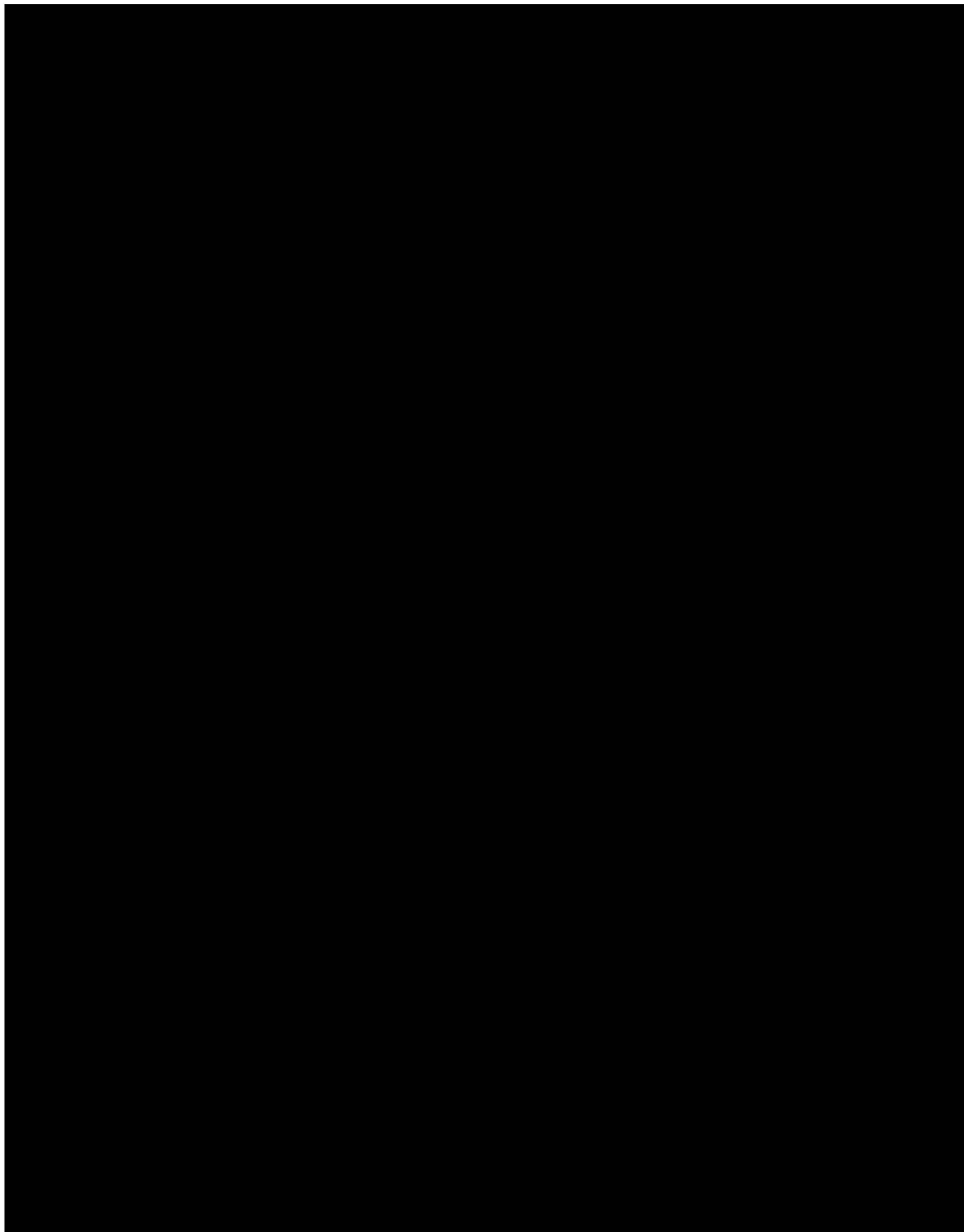


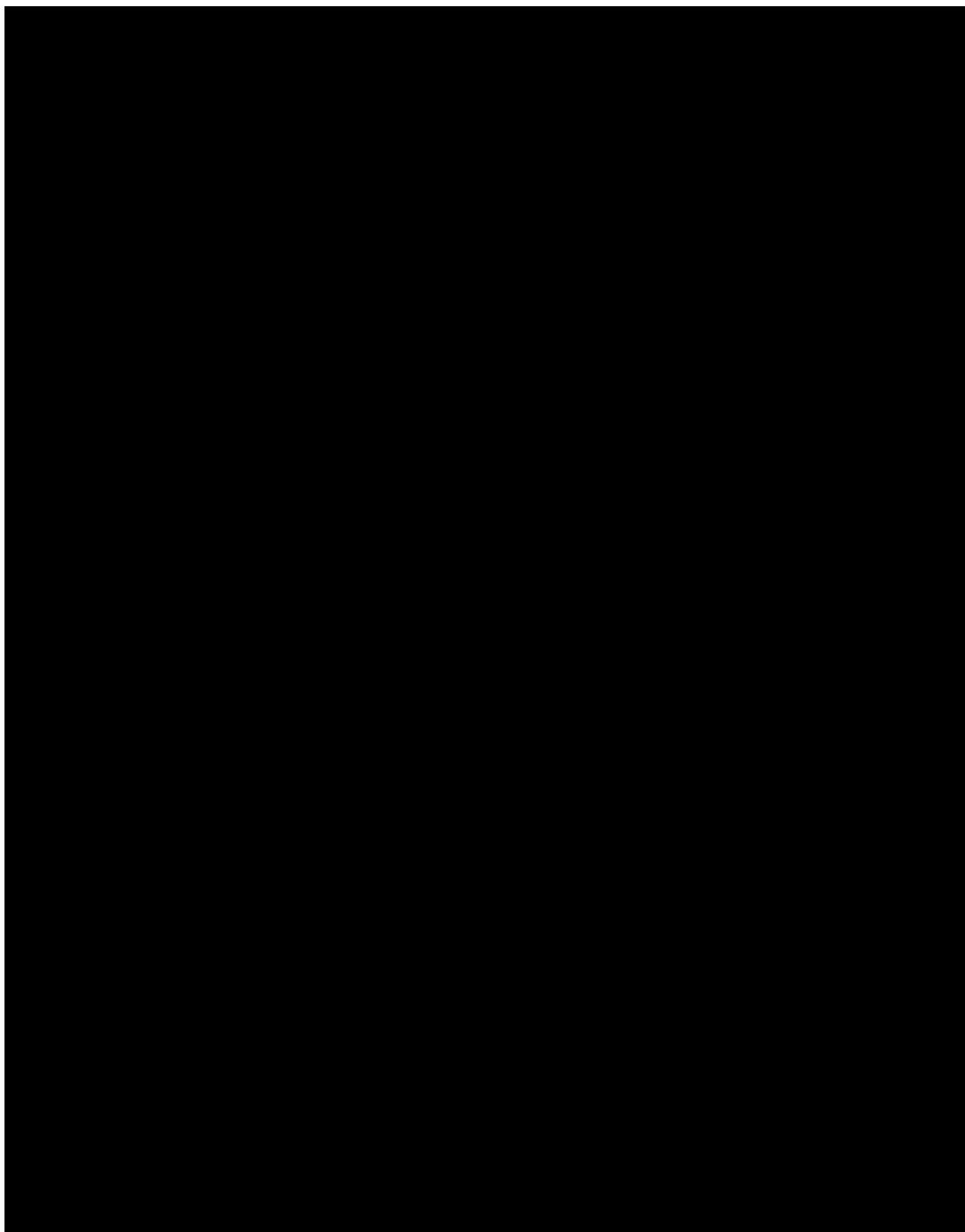




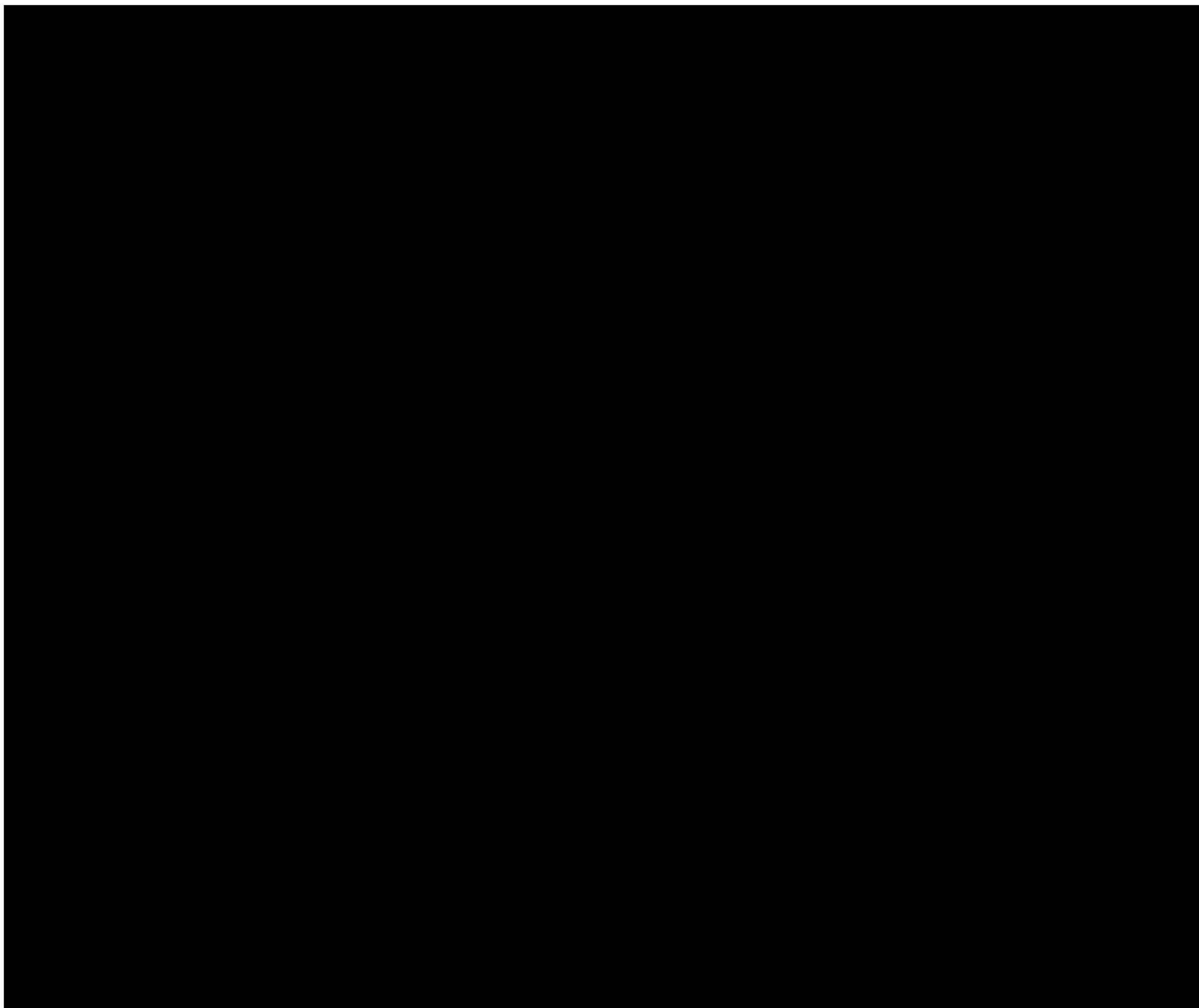












# CHEMISTRY

## A **European** Journal

### Supporting Information

#### ***meso*-Modified Cobalamins: Synthesis, Structure, and Properties**

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Valentine I. Vullev,<sup>\*[c]</sup> and Dorota Gryko<sup>\*[a]</sup>

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## S1. Nitration methods

Several procedures for the nitration of (CN)Cbl (**1**) were applied, however most of them led either to the decomposition or to the recovery of the starting material **1** (Table S .

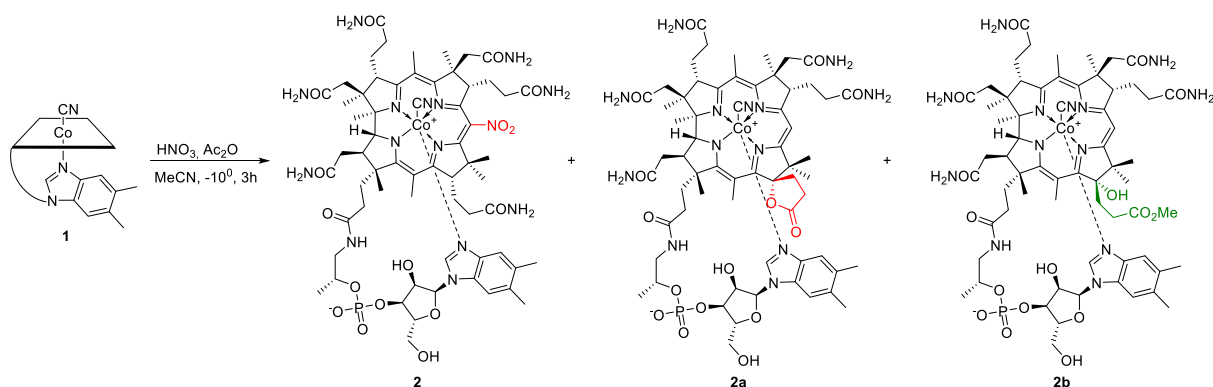
Table S1. Nitration methods.

Entry	Nitrating reagent	Observation
1	HNO <sub>3</sub> (65%), H <sub>2</sub> SO <sub>4</sub>	decomposition
2 <sup>[a]</sup>	NO <sub>2</sub> BF <sub>4</sub> , AcOH	decomposition
3 <sup>[b]</sup>	NaNO <sub>2</sub> , TFA	decomposition
4 <sup>[c]</sup>	AgNO <sub>3</sub> , AcOH	substrate recovery
5 <sup>[d]</sup>	Cu(NO <sub>3</sub> ) <sub>2</sub> ·3H <sub>2</sub> O, AcOH	complex mixture, no product
6 <sup>[e]</sup>	HNO <sub>3</sub> (99%), Ac <sub>2</sub> O	decomposition

[a] *Liebigs Ann. Chem.* **1977**, 1480 [b] *Tetrahedron* **2004**, 60, 2757; *JPP* **2011**, 15, 1087. [c] *JPP* **2006**, 10, 1319. [d] *J. Heterocyclic Chem.* **2010**, 47, 1221; *ARKIVOC* **2014**, 5, 64 [e] *Bull. Acad. Sci. USSR Div. Chem. Sci.* **1965**, 14, 90.

## S2. Determination of (CN)Cbl-*epi*-(*e*-lactone) (**2a**) structure

Nitration of cobalamin (**1**) in the optimal conditions (see manuscript: Table 1, entry 4 and Experimental Section: Synthesis of (CN)Cbl(10-NO<sub>2</sub>)) led to the formation of (CN)Cbl(10-NO<sub>2</sub>) (**2**) and compound **2a** – a main by-product. When MeOH was used during the work-up instead of H<sub>2</sub>O, product (**2b**) formed additionally (Scheme S1). Crystallization of compound **2b** by vapor diffusion of acetone into its water solution gave crystals suitable for X-ray analysis (see S4). Compound **2b** proved to be the opened form of *e*-lactone **2a** with the reversed configuration at C-13. The ring-opening occurred during the removal of crude **2** from the cotton wool with MeOH in the presence of traces of a nitrating mixture (for details see manuscript, Experimental Section). Compounds **2a** and **2b** were fully characterized by NMR and HR MS experiments. The structure of (CN)Cbl-*epi*-(*e*-lactone) (**2a**) was additionally confirmed by the 2D NMR experiments (see S19). The yield of the **2a** varies from 33 to 37% under the optimal conditions developed for the nitration of (CN)Cbl (**1**) (see manuscript, Table 1, entry 4).



Scheme S1. Nitration of cobalamin (**1**). For detailed procedure see Experimental Section of the manuscript part: Synthesis of (CN)Cbl(10-NO<sub>2</sub>).

### Characterization of by-products:

Compound **2a**: For full assignment of signals in 2D NMR spectra see S19; UV/Vis (H<sub>2</sub>O):  $\lambda^{\max}$  ( $\epsilon$ ) = 557 ( $7.7 \times 10^3$ ), 361 ( $2.0 \times 10^4$ ), 221 ( $4.5 \times 10^4 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$ ); HRMS (ESI)  $m/z$   $[M + 2\text{Na}]^{2+}$  calcd for C<sub>63</sub>H<sub>85</sub>CoN<sub>13</sub>O<sub>15</sub>PNa<sub>2</sub> 699.7571, found 699.7601; elemental analysis calcd (%) for C<sub>63</sub>H<sub>85</sub>CoN<sub>13</sub>O<sub>15</sub>P·11H<sub>2</sub>O: C 48.74, H 6.95, N 11.73, found: C 48.82, H 6.60, N 11.70;  $t_R$  (RP-HPLC, from 10 % MeCN/H<sub>2</sub>O + 0.02 % TFA to 70 % MeCN/H<sub>2</sub>O + 0.02 % TFA in 15 min): 5.80 min.

Compound **2b**: <sup>1</sup>H NMR (500 MHz, 30°C, [D<sub>4</sub>]MeOH)  $\delta$  = 7.28 (s, 1H), 7.17 (s, 1H), 6.55 (s, 1H), 6.28 (d,  $J$  = 3.4 Hz, 1H), 5.79 (s, 1H), 4.67 – 4.63 (m, 1H), 4.50 (d,  $J$  = 8.6 Hz, 1H), 4.38 – 4.30 (m, 1H), 4.28 – 4.23 (m, 2H), 4.13 – 4.08 (m, 1H), 3.89 (dd,  $J$  = 12.3, 3.1 Hz, 1H), 3.76 (dd,  $J$  = 12.3, 4.3 Hz, 1H), 3.69 (d,  $J$  = 13.8 Hz, 1H), 3.63 – 3.53 (m, 1H), 3.57 (s, 1H), 2.87 – 2.74 (m, 2H), 2.78 (s, 3H), 2.70 – 2.33 (m, 11H), 2.55 (s, 3H) 2.28 (s, 6H), 2.24 – 2.13 (m, 2H), 2.12 – 2.00 (m, 3H), 1.87 (s, 3H), 1.99 – 1.76 (m, 5H), 1.75 – 1.65 (m, 1H), 1.43 (s, 3H), 1.37 (s, 3H), 1.30 (s, 3H), 1.26 (d,  $J$  = 6.3 Hz, 3H), 1.21 – 1.11 (m, 2H), 1.14 (s, 3H), 0.39 (s, 3H); <sup>13</sup>C NMR (126 MHz, 30°C, [D<sub>4</sub>]MeOH)  $\delta$  = 181.68, 180.95, 177.35, 176.62, 175.49, 175.05, 174.99, 174.65, 174.34, 173.91, 166.89, 161.61, 144.18, 138.51, 135.78, 133.85, 131.64, 117.80, 112.48, 109.94, 109.49, 95.03, 87.39, 87.30, 86.85, 83.18, 76.56, 75.35, 73.84, 73.78, 70.87, 62.37, 60.52, 57.16, 56.30, 53.38, 52.98, 52.19, 46.74, 44.31, 43.20, 40.52, 36.20, 34.73, 33.43, 32.84, 32.78, 31.94, 30.40, 27.64, 27.12, 26.78, 20.91, 20.30, 20.21, 20.18, 20.00, 19.86, 18.63, 17.64, 16.42, 16.12, 12.65; UV/Vis (H<sub>2</sub>O):  $\lambda^{\max}$  ( $\epsilon$ ) = 544 ( $6.9 \times 10^3$ ), 522 ( $6.9 \times 10^3$ ), 361 ( $1.7 \times 10^4$ ), 220 ( $3.9 \times 10^4 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$ ); HRMS (ESI)  $m/z$   $[M - \text{H}]^-$  calcd for C<sub>64</sub>H<sub>88</sub>CoN<sub>13</sub>O<sub>16</sub>PCo 1384.5542, found 1384.5507; elemental analysis calcd (%) for C<sub>64</sub>H<sub>89</sub>CoN<sub>13</sub>O<sub>16</sub>P·10H<sub>2</sub>O: C 49.07, H 7.01, N 11.62; found: C 48.91, H 6.60, N 11.50;  $t_R$  (RP-HPLC, from 10 % MeCN/H<sub>2</sub>O + 0.02 % TFA to 70 % MeCN/H<sub>2</sub>O + 0.02 % TFA in 15 min): 6.22 min.

### S3. Purification of (CN)Cbl(10-NO<sub>2</sub>) (2)

Compound **2** was purified by RP column chromatography with MeCN/H<sub>2</sub>O (10% v/v). The first of the most intense red bands was found to be (CN)Cbl-*epi*-(*e*-lactone) (**2a**) (see S2 and S19) while the third band (of a red-brick color) comprised (CN)Cbl(10-NO<sub>2</sub>) (**2**) (Figure S1).

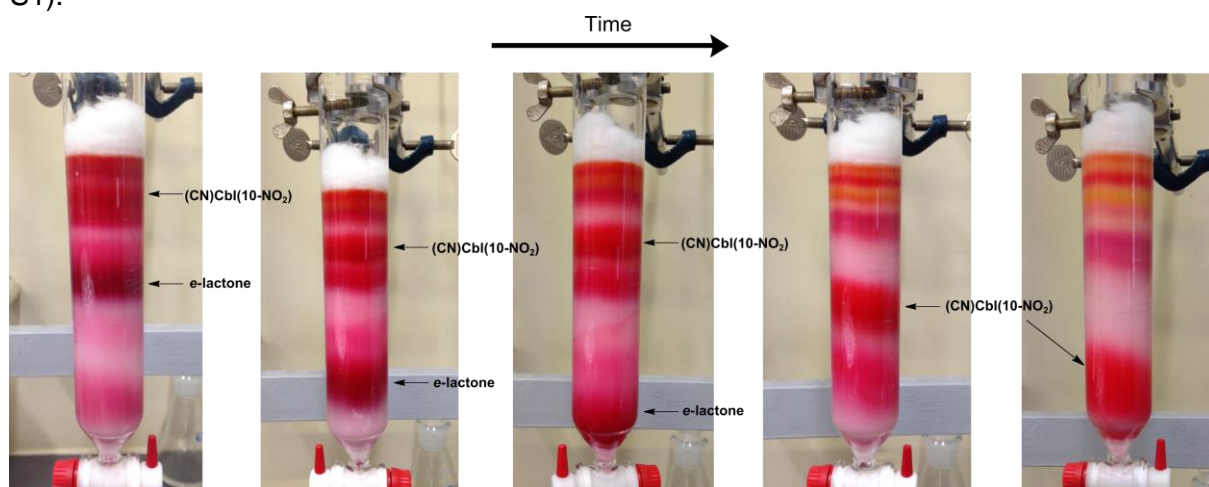


Figure S1. Purification of compound **2** with RP chromatography (stationary phase: LiChroprep® RP-18 (40-63 $\mu$ m)). Solvent system: MeCN/H<sub>2</sub>O (10% v/v).

## S4. X-ray analyses

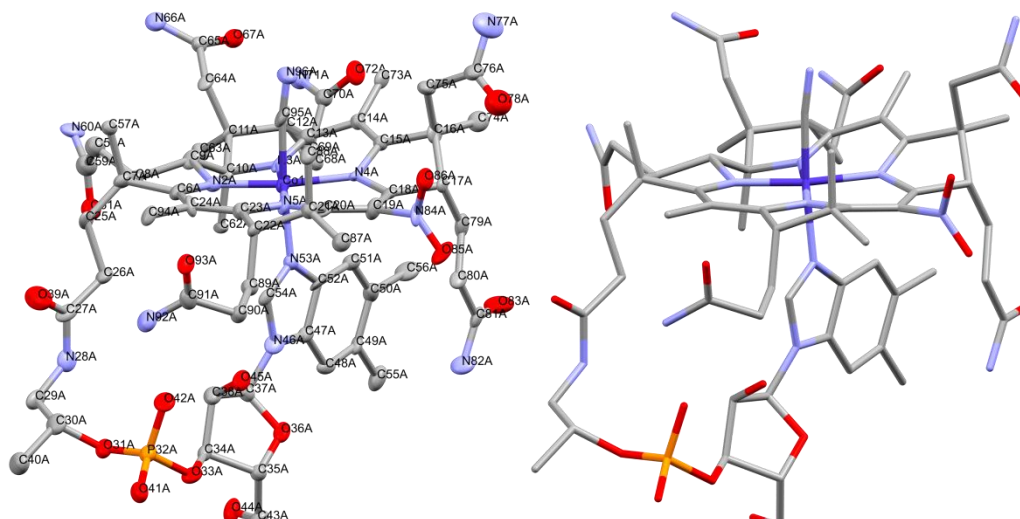
### a) (CN)Cbl(10-NO<sub>2</sub>) (2)

The single-crystal of investigated compound was selected for the X-ray diffraction experiment at  $T = 100(2)$  K. The crystal was mounted with the paratone-N oil to the MiTeGen micromount. Diffraction data were collected on the Agilent Technologies SuperNova Dual Source with the  $\text{CuK}\alpha$  radiation ( $\lambda = 1.54184$  Å). The lattice parameters were obtained by least-squares fit to the optimized setting angles of the reflections collected by using the CrysAlis CCD software.<sup>[1]</sup> Data were reduced using the CrysAlis RED program.<sup>[1]</sup> The multi-scan empirical absorption correction using spherical harmonics, implemented in SCALE3 ABSPACK scaling algorithm was applied.<sup>[1]</sup> The structural determination procedure was carried out using the SHELX package.<sup>[2]</sup> The structures were solved with direct methods, and then successive least-squares refinements were carried out based on full-matrix least-squares on  $F^2$  using the XLMP program.<sup>[2]</sup> The H-atoms bonded to O-atoms of water molecules

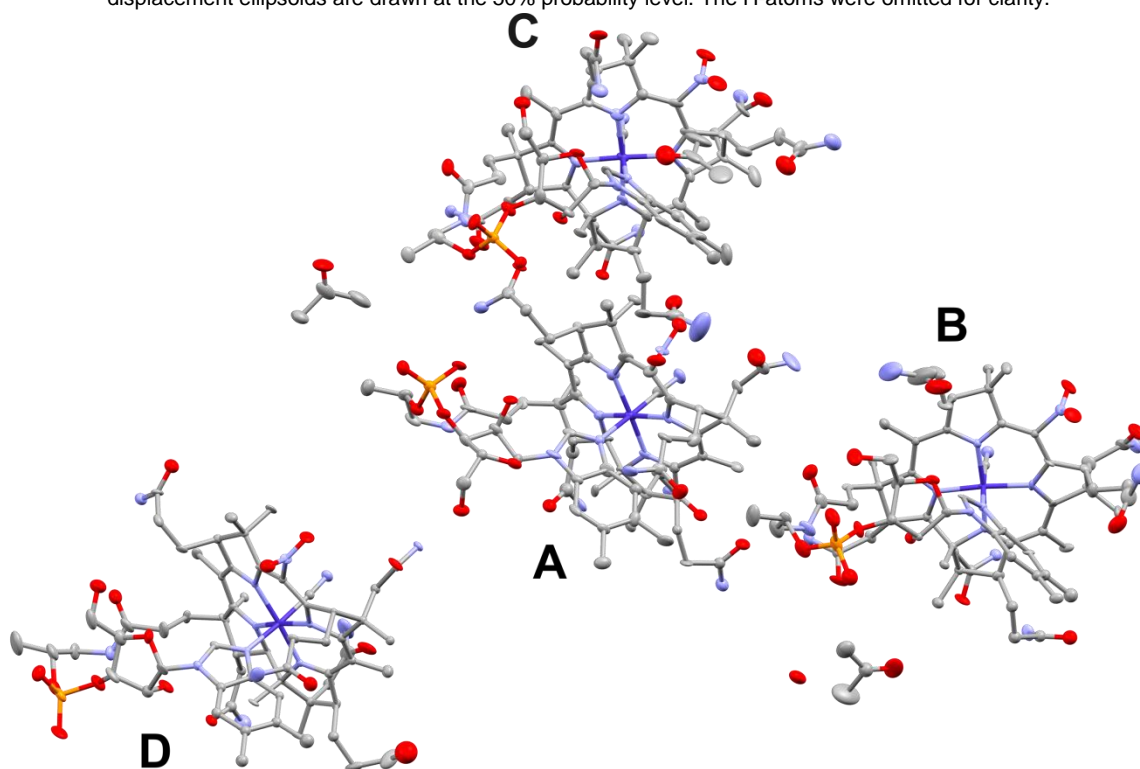
g

with  $U_{\text{iso}}(\text{H}) = 1.5U_{\text{eq}}(\text{N})$ , and were restrained with DFIX 0.86 and 1.39 Å for O–H distances and H···H distance, respectively. Remaining H-atoms bonded to O and N-atoms were positioned geometrically for the O–H and N–H bond lengths equal to 0.82 and 0.86 Å, respectively, and constrained to ride on their parent atoms with  $U_{\text{iso}}(\text{H}) = xU_{\text{eq}}(\text{O,N})$ , where  $x$  was 1.5 for the hydroxyl and 1.2 for the amine H-atoms, respectively. All the H-atoms bound to the C-atoms were positioned geometrically with the C–H bond length equal to 0.93, 0.96, 0.97 and 0.98 Å for the aromatic, methyl, methylene and methine hydrogen atoms, respectively, and constrained to ride on their parent atoms with  $U_{\text{iso}}(\text{H}) = xU_{\text{eq}}(\text{C})$ , where  $x$  was 1.5 for the methyl and 1.2 for the aromatic, methylene and methine H-atoms, respectively. The C8A–C58A and C58A–C59A distances were restrained using DFIX 1.54 Å. The distances C59A–N60A and C81A–N82A were restrained using DFIX 1.325 Å, and the C1E–O4E, C59A–O61A and C70D–O72D distances using DFIX 1.21 Å, respectively. The 148 ISOR restraints were also applied (detailed list of restraints used can be found in attached CIF-file). Several distinct peaks on the difference Fourier map are indicating the presence of additional solvent molecules. Unfortunately, all attempts to properly model these molecules failed. Therefore, this solvent contribution has been removed applying the appropriate MASK procedure in Olex2 program.<sup>[3]</sup> Calculated total solvent accessible volume/cell was 2332.0 Å<sup>3</sup> (15.3%) occupied by the 789.4 electrons/cell. The figures were prepared using Olex2 and Mercury programs.<sup>[3,4]</sup>

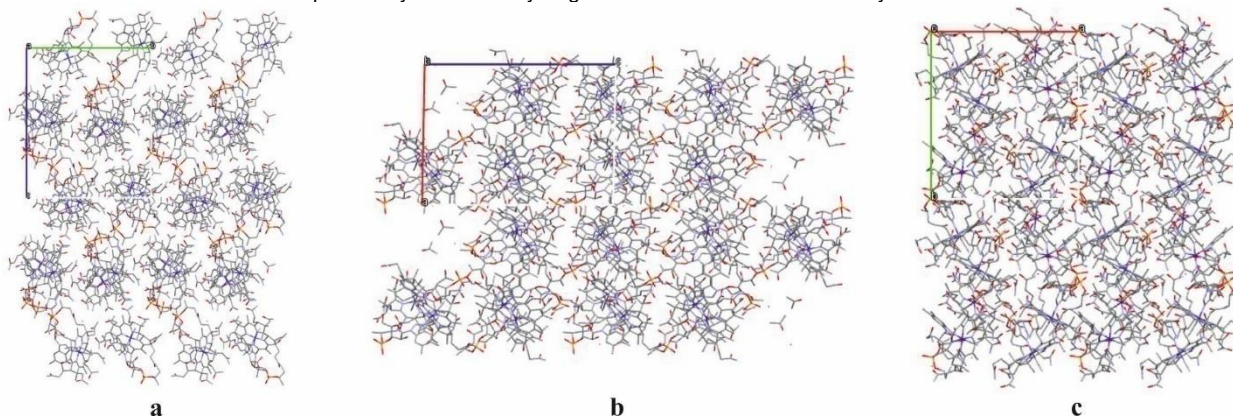
The identity of compound was proven by the single-crystal X-ray diffraction analysis (Figure S2). Compound **2** crystallizes in the monoclinic  $P2_1$  space group with four molecules of compound **2** in the asymmetric part of the crystal lattice. The asymmetric unit also possesses three molecules of acetone and two molecules of water (Figure S3). Configuration of molecules A, B, C and D of given compound is the same, however alignment of their molecular skeletons reveals that they have quite different geometry (Figure S5). The crystallographic data is summarized in the Table S2. The arrangement of molecules in the crystal of compound **2** is presented on the Figure S4. Differences between the values of the axial bond lengths and folding angles of the corrin ring for molecules A – D are given in the Table S3.



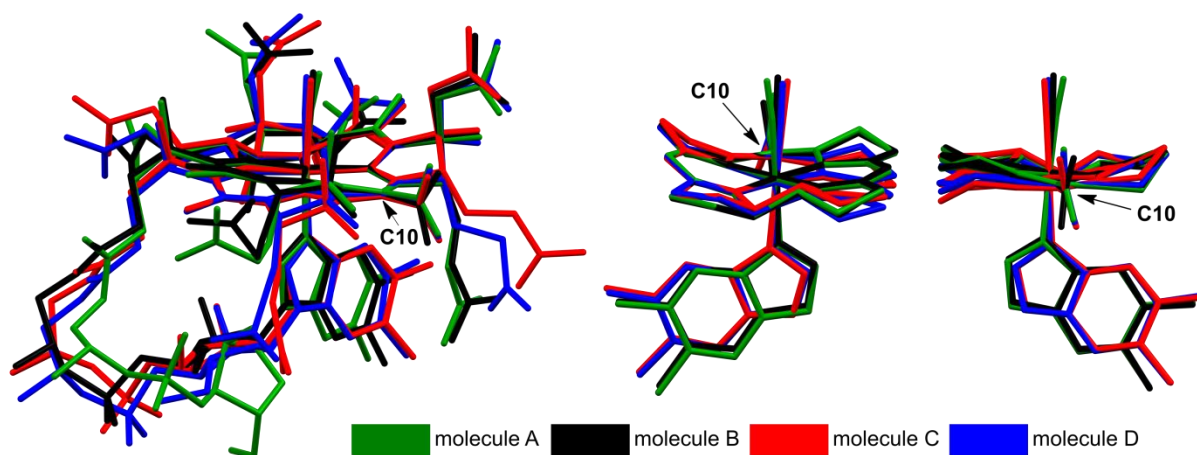
**Figure S2.** Molecular structure of the investigated compound (molecule A) showing the atom labeling scheme. Atomic displacement ellipsoids are drawn at the 50% probability level. The H-atoms were omitted for clarity.



**Figure S3.** The asymmetric part of the unit cell of the investigated compound. Displacement ellipsoids are drawn at the 50% probability level. The hydrogen atoms were omitted for clarity.



**Figure S4.** The arrangement of molecules in the crystal of investigated compound, viewed along a, b and c-direction. The H-atoms were omitted for clarity.



**Figure S5.** Superposition of molecules present in the asymmetric part of the crystal lattice of compound **2**. Corrin substituents (except for the *meso*-substituent) were omitted for clarity. Superposition at atoms: N21, C4, C5, C6, N22, C9, C10.

**Table S2.** Crystal data and structure refinement details for (CN)Cbl(10-NO<sub>2</sub>)

Compound	(CN)Cbl(10-NO <sub>2</sub> )
CCDC deposition code	CCDC 1584989
Empirical formula	4(C <sub>63</sub> H <sub>87</sub> CoN <sub>15</sub> O <sub>16</sub> P), 3(C <sub>3</sub> H <sub>6</sub> O), 2(H <sub>2</sub> O)
Formula weight	5811.77
Temperature/K	100(2)
Crystal system	monoclinic
Space group	<i>P</i> 2 <sub>1</sub>
<i>a</i> /Å	21.4297(5)
<i>b</i> /Å	24.3163(4)
<i>c</i> /Å	29.2432(7)
<i>α</i> /°	90.00
<i>β</i> /°	91.114(2)
<i>γ</i> /°	90.00
Volume/Å <sup>3</sup>	15235.5(6)
<i>Z</i>	2
$\rho_{\text{calc}}$ /cm <sup>3</sup>	1.267
$\mu$ /mm <sup>-1</sup>	2.575
<i>F</i> (000)	6152.0
Crystal size/mm	0.75 × 0.47 × 0.07
Radiation	CuK $\alpha$ ( $\lambda$ = 1.54184)
2 $\theta$ range for data collection/°	4.72 to 134.16
Index ranges	-26 ≤ <i>h</i> ≤ 25, -28 ≤ <i>k</i> ≤ 29, -34 ≤ <i>l</i> ≤ 35
Reflections collected	137327
Independent reflections	53244 [ <i>R</i> <sub>int</sub> = 0.0722, <i>R</i> <sub>sigma</sub> = 0.0927]
Data/restraints/parameters	53244/903/3650
Goodness-of-fit on <i>F</i> <sup>2</sup>	1.014
Final <i>R</i> indexes [ <i>I</i> > 2 $\sigma$ ( <i>I</i> )]	<i>R</i> <sub>1</sub> = 0.1092, <i>wR</i> <sub>2</sub> = 0.2815
Final <i>R</i> indexes [all data]	<i>R</i> <sub>1</sub> = 0.1182, <i>wR</i> <sub>2</sub> = 0.2916
Largest diff. peak/hole / e Å <sup>-3</sup>	2.90/-1.03
Flack parameter	0.086(3)

**Table S3.** The lengths of the axial bonds and value of the folding angle for compound **2**

Molecule	Co-CN [Å]	Co-N <sub>DMBI</sub> [Å]	CN-N <sub>DMBI</sub> [Å]	Folding angle [°]
A	1.855(9)	2.131(6)	3.980	
B	1.861(7)	2.075(6)	3.932	13.06
C	1.839(7)	2.075(6)	3.907	11.63
D	1.879(7)	2.026(5)	3.896	26.47



## b) Compound 2b

The data for compound **2b** was collected at 100 K using the P13 Macromolecular Crystallography beamline at the PETRA III Synchrotron in Hamburg (Research Centre of the Helmholtz Association,  $\lambda = 0.7293 \text{ \AA}$ ) and processed with XDS. The structure was solved by direct methods using SHELXS and was refined by full-matrix least squares on  $F^2$  using SHELXL.<sup>[2]</sup> All non-hydrogen atoms were refined with anisotropic displacement parameters. The hydrogen atoms were introduced at geometrically idealized coordinates with fixed isotropic displacement parameters.

Compound **2b** crystallizes in orthorhombic space group  $P2_12_12_1$ , the usual space group for  $B_{12}$  derivatives (Figure S6, Table S4). The upper  $\beta$  axial ligand–Co bond was found to be longer than for (CN)Cbl (1.882 vs. 1.861 Å) and the  $\alpha$  axial ligand–Co bond was determined to be 2.049 vs. 2.011 Å (Table S5). Significant difference for the value of the folding angle of the corrin ring when comparing it to the native CN(Cbl) was observed (24.76° vs. 18°, Table S5). This result is consistent with the data reported for cyano-8-*epi*-cobalamin.<sup>[5]</sup>

**Table S4.** Crystal data and structure refinement details for compound **2b**.

Compound	2b
CCDC deposition code	CCDC 1576552
Empirical formula	$C_{66.53}H_{112.84}CoN_{13}O_{28.76}P$
Formula weight	1645.04
Temperature/K	100
Crystal system	orthorhombic
Space group	$P2_12_12_1$
$a/\text{\AA}$	15.3500(4)
$b/\text{\AA}$	21.9700(5)
$c/\text{\AA}$	24.520(2)
$\alpha/^\circ$	90.00
$\beta/^\circ$	90.00
$\gamma/^\circ$	90.00
Volume/ $\text{\AA}^3$	8269.1(8)
$Z$	4
$\rho_{\text{calc}}/\text{g/cm}^3$	1.321
$\mu/\text{mm}^{-1}$	0.331
$F(000)$	3501
Crystal size/mm	0.17 × 0.14 × 0.14
$2\theta$ range for data collection/ $^\circ$	1.277 to 29.277
Index ranges	$-20 \leq h \leq 20, -28 \leq k \leq 28, -32 \leq l \leq 32$
Reflections collected	20532
Independent reflections	18605 [ $R_{\text{int}} = 0.0645, R_{\text{sigma}} = 0.0409$ ]
Data/restraints/parameters	20532/104/1176
Goodness-of-fit on $F^2$	1.200
Final $R$ indexes [ $I > 2\sigma(I)$ ]	$R_1 = 0.0916, wR_2 = 0.2612$
Final $R$ indexes [all data]	$R_1 = 0.0949, wR_2 = 0.2661$
Largest diff. peak/hole / $e \text{ \AA}^{-3}$	1.021/-0.537
Flack parameter	0.094(3)

**Table S5.** The lengths of the axial bonds and value of the folding angle for compound **2b**.

Co-CN [Å]	Co-N <sub>DMBI</sub> [Å]	CN- N <sub>DMBI</sub> [Å]	Folding angle [°]
1.882(5)	2.049(5)	3.930	24.76

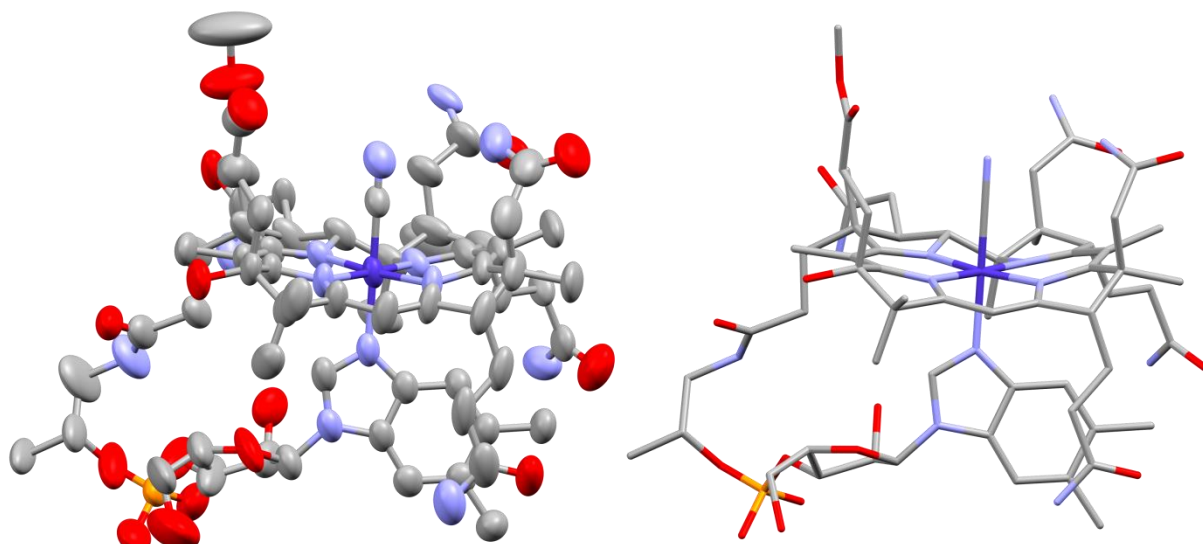


Figure S6. X-ray structure of compound **2b**.

### a) Compound **9**

The data for compound **9** was collected using the same conditions as for the compound **2b**. *Meso*-amide **9** crystallizes in  $P4_1$  (tetragonal) space group (Figure S7) with two molecules of compound **9** in the asymmetric part of the crystal lattice (Figure S8). Crystallographic and structural data for compound are summarized in Table S6 and Table S7.

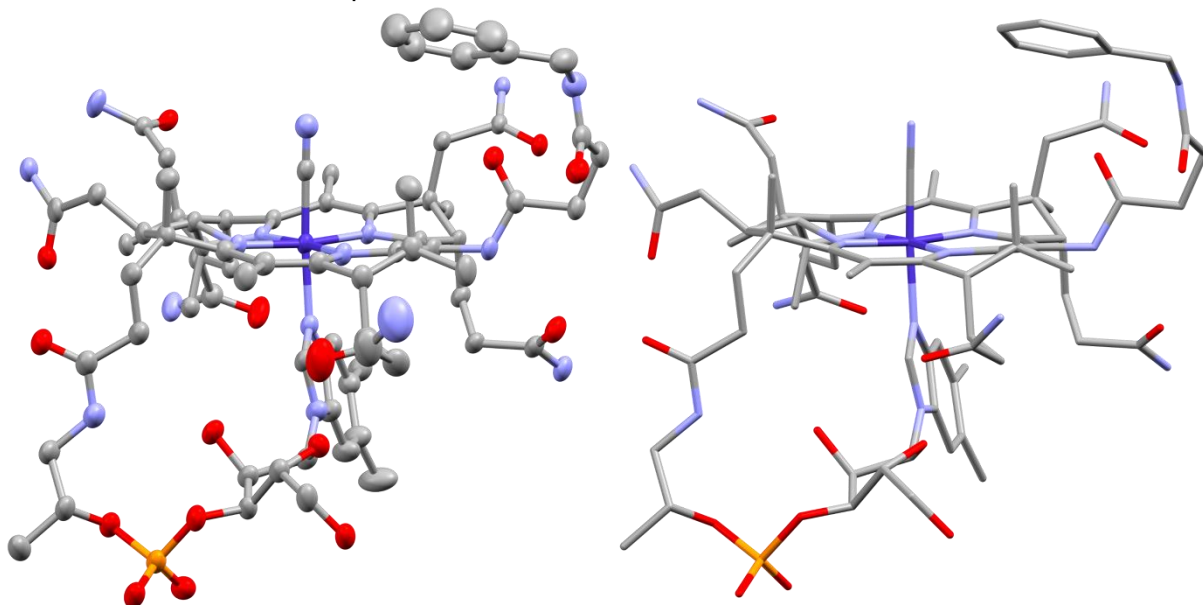
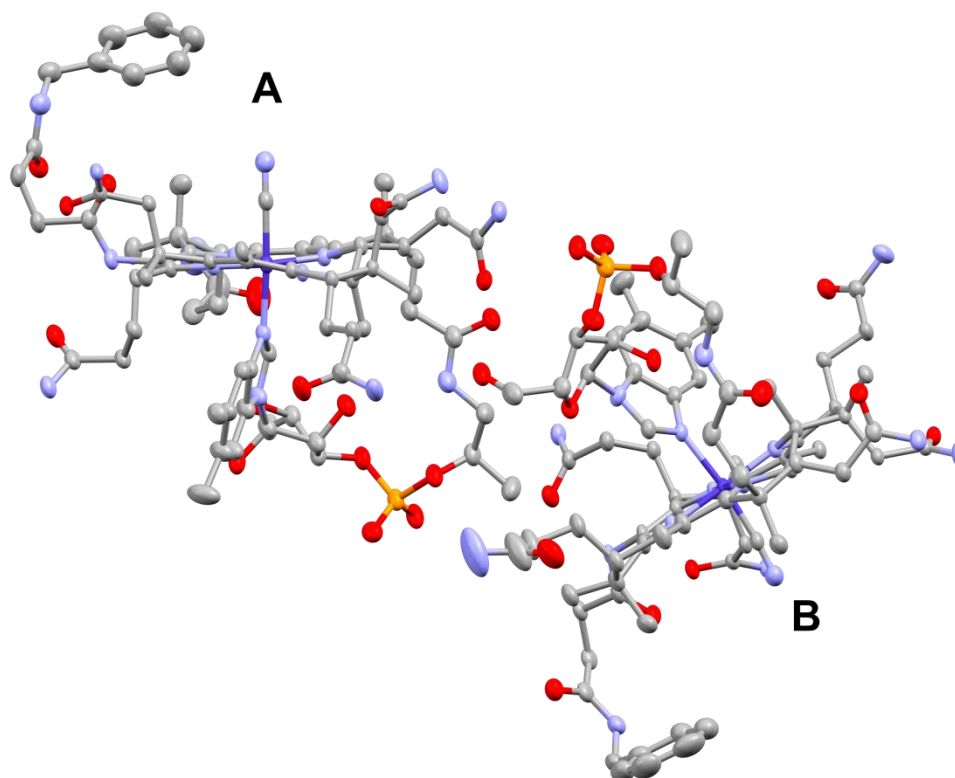


Figure S7. X-ray structure of *meso*-amide **9**.



**Figure S8.** The asymmetric part of the unit cell of the compound **9**. The hydrogen atoms and solvent molecules were omitted for clarity.

**Table S6.** Crystal data and structure refinement details for compound **9**.

Compound	<b>9</b>
CCDC deposition code	CCDC 1574294
Empirical formula	$C_{150.32}H_{260.97}CO_2N_{32}O_{61.35}P_2$
Formula weight	3678.14
Temperature/K	100
Crystal system	tetragonal
Space group	$P4_1$
$a/\text{\AA}$	34.002(7),
$b/\text{\AA}$	34.002(7),
$c/\text{\AA}$	18.744(14)
$\alpha/^\circ$	90.00
$\beta/^\circ$	90.00
$\gamma/^\circ$	90.00
Volume/ $\text{\AA}^3$	21671(18)
$Z$	4
$\rho_{\text{calc}}/\text{g/cm}^3$	1.127
$\mu/\text{mm}^{-1}$	0.262
$F(000)$	7847
Crystal size/mm	0.29 × 0.21 × 0.20
$2\theta$ range for data collection/ $^\circ$	1.229 to 29.608
Index ranges	$-45 \leq h \leq 45, -46 \leq k \leq 46, -25 \leq l \leq 25$
Reflections collected	55859
Independent reflections	52312 [ $R_{\text{int}} = 0.0513, R_{\text{sigma}} = 0.0338$ ]
Data/restraints/parameters	55859/87/2393
Goodness-of-fit on $F^2$	1.135
Final $R$ indexes [ $I \geq 2\sigma(I)$ ]	$R_1 = 0.0693, wR_2 = 0.2073$
Final $R$ indexes [all data]	$R_1 = 0.0724, wR_2 = 0.2097$
Largest diff. peak/hole / $e \text{\AA}^{-3}$	0.967/-0.648
Flack parameter	0.0886(17)

**Table S7.** The lengths of the axial bonds and value of the folding angle for compound **9**.

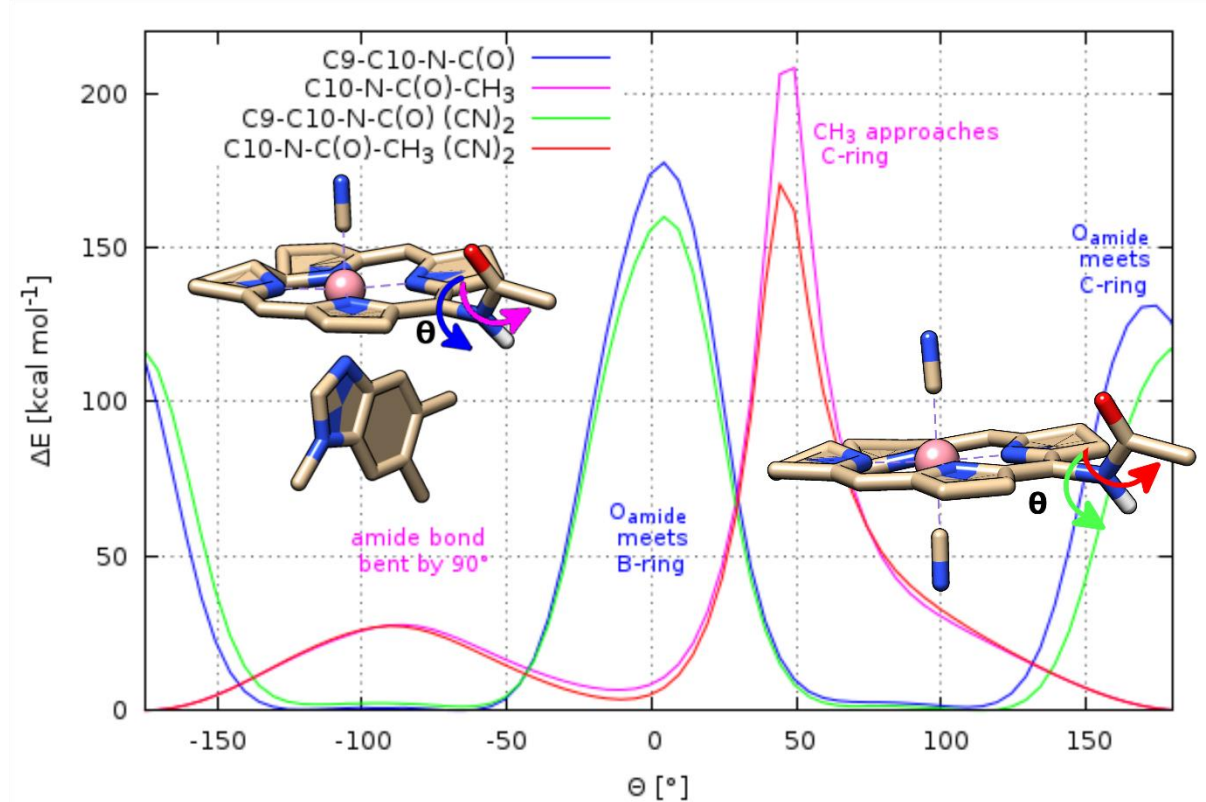
Molecule	Co-CN [Å]	Co-N <sub>DMBI</sub> [Å]	CN-N <sub>DMBI</sub> [Å]	Folding angle [°]
A	1.877(5)	2.060(4)	3.933	11.91
B	1.875(4)	2.043(4)	3.912	12.26

## References:

1. *CrysAlis CCD and CrysAlis RED*; Oxford Diffraction Ltd: Yarnton, **2008**.
2. G. M. Sheldrick, *Acta Crystallogr., Sect. A: Found. Crystallogr.* **2008**, *64*, 112–122.
3. O. V. Dolomanov, L. J. Bourhis, R. J. Gildea, J. A. K. Howard, H. Puschmann, *J. Appl. Cryst.* **2009**, *42*, 339–341.
4. C. F. Macrae, P. R. Edgington, P. McCabe, E. Pidcock, G. P. Shields, R. M. Towler, J. van de Streek, *J. Appl. Cryst.* **2006**, *39*, 453–457.
5. R. Banerjee, *Chemistry and Biochemistry of B<sub>12</sub>*, Wiley, **1999**.

## S5. DFT calculations

All calculations were performed using Gaussian 2009-E.01.<sup>1</sup> The input structure for conformer study of full structure was based on the crystal structure of amide **9** (Conformer **A**) and further optimized using BP86 functional, 6-31G(d) basis set for C, H, N, O, P and def2TZVP for Co. The second structure (Conformer **B**) was obtained by rotation of *meso*-amide bond and arranging the remaining side chain linearly.<sup>2</sup> The same conditions were used when rotation around the amide moiety for the truncated form of cobalamin was studied.<sup>3</sup> The scanning of the dihedral angle covered 72 steps of 5° (Figure S9).

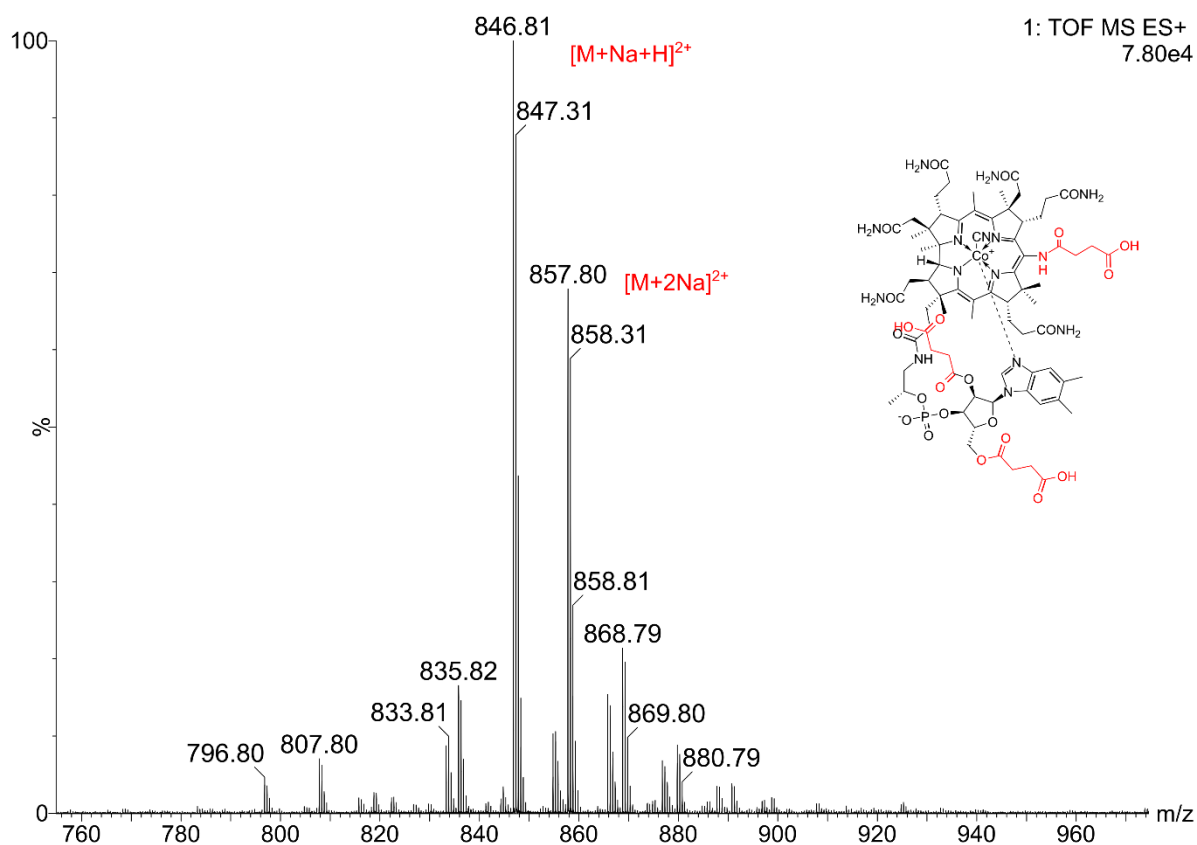


**Figure S9.** Energy change vs torsion angle between corrin ring and *meso*-amide moiety in truncated form of cobalamin.

## References:

1. M. J. Frisch et al., Gaussian'09 Revision E.01, Gaussian Inc. Wallingford CT, **2009**.
2. E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E.C. Meng, T. E. Ferrin, *J. Comp. Chem.* **2004**, *25*, 1605–1612.
3. J. Kuta, S. Patchkovskii, M. Z. Zgierski, P. M. Kozlowski, *J. Comp. Chem.* **2006**, *27*, 1429–1437.

## S6. ESI MS spectrum of amidoester 8a



## S7. <sup>13</sup>C chemical shifts for (CN)Cbl (1) and meso-modified cobalamins

**Table S8.** <sup>13</sup>C chemical shifts for (CN)Cbl and meso-modified cobalamins (solvent- [D<sub>4</sub>]MeOH)

Entry	Group	(CN)Cbl $\delta$ [ppm]	(CN)Cbl(10-NH <sub>2</sub> ) $\delta(\Delta\delta)^{[a]}$ [ppm]	(CN)Cbl(10-NO <sub>2</sub> ) $\delta(\Delta\delta)^{[b]}$ [ppm]
	C1	86.42	86.37 (-0.05)	86.73 (0.31)
2	C2	48.39	48.21 (-0.18)	48.45 (0.06)
3	C3	57.68	57.41 (-0.27)	57.80 (0.12)
4	C4	181.56	180.61 (-0.95)	182.68 (0.12)
5	C5	108.73	108.32 (-0.4)	111.82 (3.1)
6	C6	166.87	166.45 (-0.4)	165.74 (-1.1)
7	C7	52.55	52.93 (0.38)	52.93 (0.38)
8	C8	56.94	55.49 (-1.45)	55.49 (-1.45)
9	C9	175.57	172.35 (-3.2)	171.13 (-4.4)
10	C10	95.64	nd	131.36 (35.7)
11	C11	177.58	171.52 (-6.1)	169.16 (-8.4)
12	C12	49.44	51.74 (2.3)	52.03 (2.59)
13	C13	55.06	56.90 (-1.8)	56.93 (-1.9)
14	C14	167.12	167.19 (0.1)	165.48 (-1.6)
15	C15	105.11	104.37 (-0.7)	108.31 (3.2)
16	C16	180.11	178.67 (-1.4)	181.01 (0.9)
17	C17	60.36	59.85 (-0.51)	60.90 (0.54)
18	C18	40.13	40.14 (0.01)	40.01 (-0.12)
19	C19	76.34	75.81 (0.5)	76.93 (-1.1)
20	C20	20.47	20.52 (0.05)	20.51 (0.04)
21	C25	17.50	17.57 (0.07)	17.46 (-0.04)
22	C35	16.38	16.60 (0.22)	16.55 (0.17)
23	C36	19.90	20.19 (0.29)	19.46 (-0.44)
24	C46	32.31	30.16 (-2.2)	30.40 (-1.9)
25	C47	20.33	23.29 (3.0)	18.78 (-1.6)
26	C53	16.10	16.42 (0.32)	16.53 (0.43)
27	C54	17.09	17.05 (-0.4)	17.22 (0.13)

[a]  $\Delta\delta = \bar{\delta}_{(\text{CN})\text{Cbl}(10\text{-NH}_2)} - \bar{\delta}_{(\text{CN})\text{Cbl}}$ . [b]  $\Delta\delta = \bar{\delta}_{(\text{CN})\text{Cbl}(10\text{-NO}_2)} - \bar{\delta}_{(\text{CN})\text{Cbl}}$ .

## S8. NMR experiments

NMR spectra for compound **8** recorded in the temperature range of 253 – 333K (for clarity only aromatic fragment of spectra is presented):

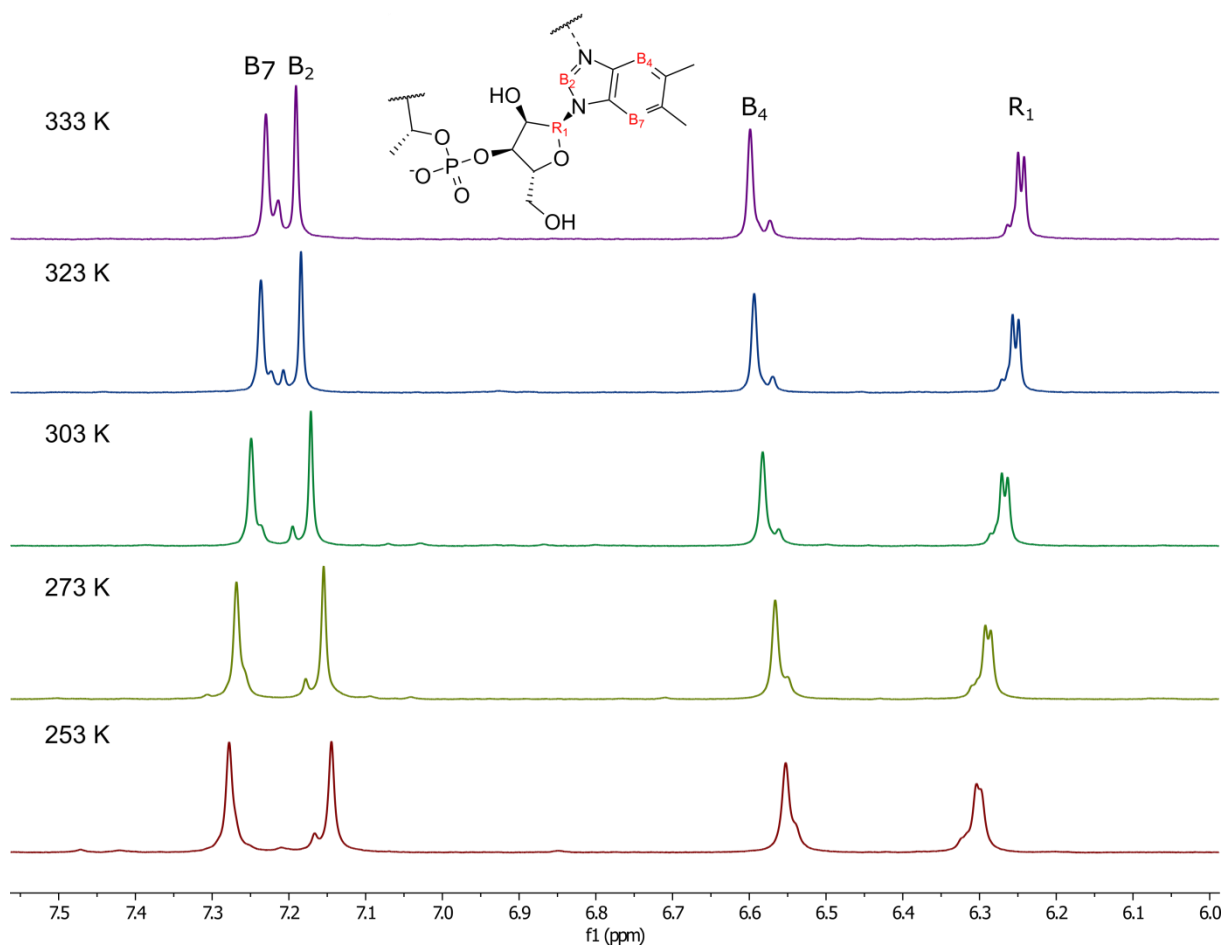
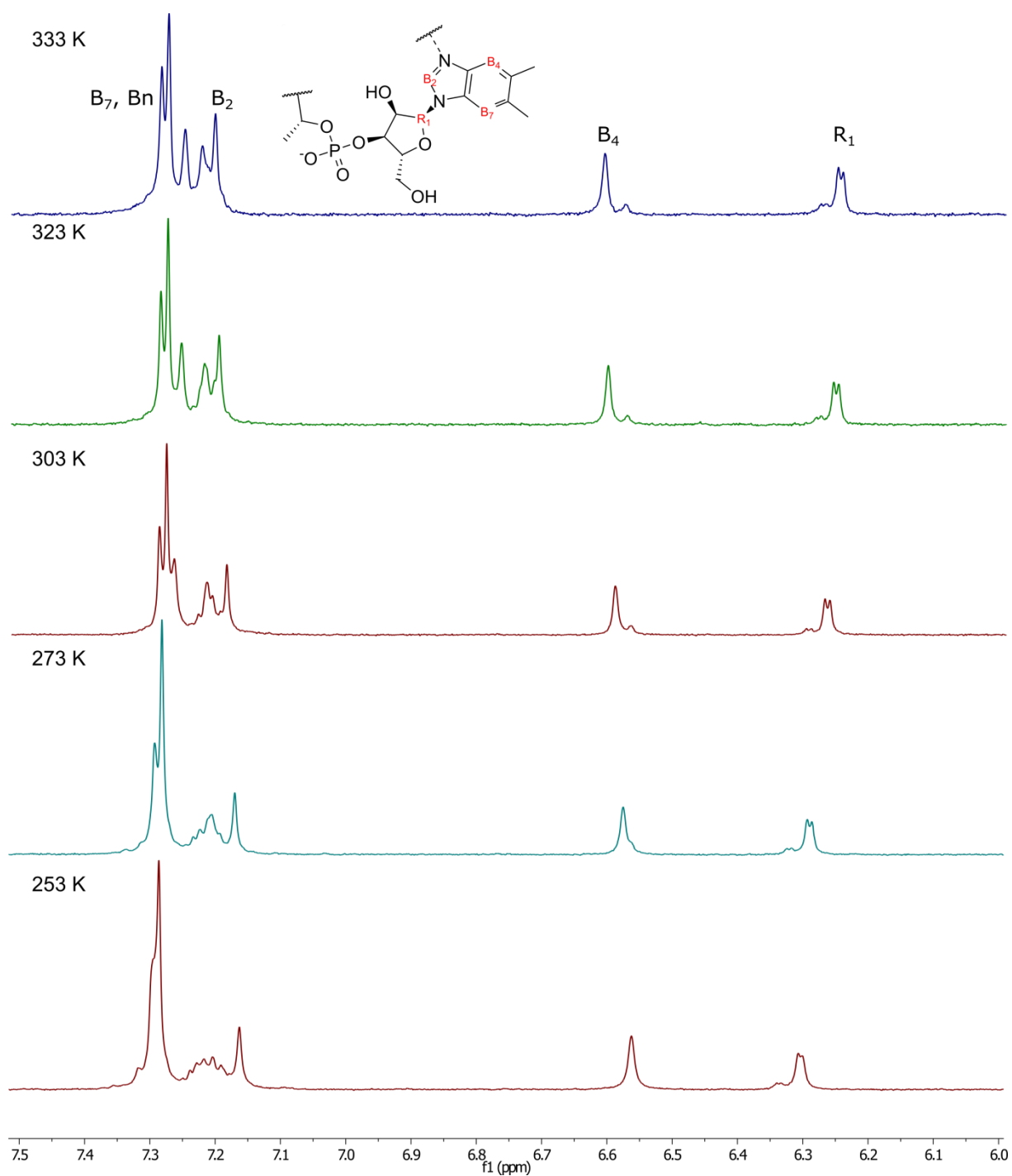


Figure S10. NMR spectra for compound **8** (solvent [D<sub>4</sub>]MeOH, 400MHz).

NMR spectra for compound **9** recorded in the temperature range of 253 – 333K (for clarity only aromatic fragment of spectra is presented):

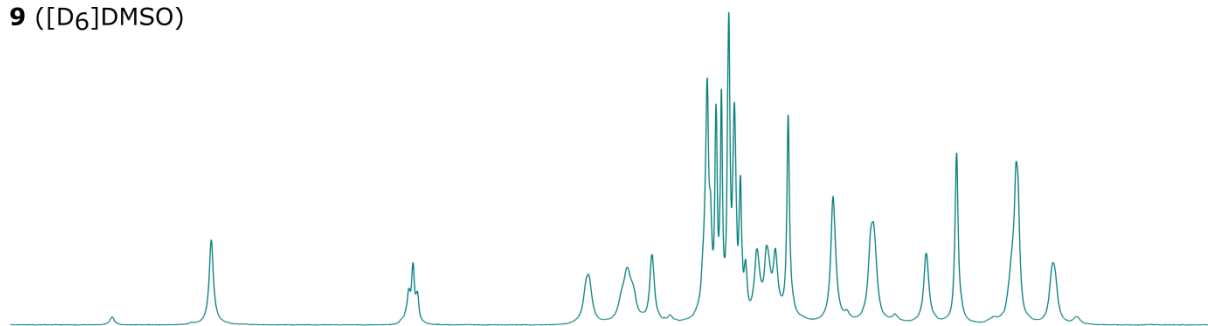


**Figure S11.** NMR spectra for compound **9** (solvent  $[D_4]MeOH$ , 400MHz).

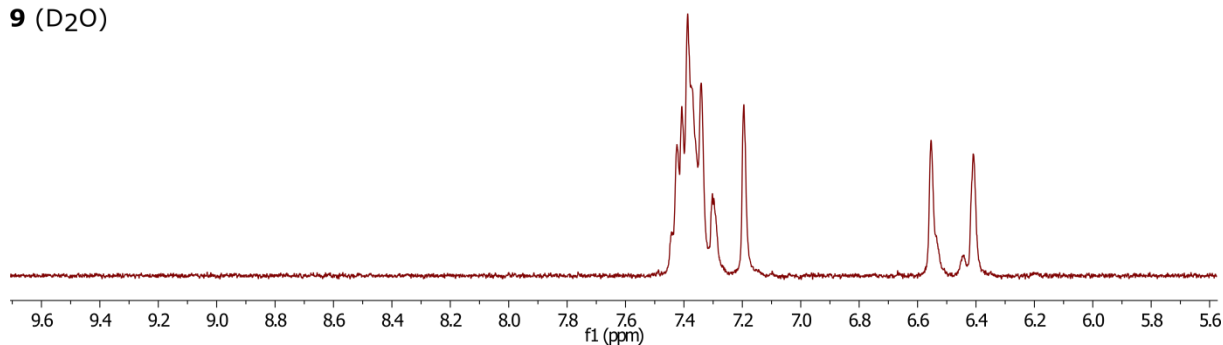


NMR spectra of compound **9** recorded for [D<sub>6</sub>]DMSO and D<sub>2</sub>O (for clarity only aromatic fragment of spectra is presented):

**9** ([D<sub>6</sub>]DMSO)

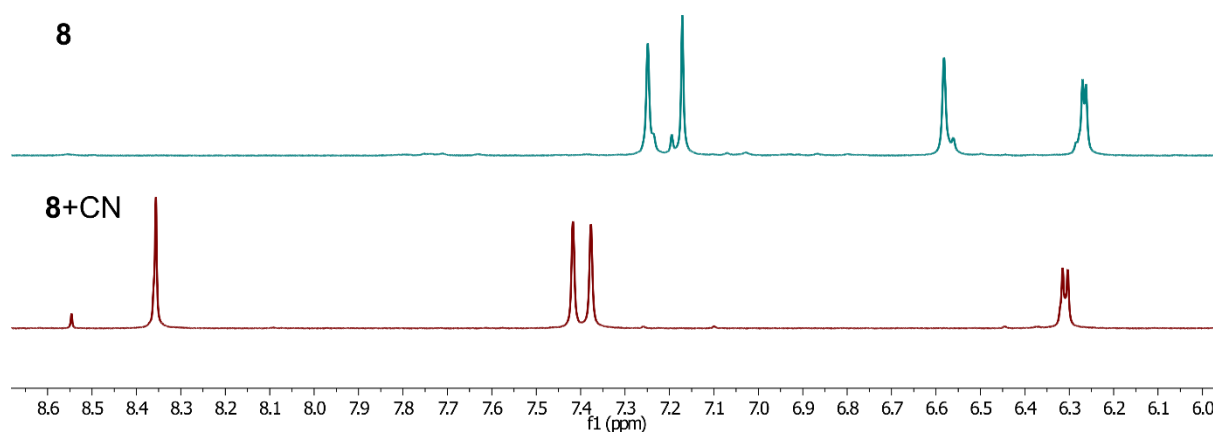


**9** (D<sub>2</sub>O)



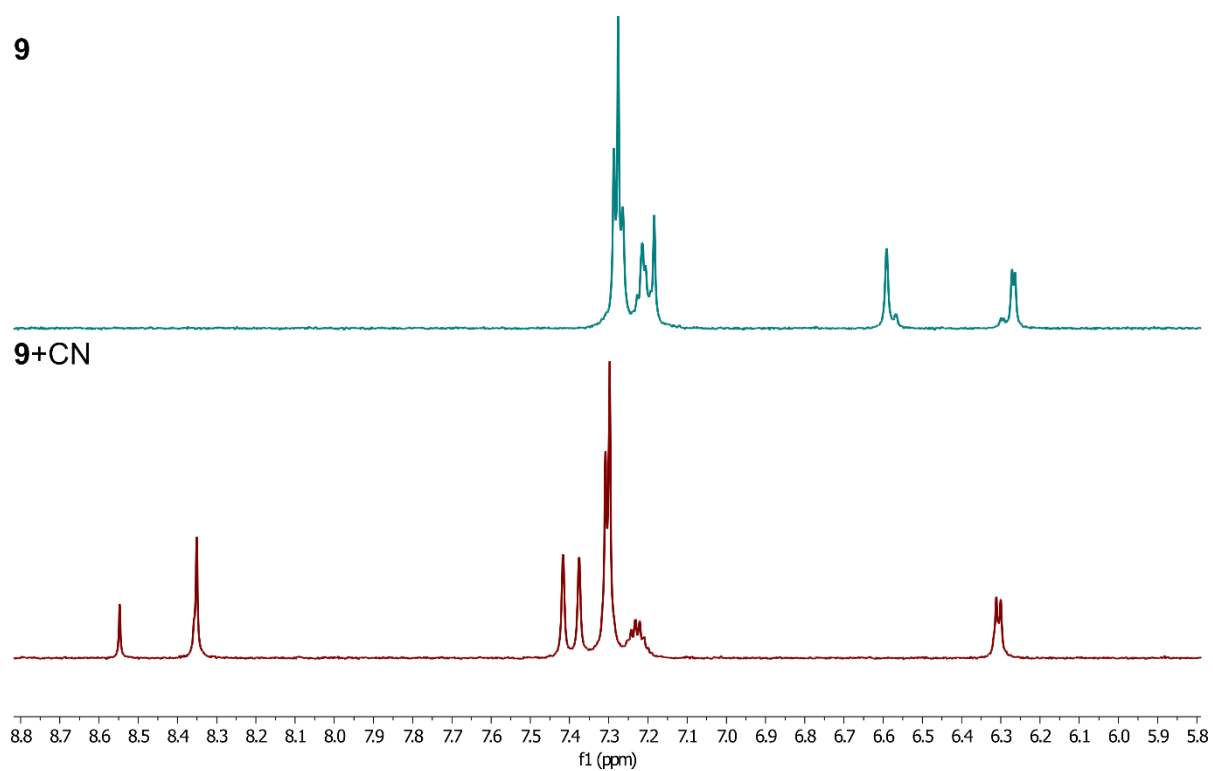
**Figure S12.** NMR spectra for compound **9** recorded for [D<sub>6</sub>]DMSO and D<sub>2</sub>O (400 MHz).

NMR spectra of compound **8** recorded with and without an addition of NaCN (for clarity only aromatic fragment of spectra is presented):



**Figure S13.** NMR spectra for compound **8** (solvent [D<sub>4</sub>]MeOH, 400MHz).

NMR spectra for compound **9** recorded with and without an addition of NaCN (for clarity only aromatic fragment of spectra is presented):



**Figure S14.** NMR spectra for compound **9** (solvent [D<sub>4</sub>]MeOH, 400MHz).

## S9. UV-Vis studies



Figure S15. Solutions of cobalamins used in UV-Vis studies.

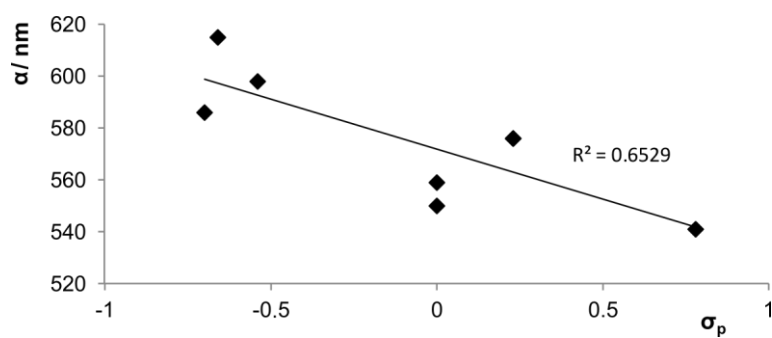


Figure S16. Correlation between the  $\alpha$  band and the  $\sigma_p$  value for the substituent at the *meso*-position.

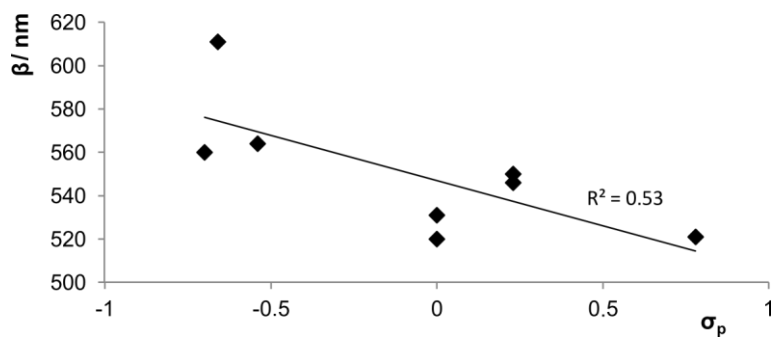


Figure S17. Correlation between the  $\beta$  band and the  $\sigma_p$  value for the substituent at the *meso*-position.

## S10. CN stretching frequencies

**Table S9.** CN stretching frequencies from IR spectra for compounds **1**, **2**, **3**, **5**, **7** and **9**.

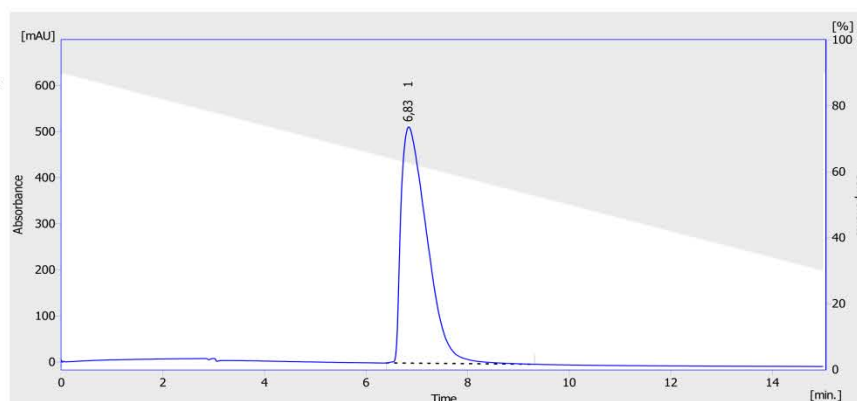
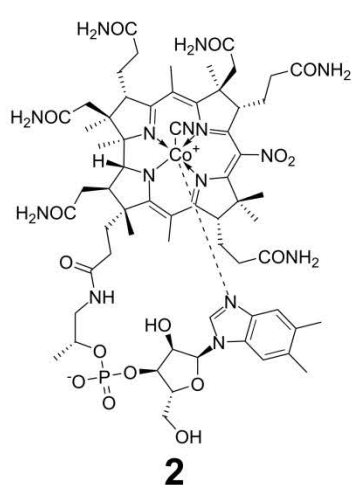
Entry	Cbl	$\sigma_p^{[a]}$	Wavenumber [ $\text{cm}^{-1}$ ]
	<b>1</b>	0	2135
2	<b>2</b>	0.78	2139
3	<b>3</b>	-0.66	2133
4	<b>5</b>	-0.54	2135
5	<b>7</b>	-0.70 <sup>[b]</sup>	2134
6	<b>9</b>	0 <sup>[c]</sup>	2136

[a] C. Hansch, A. Leo, R. W. Taft, *Chem. Rev.* **1991**, *91*, 165–195

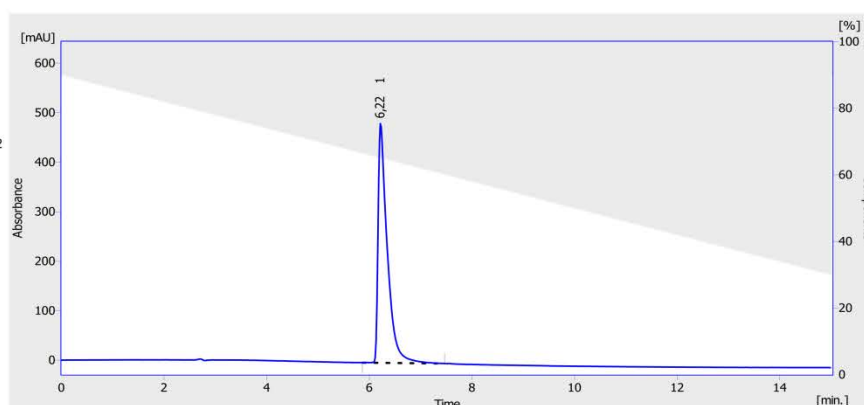
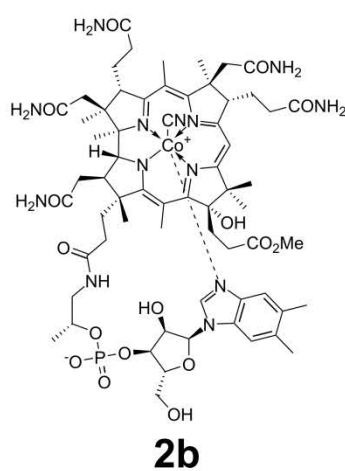
[b]  $\sigma$  values for substituent: NHMe. [c]  $\sigma$  values for substituent: NHC(O)Me.

## S11. HPLC chromatograms

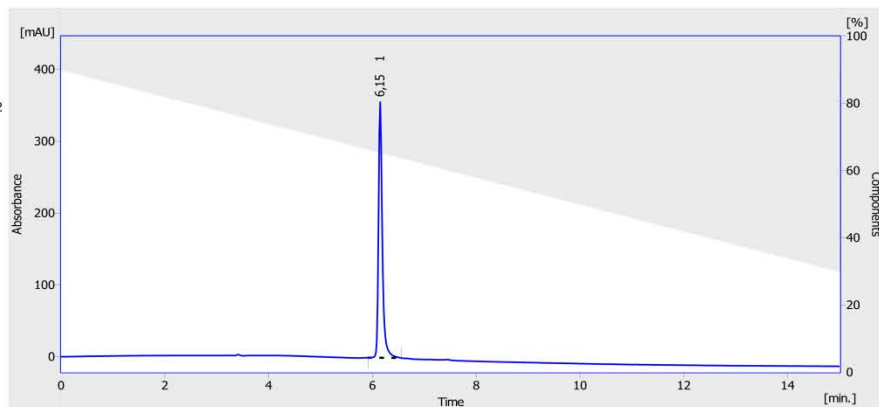
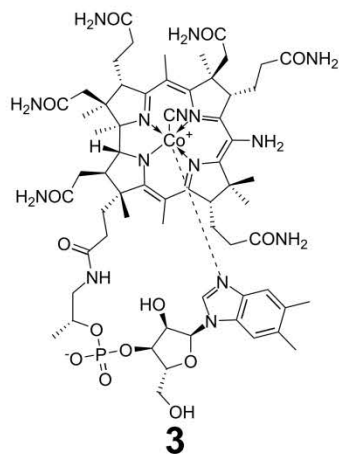
All measurements were carried according to the HPLC method described in the manuscript (Experimental Section, Table 8).



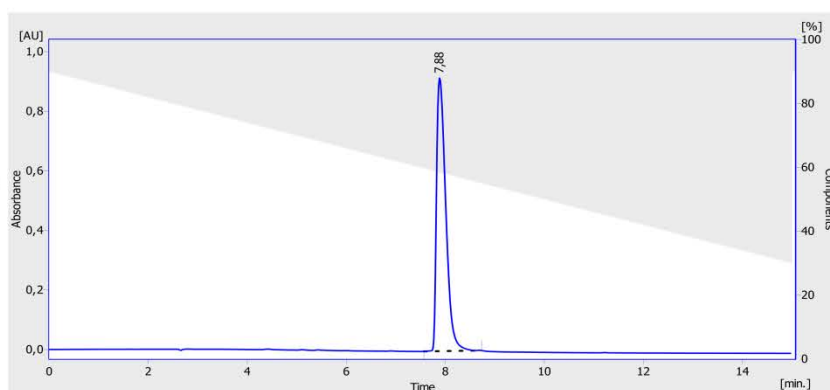
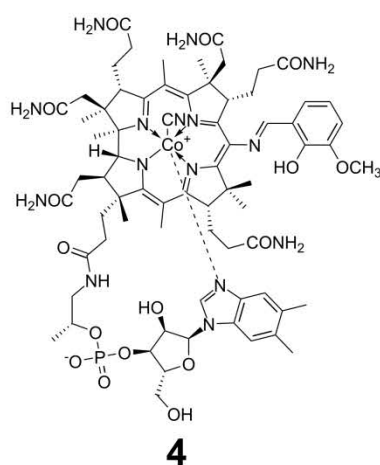
	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	6,833	18210,999	512,386	100,0	100,0	0,58	418
	Total	18210,999	512,386	100,0	100,0		



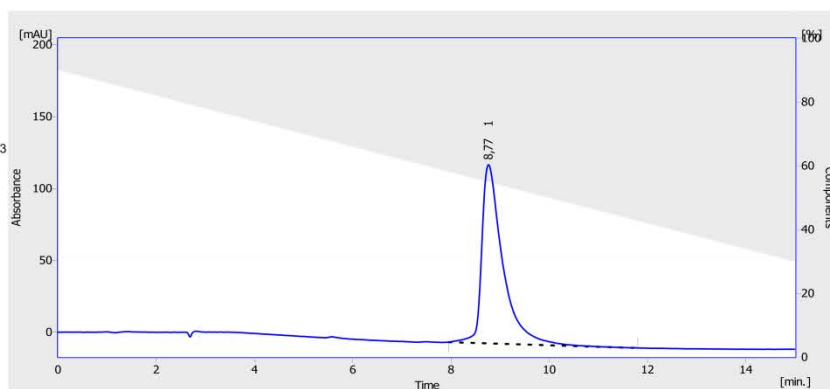
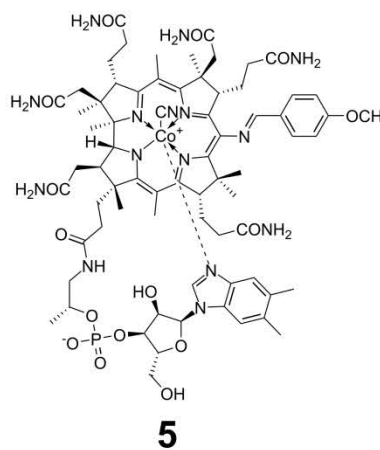
	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	6,217	6030,595	483,438	100,0	100,0	0,18	517
	Total	6030,595	483,438	100,0	100,0		



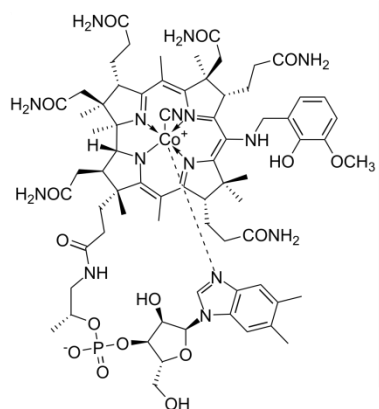
	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	6,150	1826,824	355,971	100,0	100,0	0,10	617
	Total	1826,824	355,971	100,0	100,0		



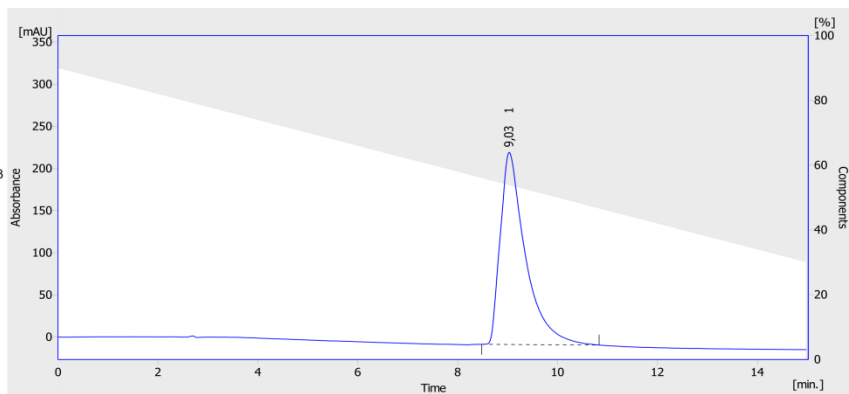
	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	7,883	11884,936	916,623	100,0	100,0	0,22	0
	Total	11884,936	916,623	100,0	100,0		



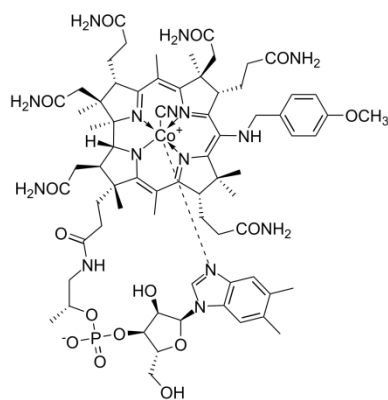
	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	8,767	3703,808	124,263	100,0	100,0	0,42	544
	Total	3703,808	124,263	100,0	100,0		



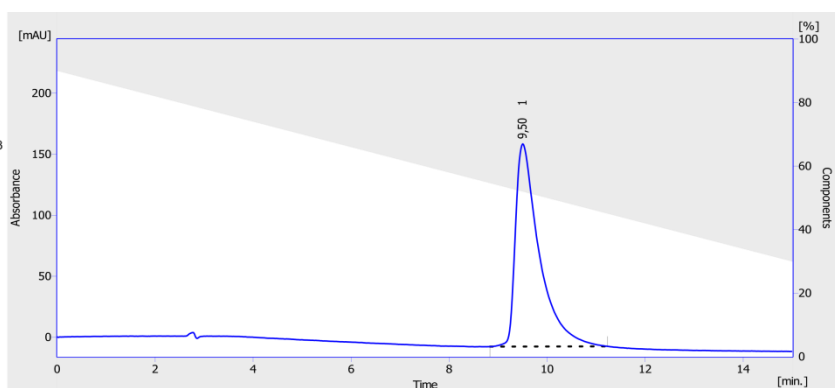
**6**



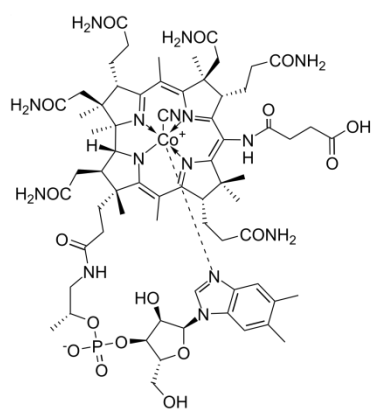
	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	9,033	7964,351	228,112	100,0	100,0	0,53	624
Total		7964,351	228,112	100,0	100,0		



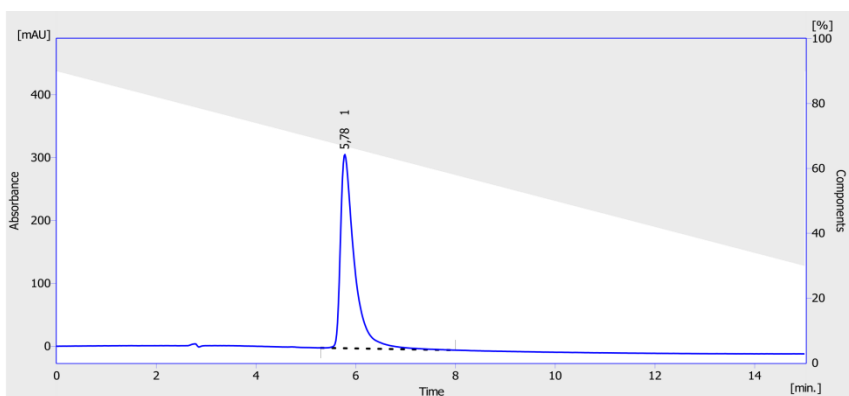
**7**



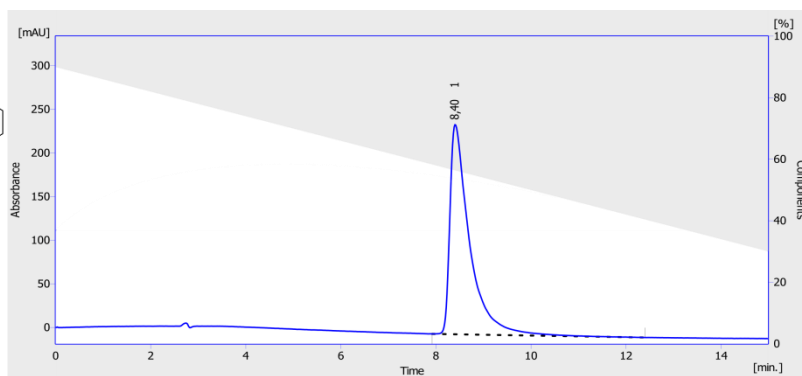
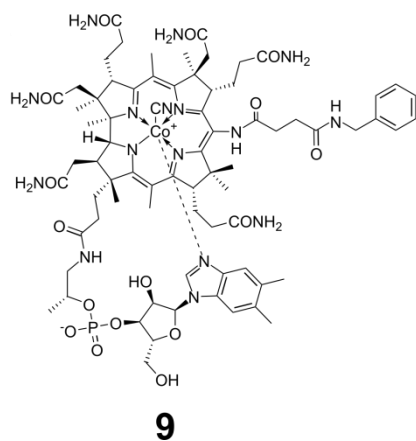
	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	9,500	5605,774	166,139	100,0	100,0	0,48	578
Total		5605,774	166,139	100,0	100,0		



**8**



	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	5,783	5994,102	308,193	100,0	100,0	0,28	623
Total		5994,102	308,193	100,0	100,0		



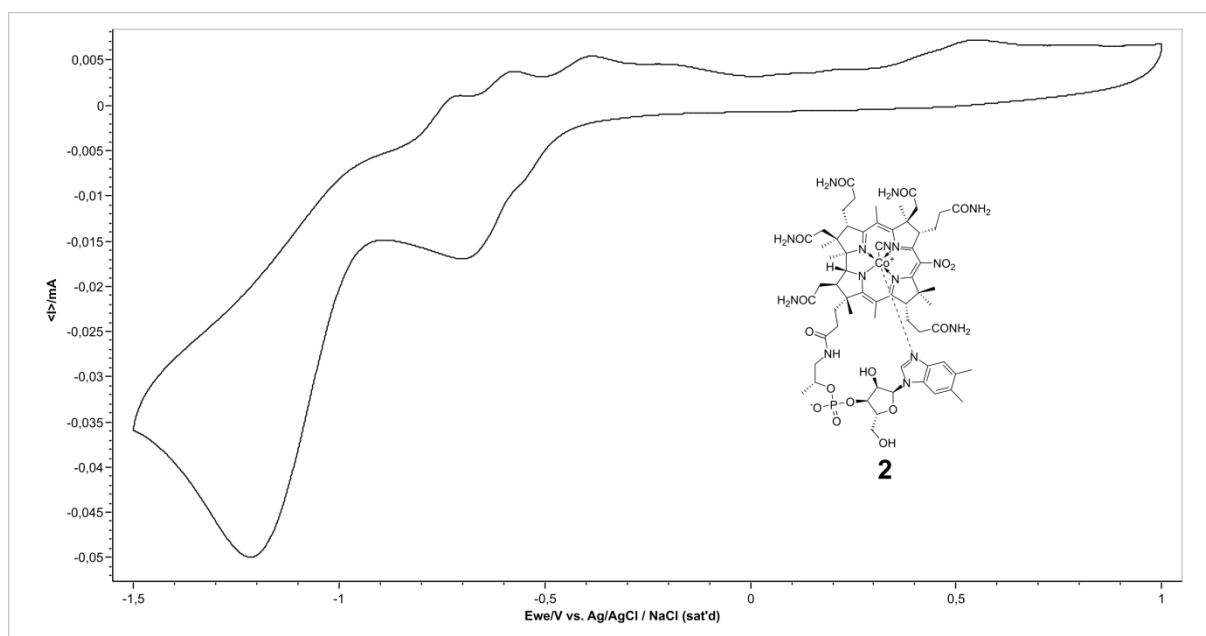
	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	8,400	6936,527	240,316	100,0	100,0	0,40	647
	Total	6936,527	240,316	100,0	100,0		

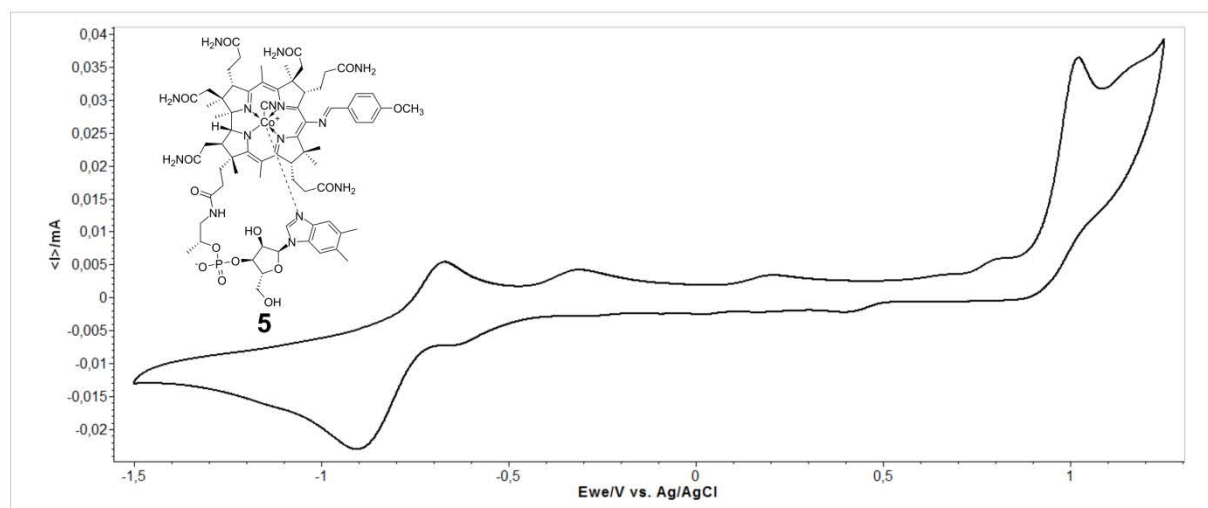
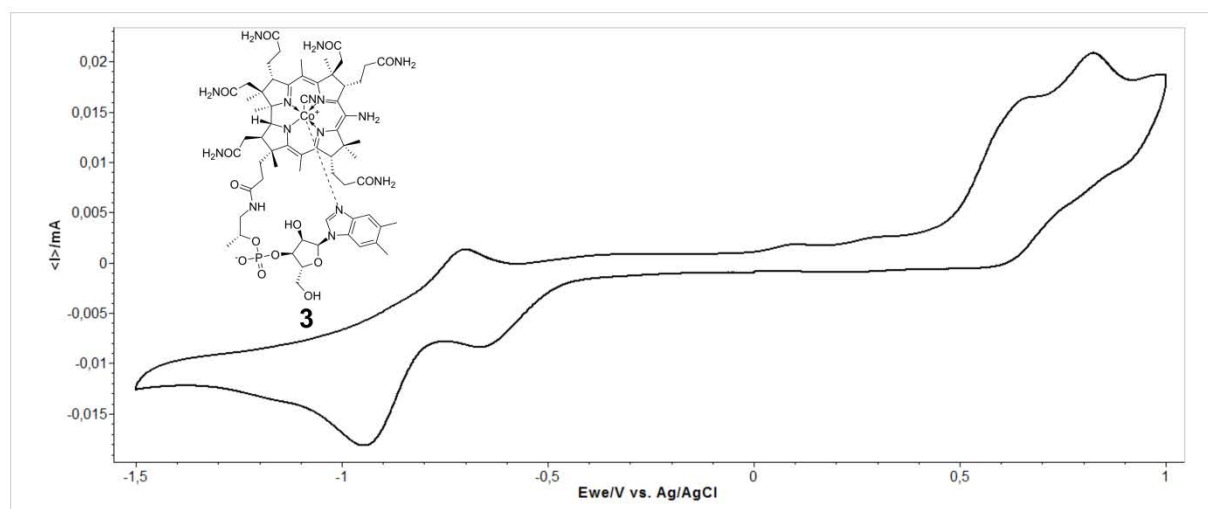
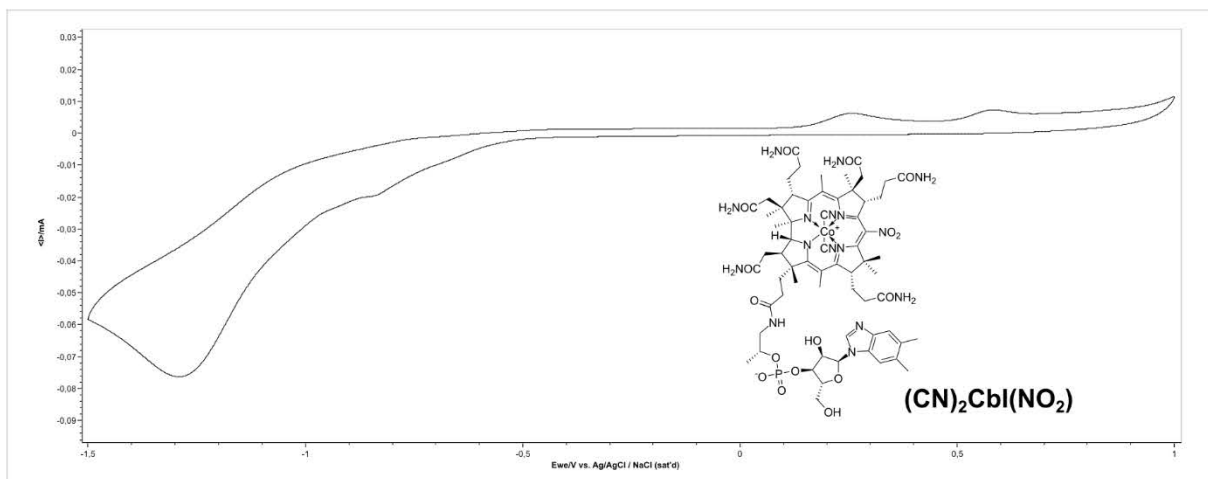


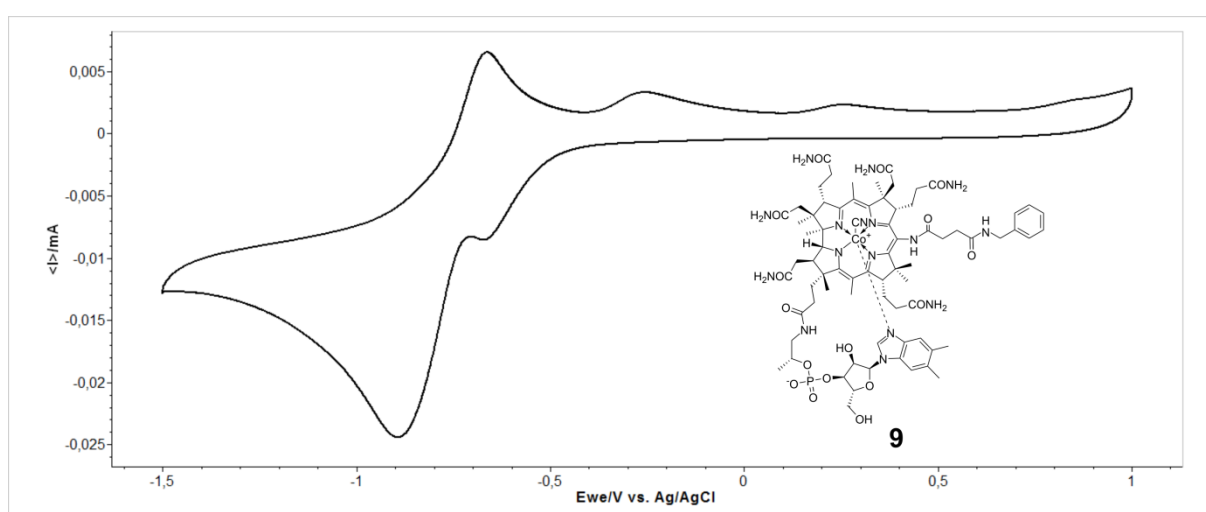
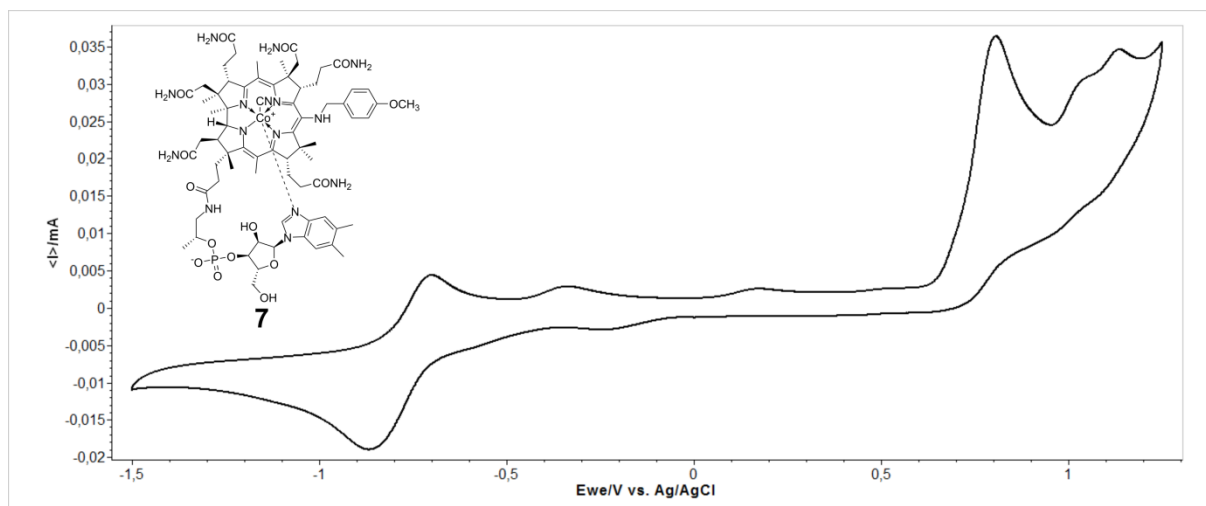
## S12. Electrochemical measurements

For all CV measurements a cylindrical three-electrode cell that was equipped with a glassy carbon working electrode, a 25 mm platinum wire as the counter electrode and an Ag/AgCl (1.0 M NaCl) electrode as the reference electrode were used. The scan rate for a typical experiment was  $100 \text{ mV s}^{-1}$ . The solution of each *meso*-derivative ( $c = 1 \text{ mM}$ ) and electrolyte  $\text{NBu}_4\text{ClO}_4$  ( $c = 0.1 \text{ M}$ ) was deaerated by purging with Ar gas prior each measurement, and the cyclic voltammetry was carried out under an Ar gas atmosphere at room temperature.

## S13. Cyclic voltammograms







#### S14. Estimation of the $\mathcal{E}_{00}$ from the red edge of an absorption spectrum

The values of the zero-to-zero energy,  $\mathcal{E}_{00}$ , are estimated from the onset of absorption from the low energy side as the intersection of the extrapolations of the linear parts of a spectrum (Figure S18). The wavelength at that point is subsequently converted into energy via the formula:  $\mathcal{E}[\text{eV}] = (h[\text{eV s}] \times c[\text{m s}^{-1}]) / \lambda[\text{m}] = 1239.8 [\text{eV nm}] / \lambda[\text{nm}]$ .

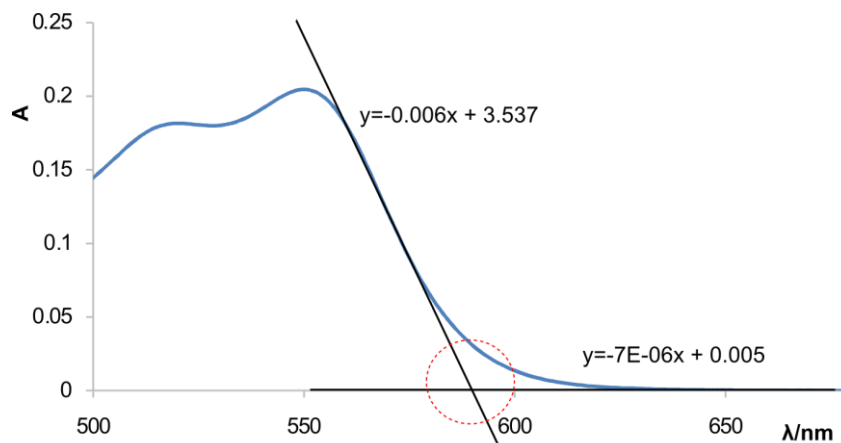
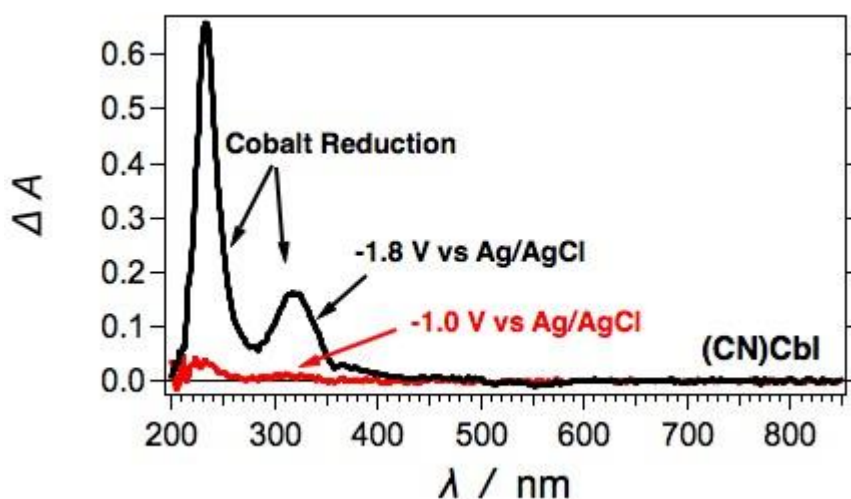


Figure S18. Estimation of the  $\mathcal{E}_{00}$  from the onset of absorption.

## S15. Spectroelectrochemistry

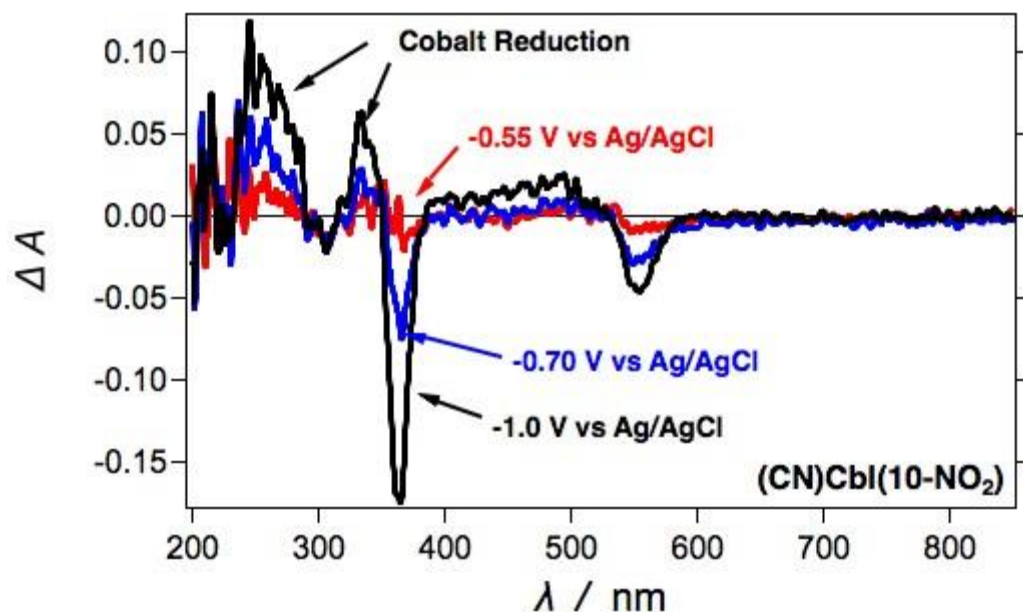
For spectroelectrochemistry (SE) measurements we employ Reference 600 Potentiostat/Galvanostat/ZRA (Gamry Instruments, Inc., Warminster, PA, USA) equipped with a three-electrode SE honeycomb quartz cell (Pine Research Instrumentation, Durham, NC, USA) and Gamry Instruments light source and a spectrometer (200 – 900 nm). After the light intensity recorded through the platinum honeycomb working electrode, the integration time, and the potential of reference electrode are calibrated to Ag/AgCl, we sweep the voltage and record the spectral distribution of the transmitted light intensity at predetermined intervals of time,  $I(\lambda, t)$ . The intensity spectra,  $I(\lambda, t)$ , allow for determining the differential absorbance,  $\Delta A$ , that represent the spectral features of the species that are electrochemically formed on the surface of the working electrode, i.e.,  $\Delta A(\lambda, t) = \lg(I(\lambda, 0) / I(\lambda, t))$ . The positive  $\Delta A$  values correspond to the absorption spectra of the formed species, and the negative  $\Delta A$  values account for the decrease in the absorption due to the depletion of the analyte that is reduced (or oxidized). The  $\Delta A(\lambda, t)$  features represent the sum between the overlapping positive and negative  $\Delta A$  bands. Prior to each scan, the sample was purged with Ar gas and then kept blanketed under Ar. The cell was set at the initial potential and held there for 30 s to ensure equilibration prior to the voltage sweep. We use 20 mV/s scan rate to ensure the accumulation of a sufficient amount of electrochemically formed species at the applied potential and recorded intensity spectra at a 50-mV step size, i.e., every 2.5 s. All measurements were carried out in degassed MeOH in the presence of electrolyte – 100 mM NBu<sub>4</sub>PF<sub>6</sub>.

The parent compound, (CN)Cbl, shows two distinct  $\Delta A$  bands in the UV region as the voltage sweeps to -1.8 V vs. Ag/AgCl (over the cathodic peak). We ascribe these absorption bands to the product of Co(III) electrochemical reduction (Figure S19).



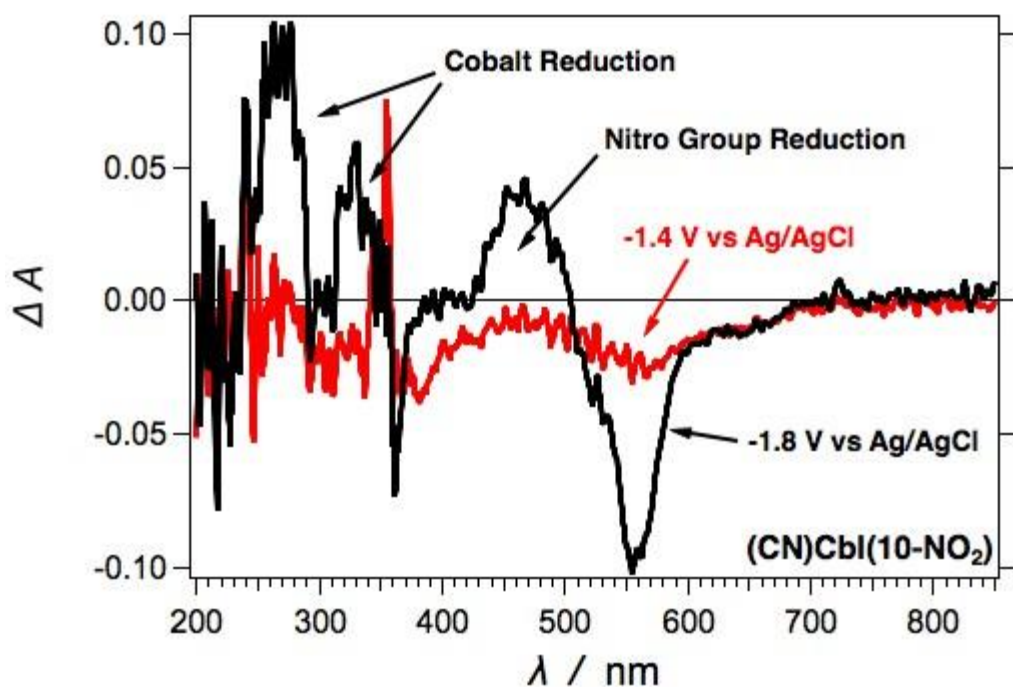
**Figure S19.** Differential absorption spectra for (CN)Cbl recorded for the maximum accumulation of electroreduced species on the working electrode (i.e., the strongest signal) during a voltage sweep from -0.2 to -1.8 V vs. Ag/AgCl.

When sweeping the voltage only over the first cathodic peak (from -0.4 to -1.0 V vs. Ag/AgCl), the nitrated complex, (CN)Cbl(10-NO<sub>2</sub>), shows similar absorption UV bands, resulting from the Co(III) reduction, that overlap with a bleach band (i.e.,  $\Delta A < 0$ ) at about 360 nm. An additional bleach band at about 550 nm is also apparent (Figure S20).

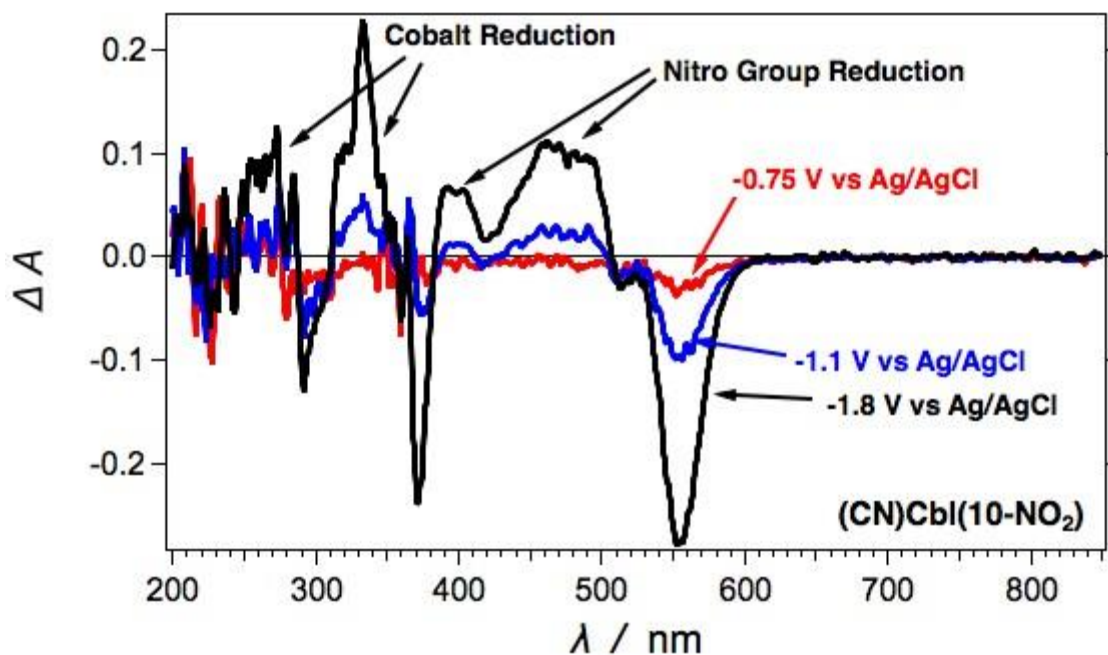


**Figure S20.** Differential absorption spectra for (CN)Cbl(10-NO<sub>2</sub>) recorded for the maximum accumulation of electroreduced species on the working electrode (i.e., the strongest signal) during a voltage sweep from -0.4 to -1.0 V vs. Ag/AgCl.

When we expand the voltage sweep over the second cathodic peak (from -0.4 to -1.8 V vs. Ag/AgCl) for (CN)Cbl(10-NO<sub>2</sub>), the 550-nm bleach becomes pronounced and new absorption bands rise in the visible region of the spectrum, which we ascribe to the products of the reduction of the nitro group (Figure S21 and S22).

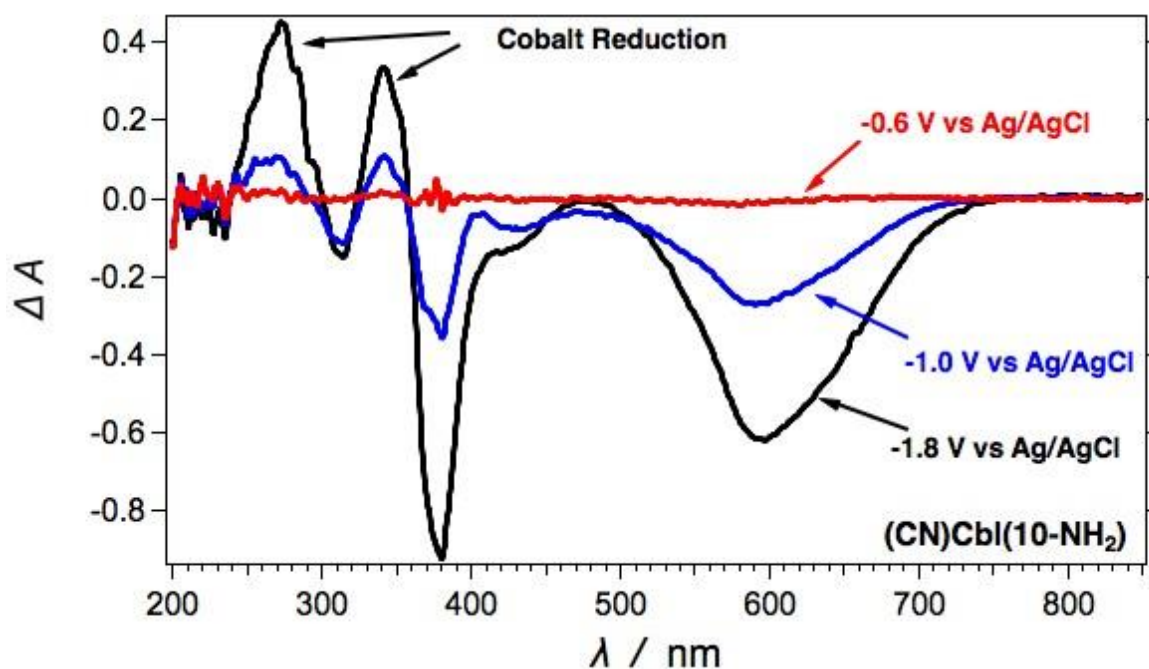


**Figure S21.** Differential absorption spectra for (CN)Cbl(10-NO<sub>2</sub>) recorded for the maximum accumulation of electroreduced species on the working electrode (i.e., the strongest signal) during a voltage sweep from -1.0 to -1.8 V vs. Ag/AgCl.



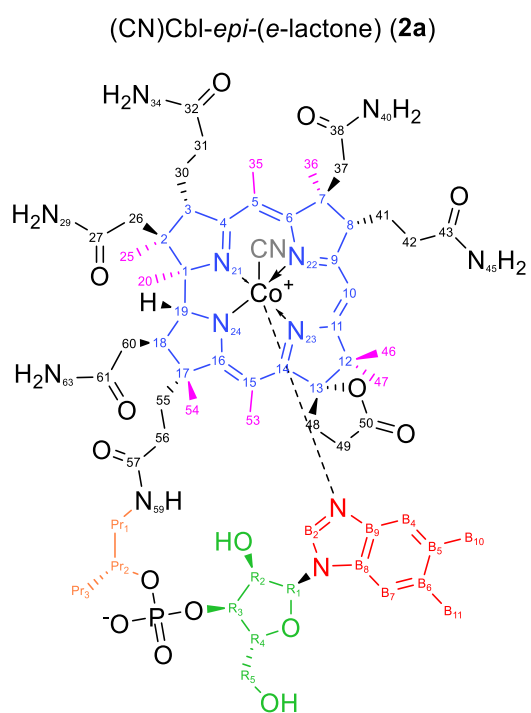
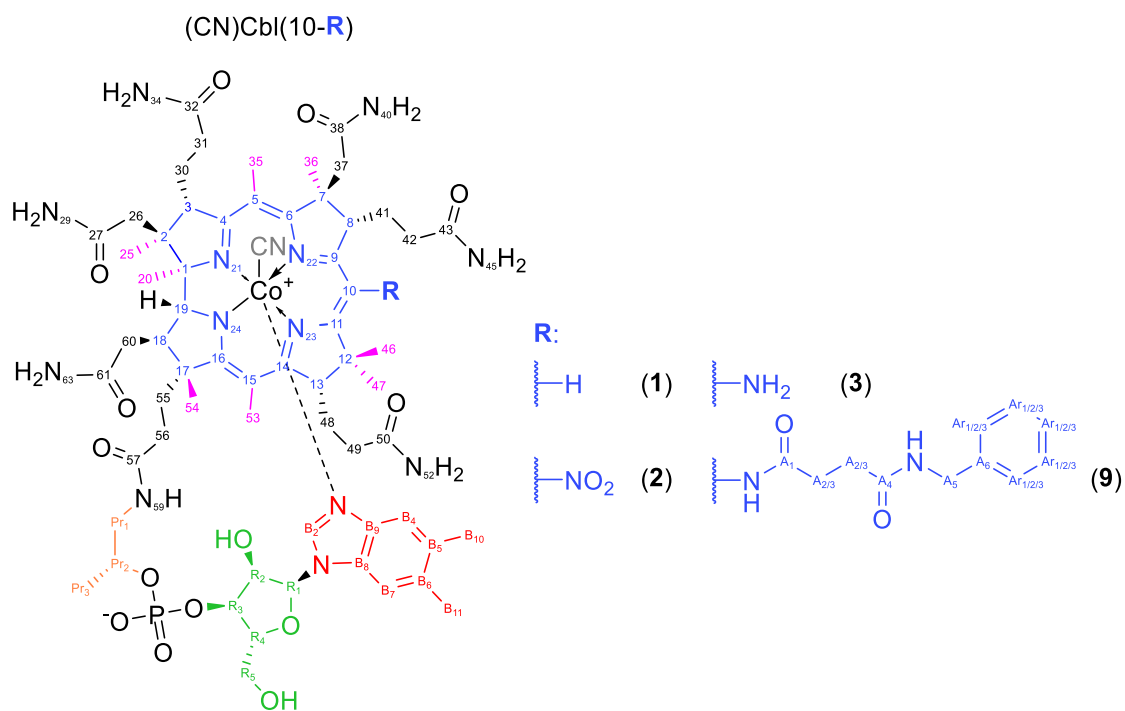
**Figure S22.** Differential absorption spectra for (CN)Cbl(10-NO<sub>2</sub>) recorded for the maximum accumulation of electroreduced species on the working electrode (i.e., the strongest signal) during a voltage sweep from -0.4 to -1.8 V vs. Ag/AgCl.

Meanwhile, the electrochemical reduction of the amine complex, (CN)Cbl(10-NH<sub>2</sub>), produces the same absorption bands in the UV (ascribed to the Co(III) reduction) along with the bleach bands in the near UV and in the visible spectral regions, which most likely are due to spectral changes in the aminated ligand upon the reduction of the metal ion (Figure 23). The absorption bands,  $\Delta A > 0$ , in the visible spectral region, however, are unique for the reduction of the nitrated complex. Similar to the amino complex, the electrochemically reduced nitrated cobalamin shows bleach bands in the visible and in the near-UV regions because the central ion is already reduced at these potentials (of -1.8 V vs. Ag/AgCl).



**Figure 23.** Differential absorption spectra for (CN)Cbl(10-NH<sub>2</sub>) recorded for the maximum accumulation of electroreduced species on the working electrode (i.e., the strongest signal) during a voltage sweep from -0.2 to -1.8 V vs. Ag/AgCl.

## S16. Numbering of atoms in cobalamins



**S17. 2D NMR assignments of (CN)Cbl (1) (600 MHz, [D<sub>4</sub>]MeOH)**

Nr	Assignment	<sup>13</sup> C Shift [ppm]	HSQC [ppm]	HMBC	COSY
1	C4	181.6		C3, C30, C35	
2	C16	180.1		C19, C53, C54, C56	
3	C11	177.6		C46, C47	
4	C50	177.5		C13, C48, C49	
5	C32	177.3		C31	
6	C43	176.6		C41, C42	
7	C9	175.6		C8, C41	
8	C27	175.5		C26	
9	C61	175.3		C18, C60	
10	C38	174.6		C37	
11	C57	174.2		C55, Pr1	
12	C14	167.1		C13, C19, C53	
13	C6	166.9		C8, C35, C37	
14	B2	143.4	7.14	R1	
15	B9	138.2		B2, B7	
16	B6	135.6		B4, B11	
17	B5	133.8		B7, B10	
18	B8	131.5		B2, B4	
19	B4	117.8	6.58	B7, B10	B10
20	B7	112.5	7.26	B2, B4, B11	B11
21	C5	108.7		C35	
22	C15	105.1		C13, C19, C53	
23	C10	95.6	6.05	C8	
24	R1	88.0	6.28	R2	R2
25	C1	86.4		C3, C18, C19, C20, C25, C26	
26	R4	83.71, 83.67	4.08	R2, R3, R5	R5
27	C19	76.3	4.13	C18, C20, C60	C18, C20, C26
28	R3	75.51, 75.48	4.67	R4, R5	R2, R4
29	Pr2	73.60, 73.56	4.33	Pr1, Pr3	Pr1, Pr3
30	R2	70.73, 70.71	4.18	R3	R1, R3
31	R5	62.7	3.91, 3.76	R3, R4	R4
32	C17	60.4		C54, C19, C55, C56, C60	
33	C3	57.7	4.50	C25	C25, C26, C30
34	C8	56.9	3.63	C37, C36	C41
35	C13	55.1	3.32	C46, C47, C48, C49	C48
36	C7	52.6		C8, C37, C36, C41	
37	C12	49.4		C10, C13, C46, C48, C47	
38	C2	48.4		C3, C19, C20, C25, C26, C30	
39	Pr1	46.80, 46.77	3.68, 2.86	Pr3	Pr2
40	C37	43.9	2.54, 2.07	C8, C36	
41	C26	43.0	2.40-2.33	C3, C25	C19, C25
42	C18	40.1	2.86	C19, C60, C54	C19, C20, C60
43	C31	36.2	2.52-2.39	C3, C30	C30
44	C49	35.3	2.54	C13, C48	C48
45	C56	33.5	1.83, 2.65	C18, C54, C55	C55
46	C42	32.99	1.74, 1.30	C8, C41	C41
47	C55	32.95	2.16, 2.56	C56	C56
48	C60	32.4	2.67-2.55	C18, C19	C18
49	C46	32.3	1.20	C13, C47	
50	C48	29.6	2.06, 1.89	C13, C49	C49
51	C41	27.4	2.00, 1.13	C8, C36, C42	C8, C42
52	C30	27.4	2.03, 1.90	C3, C31	C31
53	B10	20.9	2.29	B4	
54	C20	20.5	0.46	C19	C18, C19
55	C47	20.33	1.46	C46	
56	B11	20.26	2.28	B7	
57	Pr3	20.17, 20.15	1.26	Pr1	Pr2
58	C36	19.9	1.89	C37	
59	C25	17.5	1.38	C26	C26
60	C54	17.1	1.37	C18	
61	C35	16.4	2.58	C36	
62	C53		2.59		



**S18. 2D NMR assignments of (CN)Cbl(10-NO<sub>2</sub>) (2) (600 MHz, [D<sub>4</sub>]MeOH)**

Nr	Assignment	<sup>13</sup> C Shift [ppm]	HSQC [ppm]	HMBC	COSY
1	C4	182.7		C3, C35	
2	C16	181.0		C19, C53, C54	
3	C50	177.2		C48, C49	
4	C32	177.1		C31	
5	C43	176.2		C41, C42	
6	C27	175.2		C26	
7	C61	175.1		C18, C60	
8	C38	174.0		C37	
9	C57	174.0		C55, Pr1	
10	C9	171.1		C8, C41	
11	C11	169.2		C13, C46, C47	
12	C6	165.7		C8, C35, C36, C37	
13	C14	165.5		C13, C53	
14	B2	143.1	7.21	R1	R1
15	B9	138.0		B2, B7	
16	B6	136.2		B4, B10	
17	B5	134.5		B7, B11	
18	B8	131.5		B2, B4	
19	C10	131.4		C8	
20	B4	117.5	6.56	B7, B10	B7, B10
21	B7	112.9	7.31	B4, B11	B4, B11
22	C5	111.8		C35	
23	C15	108.3		C53	
24	R1	88.3	6.33	R2	B2, R2
25	C1	86.7		C3, C18, C19, C20, C25	
26	R4	84.0, 83.9	4.10	R2, R3, R5	R3, R5
27	C19	76.9	4.11	C18, C20, C60	C18
28	R3	75.5, 75.4	4.69	R4, R5	R2, R4
29	Pr2	73.5, 73.5	4.33	Pr1, Pr3	Pr1, Pr3
30	R2	70.7	4.22	R1	R1, R3
31	R5	62.7	3.93, 3.76	R3	R4
32	C17	60.9		C18, C53, C54, C56, C60	
33	C3	57.8	4.62	C25, C26, C30, C31	C30
34	C13	56.9	3.39	C46, C47, C49	C48
35	C8	55.5	3.72	C36, C37, C41, C42	C41
36	C7	52.9		C8, C36, C37, C41	
37	C12	52.0		C13, C46, C47	
38	C2	48.5		C3, C19, C20, C25, C26, C30	
39	Pr1	46.9, 46.8	3.67, 2.82	Pr3	Pr2
40	C26	43.0	2.33	C3, C25	
41	C37	42.9	2.61, 2.08	C8, C36	
42	C18	40.0	2.90	C19, C54, C60	C19, C60
43	C31	36.1	2.48, 2.45	C3, C30	C30
44	C49	35.1	2.54	C13, C48	C48
45	C42	34.6	2.10, 1.52	C8, C41	C41
46	C56	33.6	2.67, 1.85	C54, C55	C55
47	C55	33.1	2.56, 2.16	C56	C56
48	C60	32.3	2.64, 2.58	C18, C19	C18
49	C46	30.4	1.32	C13, C47	
50	C48	30.0	1.96	C49	C13, C49
51	C41	28.9	1.88, 1.18	C8, C42	C8, C42
52	C30	27.3	2.03, 1.90	C3, C31	C3, C31
53	B10	21.1	2.31	B4	B4
54	C20	20.5	0.45	C19	
55	B11	20.3	2.30	B7	B7
56	Pr3	20.19, 20.17	1.26	Pr1	Pr2
57	C36	19.5	1.95	C37	
58	C47	18.8	1.40	C46	
59	C25	17.5	1.37	C26	
60	C54	17.2	1.37	C18	
61	C35	16.6	2.62		
62	C53	16.5	2.65		

**S19. 2D NMR assignments of (CN)Cbl-*epi*-( $\epsilon$ -lactone) (2b) (600 MHz, [D<sub>4</sub>]MeOH)**

Nr	Assignment	<sup>13</sup> C Shift [ppm]	HSQC [ppm]	HMBC	COSY
1	C4	182.0		C3, C35	
2	C16	181.0		C19, C53, C54	
3	C32	177.2		C31	
4	C50	177.1		C49	
5	C43	176.5		C42	
6	C27	175.4		C26	
7	C9	175.1		C8, C10	
8	C61	175.1		C18, C60	
9	C38	174.5		C37	
10	C57	174.1		C56, Pr1	
11	C11	172.6		C10, C46, C47	
12	C6	166.7		C8, C35, C36, C37	
13	C14	159.8		C10, C19, C48, C53	
14	B2	143.9	7.15	R1	
15	B9	138.5		B2, B7	
16	B6	135.9		B4, B10, B11	
17	B5	134.0		B7, B10, B11	
18	B8	131.5		B2, B4	
19	B4	117.6	6.54	B2, B7	B10
20	B7	112.6	7.29	B2, B4	B11
21	C5	110.8		C35	
22	C15	106.4		C19, C53	
23	C10	95.5	5.89	C8	
24	C13	95.1		C46, C47, C48, C49	
25	R1	87.6	6.29		R2
26	C1	86.9		C3, C18, C19, C20, C25	
27	R4	83.22, 83.17	4.05	R2, R5	R3, R5
28	C19	76.8	4.26	C18, C20, C60	C18
29	R3	75.03, 75.00	4.73	R4, R5	R2, R4
30	Pr2	73.71, 73.66	4.34	Pr1, Pr3	Pr1, Pr3
31	R2	70.89, 70.87	4.27	R1	R1, R3
32	R5	61.5	3.89, 3.82	R3	R4
33	C17	60.5		C18, C54, C60	
34	C3	57.3	4.55	C25, C26	C30
35	C8	56.4	3.66	C10, C36, C37, C42	C41, C42
36	C12	53.3		C10, C46, C47, C48	
37	C7	52.9		C8, C36, C37	
38	C2	48.9		C3, C19, C20, C25, C26, C30	
39	Pr1	46.9, 46.8	3.67, 2.83	Pr3	Pr2
40	C37	44.2	2.52, 2.04	C8, C36	
41	C26	43.2	2.39, 2.46	C3, C25	
42	C18	40.7	2.81	C19, C54, C60	C19, C60
43	C31	36.2	2.44-2.52	C3, C30	C30
44	C55	33.9	1.85, 2.62	C18, C54, C56	C56
45	C56	32.9	2.15, 2.54	C55	C55
46	C42	32.8	1.71, 1.19	C8, C41	C8, C41
47	C60	31.9	2.57, 2.67	C18, C19	C18
48	C48	30.7	2.66, 2.37	C49	C49
49	C49	28.8	2.76-2.87	C48	C48
50	C30	27.6	2.08, 1.91	C3, C31	C31
51	C41	27.2	1.96, 1.19	C8, C42	C8, C42
52	C46	26.1	1.19	C47	
53	B10	20.9	2.28	B4	
54	B11	20.3	2.28	B7	
55	Pr3	20.21, 20.19	1.25	Pr1	Pr2
56	C20	20.1	0.43	C18, C19	
57	C36	19.8	1.87	C37	
58	C47	19.6	1.43	C46	
59	C25	17.6	1.39	C26	
60	C54	16.7	1.42	C18	
61	C35	16.2	2.57		
62	C53	13.0			

**S20. 2D NMR assignments of (CN)Cbl(10-NH<sub>2</sub>) (3) (600 MHz, [D<sub>4</sub>]MeOH)**

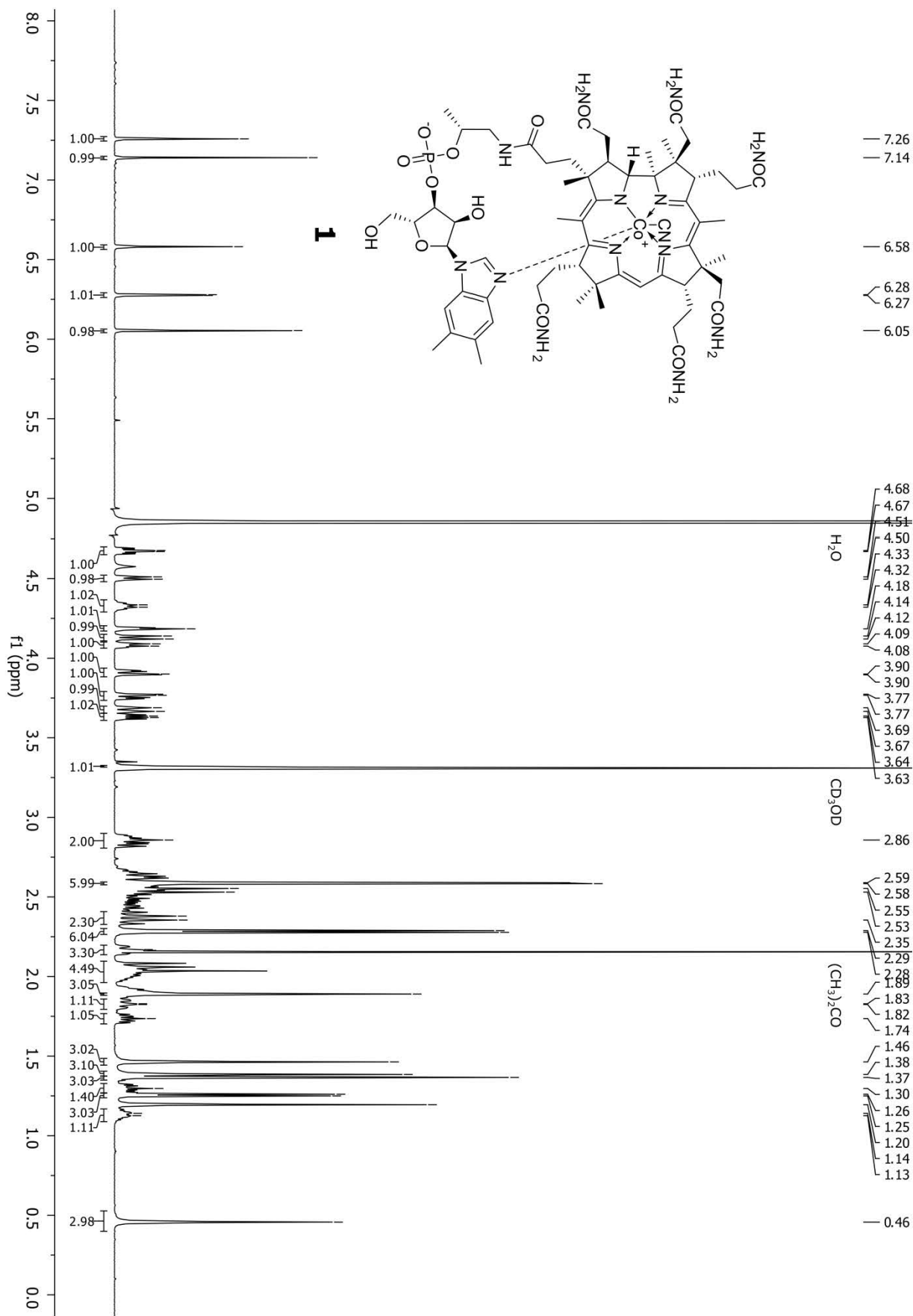
Nr	Assignment	<sup>13</sup> C Shift [ppm]	HSQC [ppm]	HMBC	COSY
1	C4	180.6		C3, C30, C35,	
2	C16	178.7		C19, C53, C54, C56	
3	C50	177.6		C48	
4	C32	177.4		C31	
5	C43	177.2		C41, C42	
6	C27	175.6		C26	
7	C61	175.4		C18, C60	
8	C38	174.7		C37	
9	C57	174.4		Pr1, C55	
10	C9	172.4		C8, C41	
11	C11	171.5		C13, C46, C47	
12	C14	167.2		C13, C48, C53	
13	C6	166.5		C8, C36, C37	
14	B2	143.2		R1	
15	B9	138.3		B2, B7	
16	B6	135.6		B4, B10	
17	B5	133.7		B7, B11	
18	B8	131.5		B2, B4	
19	B4	118.1	6.55	B7, B10	B10
20	B7	112.4	7.23	B4, B11	B11
21	C5	108.3		C35	
22	C15	104.4		C19, C53	
23	R1	88.0	6.25	R2	R2
24	C1	86.4		C3, C18, C19, C20, C25, C26	
25	R4	83.72, 83.68	4.09	R2, R3, R5	R3, R5
26	C19	75.8	4.09	C18, C20, C60	C18
27	R3	75.62, 75.60	4.68	R4, R5	R2, R4
28	Pr2	73.6, 73.5	4.33	Pr1, Pr3	Pr1, Pr3
29	R2	70.77, 70.76	4.18	R1, R3	R1, R3
30	R5	62.8	3.90, 3.76	R3, R4	R4
31	C17	59.9		C18, C54, C60, C56	
32	C3	57.4	4.50	C25, C26, C30, C31	C25, C26, C30
33	C13	56.9	3.24	C46, C47, C48	C48
34	C8	54.2	4.12	C36, C37, C41, C42,	C41
35	C7	52.1		C8, C37, C36, C41	
36	C12	51.7		C13, C46, C47, C48	
37	C2	48.2		C3, C19, C20, C25, C26, C30	
38	Pr1	46.80, 46.76	3.67, 2.85	Pr3	Pr2
39	C37	44.2	2.51, 2.03	C8, C36	
40	C26	43.2	2.40-2.30	C3, C25	C3
41	C18	40.1	2.81	C19, C54, C60	C19
42	C31	36.3	2.53-2.38	C3, C30	C30
43	C49	36.1	2.58	C13, C48	C48
44	C56	33.7	2.63, 1.83	C18, C54, C55	C55
45	C42	33.2	2.06, 1.51	C8, C41	C41
46	C55	33.0	2.55, 2.16	C56	C56
47	C60	32.4	2.66-2.54	C18, C19	C18
48	C46	30.2	1.26	C47	
49	C48	30.1	2.00, 2.16	C13, C47, C49	C13
50	C30	27.70	2.06, 1.89	C3, C31	C3
51	C41	27.68	1.22, 1.86	C8, C42	C8
52	C47	23.3	1.84	C46	
53	B10	20.9	2.29	B4	B4
54	C20	20.5	0.39	C19	
55	B11	20.3	2.28	B7	B7
56	C36	20.2	1.94	C37	
57	Pr3	20.17, 20.19	1.25	Pr1	Pr2
58	C25	17.6	1.37	C26	C3
59	C54	17.1	1.36	C18	
60	C35	16.6	2.59	C36	
61	C53	16.4	2.58		

## S21. 2D NMR assignments of amide 9 (600 MHz, [D<sub>4</sub>]MeOH)

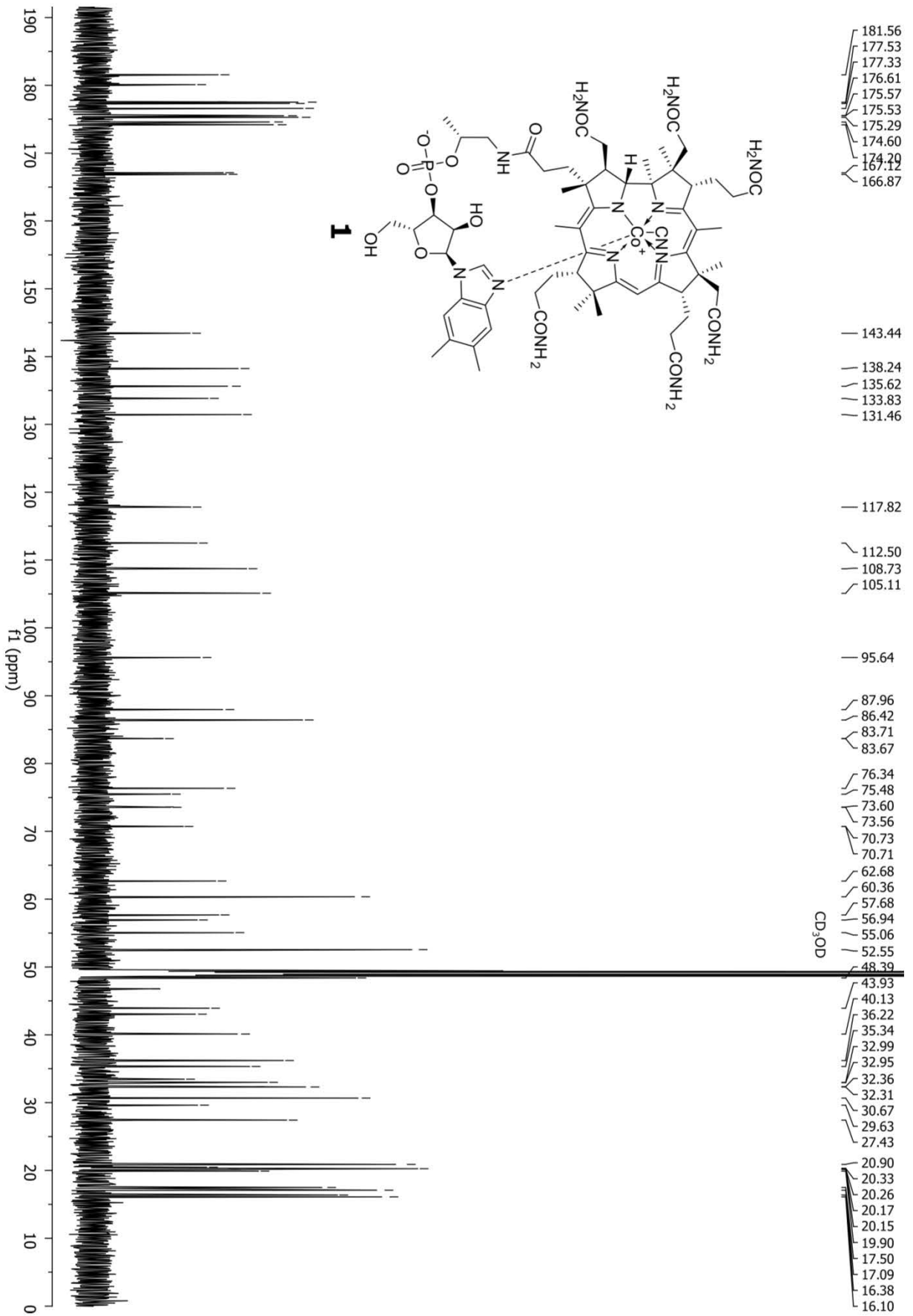
Note: Symbols 1<sup>st</sup> and 2<sup>nd</sup> in parentheses refer to the major and minor conformer of amide 9, respectively.

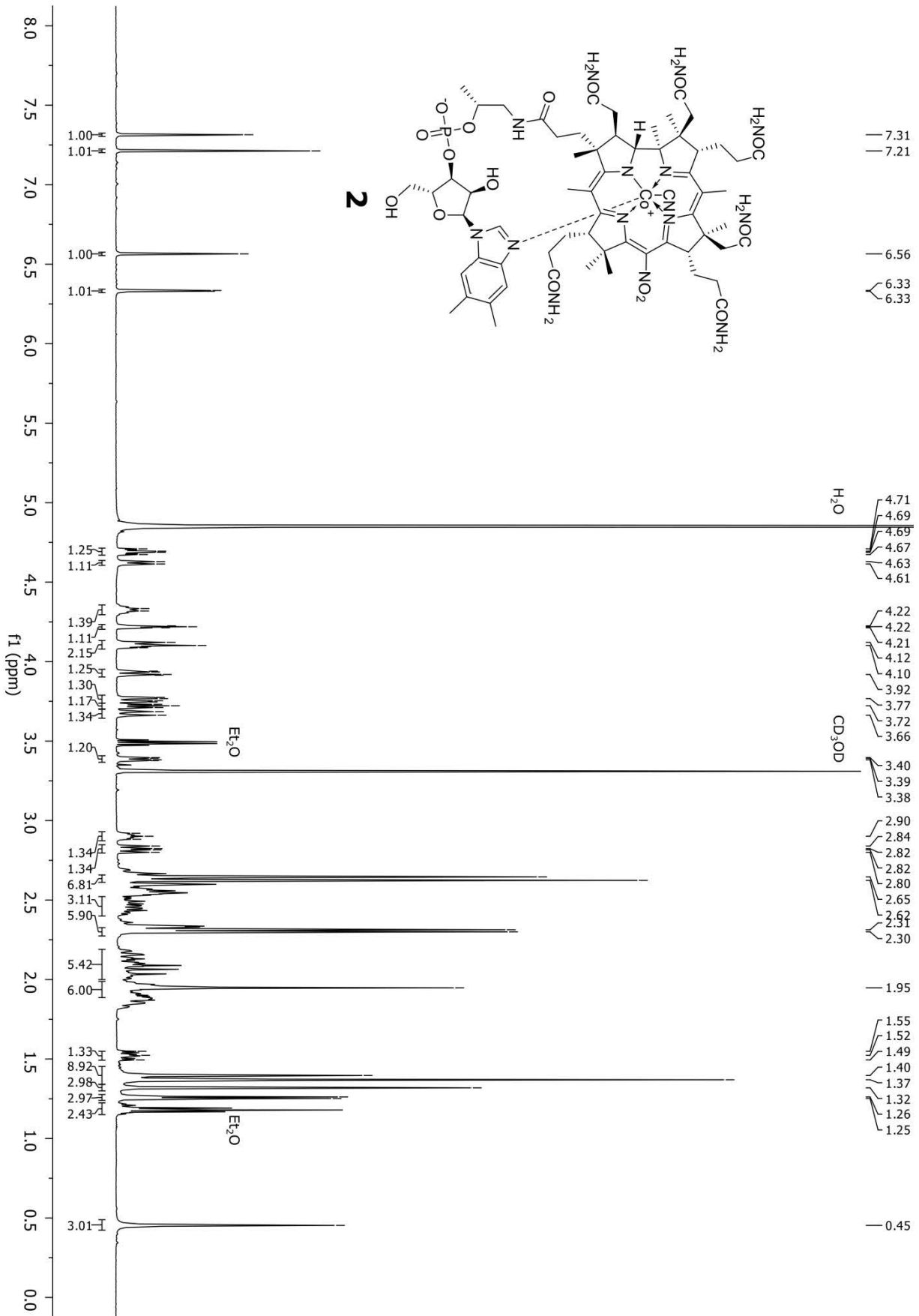
Nr	Assignment	<sup>13</sup> C Shift [ppm]	HSQC [ppm]	HMBC	ROESY	COSY
1	C4	181.6		C3, C30, C35		
2	C16	179.8		C19, C53, C54, C56		
3	C9	178.2		C8, C41		
4	C50	177.5		C48, C49		
5	C32	177.3		C31		
6	C43	177.0		C41, C42		
7	C11	175.5		C13, C46, C47		
8	C27	175.5		C26		
9	A1	175.4		A2, A3		
10	C61	175.3		C18, C60		
11	C38	174.7		C37		
12	A6	174.3		A2, A3, A5		
13	C57	174.2		C55, Pr1		
14	C14	167.2		C13, C48		
15	C6	167.0		C8, C36, C35, C37		
16	B2	143.3	7.18	R1	R4, C20, C48, C53, C55	R1
17	A4	140.0		Ar1/2, Ar3, A5		
18	B9	138.2		B7, B2, B11		
19	B6	135.8		B4, B11		
20	B5	134.0		B7, B10		
21	B8	131.5		B4, B2, B11		
22	Ar1/2 (1 <sup>st</sup> )	129.5, 128.6	7.28	Ar3, A5		A5, Ar3
23	Ar1/2 (2 <sup>nd</sup> )	129.5, 128.5	nd			
24	Ar3	128.2	7.21	Ar1/2		A5, Ar1/2
25	B4 (1 <sup>st</sup> )	119.0	6.59	B10	B10, C20, C30, C35, C36, C41	B7, B10
26	B4 (2 <sup>nd</sup> )	118.0	6.57	B10		
27	B7	112.5	7.27		R1, R2, B11	B4, B11
28	C5	109.7		C35		
29	C10	107.9		C8		
30	C15	105.6		C53, C19		
31	R1(2 <sup>nd</sup> )	88.2	6.30			R2 (2 <sup>nd</sup> )
32	R1(1 <sup>st</sup> )	88.1	6.27	R2	B7, R2, R3	R2, B2
33	C1	86.5		C3, C18, C19, C20, C25, C26		
34	R4	83.8	4.09	R2, R5	B2, C48, C55, R3, R5	R3, R5
35	C19(1 <sup>st</sup> )	76.3	4.12	C18, C60	C18, C26, C54, C60	C18, C20, C26, C53, C54
36	C19 (2 <sup>nd</sup> )	76.3	4.05		C54 (2 <sup>nd</sup> )	C18, C20 (2 <sup>nd</sup> ), C53
37	R3 (2 <sup>nd</sup> )	75.6	4.67			R2 (2 <sup>nd</sup> )
38	R3 (1 <sup>st</sup> )	75.6	4.63	R5, R4	R1, R2, R4, R5	R2, R4
39	Pr2	73.6, 73.5	4.31	Pr1, Pr3	Pr1, Pr3	Pr1, Pr3
40	R2 (1 <sup>st</sup> )	70.7	4.19	R1	B7, R1, R3	R1, R3
41	R2 (2 <sup>nd</sup> )	70.7	4.23	R1(2 <sup>nd</sup> )	R1 (2 <sup>nd</sup> )	R1 (2 <sup>nd</sup> ), R3 (2 <sup>nd</sup> )
42	R5	62.8	3.92, 3.76		R3, R4	R4
43	C17	60.3		C18, C54, C56, C60		
44	C3 (2 <sup>nd</sup> )	57.7	4.54			C25, C30
45	C3 (1 <sup>st</sup> )	57.7	4.49	C25, C26, C30, C31	C25, C30, C31, C35	C25, C26, C30
46	C13	56.8	3.24	C46, C47, C48, C49	C46, C47, C49, C53	C47, C48, C49
47	C8	56.4	3.45	C36, C37, C41, C42	A3, C36, C37, C41, C42	C41
48	C12	52.1		C13, C46, C47		
49	C7	51.7		C8, C36, C37		

Nr	Assignment	<sup>13</sup> C Shift [ppm]	HSQC [ppm]	HMBC	ROESY	COSY
51	Pr1	46.84, 46.83	3.68, 2.82	Pr3	Pr3, Pr2	Pr2
52	A5	44.2	4.40, 4.31	Ar1/2		Ar1/2, Ar3
53	C37	44.1	2.52, 2.35	C8, C36	C8, C35	C36
54	C26	43.0	2.37, 2.32	C3, C25	C19, C25	C3, C19
55	C18	40.0	2.85	C19, C54, C60	C19, C20, C54, C55, C56	C19, C20, C60
56	C31	36.2	2.53-2.38	C3, C30	C3, C25, C30	C30
57	C49	35.6	2.56	C13, C48	C13, C47	C13, C48
58	C42	34.7	2.09, 1.55	C8, C41	B10, C8, C36, C41	C41
59	C56	33.7	2.65, 1.82	C18, C54, C55	C18, C55	C55
60	C55	33.1	2.55, 2.16	C56	B2, C18, C56, C53, R4	C56
61	C60	32.4	2.66-2.53	C18, C19	C19	C18
62	A2	32.0	2.69	A3	C47 C8	A3
64	A3	31.3	2.59	A2	C47	A2
65	C46	31.2	1.12	C13, C47	C13, C47	
66	C48	30.1	2.05, 1.97	C13, C49	B2, C47, R4	C13, C49
67	C41	28.7	1.78, 1.11	C8, C42	B4, B10, C8, C36, C42	C8, C42
68	C30	27.4	2.03, 1.87	C3, C31	B4, C3, C20, C25, C31	C3, C31
69	C47	22.8	1.63	C46	C13, C46, C48, C49, A2, A3	C13
70	B10	21.0	2.31	B4	B4, C36, C41, C42	B4
71	C20	20.5	0.41, 0.40 (2 <sup>nd</sup> )	C19	B2, B4, C18, C30, C25	C18, C19
72	B11	20.3	2.29	B7	B7	B7
73	Pr3	20.18, 20.16	1.26	Pr1	Pr1, Pr2	Pr2
74	C36	19.8	1.89	C37	B4, B10, C8, C35, C41, C42	C37
75	C25	17.4	1.37	C20, C26	C3, C20, C26, C30, C31	C3, C26
76	C54	17.1	1.36, 1.35 (2 <sup>nd</sup> )	C18	C18, C53	C19
77	C35	16.7	2.59	C36	B4, C3, C37, C36	
78	C53	16.4	2.60		B2, C13, C54, C55	C19

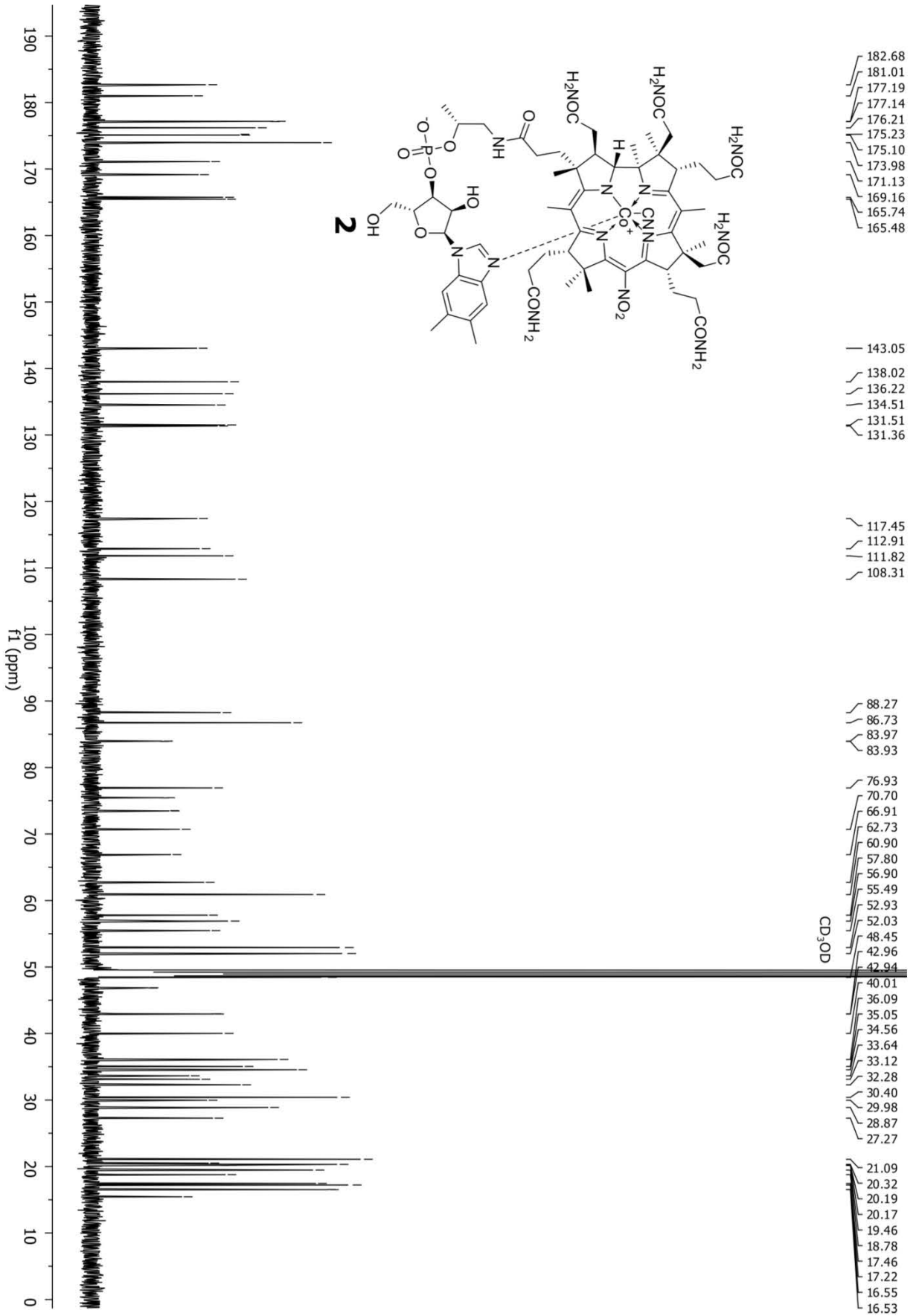


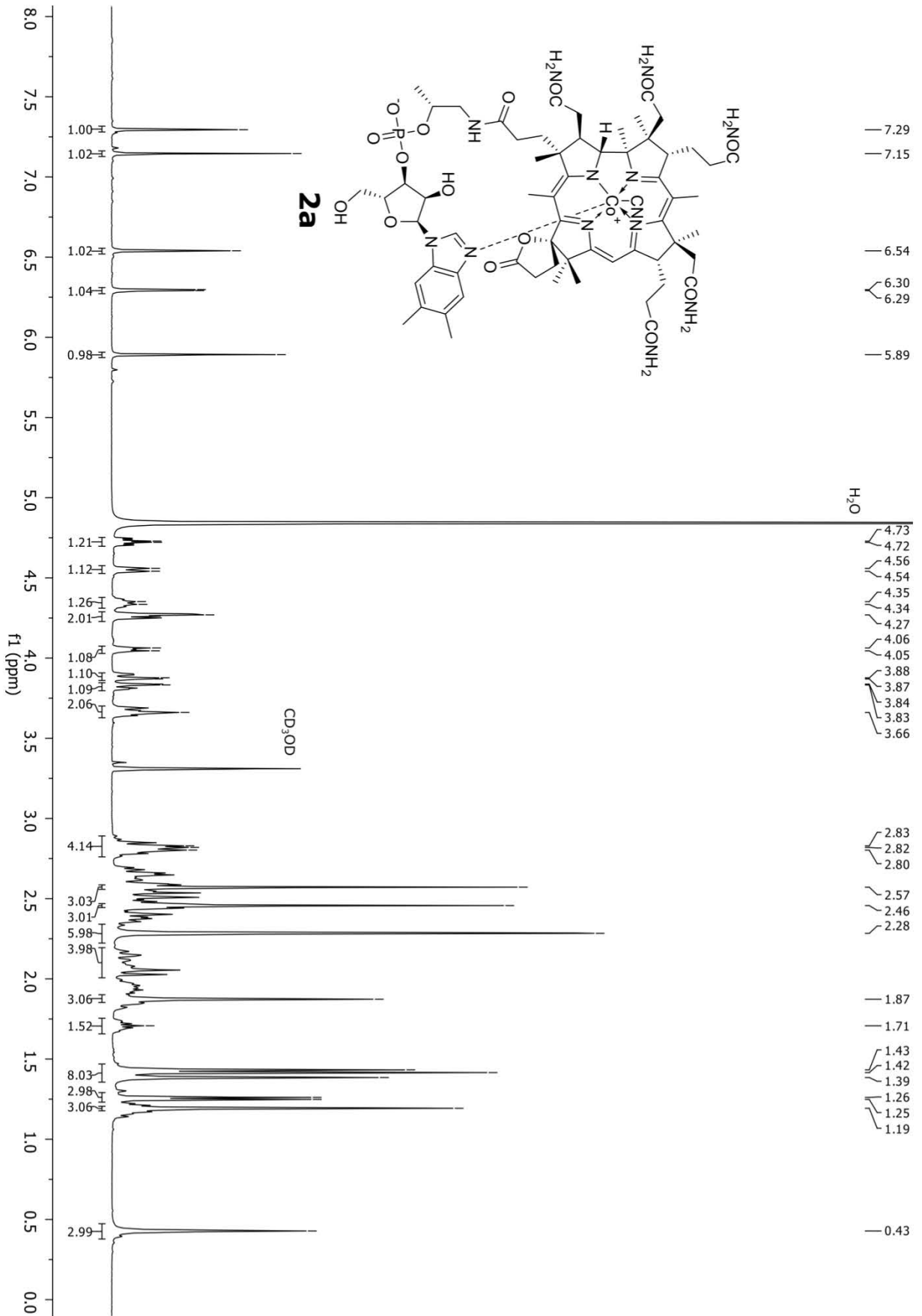
**S22. NMR spectra (500 or 600 MHz, [D<sub>4</sub>]MeOH)**  
 Note: For clarity only selected signals were integrated in <sup>1</sup>H NMR spectra.

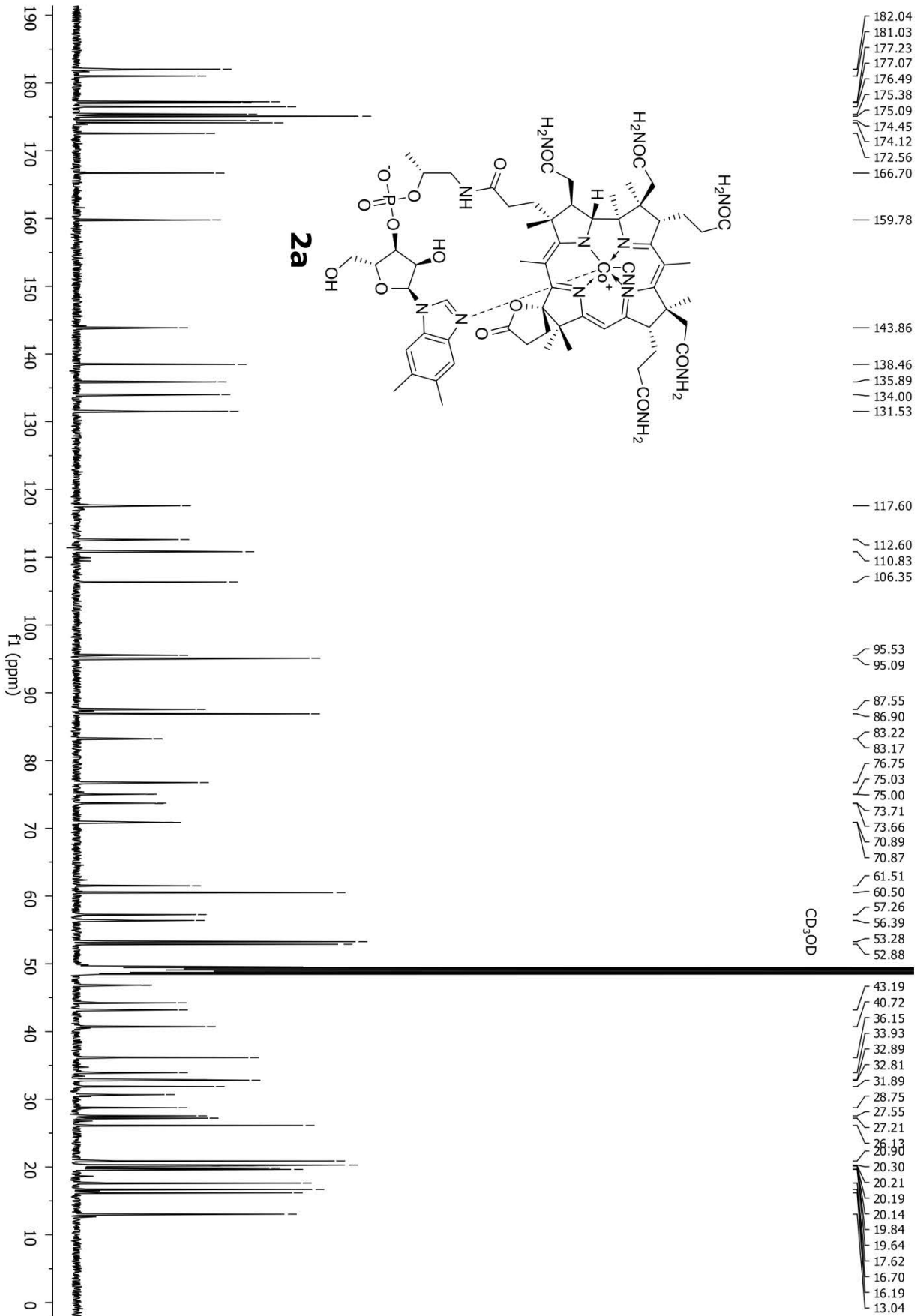


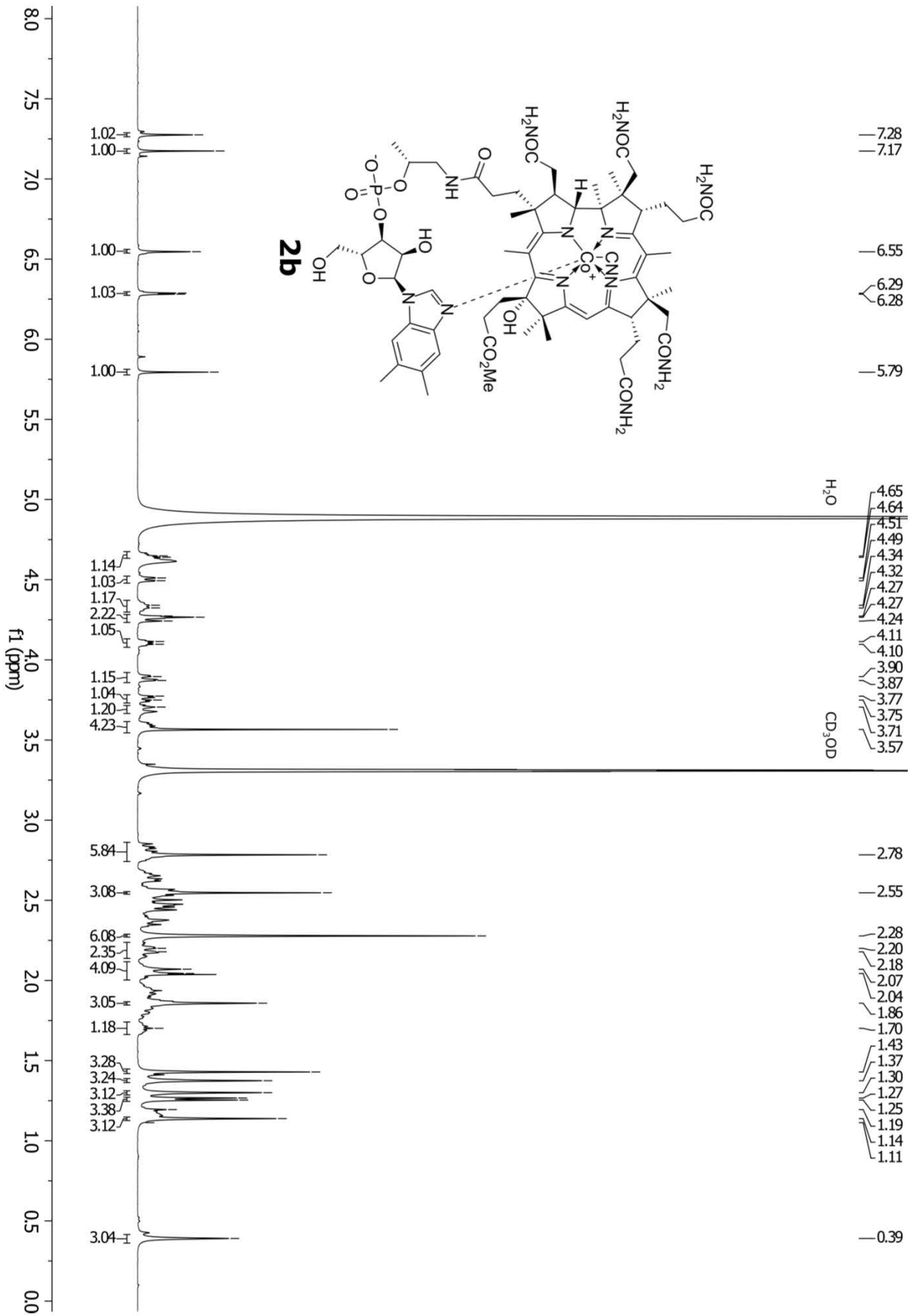


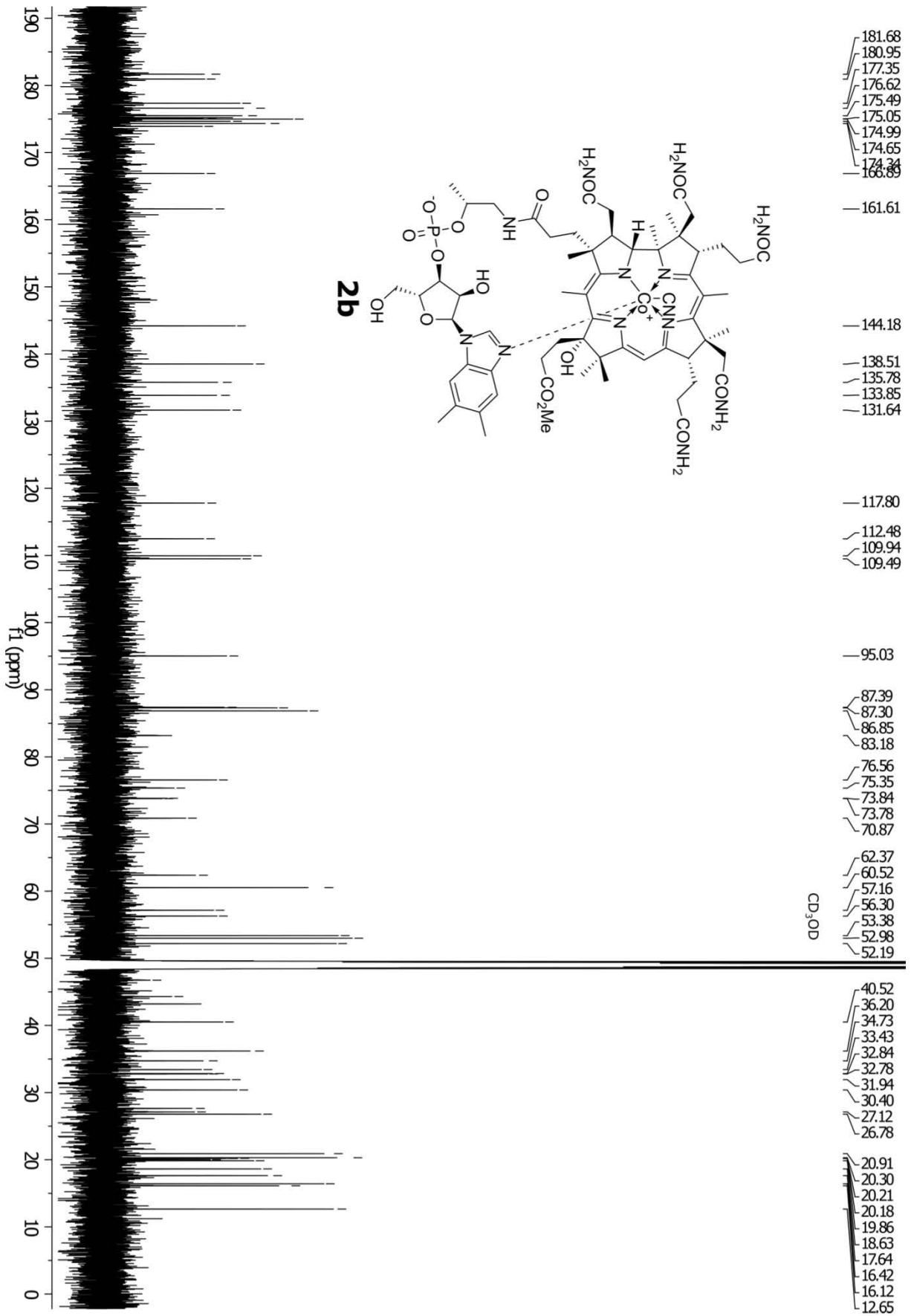


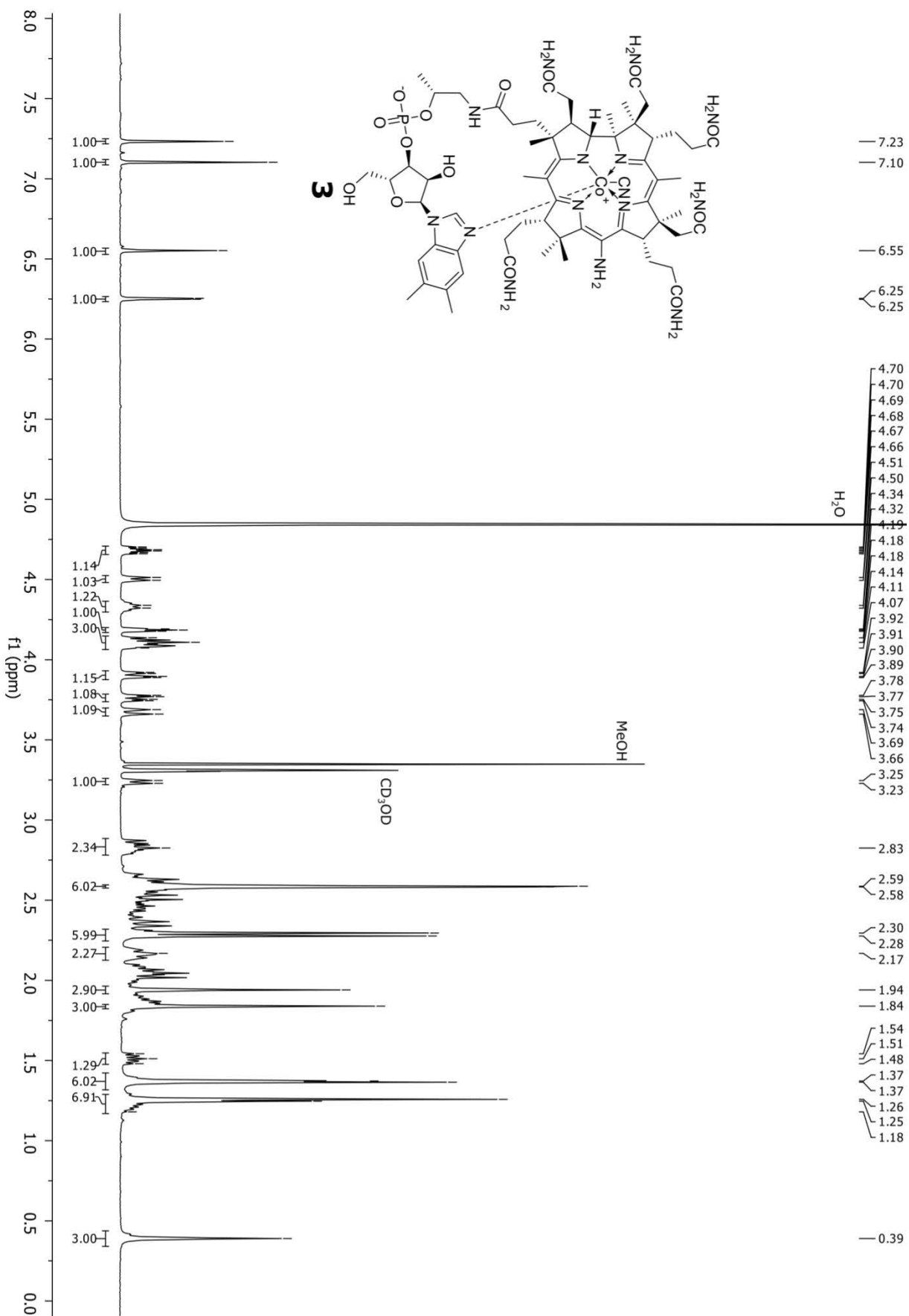


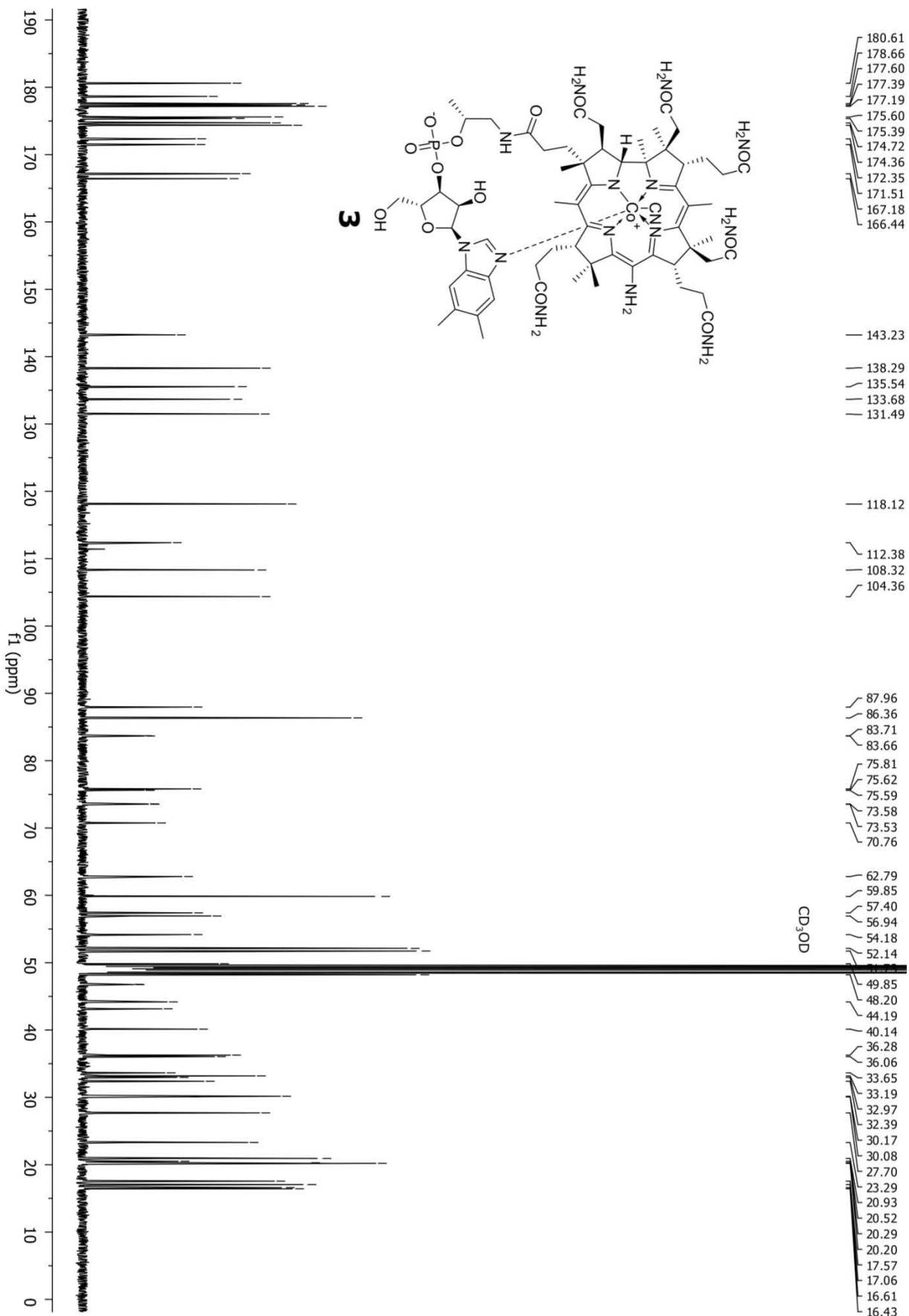


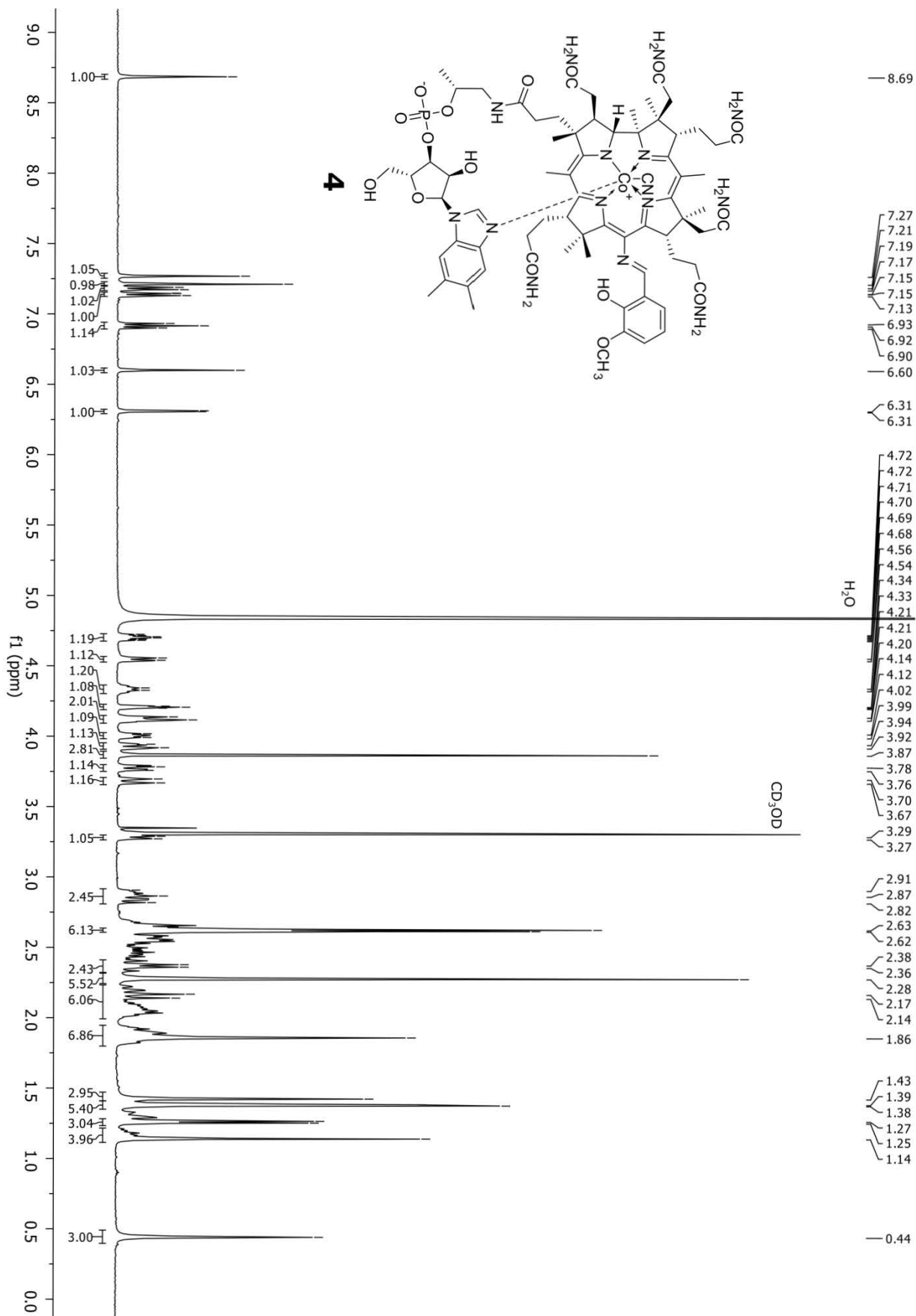




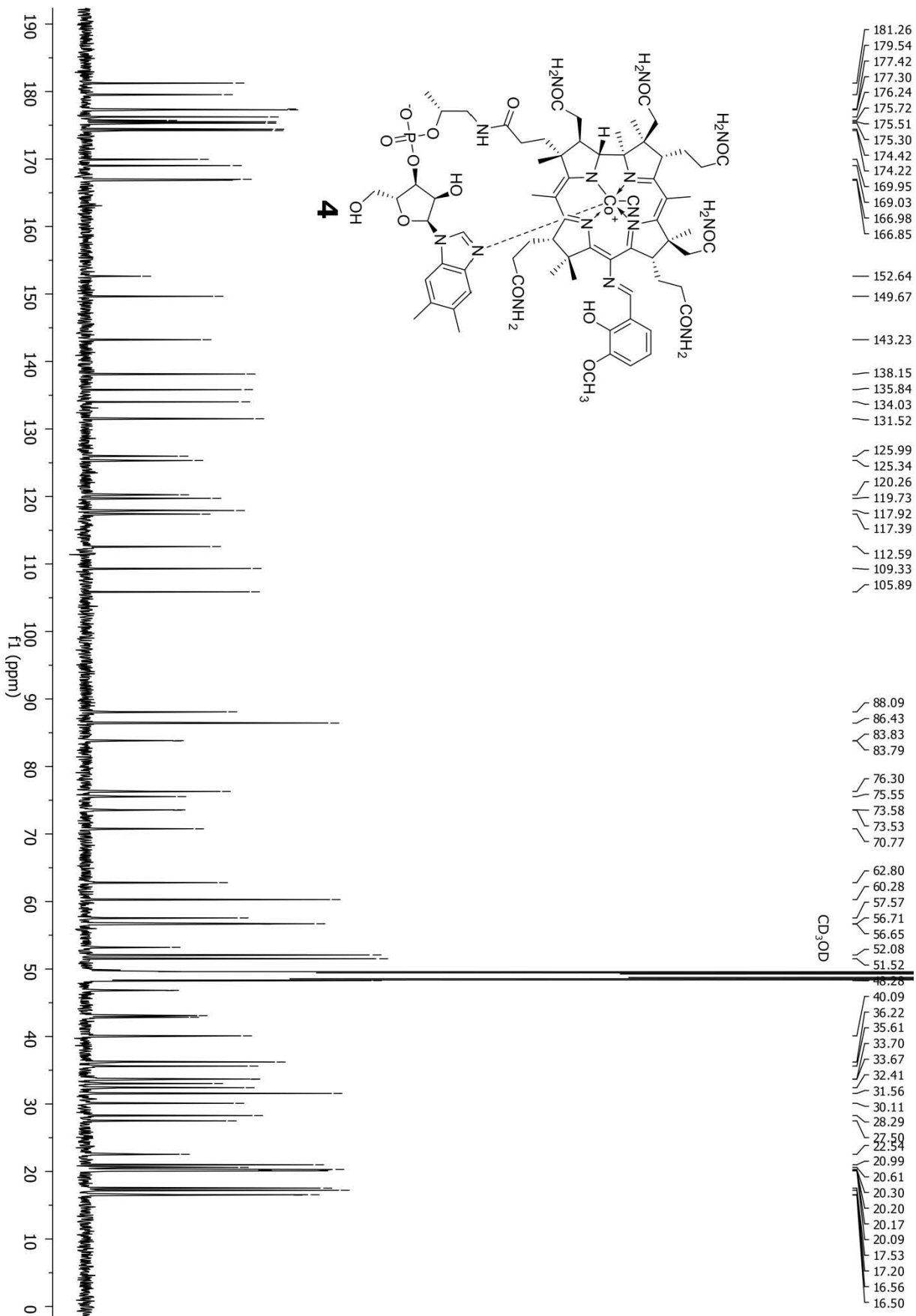


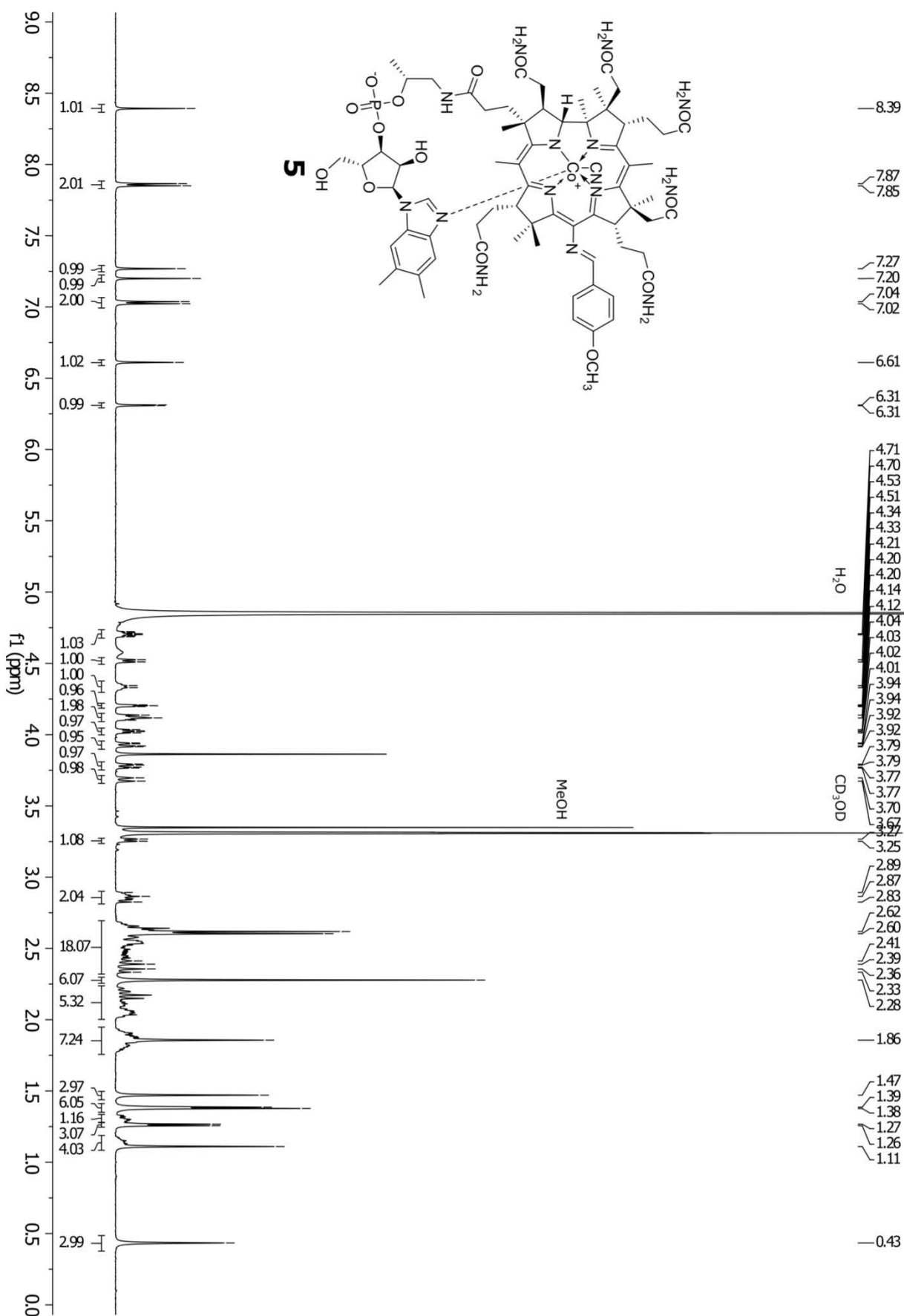


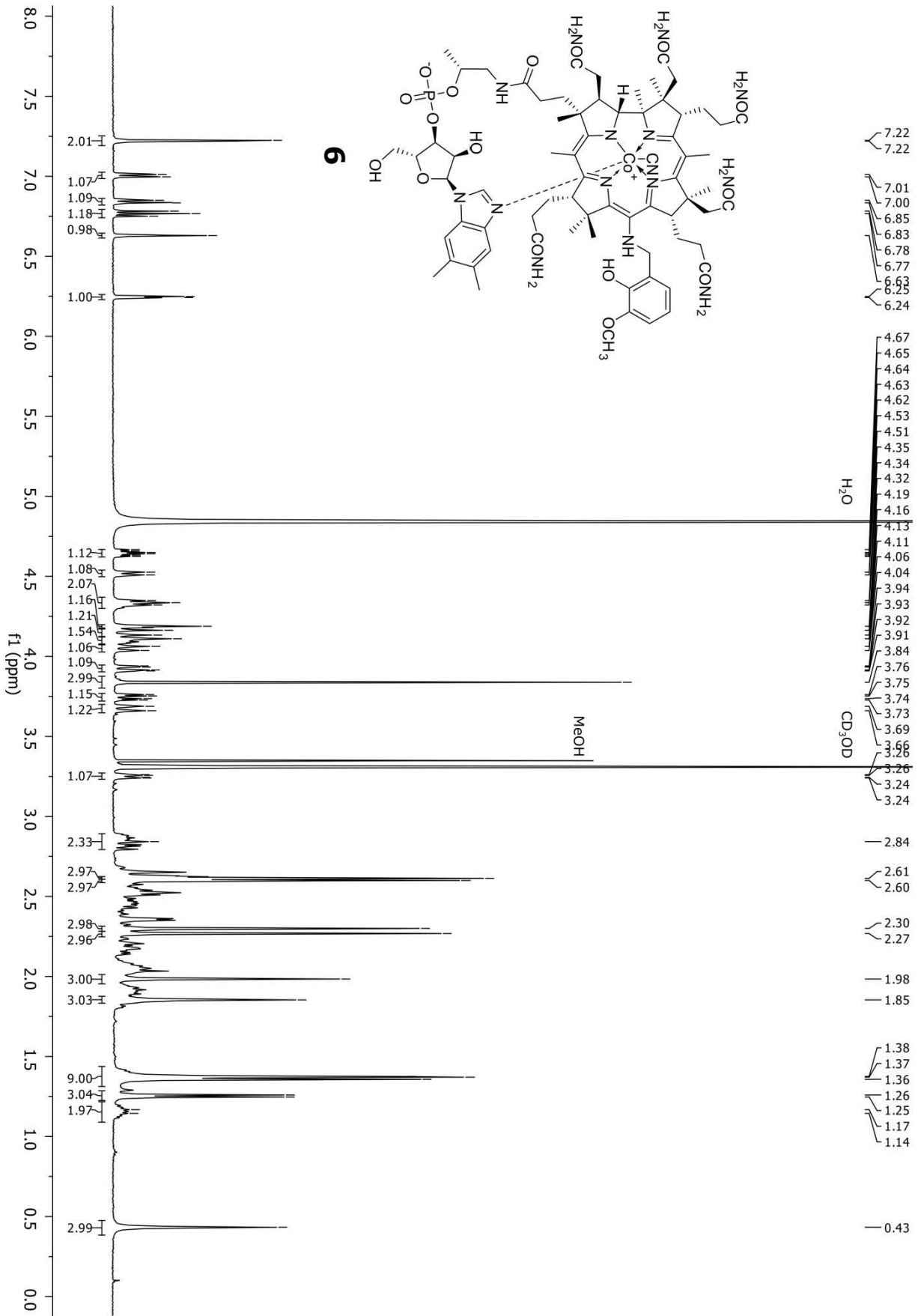


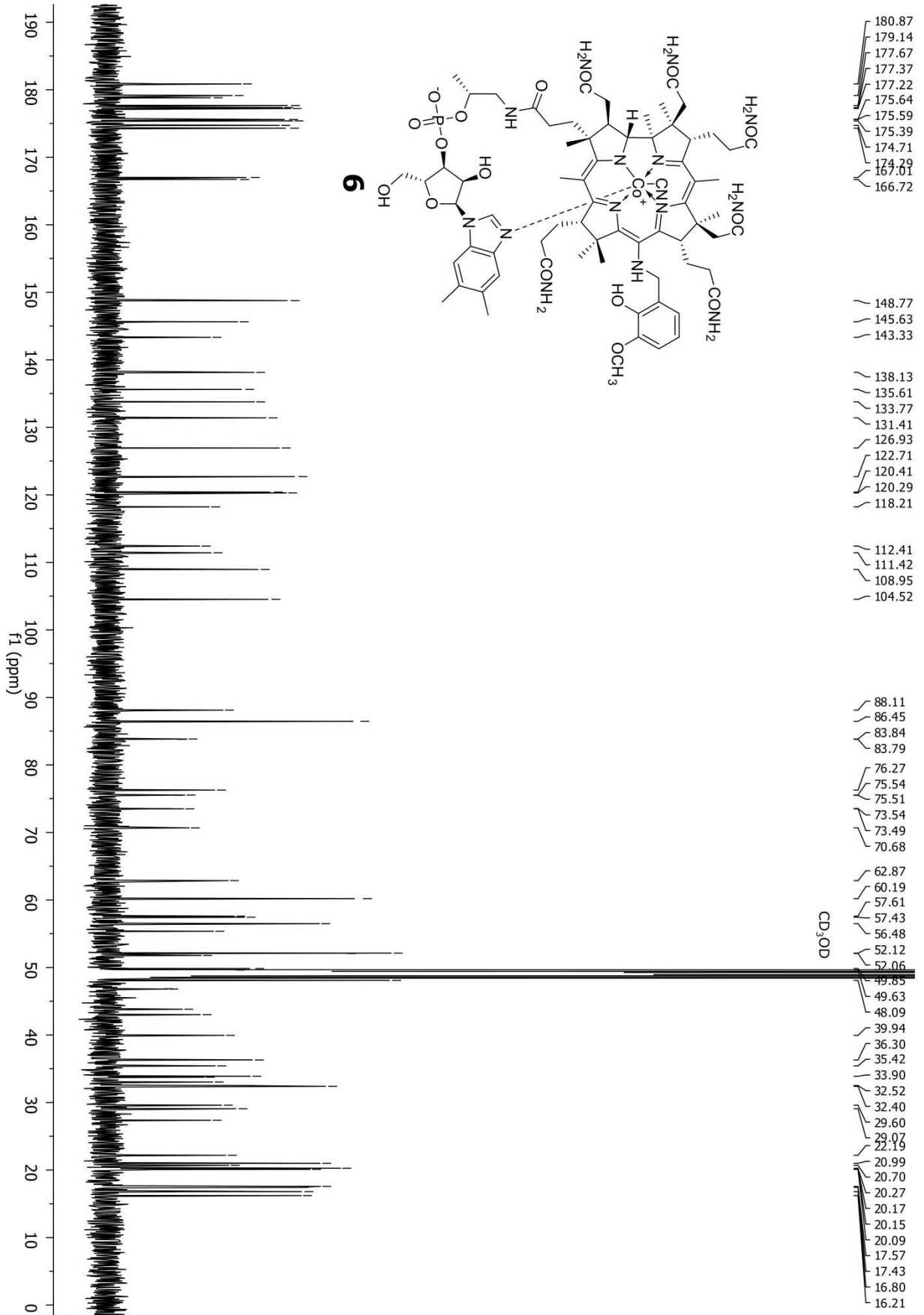


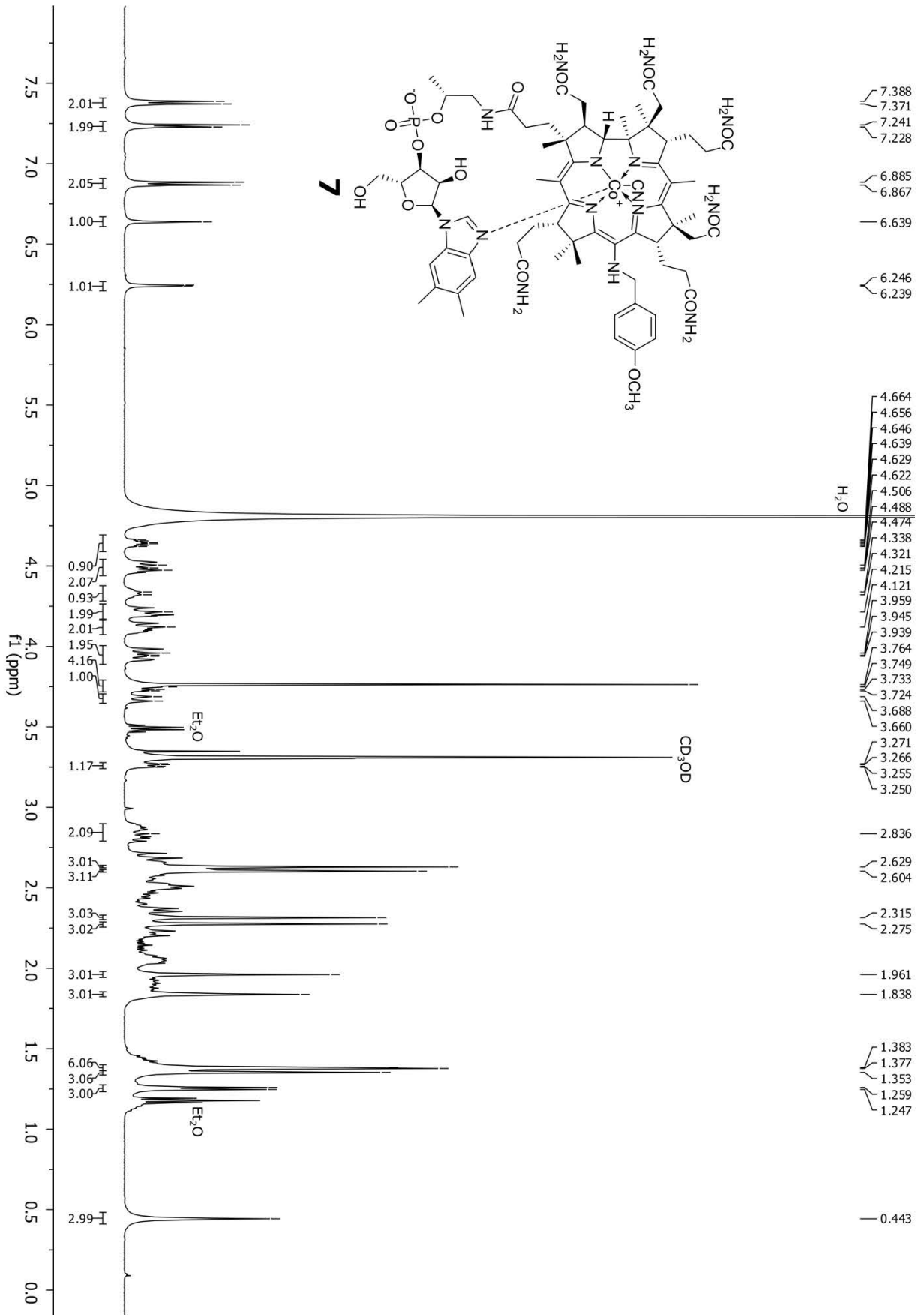


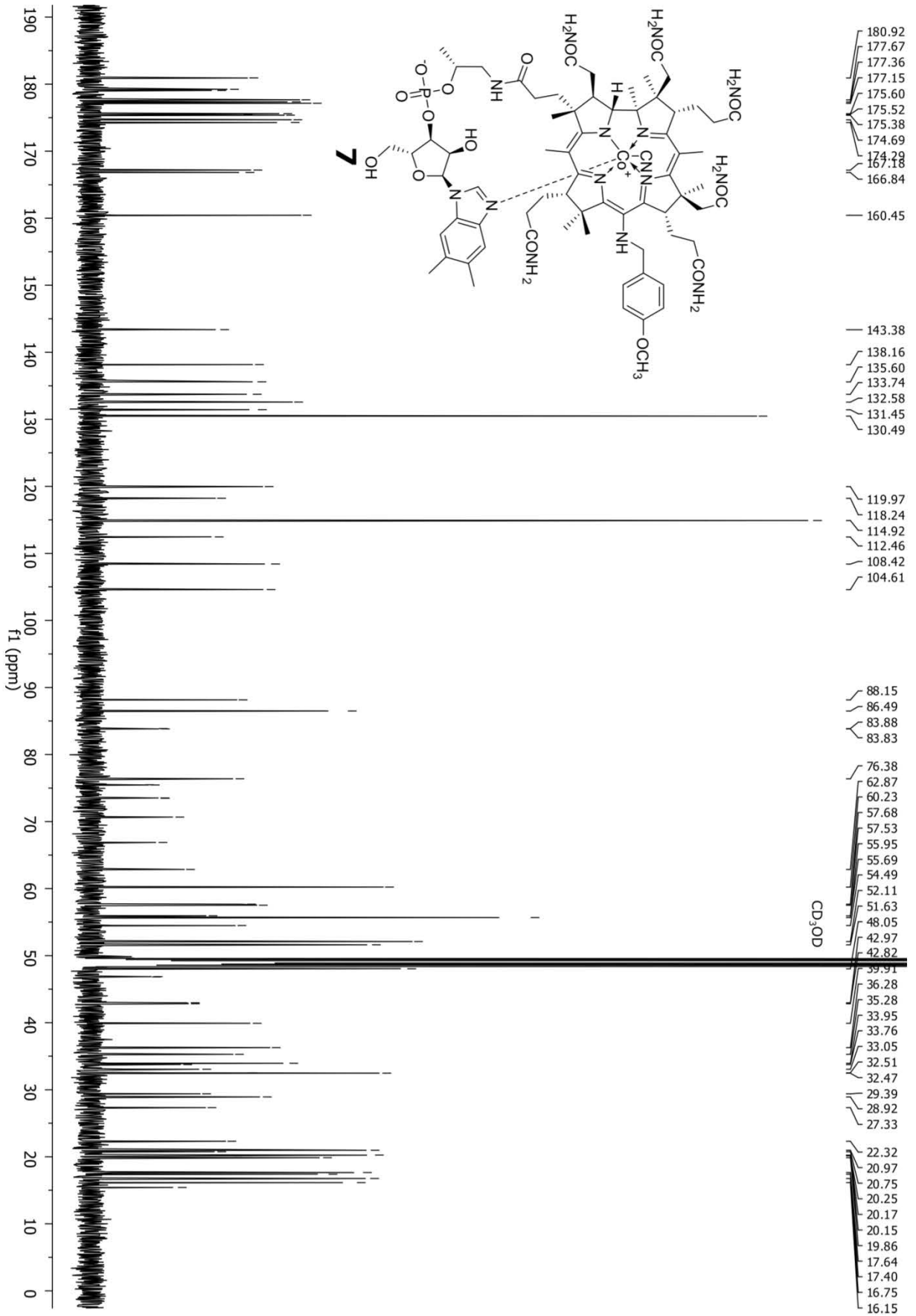


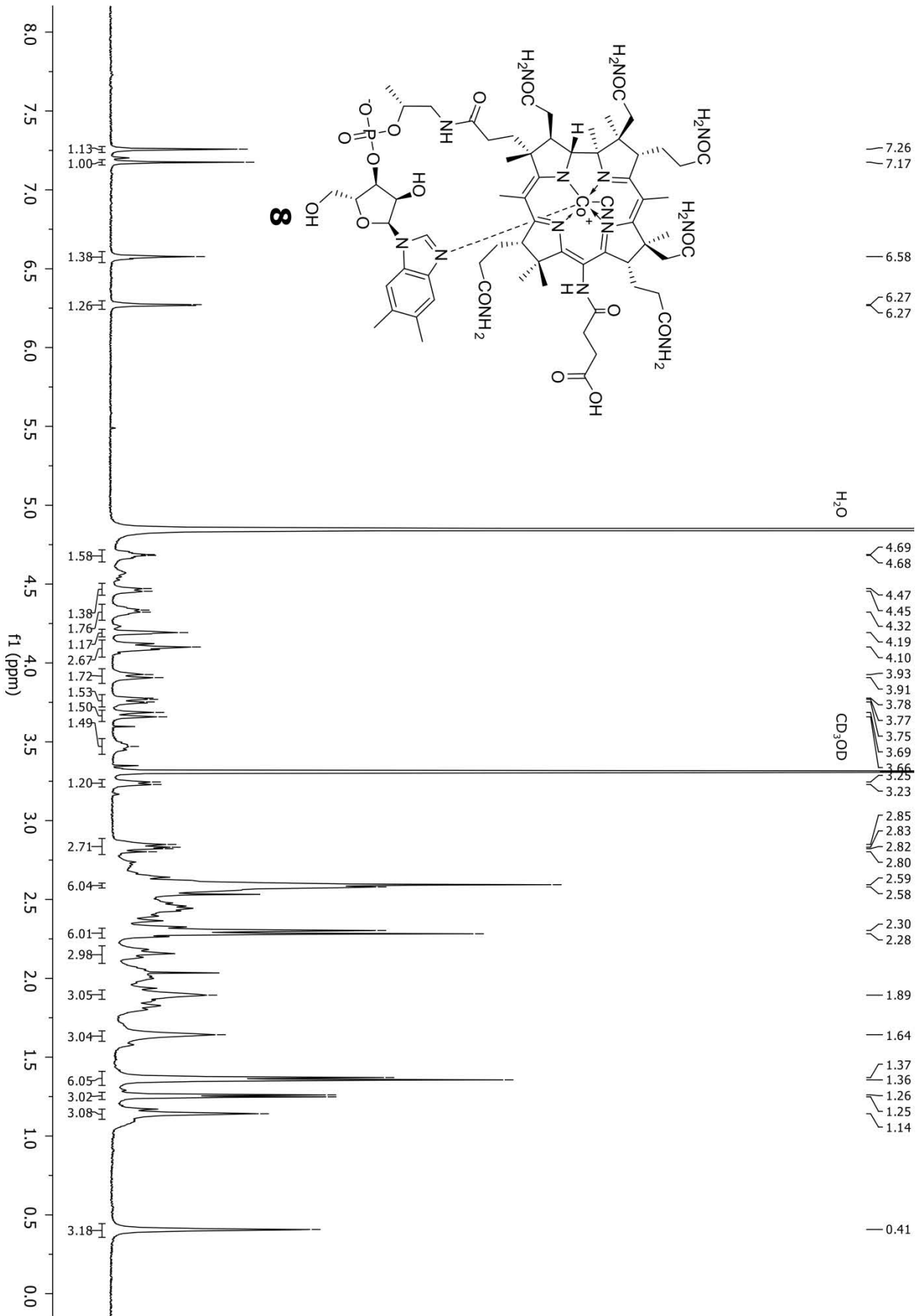


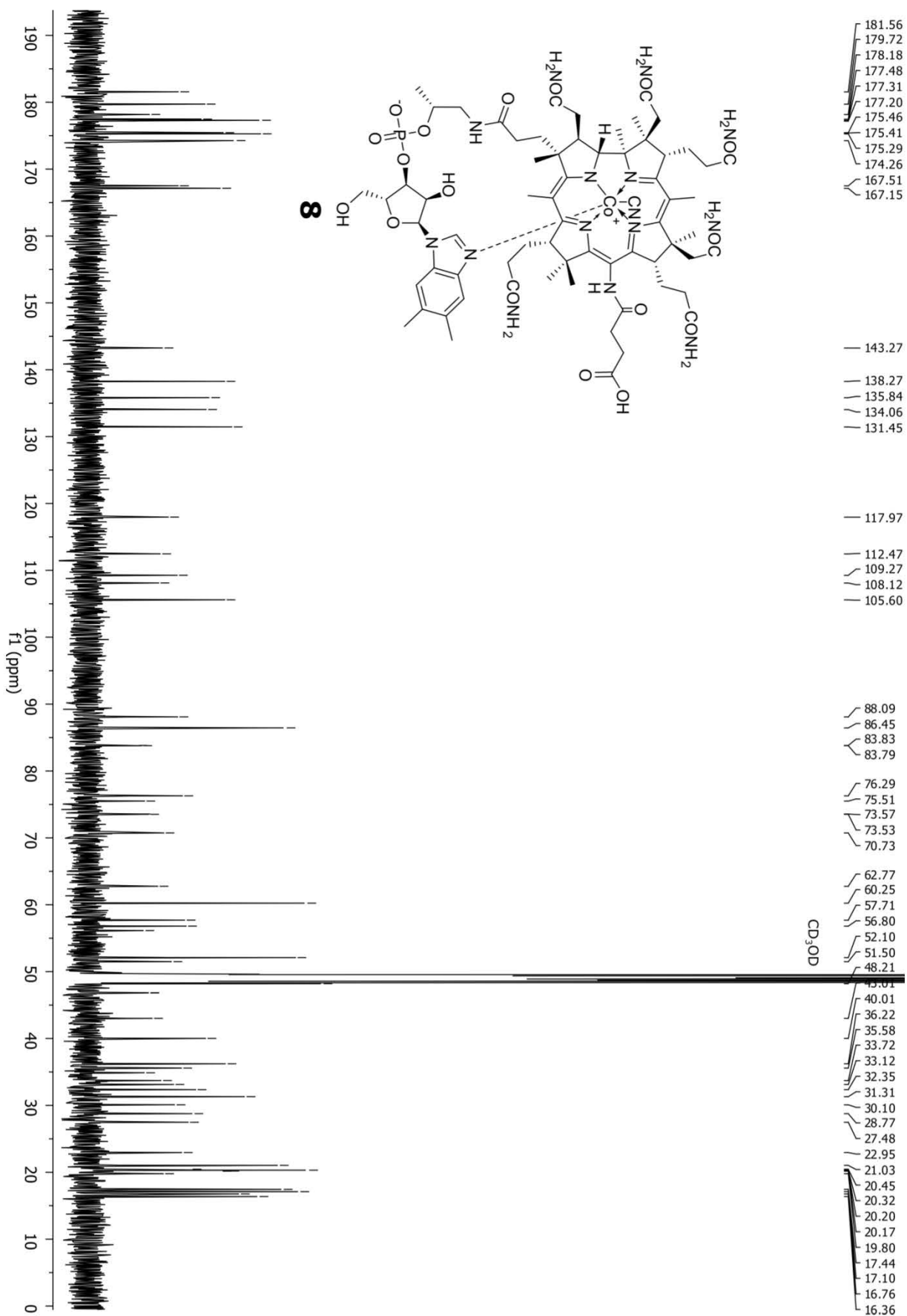




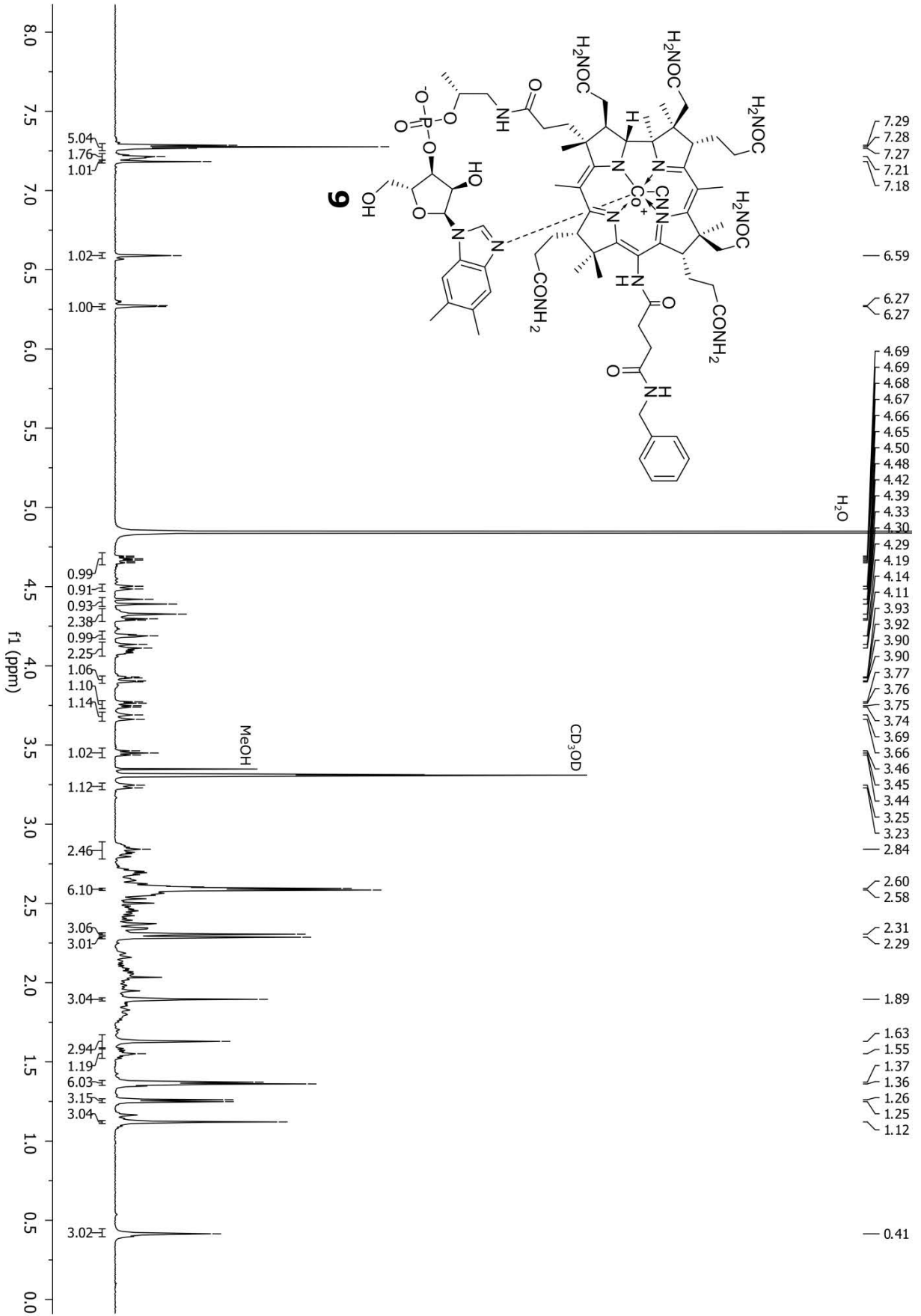


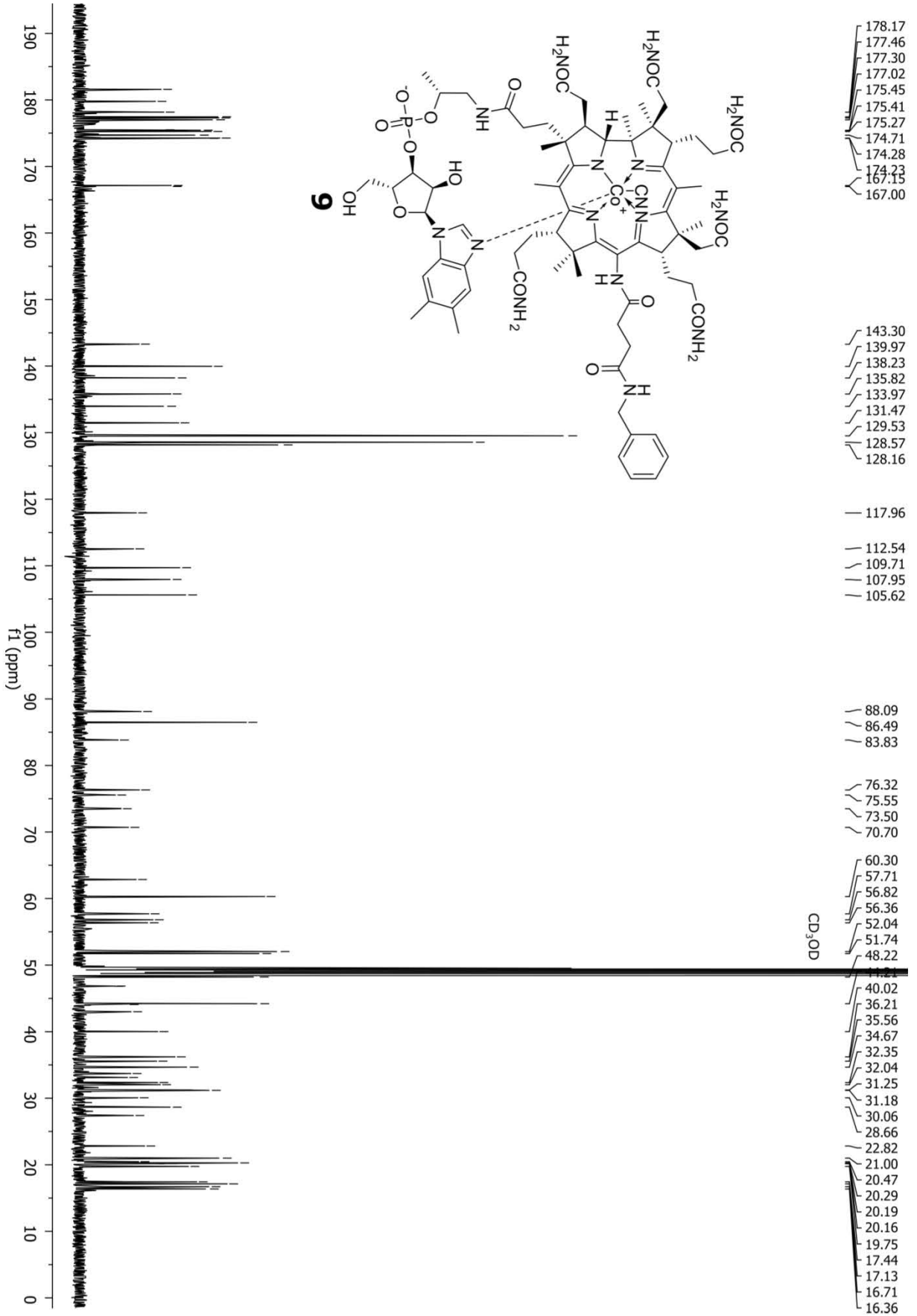




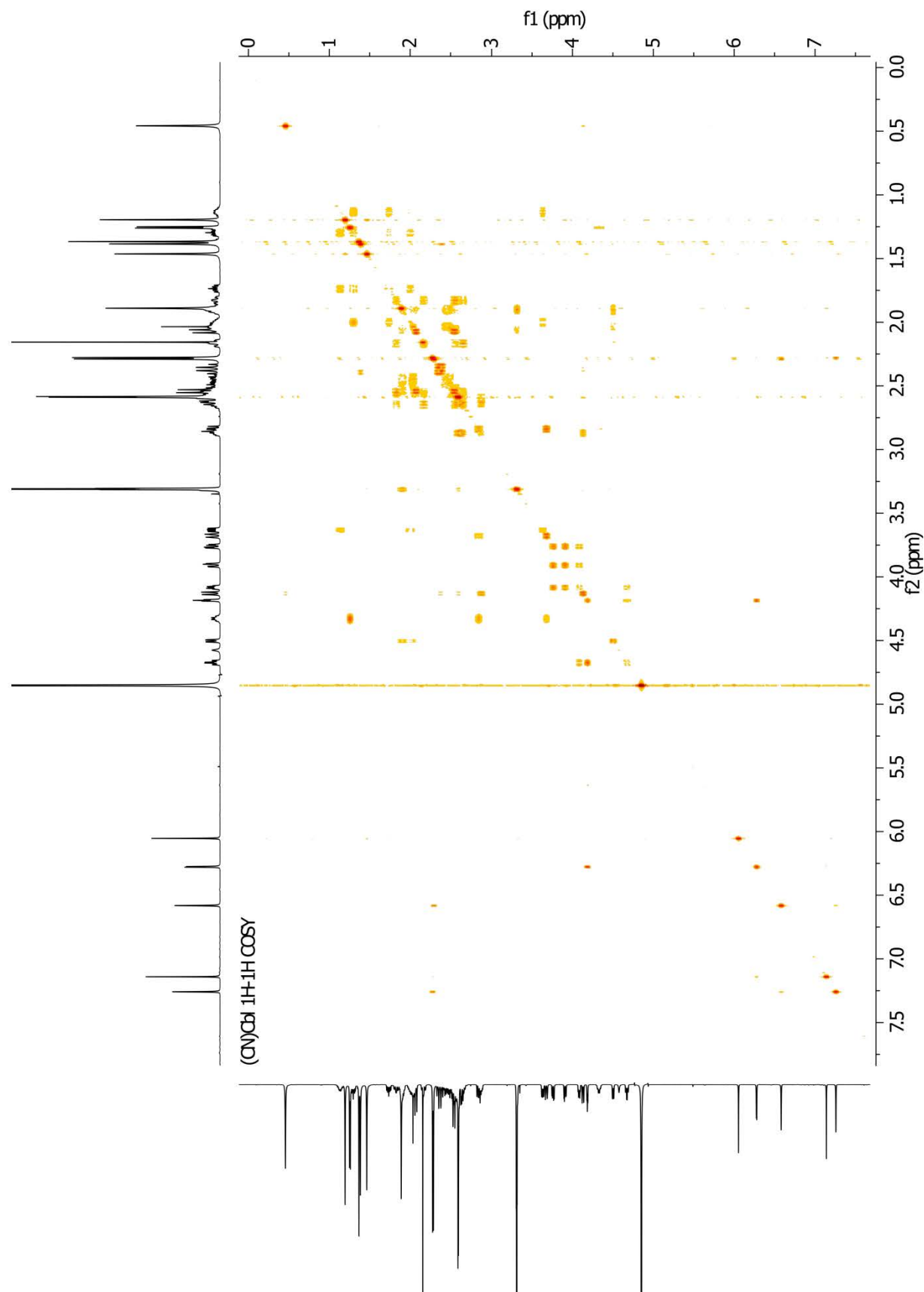


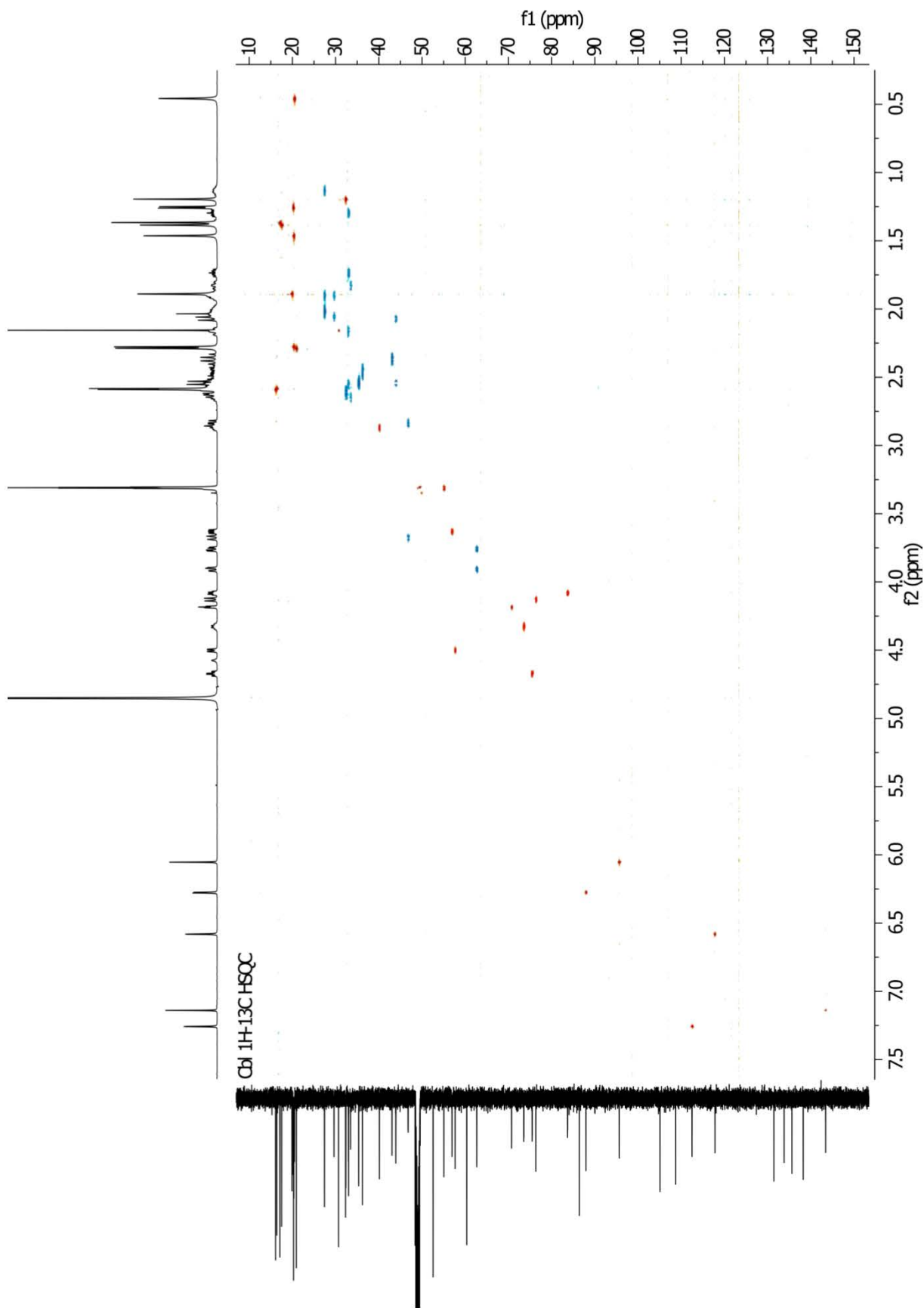


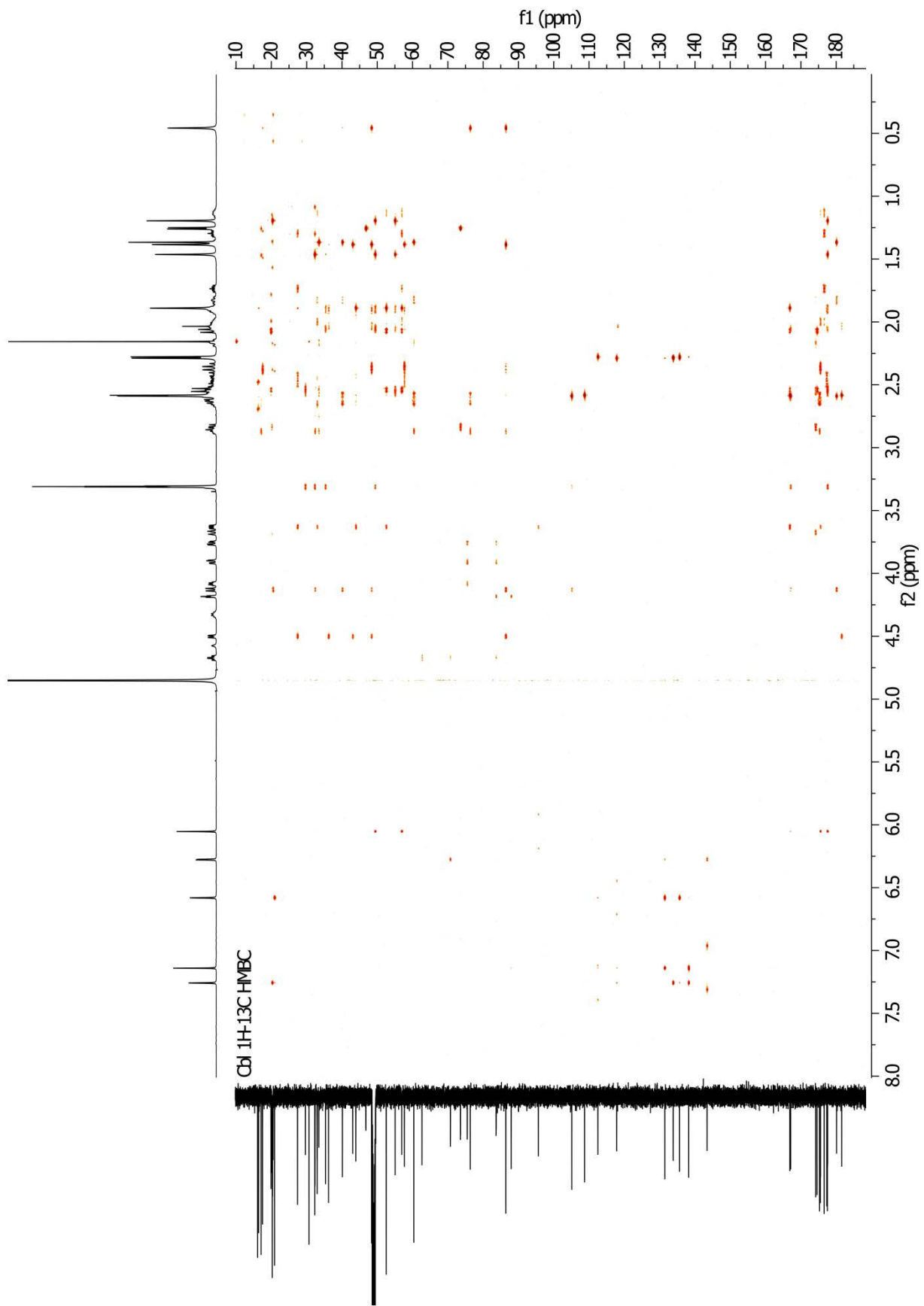


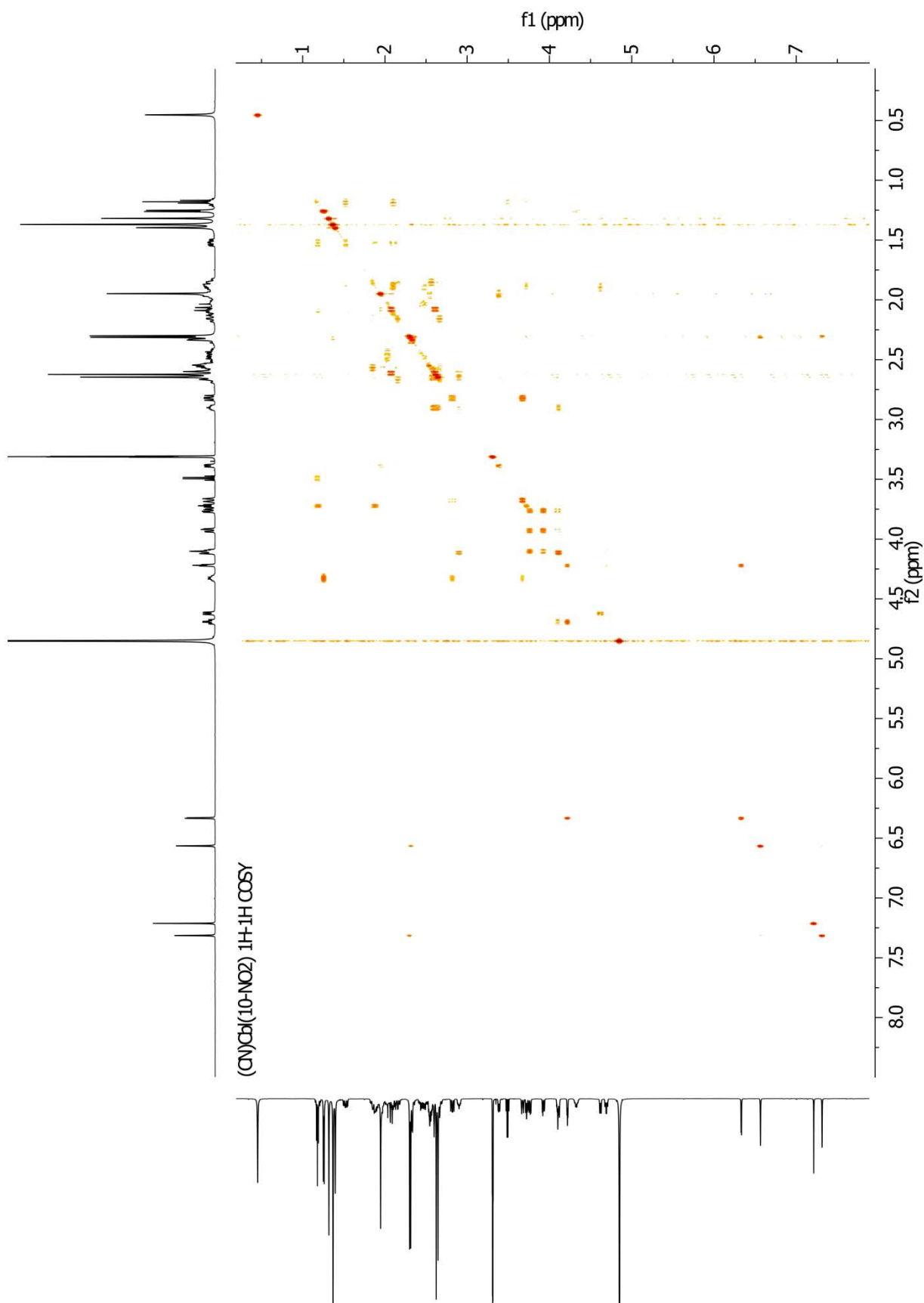


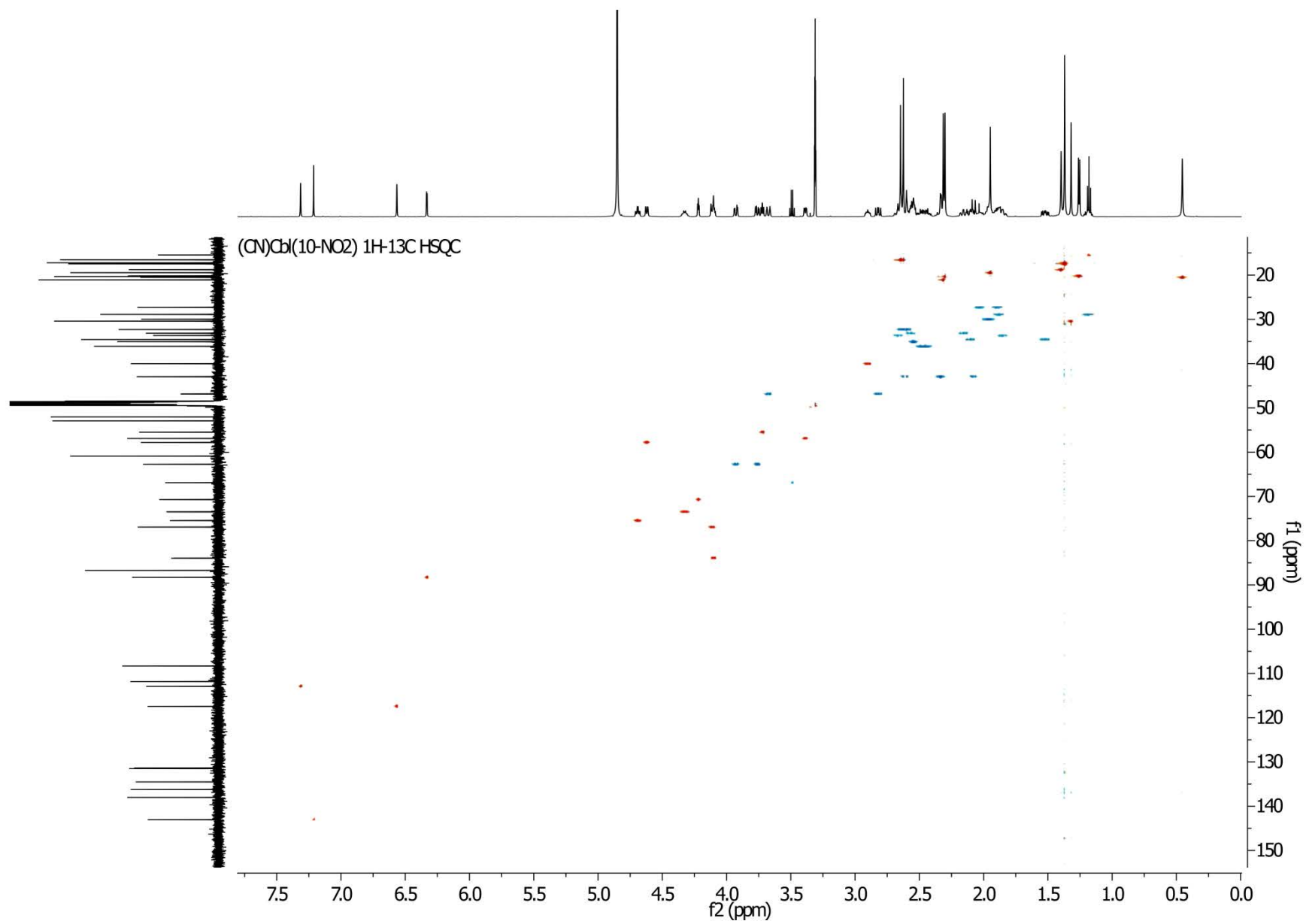
## S23. 2D NMR spectra

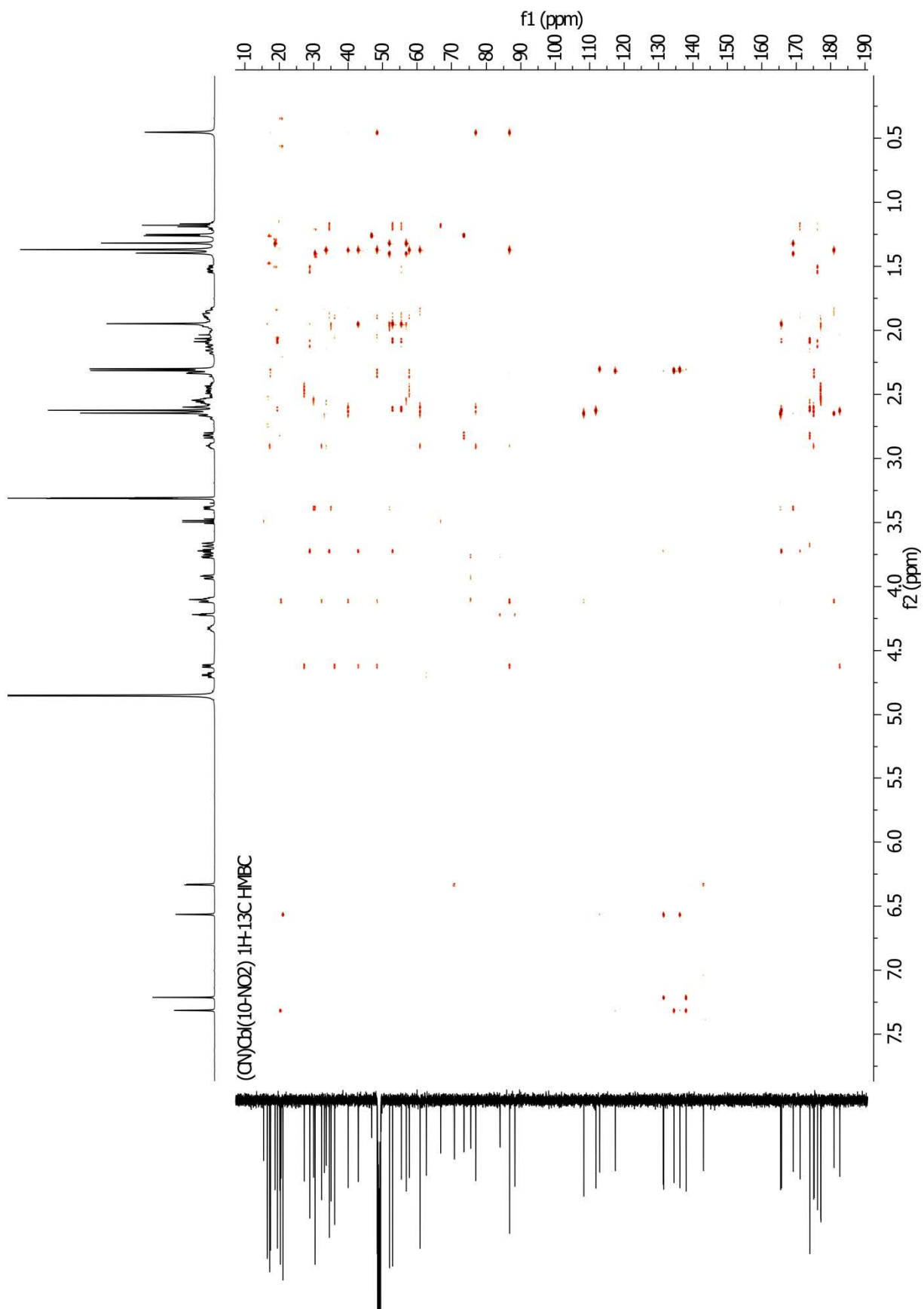




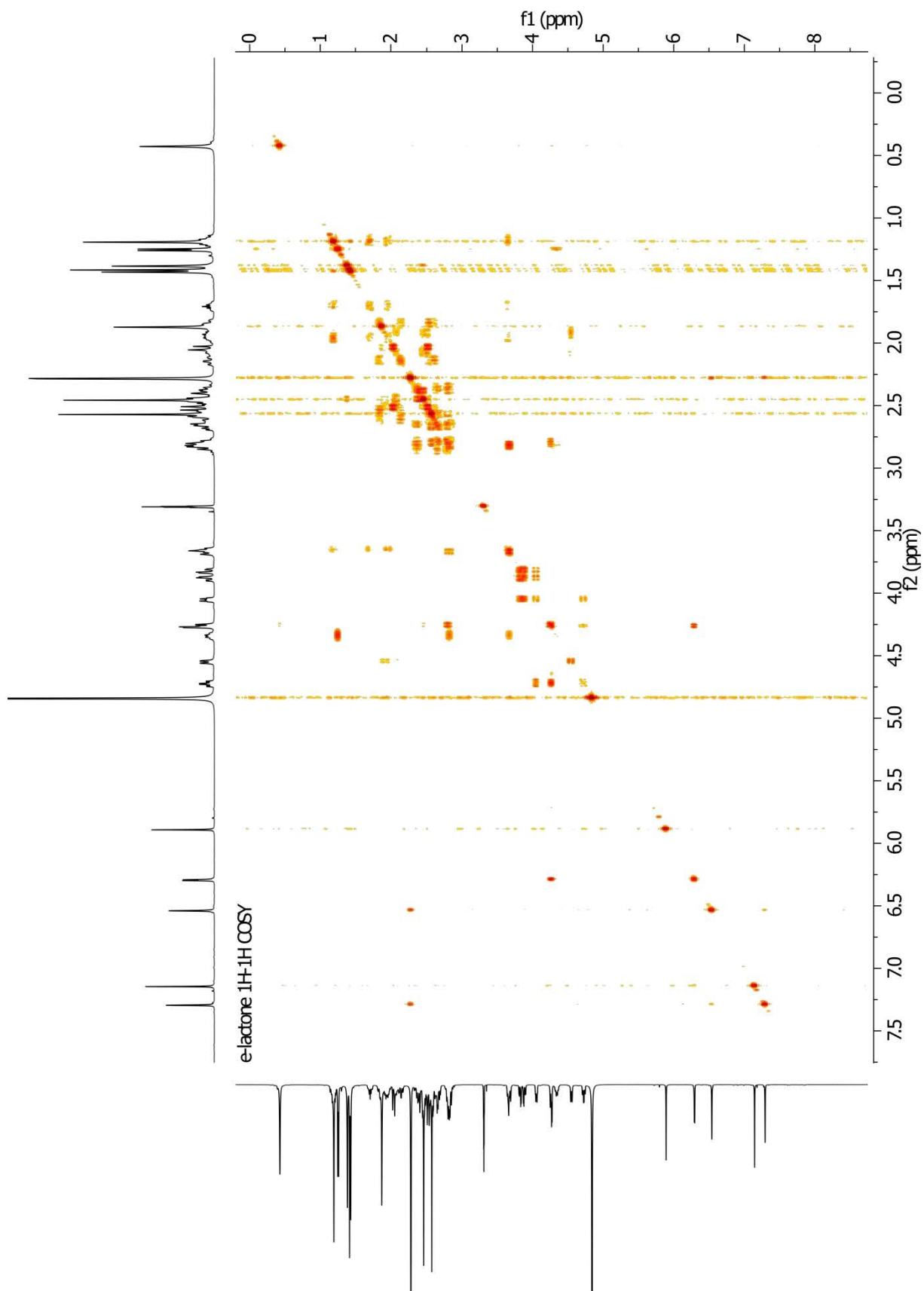


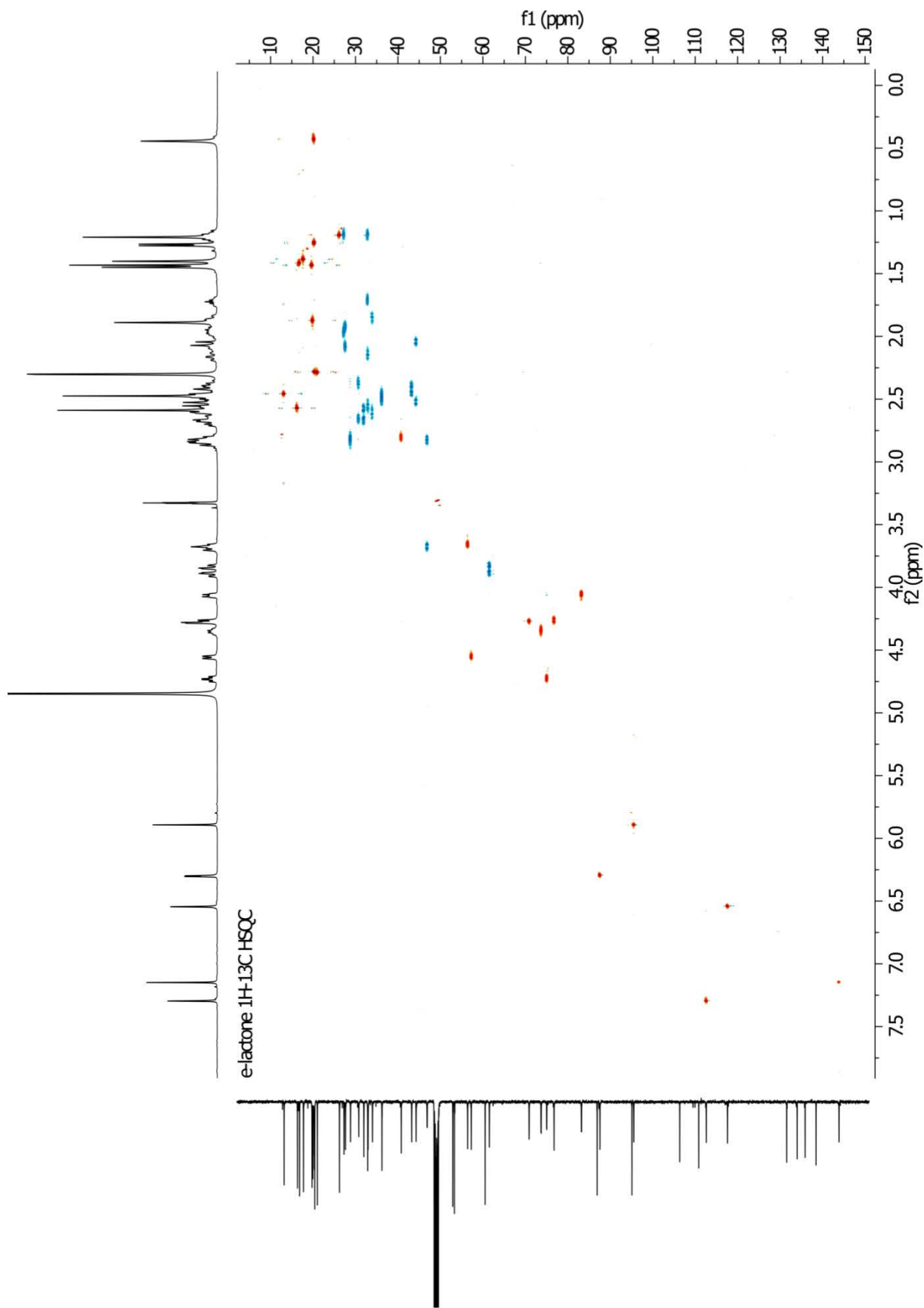






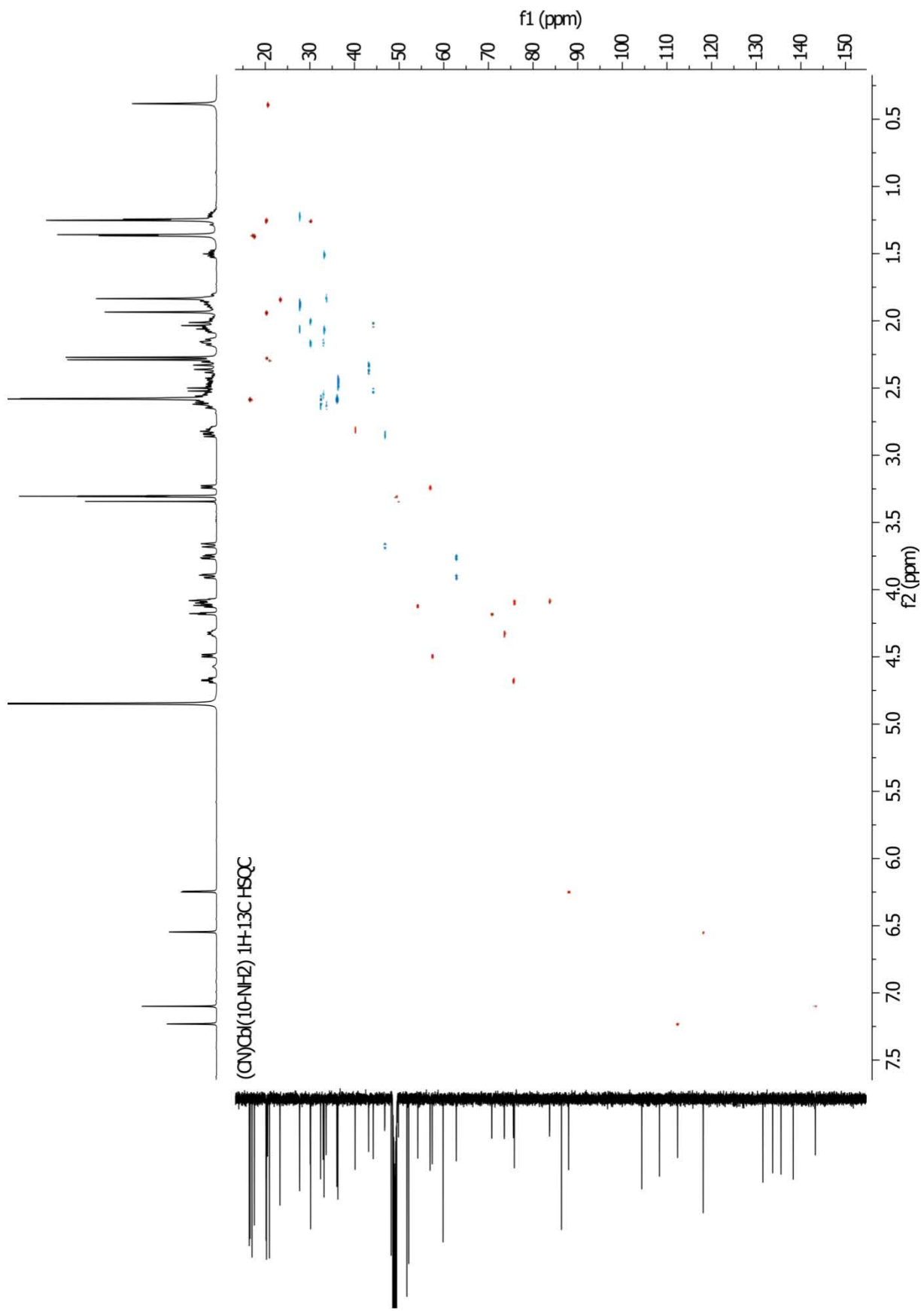


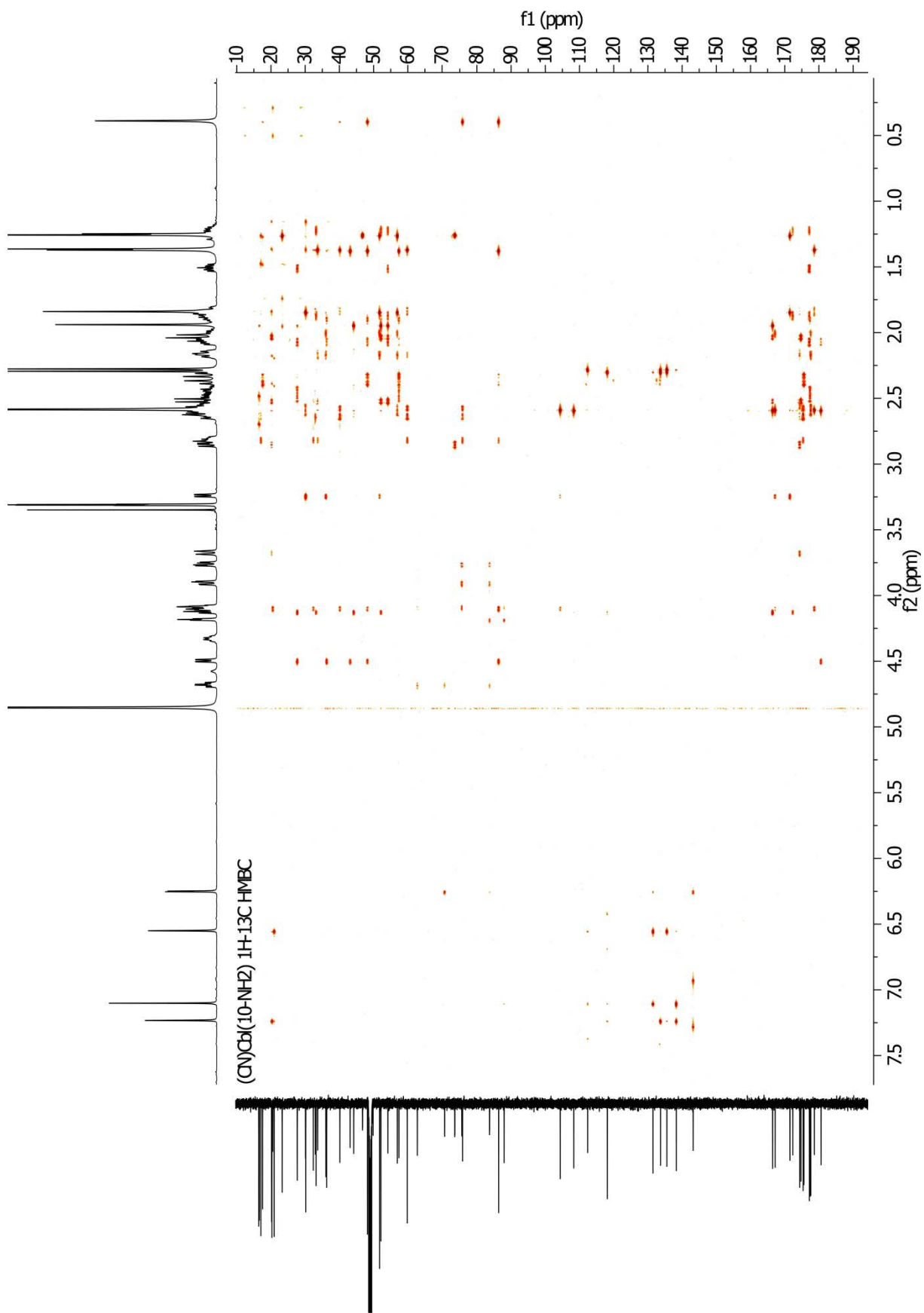


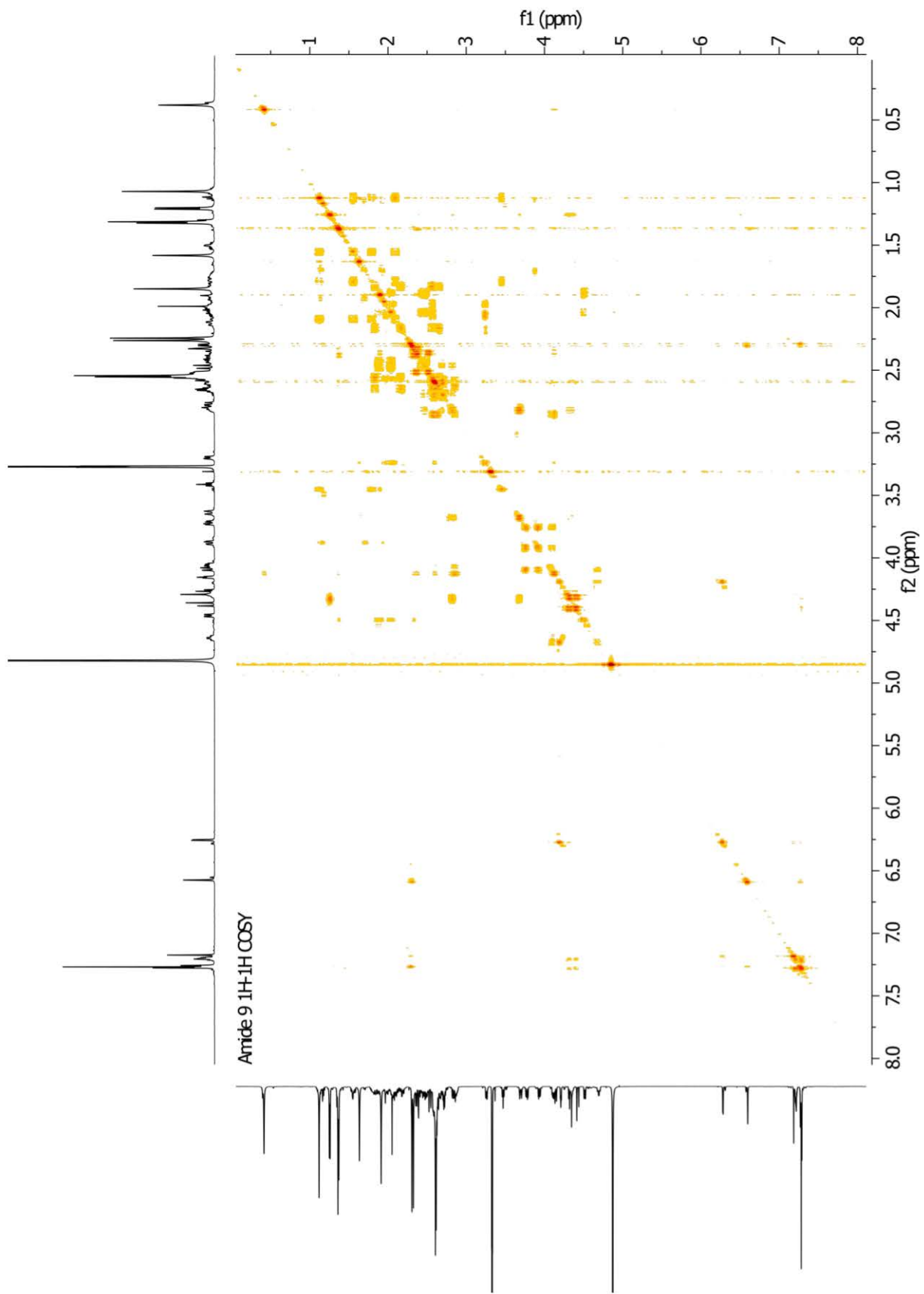


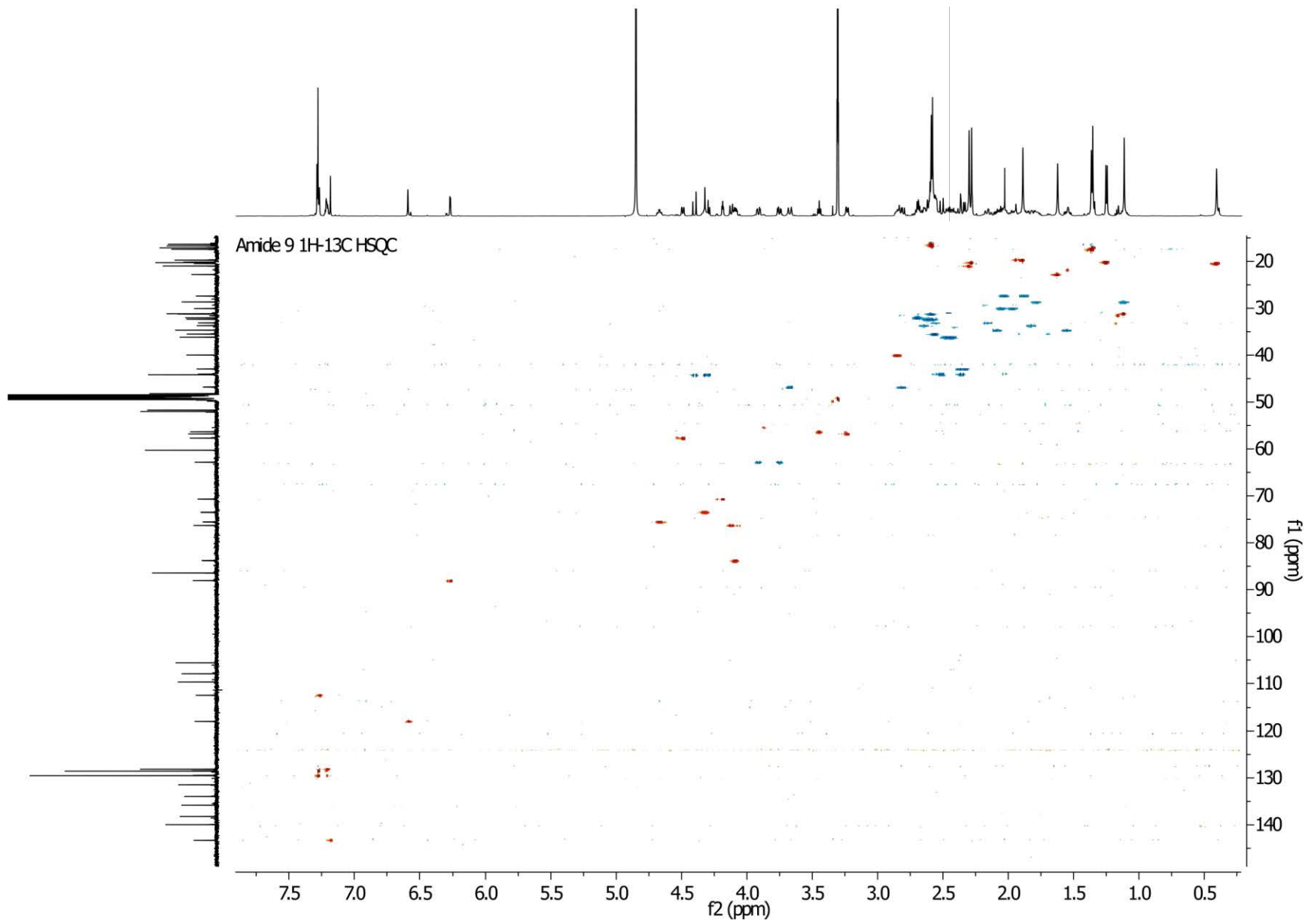




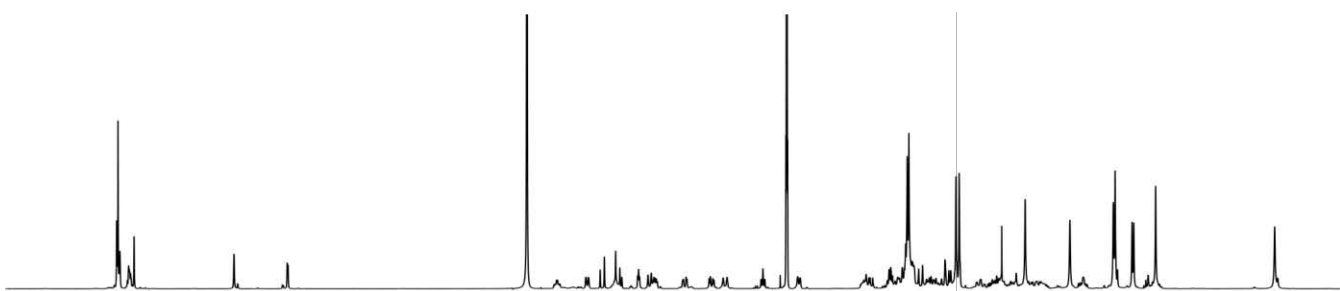




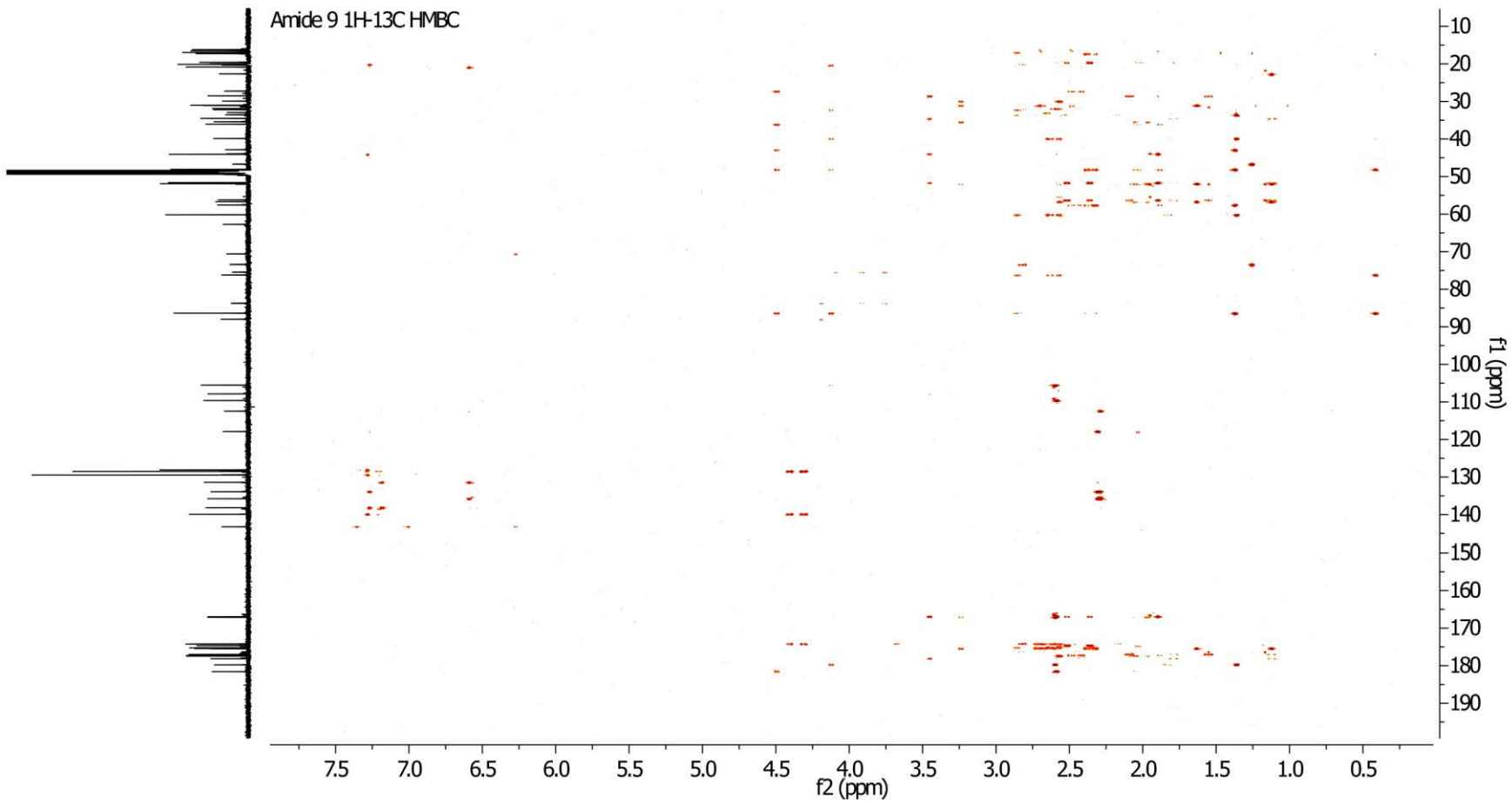


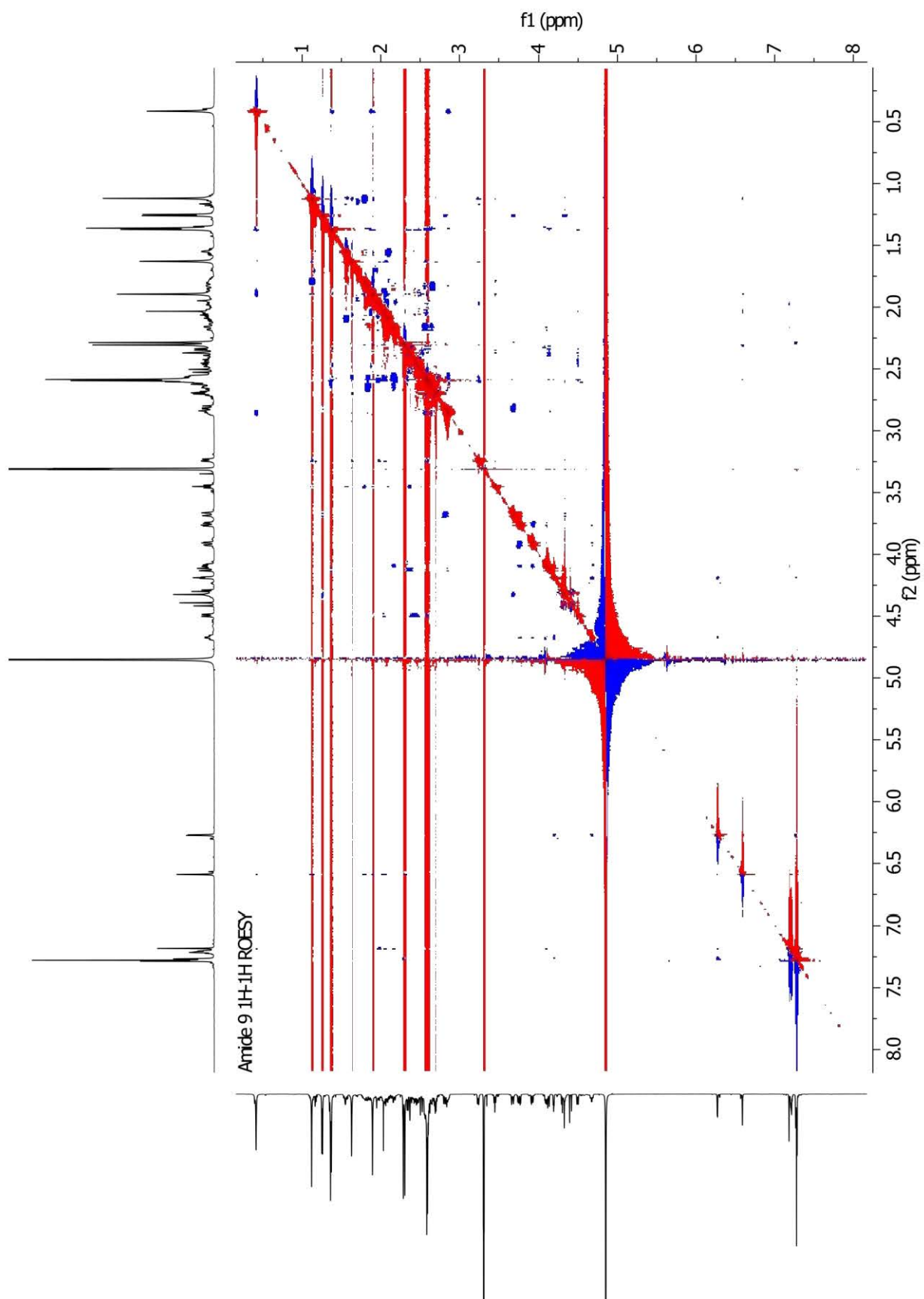






Amide 9 1H-13C HMBC

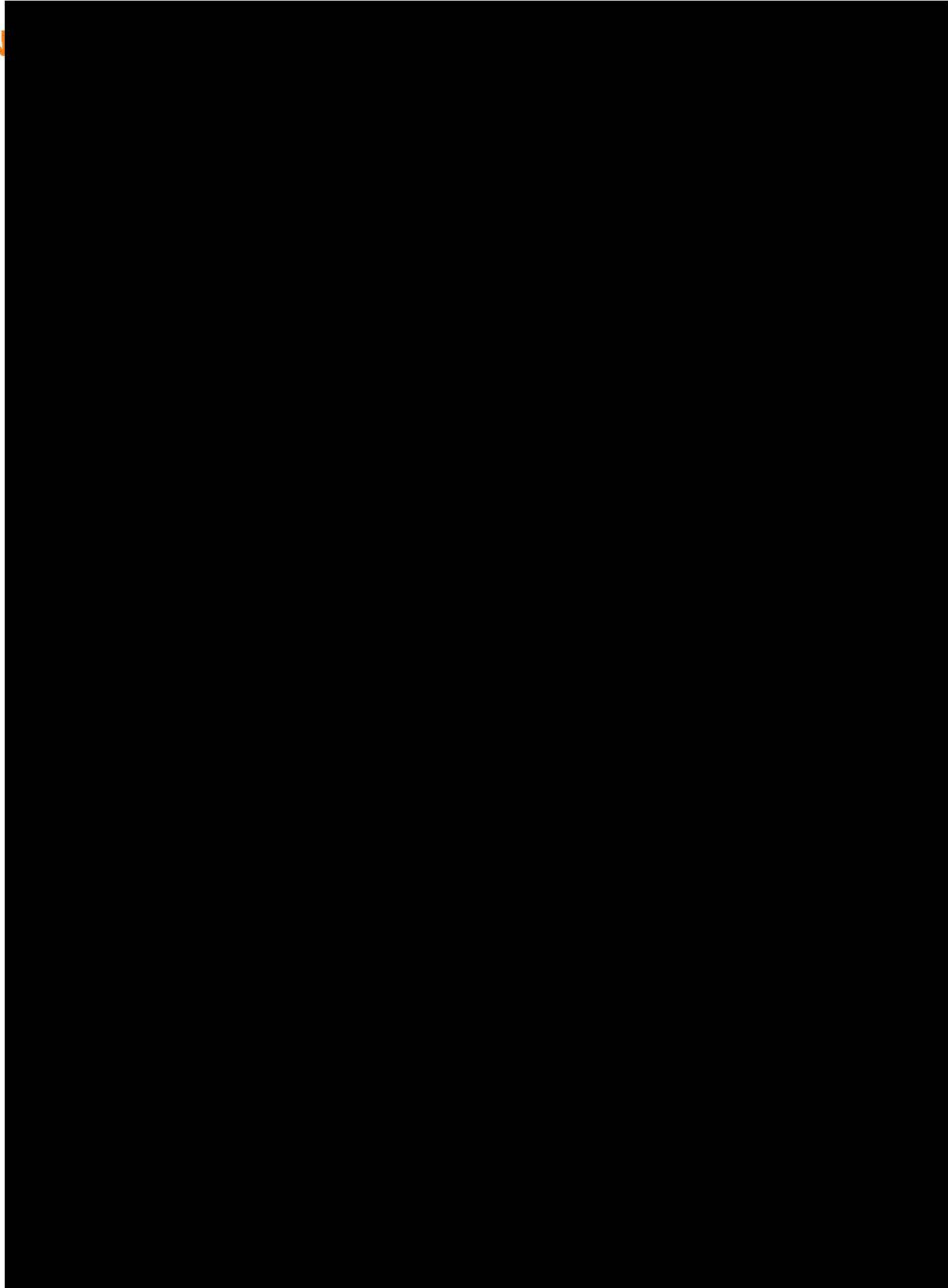




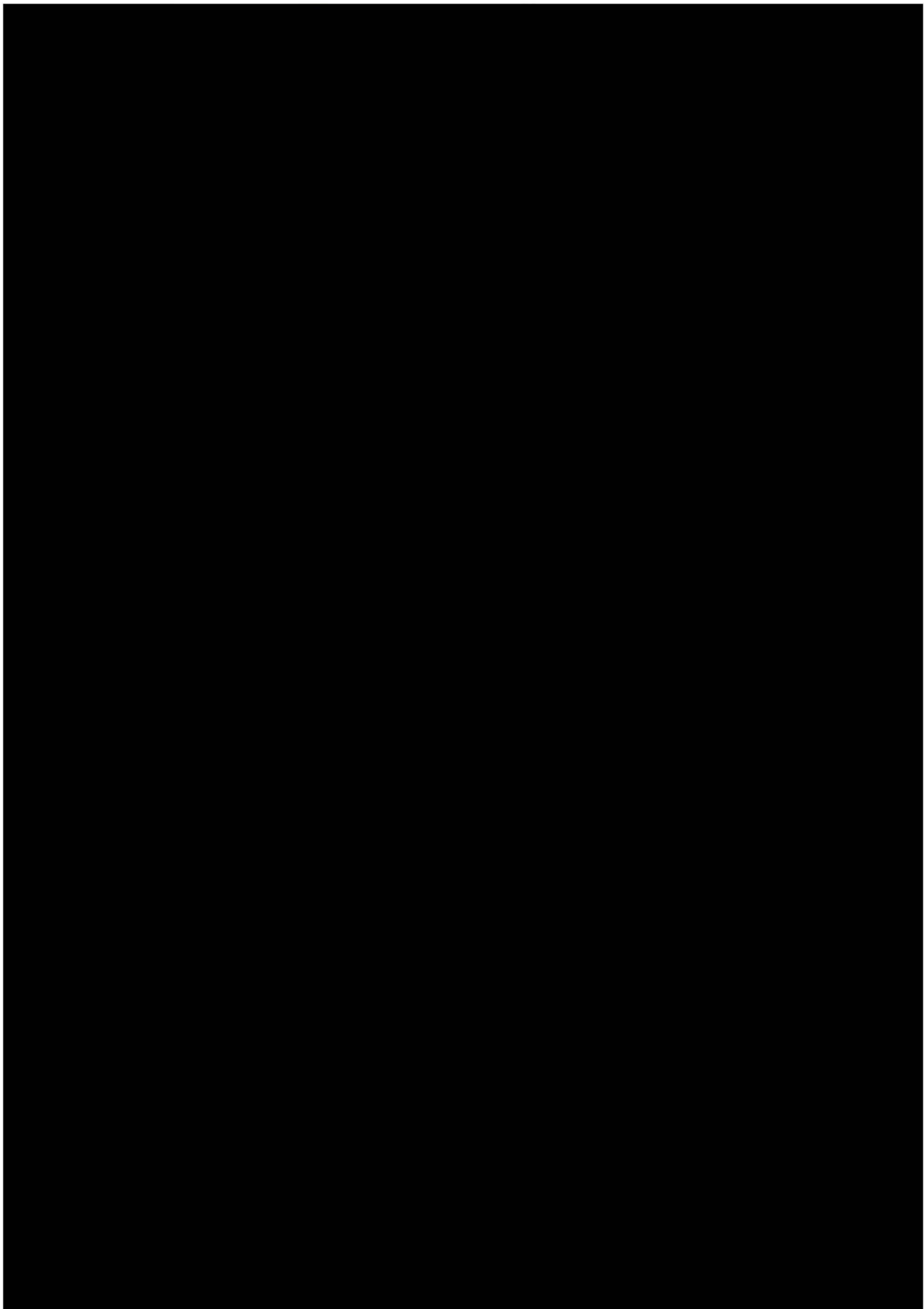
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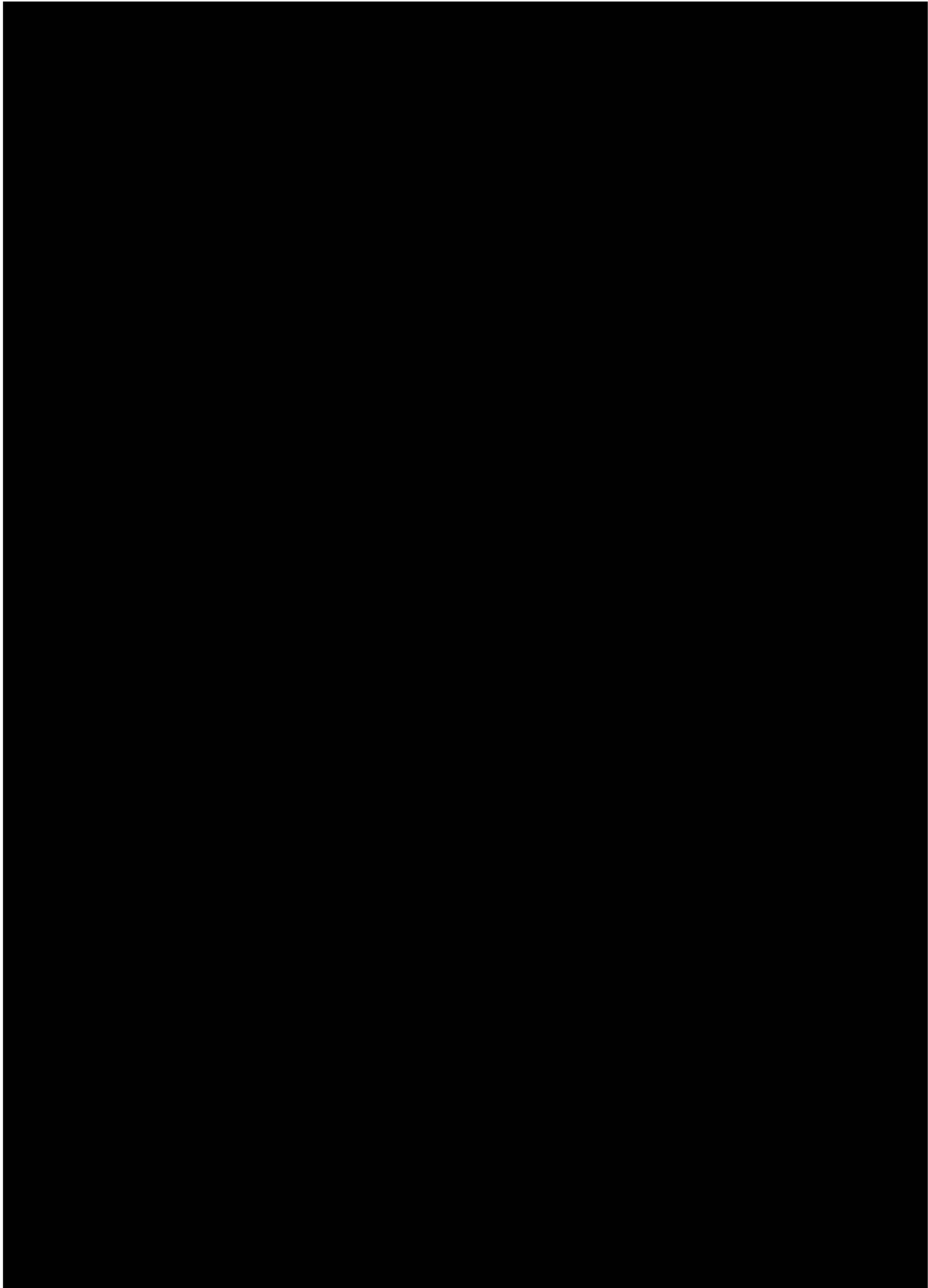


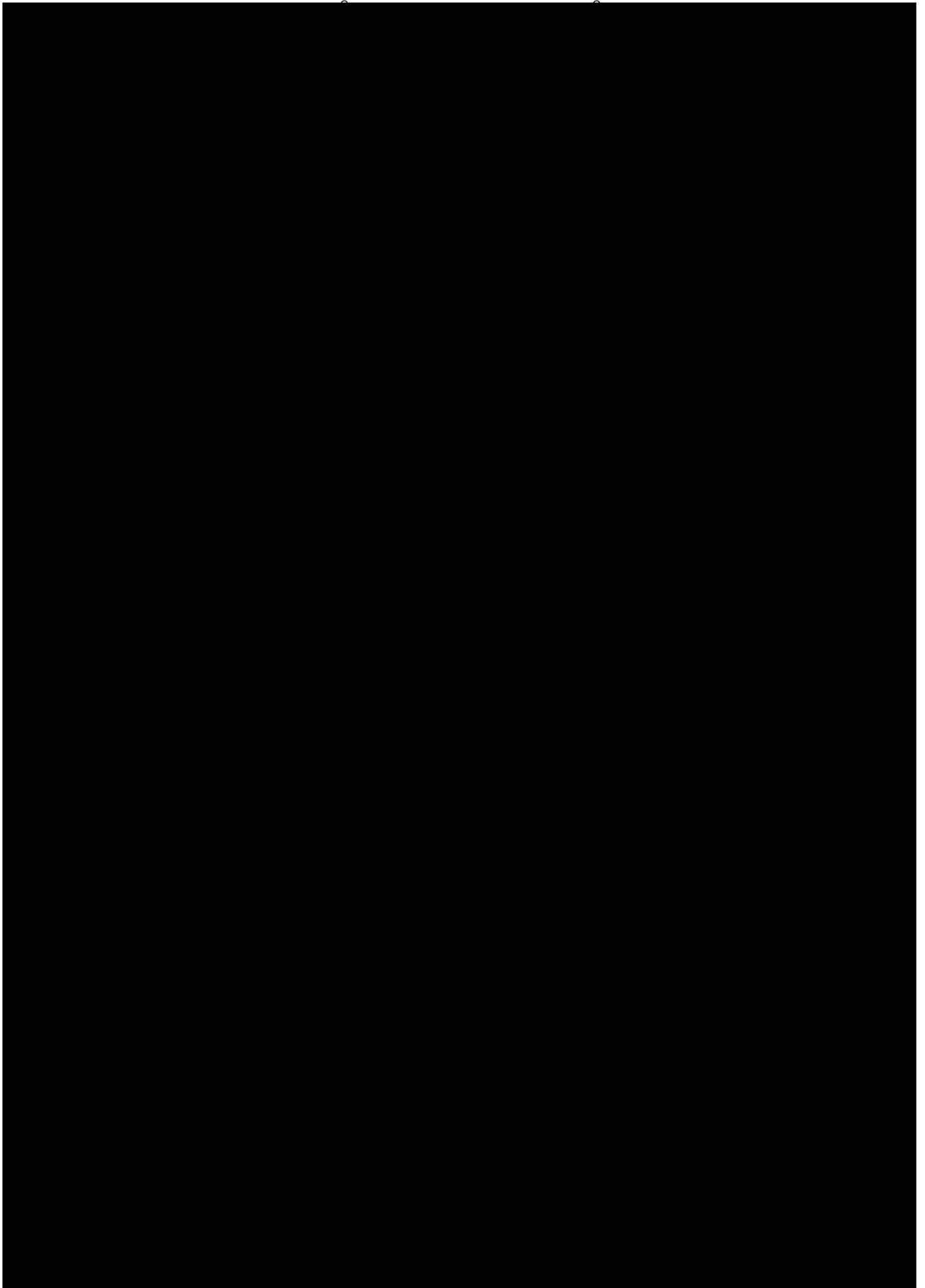
OPEN

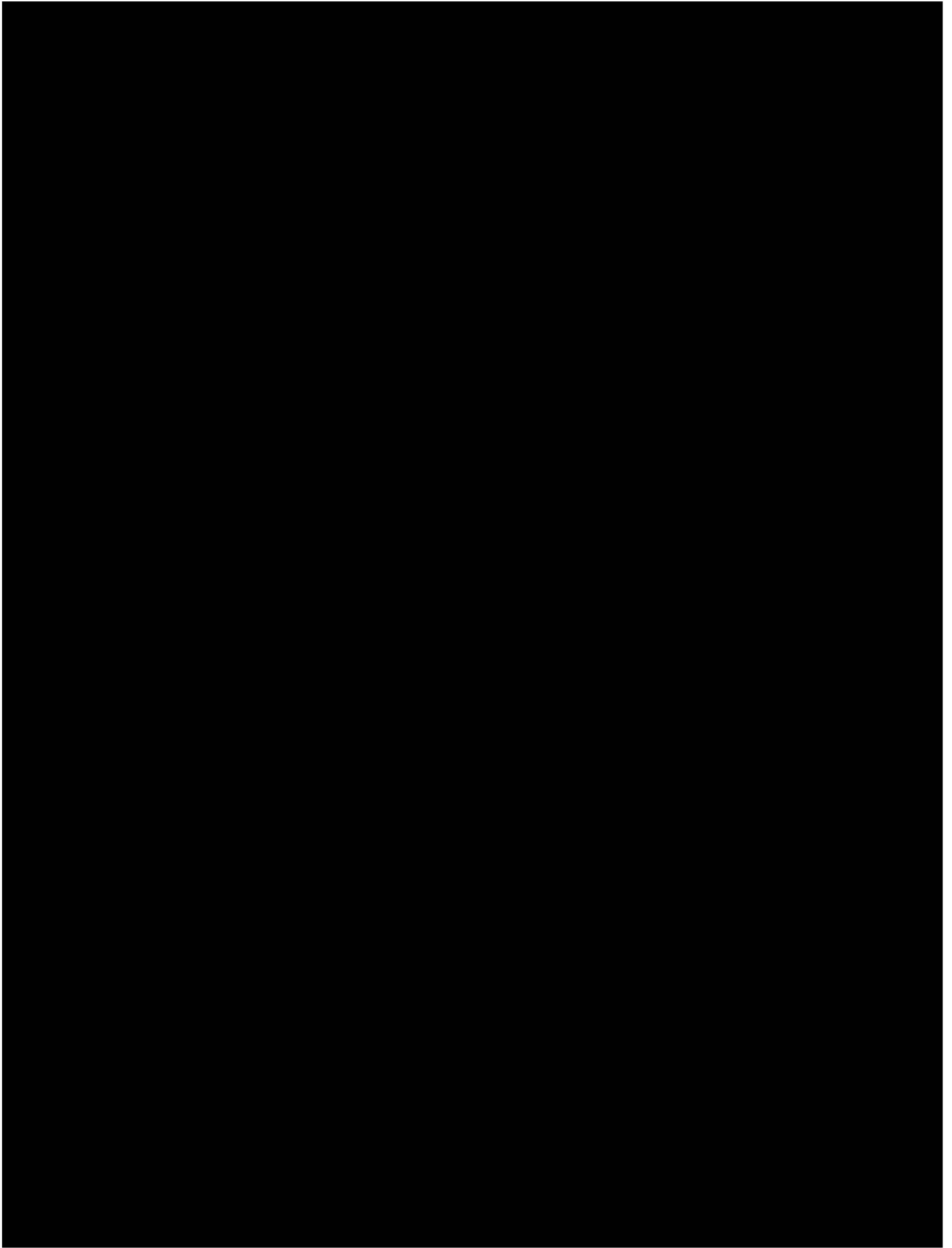


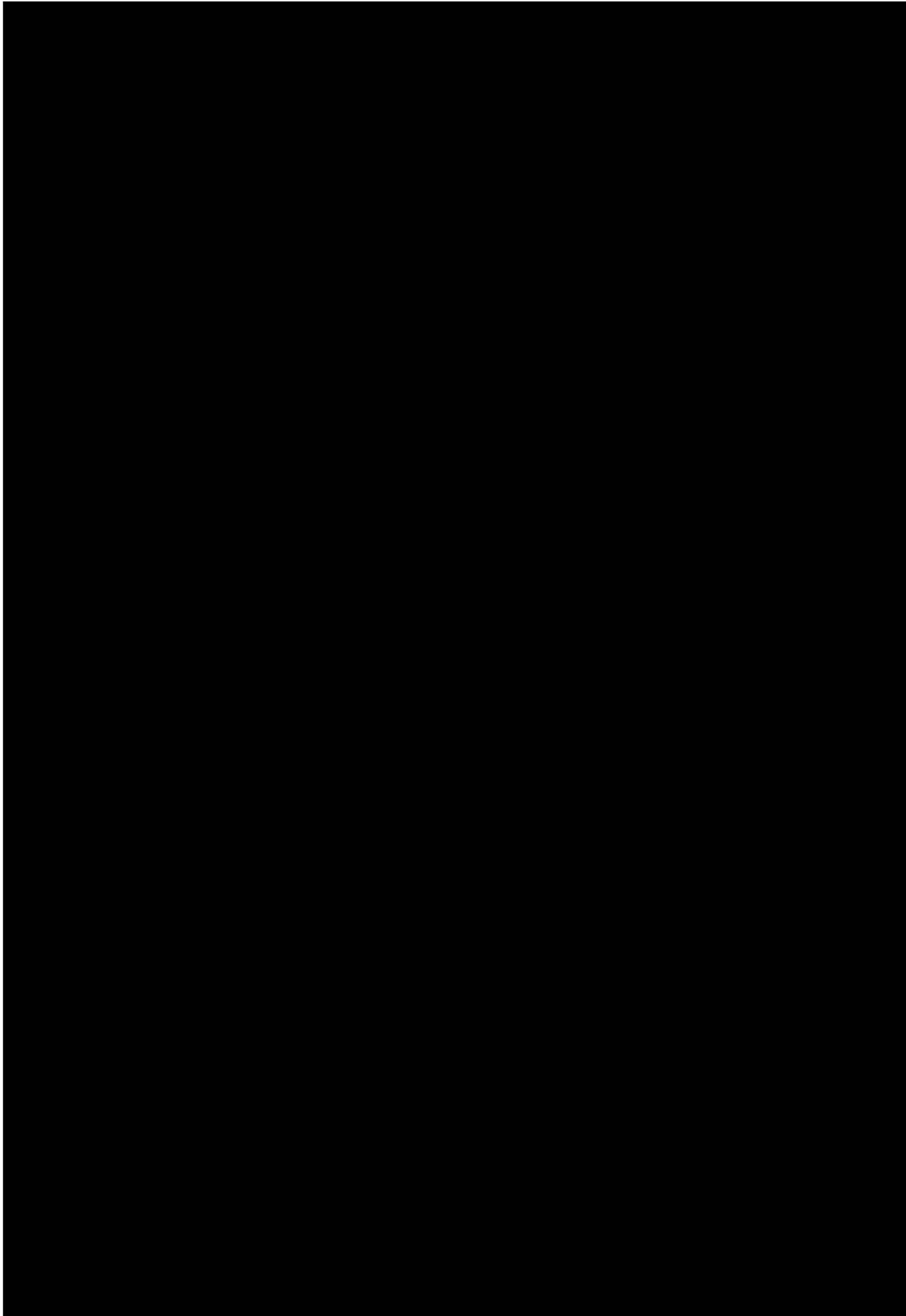
Received: 13 March 2017  
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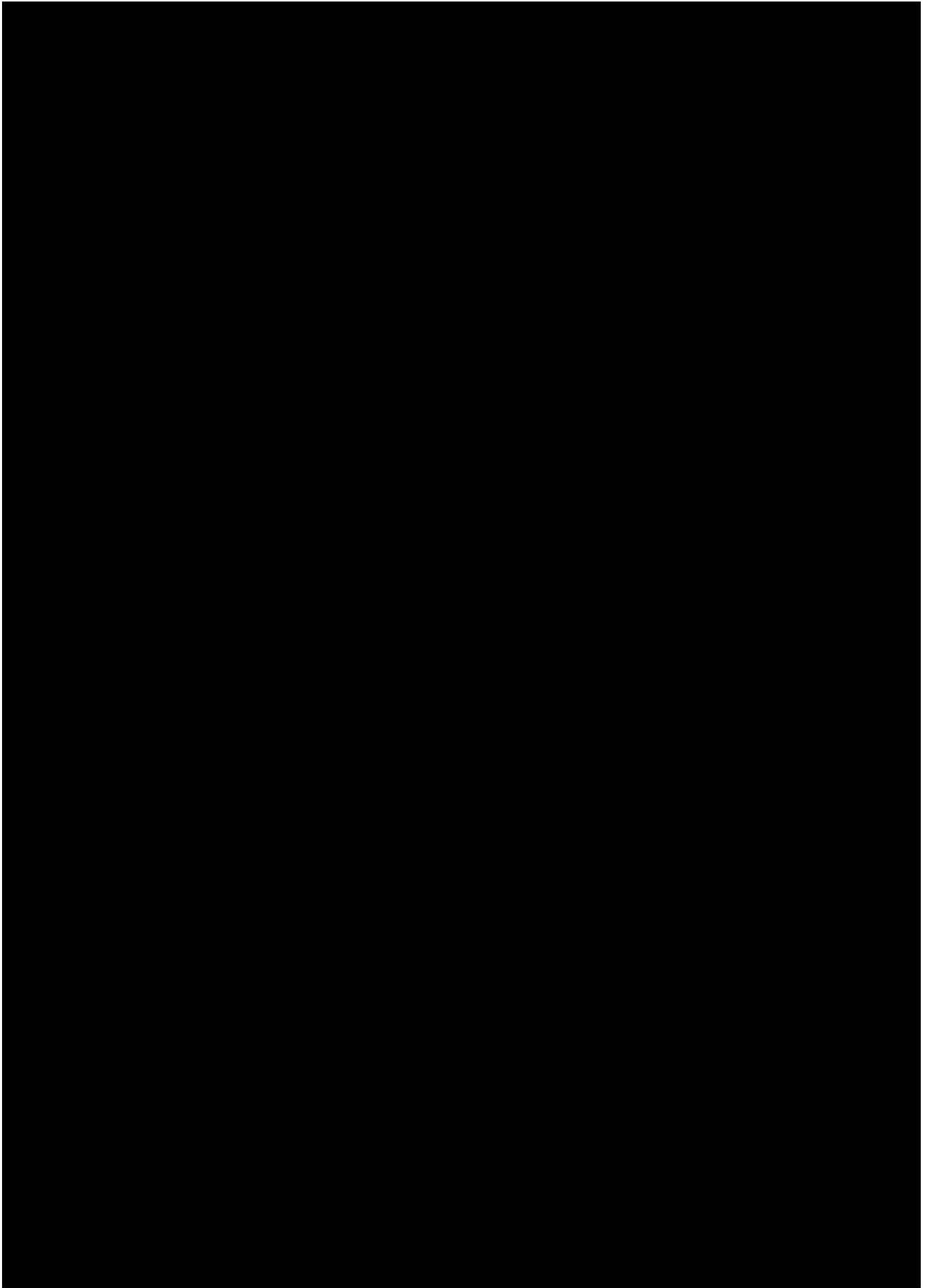


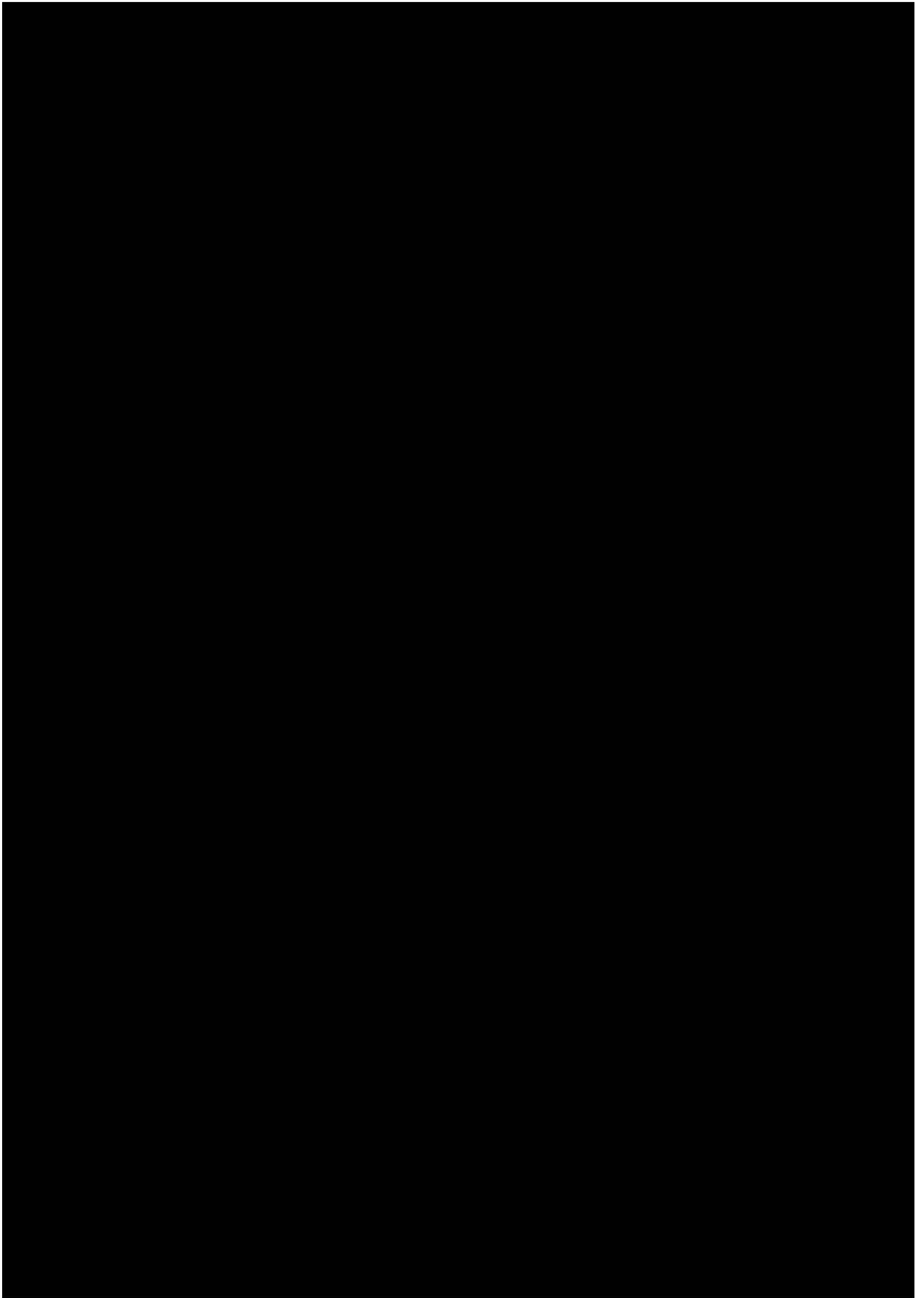


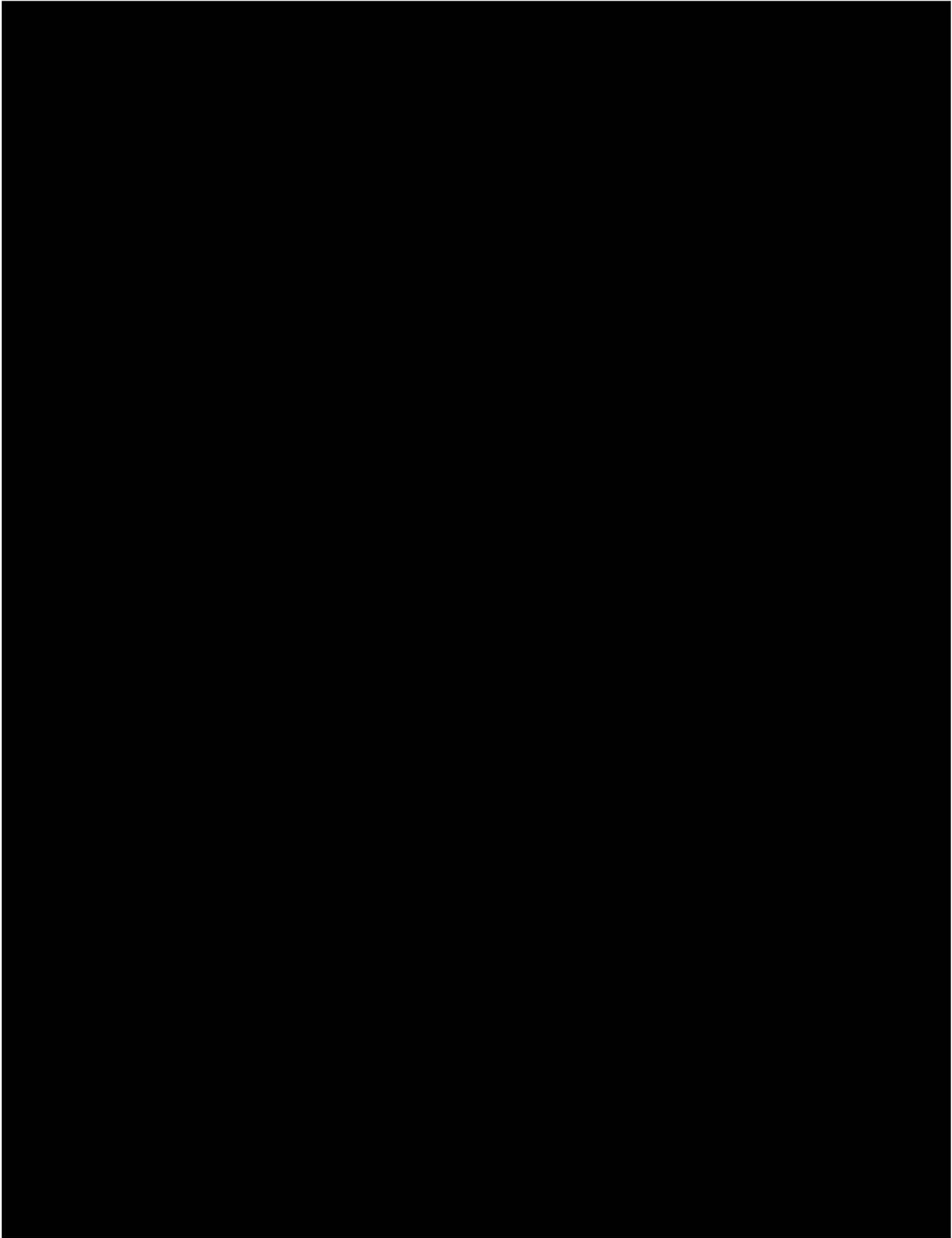


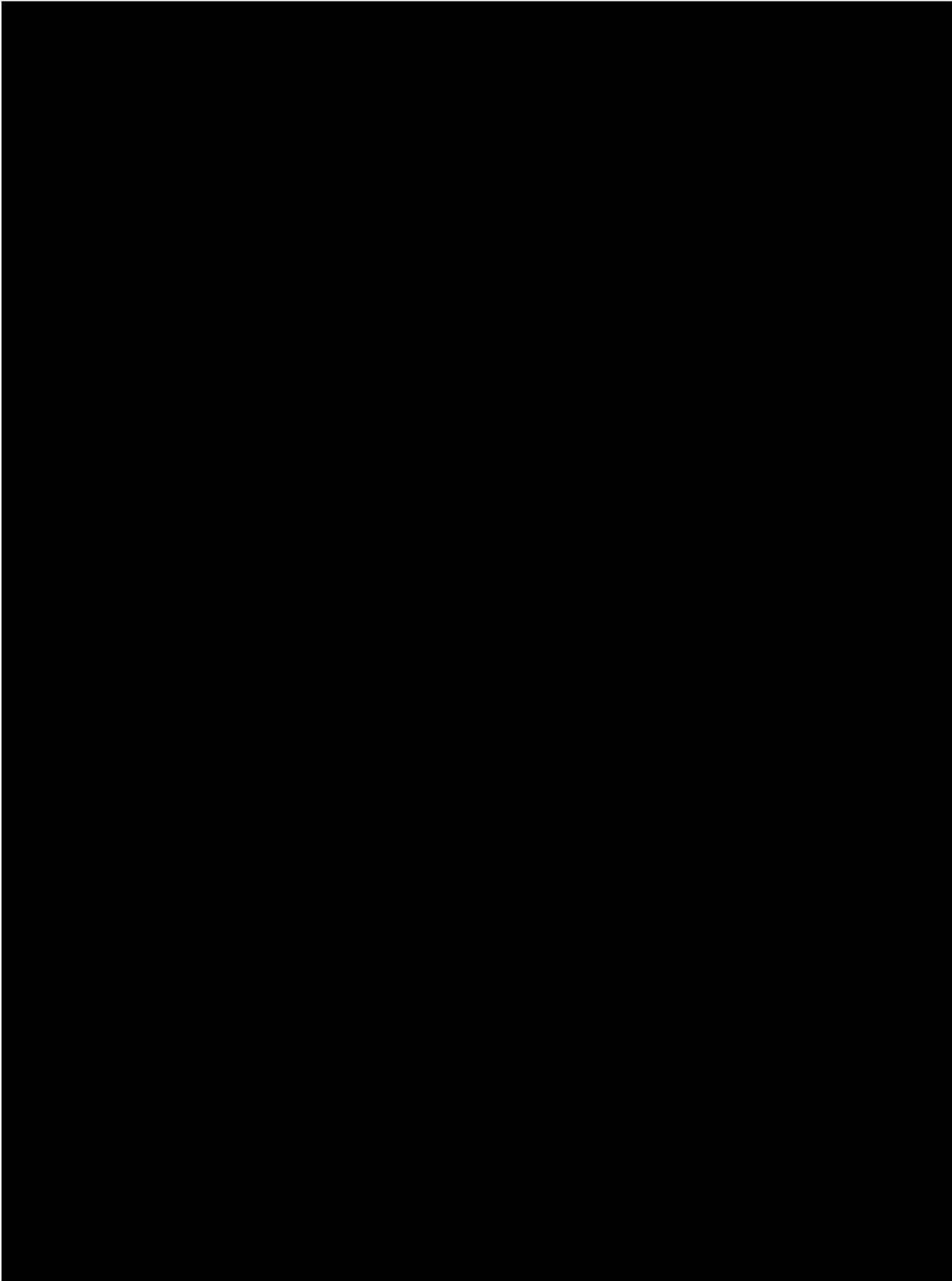














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## Supporting Information for

### Vitamin B<sub>12</sub> as a carrier of peptide nucleic acid (PNA) into bacterial cells

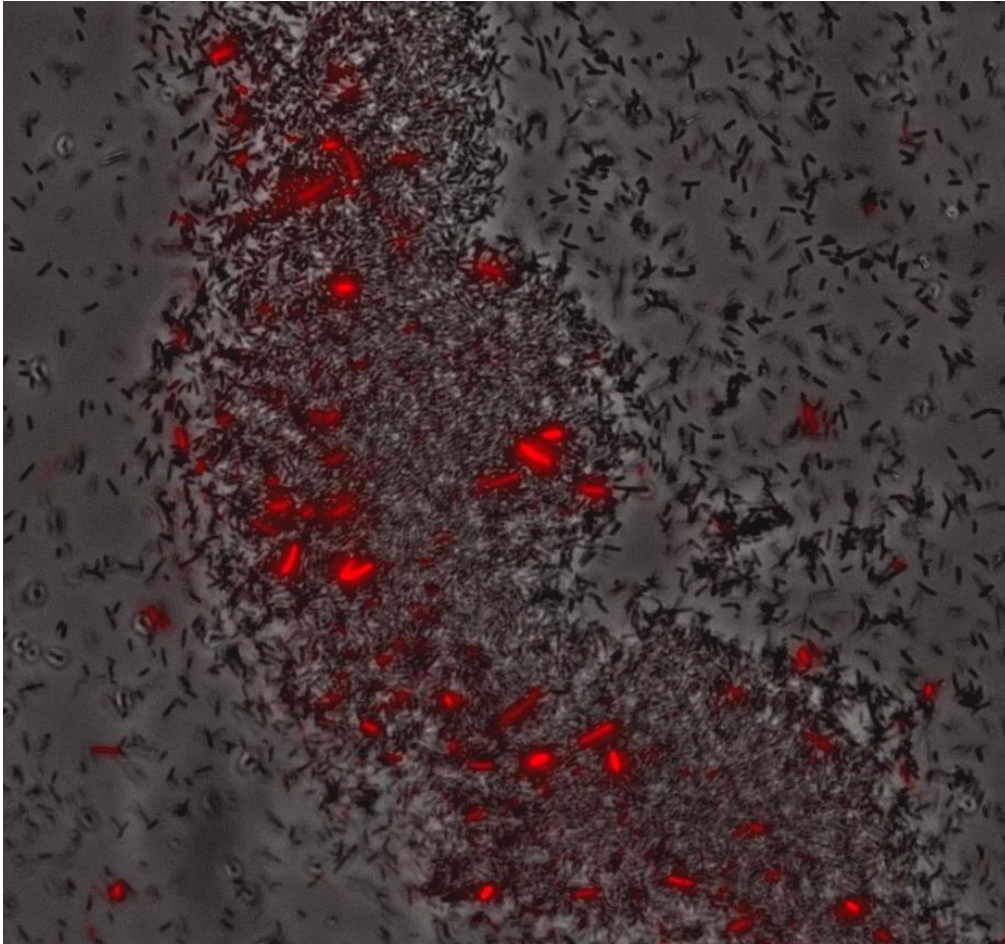
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**Figure S1.** Two superimposed pictures: 1<sup>st</sup> from light microscopy (showing the general amount of *E. coli* cells in the microscopic slide) and from fluorescence microscopy showing strong inhibition of red cellular fluorescence after overnight treatment with 16  $\mu\text{M}$  B<sub>12</sub>-(CH<sub>2</sub>)<sub>12</sub>-PNA targeted at mRNA encoding RFP.

*E. coli*

$\mu\text{M}$	Relative Fluorescence Units [RFU]											
	(KFF) <sub>3</sub> K-PNA			(KFF) <sub>3</sub> K-PNA (scrambled)			(KFF) <sub>3</sub> K only			PNA only		
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.125	0.42	0.29	0.30	0.91	1.11	0.81	0.89	1.07	0.93	1.31	1.09	1.24
0.25	0.46	0.29	0.40	1.21	0.86	0.89	0.87	0.82	0.72	1.59	1.45	1.47
0.5	0.48	0.29	0.34	1.21	0.87	0.97	0.97	0.72	1.04	1.19	1.11	1.19
1	0.38	0.28	0.32	1.17	0.93	0.72	1.26	0.70	0.83	1.48	1.07	1.48
2	0.35	0.28	0.31	0.90	0.82	0.97	1.06	0.88	1.00	1.37	0.89	1.37
4	0.28	0.29	0.36	0.78	0.93	0.69	1.11	0.86	0.81	1.31	0.93	1.31
8	0.28	0.27	0.38	0.76	0.79	0.84	0.84	0.81	0.83	1.41	1.31	1.41
16	0.37	0.26	0.24	0.75	0.80	0.84	0.85	0.79	0.79	1.22	1.16	1.22

*S. Typhimurium*

$\mu\text{M}$	Relative Fluorescence Units [RFU]											
	(KFF) <sub>3</sub> K-PNA			(KFF) <sub>3</sub> K-PNA (scrambled)			(KFF) <sub>3</sub> K only			PNA only		
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.125	0.93	0.86	0.86	0.86	0.67	1.26	1.14	1.02	1.15	1.08	1.11	1.25
0.25	0.77	0.82	0.78	1.15	0.82	0.96	1.19	1.16	1.02	1.26	1.08	0.90
0.5	0.67	0.62	0.62	0.94	0.83	1.14	0.90	1.41	0.96	0.73	0.83	0.92
1	0.33	0.47	0.52	0.98	1.04	0.97	1.09	0.93	1.17	0.90	1.02	1.00
2	0.14	0.38	0.39	1.02	1.04	0.92	1.06	1.19	1.06	0.87	1.05	0.85
4	0.07	0.13	0.14	0.91	1.06	1.10	0.99	0.89	1.08	0.84	0.83	1.05
8	0.03	0.03	0.03	0.99	0.75	1.05	0.80	0.88	1.04	0.79	0.95	0.89
16	0.02	0.02	0.01	0.91	0.83	1.00	0.86	0.92	0.74	1.13	1.04	0.90

**Table S1.** Extended data for Figure 4 of the main text. 1.00 stands for normalized fluorescence units for the untreated cells. Colors of the compounds are as in the legend of Figure 4.



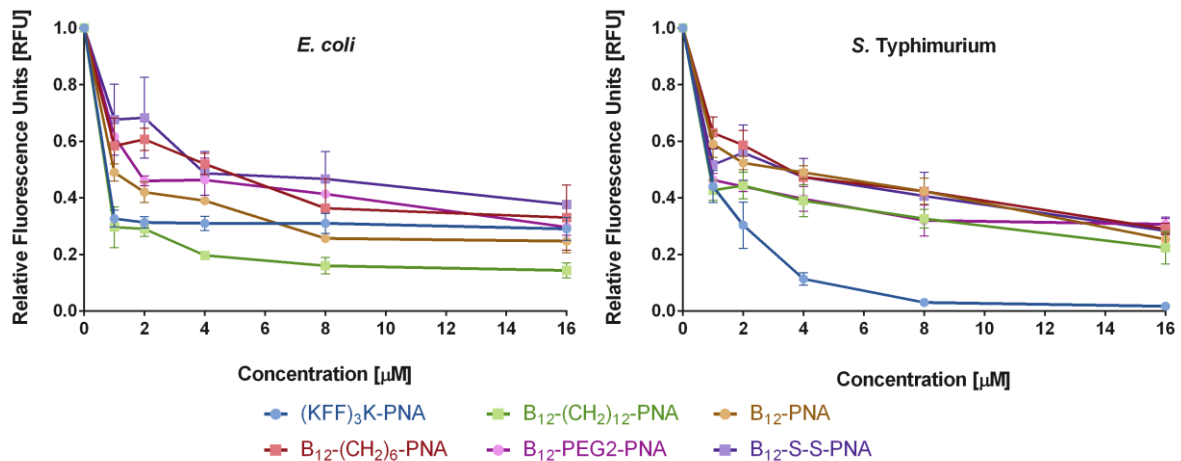
*E. coli*

$\mu\text{M}$	Relative Fluorescence Units [RFU]																	
	B <sub>12</sub> -PNA			B <sub>12</sub> -(CH <sub>2</sub> ) <sub>12</sub> -PNA			B <sub>12</sub> -(CH <sub>2</sub> ) <sub>6</sub> -PNA			B <sub>12</sub> -S-S-PNA			B <sub>12</sub> -PEG2-PNA			B <sub>12</sub> -(CH <sub>2</sub> ) <sub>12</sub> -PNA (scrambled)		
0*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.125	0.60	0.65	0.73	0.54	0.73	0.76	1.16	1.03	0.88	0.89	0.72	0.98	0.76	0.55	0.87	1.28	1.22	1.13
0.25	0.52	0.57	0.63	0.46	0.48	0.61	0.80	0.61	1.00	1.00	0.79	0.69	0.92	1.01	0.51	1.14	1.30	0.80
0.5	0.42	0.48	0.57	0.31	0.31	0.46	0.77	0.90	0.93	0.97	0.73	0.65	0.65	0.63	0.50	1.28	1.22	1.13
1	0.43	0.51	0.53	0.21	0.24	0.44	0.40	0.74	0.61	0.92	0.61	0.50	0.64	0.62	0.59	0.96	0.97	1.25
2	0.35	0.44	0.47	0.24	0.30	0.33	0.63	0.66	0.53	0.95	0.64	0.46	0.46	0.49	0.43	0.96	1.04	1.05
4	0.36	0.40	0.41	0.18	0.20	0.21	0.51	0.46	0.59	0.43	0.39	0.64	0.31	0.53	0.55	1.31	0.82	0.82
8	0.23	0.27	0.27	0.11	0.16	0.21	0.27	0.25	0.57	0.65	0.32	0.43	0.43	0.42	0.39	1.00	1.06	1.11
16	0.17	0.26	0.31	0.10	0.14	0.19	0.20	0.23	0.56	0.40	0.35	0.38	0.34	0.24	0.31	0.89	0.90	0.92

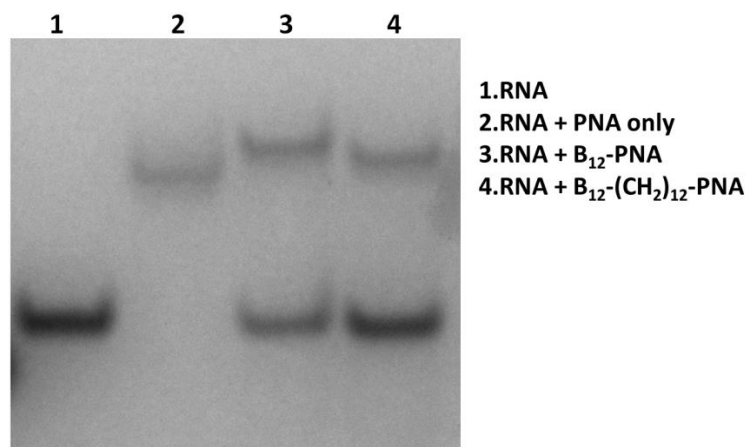
*S. Typhimurium*

$\mu\text{M}$	Relative Fluorescence Units [RFU]																	
	B <sub>12</sub> -PNA			B <sub>12</sub> -(CH <sub>2</sub> ) <sub>12</sub> -PNA			B <sub>12</sub> -(CH <sub>2</sub> ) <sub>6</sub> -PNA			B <sub>12</sub> -S-S-PNA			B <sub>12</sub> -PEG2-PNA			B <sub>12</sub> -(CH <sub>2</sub> ) <sub>12</sub> -PNA (scrambled)		
0*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.125	0.74	0.68	0.74	0.62	0.59	0.53	0.82	0.77	0.76	0.57	0.76	0.83	0.81	0.81	0.78	0.95	0.78	0.97
0.25	0.67	0.64	0.73	0.43	0.63	0.59	0.82	0.69	0.63	0.68	0.46	0.62	0.61	0.82	0.83	1.00	0.85	1.03
0.5	0.63	0.61	0.74	0.49	0.49	0.45	0.75	0.61	0.59	0.65	0.45	0.65	0.56	0.70	0.79	1.03	1.11	0.87
1	0.52	0.68	0.57	0.42	0.37	0.49	0.74	0.57	0.58	0.62	0.43	0.50	0.47	0.50	0.42	0.92	1.03	0.95
2	0.51	0.57	0.49	0.37	0.43	0.53	0.69	0.54	0.53	0.50	0.43	0.75	0.47	0.44	0.41	1.08	0.98	1.24
4	0.53	0.45	0.49	0.36	0.50	0.31	0.52	0.43	0.47	0.36	0.47	0.59	0.38	0.48	0.33	0.92	0.96	0.97
8	0.51	0.41	0.35	0.31	0.39	0.28	0.42	0.44	0.41	0.47	0.24	0.51	0.41	0.22	0.33	0.97	1.05	0.92
16	0.27	0.28	0.21	0.29	0.27	0.11	0.27	0.27	0.33	0.24	0.23	0.38	0.34	0.31	0.27	1.08	0.95	0.82

**Table S2.** Extended data for Figure 6 of the main text. 1.00 stands for normalized fluorescence for the untreated cells. Colors of the compounds correspond with the legend of Figure 6.



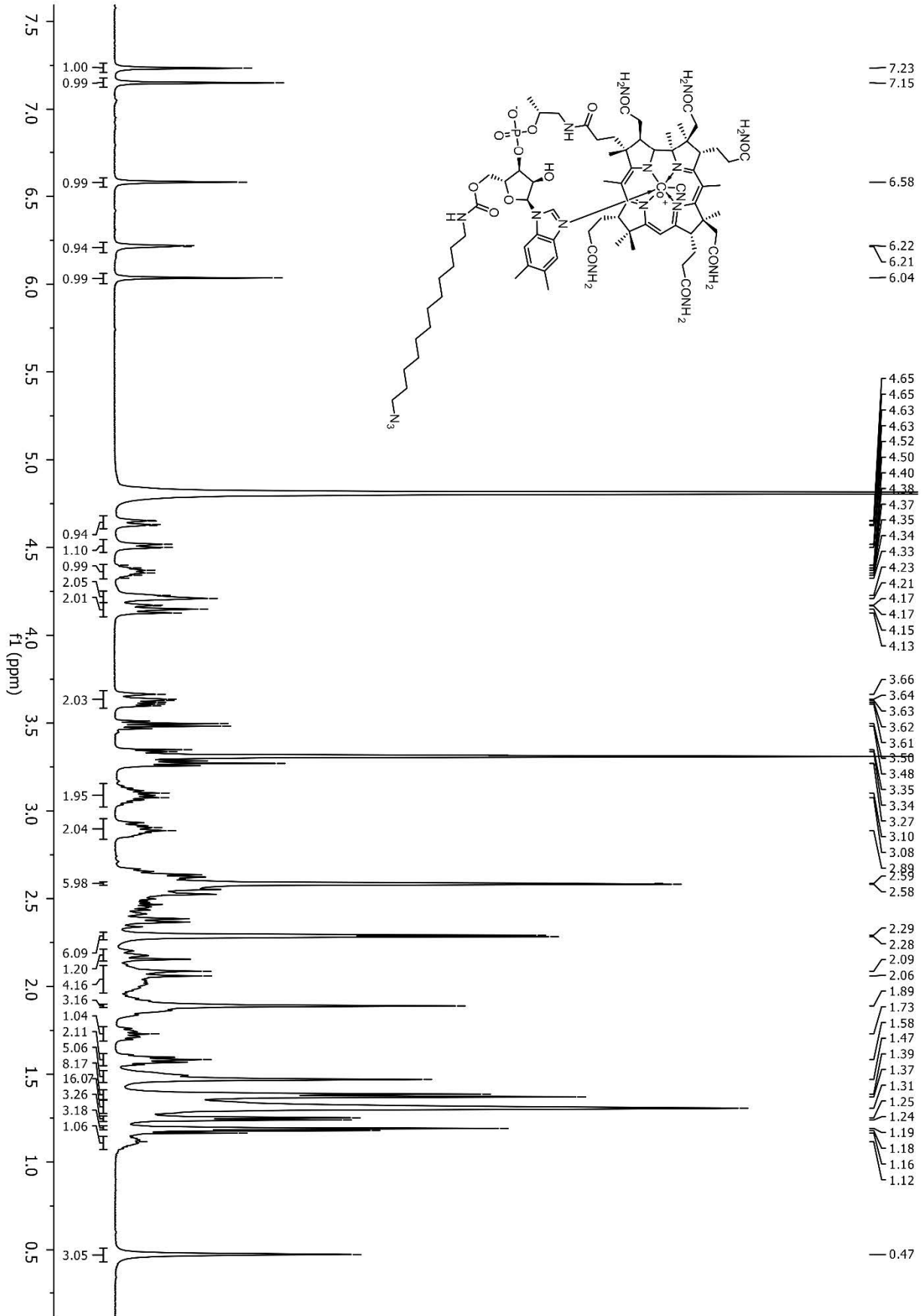
**Figure S2.** Comparison of the effect of anti-*rfp* vitamin B<sub>12</sub>-PNA conjugates and (KFF)<sub>3</sub>K-PNA on the fluorescence of *E. coli* and *S. Typhimurium* cells. The differences in the fluorescence between *E. coli* cells treated with (KFF)<sub>3</sub>K-PNA and treated with B<sub>12</sub>-(CH<sub>2</sub>)<sub>6</sub>-PNA, B<sub>12</sub>-PEG2-PNA or B<sub>12</sub>-S-S-PNA are statistically significant ( $P \leq 0.01$ ). In *S. Typhimurium* the differences in fluorescence were significant ( $P \leq 0.01$ ) between (KFF)<sub>3</sub>K-PNA and all tested vitamin B<sub>12</sub>-PNA conjugates.



**Figure S3.** The results of polyacrylamide gel electrophoresis experiments in non-denaturing conditions. RNA denotes the mRNA fragment of a sequence  $5'AGGAGAAAUACUAGAUGGCU3'$  with the region complementary to the 14-mer PNA underlined. RNA was incubated in water solution containing either PNA or the B<sub>12</sub>-PNA and B<sub>12</sub>-(CH<sub>2</sub>)<sub>12</sub>-PNA conjugates.

## Section S1: Experimental details and complete characterization of vitamin B<sub>12</sub> derivatives with aminoazide linkers at 5' position

**B<sub>12</sub>-(CH<sub>2</sub>)<sub>12</sub>-N<sub>3</sub>**: red powder; yield: 54%; compound was purified by RP column chromatography gradually with MeCN (acetonitrile) /H<sub>2</sub>O (from 15 to 35% v/v). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD (deuterated methanol)) δ 7.23 (s, 1H), 7.15 (s, 1H), 6.58 (s, 1H), 6.22 (d, *J* = 2.6 Hz, 1H), 6.04 (s, 1H), 4.64 (dd, *J* = 12.0, 2.1 Hz, 1H), 4.51 (d, *J* = 8.8 Hz, 1H), 4.40 – 4.32 (m, 1H), 4.23 – 4.09 (m, 2H), 4.17 – 4.13 (m, 2H), 3.68 – 3.59 (m, 2H), 3.14 – 3.04 (m, 2H), 2.93 – 2.85 (m, 2H), 2.67 – 2.41 (m, 12H), 2.58 (s, 3H), 2.58 (s, 3H), 2.41 – 2.34 (m, 2H), 2.29 (s, 3H), 2.28 (s, 3H), 2.25 – 2.18 (m, 1H), 2.12 – 1.96 (m, 4H), 1.95 – 1.82 (m, 3H), 1.89 (s, 3H), 1.76 – 1.70 (m, 1H), 1.61 – 1.55 (m, 2H), 1.53 – 1.48 (m, 2H), 1.47 (s, 3H), 1.39 (s, 3H), 1.39 – 1.37 (m, 2H), 1.37 (s, 3H), 1.36 – 1.27 (m, 16H), 1.25 (d, *J* = 6.2 Hz, 3H), 1.19 (s, 3H), 1.14 – 1.08 (s, 1H), 0.47 (s, 3H). <sup>13</sup>C NMR (126 MHz, CD<sub>3</sub>OD) δ 181.6, 180.2, 177.6, 177.4, 177.3, 176.6, 175.5, 175.3, 174.6, 174.1, 167.2, 166.9, 158.7, 143.4, 138.3, 135.7, 133.9, 131.4, 117.9, 112.4, 108.7, 105.2, 95.6, 88.2, 86.4, 81.4, 76.4, 75.2, 73.4, 70.6, 66.9, 64.1, 60.3, 57.7, 56.9, 55.0, 52.6, 52.5, 46.6, 43.9, 43.0, 41.9, 40.4, 36.5, 35.1, 33.2, 33.0, 32.6, 32.4, 32.3, 30.9, 30.7, 30.6, 30.6, 30.5, 30.3, 29.9, 29.5, 27.9, 27.8, 27.4, 27.3, 20.9, 20.5, 20.5, 20.3, 20.2, 20.1, 19.9, 17.5, 17.1, 16.4, 16.1, 15.4. UV/vis (H<sub>2</sub>O) λ<sub>max</sub> (nm) (ε, L mol<sup>-1</sup> cm<sup>-1</sup>) 546 (8.4 × 10<sup>3</sup>), 519 (7.5 × 10<sup>3</sup>), 361 (2.6 × 10<sup>3</sup>), 278 (1.5 × 10<sup>4</sup>), 221 (4.6 × 10<sup>4</sup>). HRMS-ESI (High Resolution Mass Spectrometry Electrospray Ionization) *m/z* [M + 2Na]<sup>2+</sup> calculated for C<sub>76</sub>H<sub>112</sub>N<sub>18</sub>O<sub>15</sub>PCoNa<sub>2</sub> 826.37043, found 826.37040. Analysis was calculated for C<sub>76</sub>H<sub>112</sub>N<sub>18</sub>O<sub>15</sub>PCo·5H<sub>2</sub>O: C, 53.77; H, 7.24; N, 14.85. Found: C, 53.67; H, 7.32; N, 15.04. *t<sub>R</sub>* (RP-HPLC, from 10 % MeCN/H<sub>2</sub>O + 0.05 % TFA (trifluoroacetic acid) to 70 % MeCN/H<sub>2</sub>O + 0.05 % TFA in 15 min and 70 % MeCN/H<sub>2</sub>O + 0.05 % TFA in 10 min): 14.50 min.



**Figure S4.** NMR spectra of compound  $B_{12}-(CH_2)_{12}-N_3$  recorded in  $CD_3OD$  (deuterated methanol)

**B<sub>12</sub>-5'-PEG2-N<sub>3</sub>**: red powder; yield: 66%; compound was purified by RP column chromatography with MeCN/H<sub>2</sub>O (10% v/v). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ 7.25 (s, 1H), 7.15 (s, 1H), 6.58 (s, 1H), 6.23 (d, *J* = 2.6 Hz, 1H), 6.04 (s, 1H), 4.66 (d, *J* = 9.9 Hz, 1H), 4.51 (d, *J* = 8.2 Hz, 1H), 4.40 – 4.32 (m, 1H), 4.24 – 4.20 (m, 2H), 4.17 (dd, *J* = 12.20, 2.38 Hz, 1H), 4.13 (d, *J* = 11.50 Hz, 1H), 3.60 – 3.67 (m, 7H), 3.54 (t, *J* = 5.57 Hz, 2H), 3.36 (t, *J* = 5.57 Hz, 2H), 2.93 – 2.85 (m, 2H), 2.59 (s, 3H), 2.58 (s, 3H), 2.67 – 2.42 (m, 12H), 2.41 – 2.34 (m, 2H), 2.29 (s, 3H), 2.28 (s, 3H), 2.21 – 2.14 (m, 1H), 2.12 – 1.96 (m, 4H), 1.94 – 1.82 (m, 3H), 1.89 (s, 3H), 1.77 – 1.70 (m, 1H), 1.47 (m, 3H), 1.39 (s, 3H), 1.39 – 1.37 (m, 2H), 1.37 (s, 3H), 1.30 – 1.26 (m, 1H), 1.25 (d, *J* = 6.27 Hz, 3H), 1.19 (s, 3H), 1.16 – 1.08 (m, 1H), 0.47 (s, 3H). <sup>13</sup>C NMR (126 MHz, CD<sub>3</sub>OD) δ 180.1, 178.7, 176.1, 176.0, 175.9, 175.1, 174.1, 174.1, 173.8, 173.2, 172.6, 165.7, 165.5, 157.2, 141.9, 136.8, 134.2, 132.5, 129.9, 116.5, 111.0, 107.3, 103.8, 94.2, 86.8, 85.0, 79.9, 74.9, 73.7, 72.0, 72.0, 70.1, 70.0, 69.7, 69.6, 69.1, 62.8, 58.9, 56.2, 55.5, 53.6, 51.1, 50.3, 45.2, 42.5, 41.6, 40.4, 38.7, 34.8, 33.7, 31.8, 31.5, 31.2, 30.9, 30.9, 28.1, 26.0, 25.9, 19.5, 19.1, 19.0, 18.9, 18.7, 18.7, 18.5, 16.1, 15.7, 14.9, 14.7. UV/vis (H<sub>2</sub>O) λ<sub>max</sub> (nm) (ε, L mol<sup>-1</sup> cm<sup>-1</sup>) 551 (7.8 × 10<sup>3</sup>), 522 (6.8 × 10<sup>3</sup>), 361 (2.4 × 10<sup>4</sup>), 278 (1.3 × 10<sup>4</sup>), 222 (4.2 × 10<sup>4</sup>). HRMS-ESI *m/z* [M + Na]<sup>+</sup> calculated for C<sub>70</sub>H<sub>100</sub>N<sub>18</sub>O<sub>17</sub>PCoNa 1577.6481, found 1577.6455. Analysis calculated for C<sub>70</sub>H<sub>100</sub>N<sub>18</sub>O<sub>17</sub>PCo · 6H<sub>2</sub>O: C, 50.54; H, 6.79; N, 15.15. Found: C, 50.62; H, 7.03; N, 14.95. *t<sub>R</sub>* (RP-HPLC, from 1 % MeCN/H<sub>2</sub>O + 0.05 % TFA to 70 % MeCN/H<sub>2</sub>O + 0.05 % TFA in 15 min): 10.88 min.

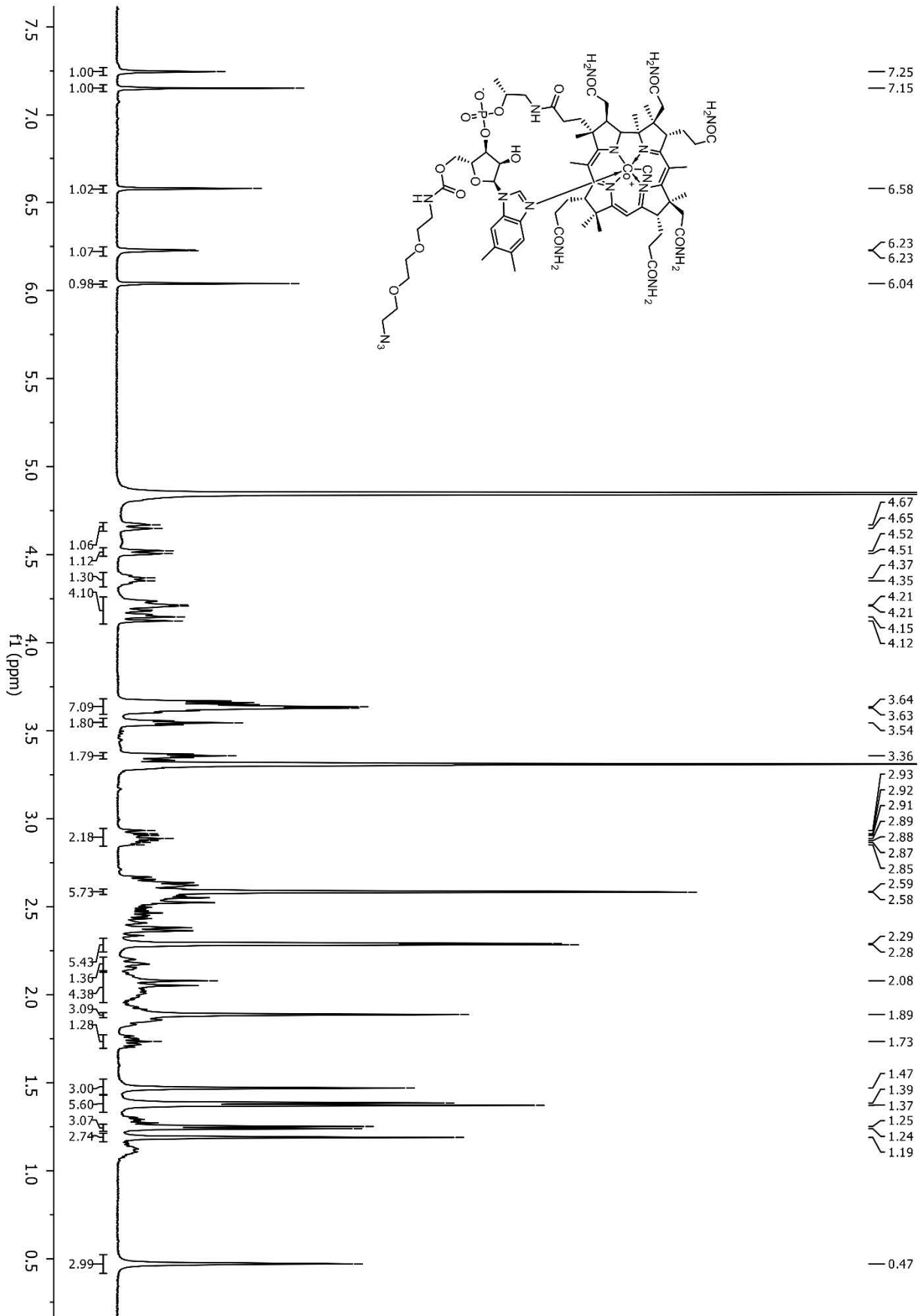
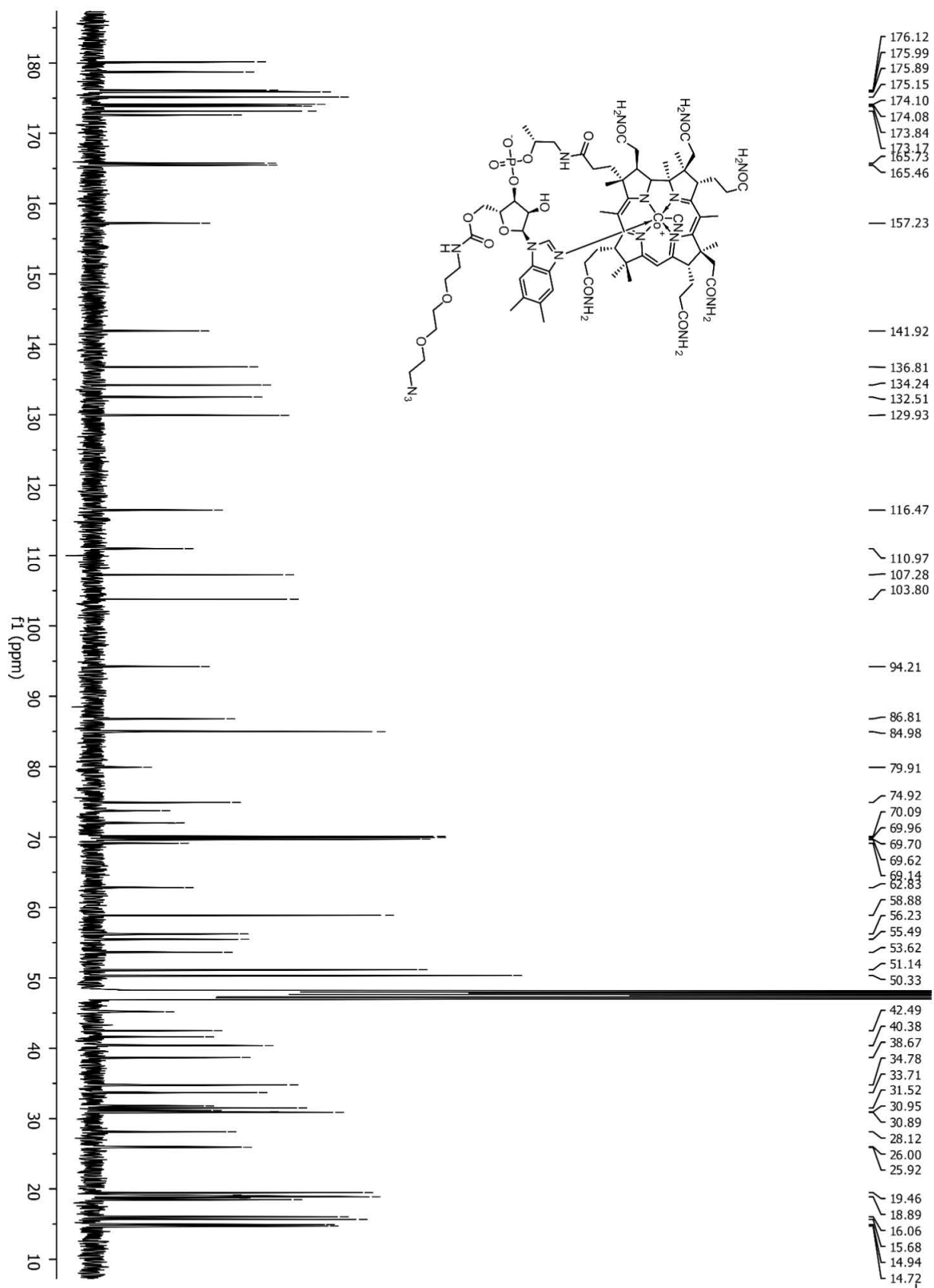


Figure S5. NMR spectra of compound B<sub>12</sub>-PEG2-N<sub>3</sub> recorded in CD<sub>3</sub>OD





## Section S2: Mass spectra and RP-HPLC chromatograms of PNA conjugates with vitamin B<sub>12</sub> and (KFF)<sub>3</sub>K

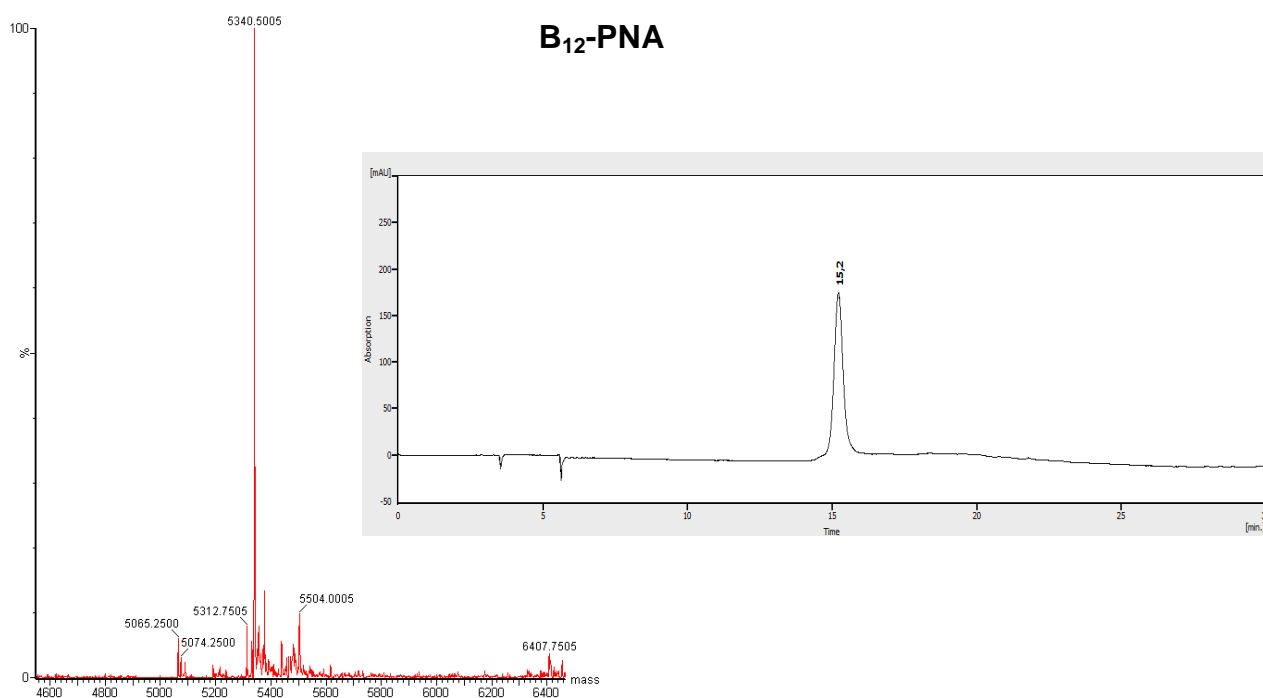


Figure S6. Mass spectrum and RP-HPLC chromatogram of B<sub>12</sub>-PNA

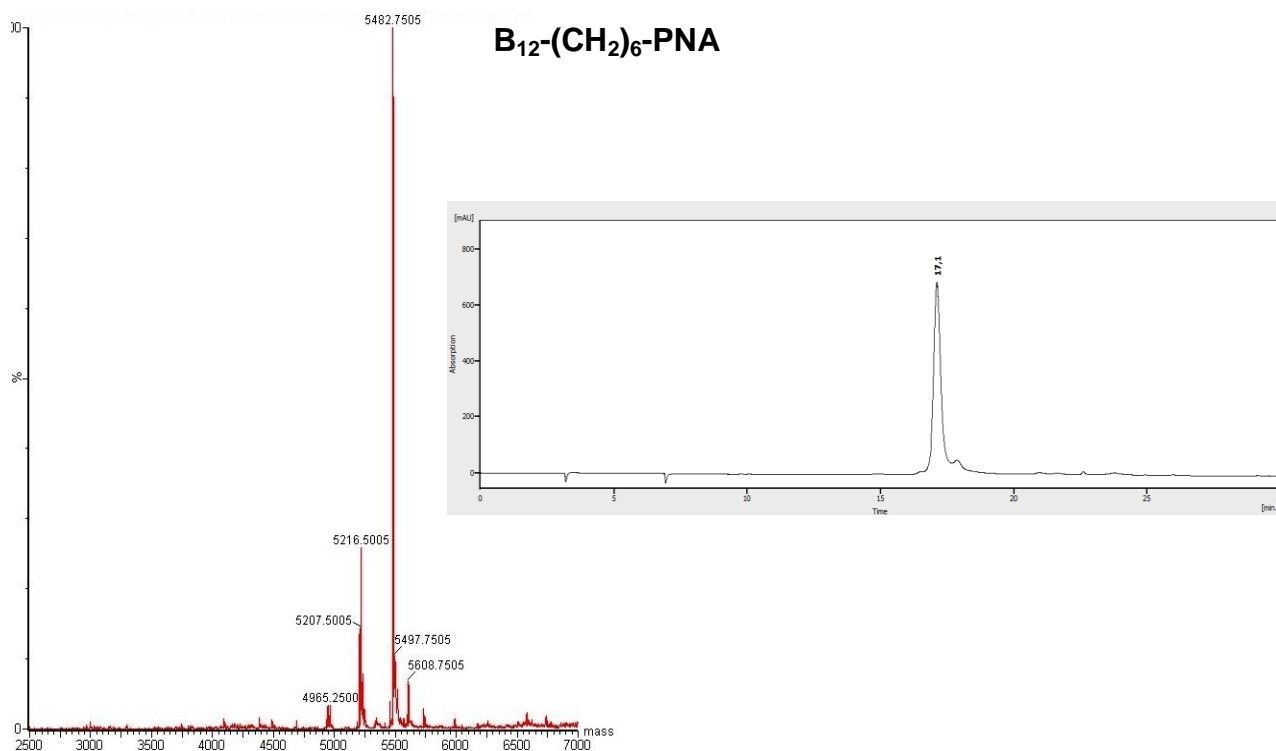
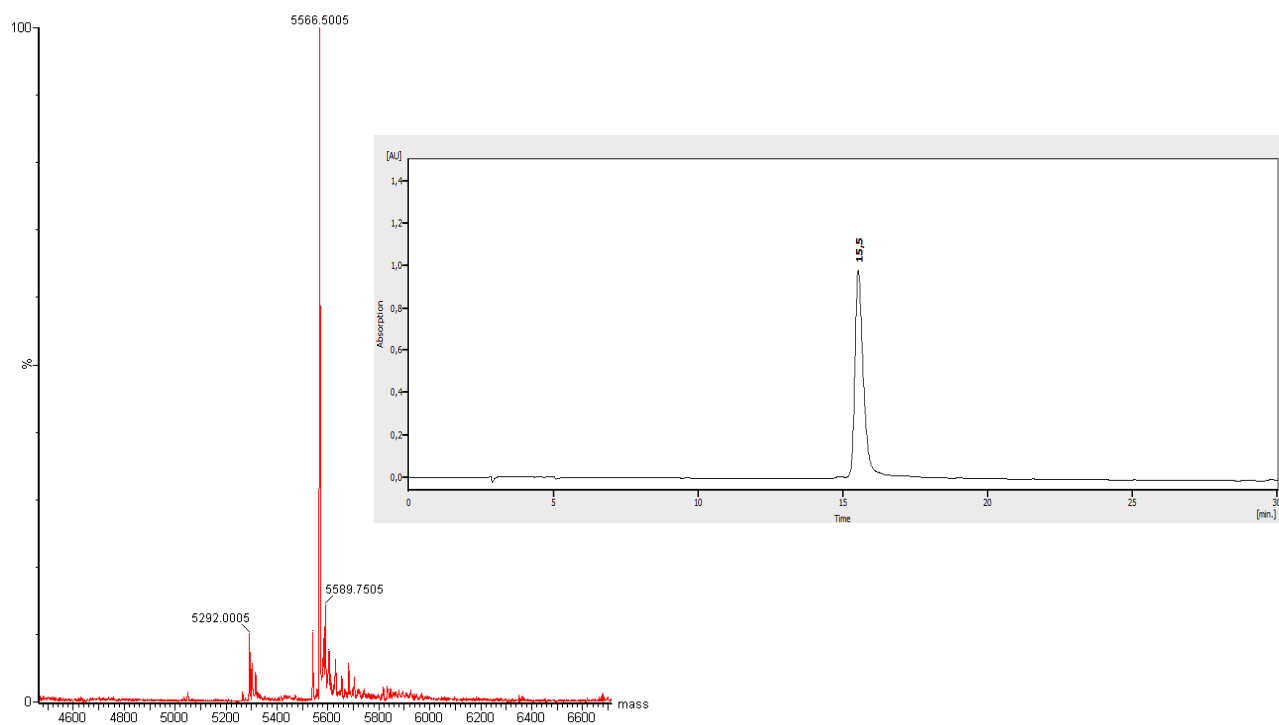
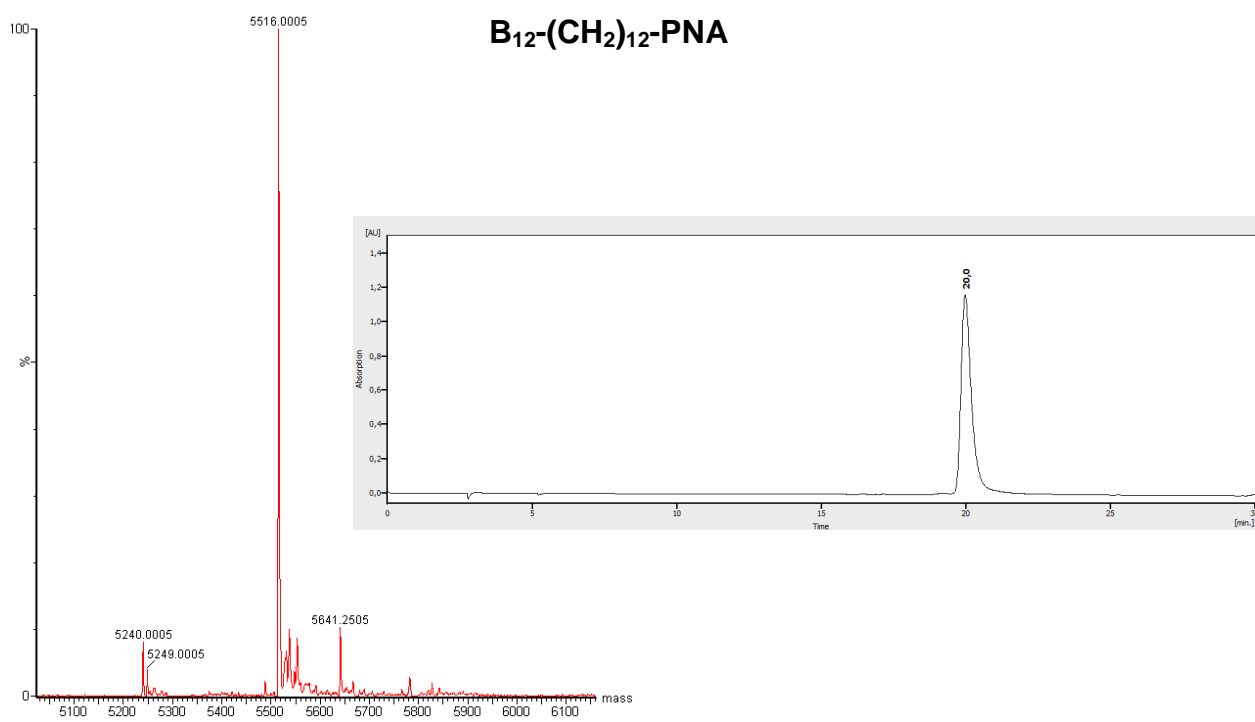


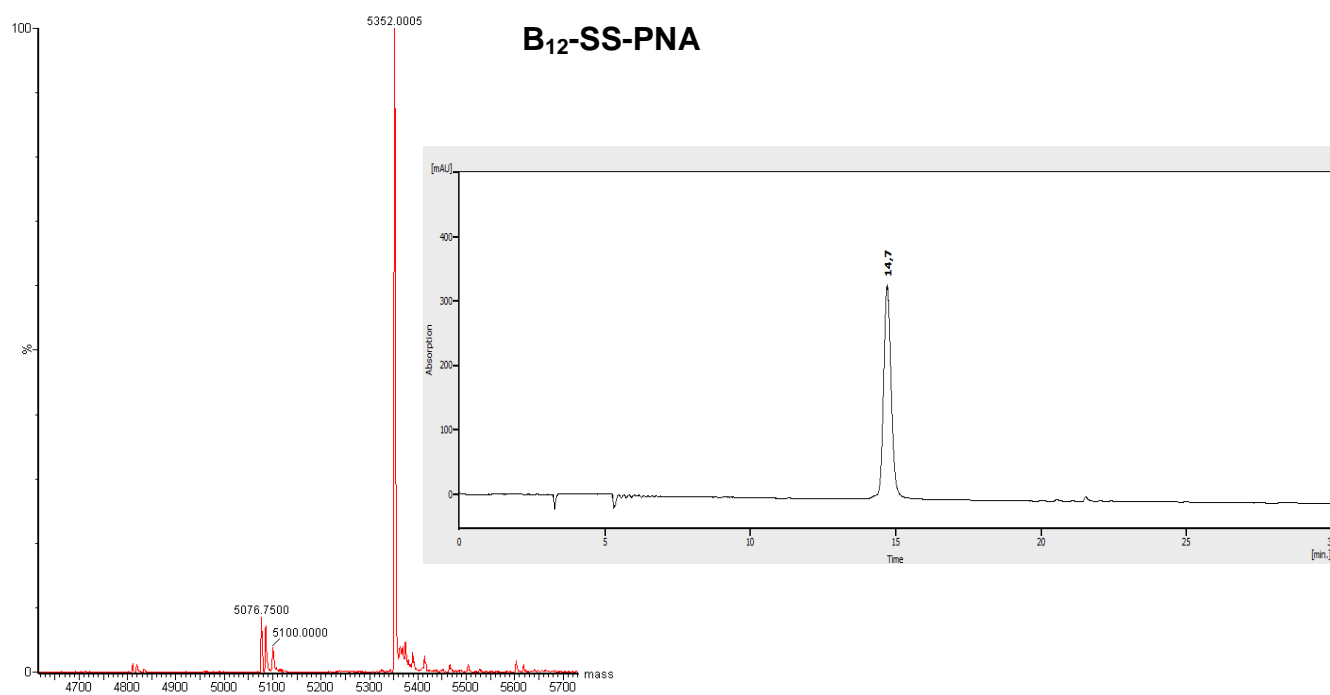
Figure S7. Mass spectrum and RP-HPLC chromatogram of B<sub>12</sub>-(CH<sub>2</sub>)<sub>6</sub>-PNA



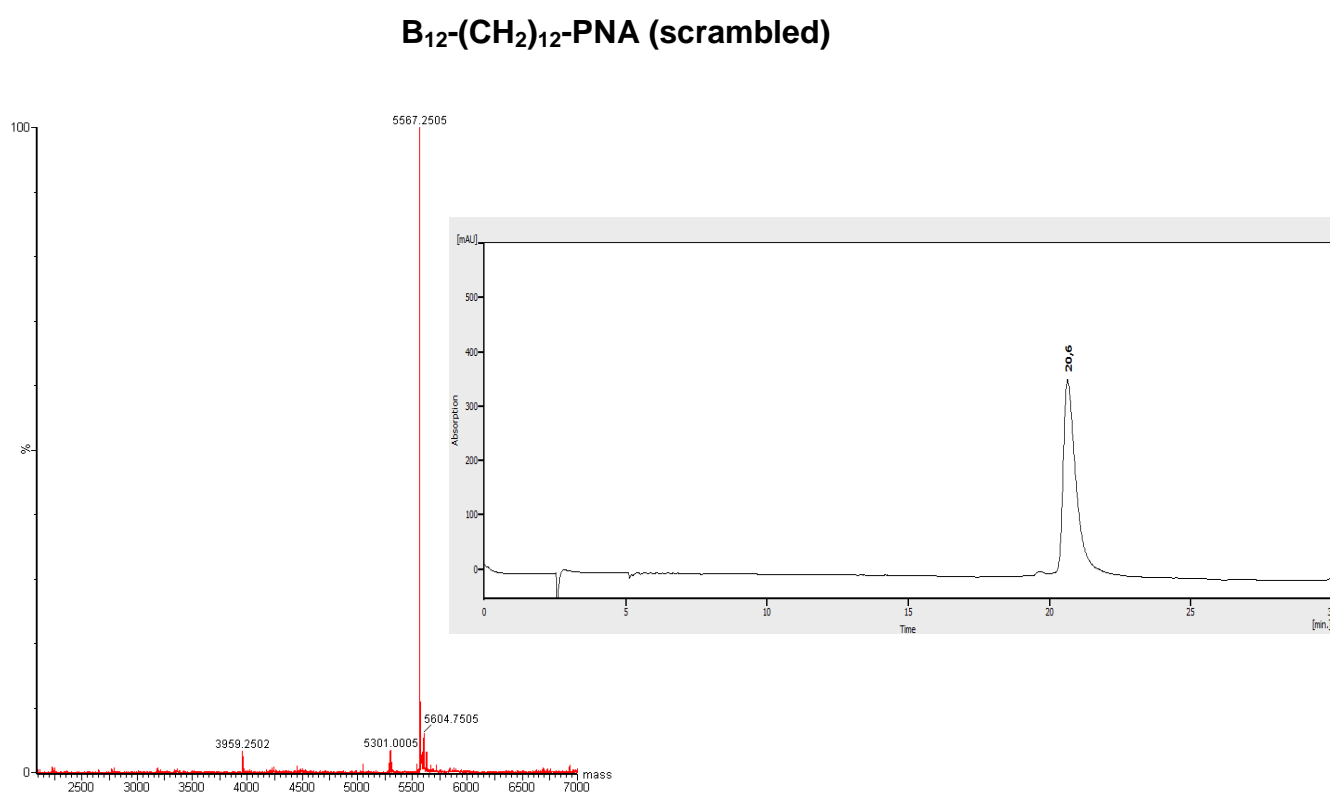
**Figure S8.** Mass spectrum and RP-HPLC chromatogram of B<sub>12</sub>-PEG<sub>2</sub>-PNA



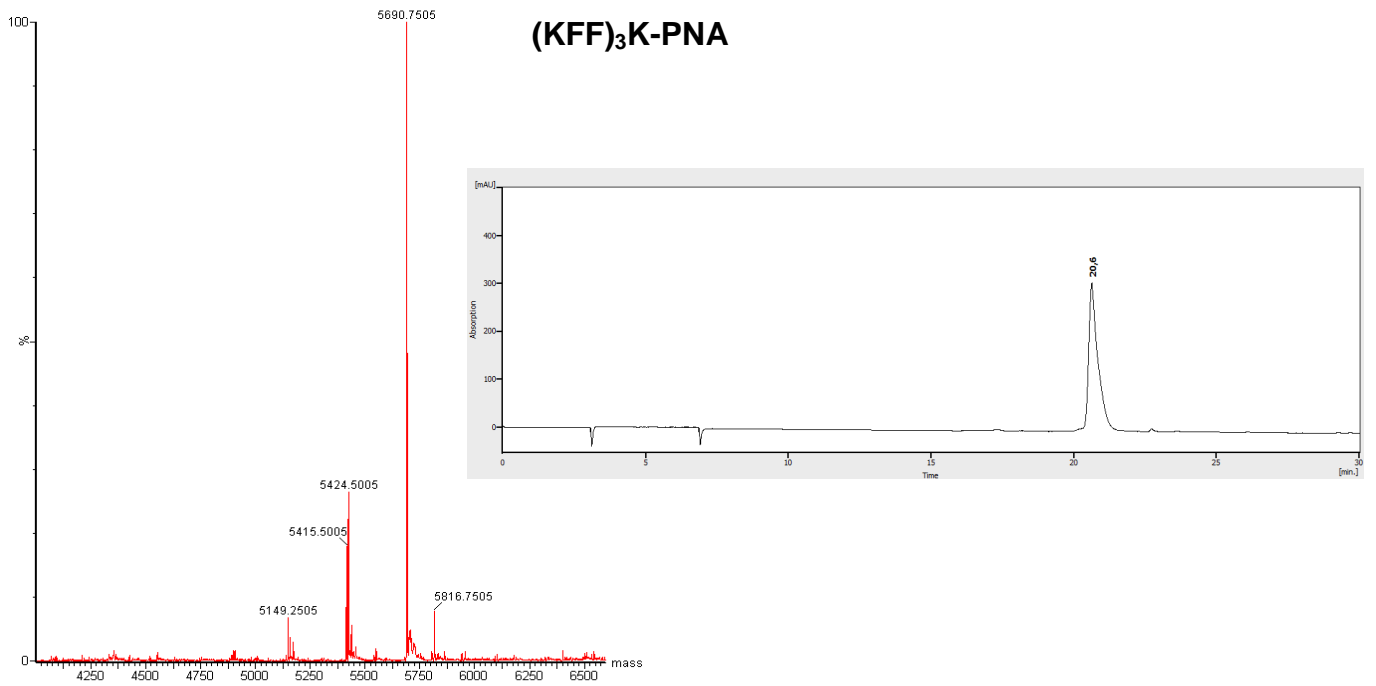
**Figure S9.** Mass spectrum and RP-HPLC chromatogram of B<sub>12</sub>-(CH<sub>2</sub>)<sub>12</sub>-PNA



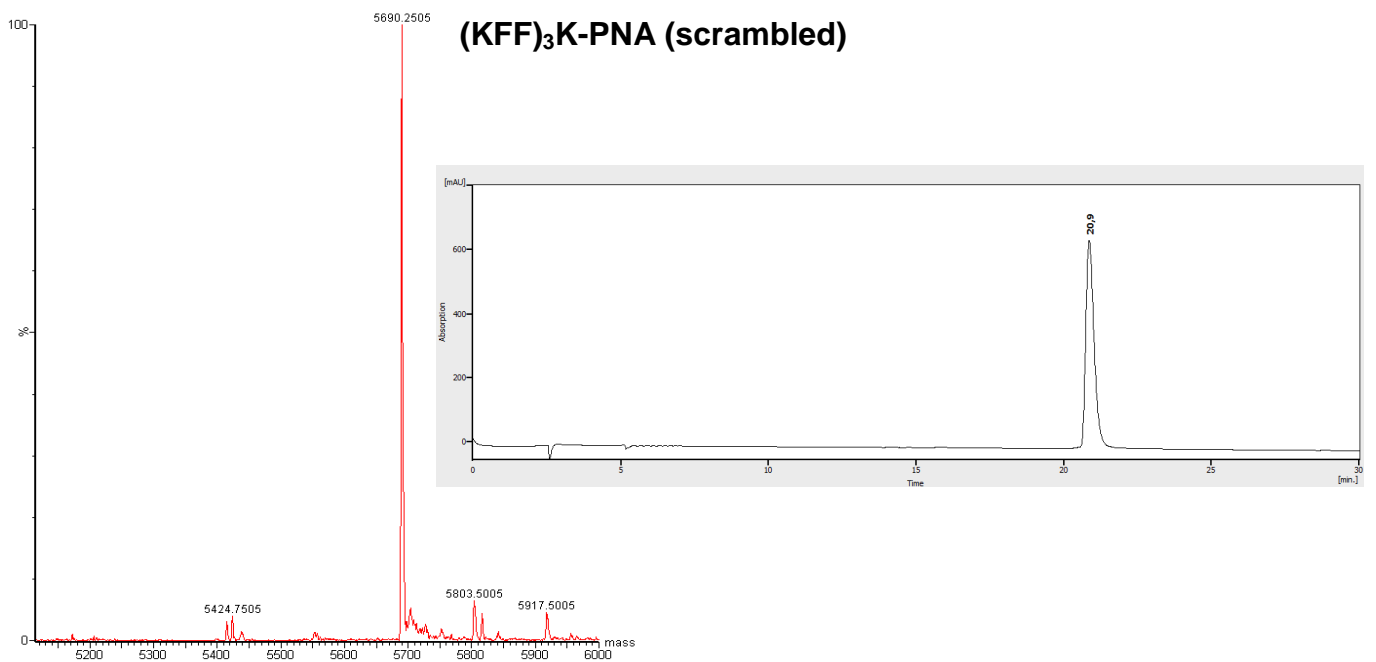
**Figure S10.** Mass spectrum and RP-HPLC chromatogram of B<sub>12</sub>-SS-PNA



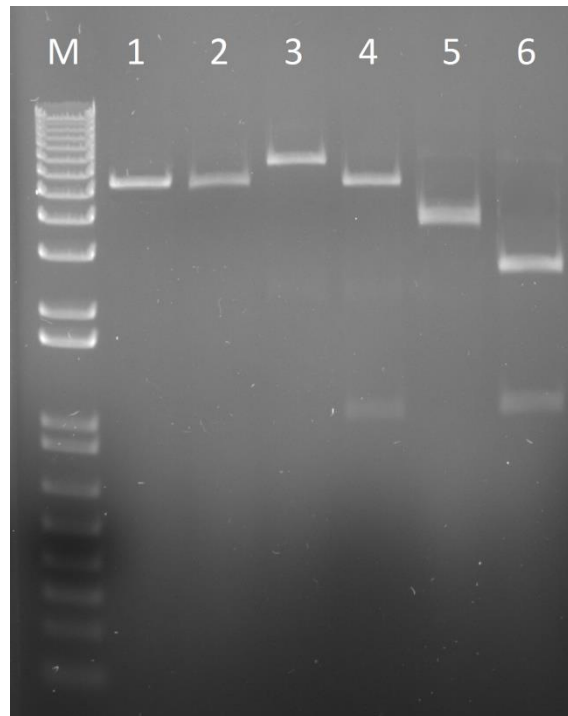
**Figure S11.** Mass spectrum and RP-HPLC chromatogram of B<sub>12</sub>-(CH<sub>2</sub>)<sub>6</sub>-PNA (scrambled)



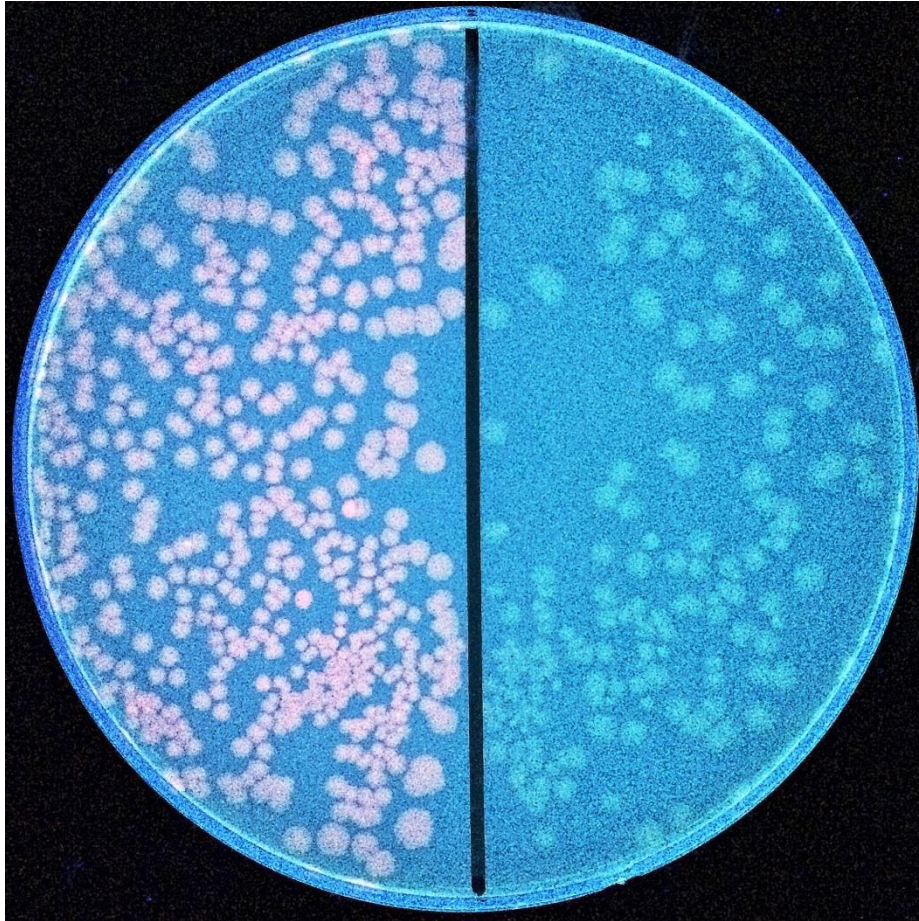
**Figure S12.** Mass spectrum and RP-HPLC chromatogram of (KFF)<sub>3</sub>K-PNA



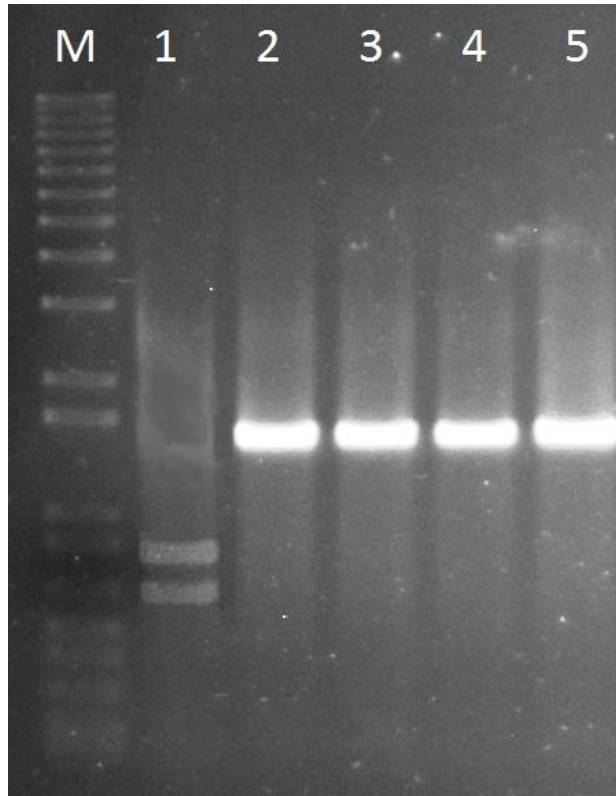
**Figure S13.** Mass spectrum and RP-HPLC chromatogram of (KFF)<sub>3</sub>K-PNA (scrambled)



**Figure S14.** Verification of the presence of the pBBR(rfp) plasmid by the restriction digest analysis. M – 1Kb Plus DNA Ladder (Thermo Scientific™, No: 10787018); 1 – pBBR1MCS2 – EcoRI (5144 bp); 2 – pBBR1MCS2 – EcoRI/SpeI (5120 + 24 bp (not visible due to low mass)); 3 – pBBR(rfp) – EcoRI (6212 bp); 4 – pBBR(rfp) – EcoRI/SpeI (5120 + 1092 bp); 5 – pSB3K3 – EcoRI (3819 bp); 6 – pSB3K3 – EcoRI/SpeI (2727 + 1092 bp).



**Figure S15.** The primary detection of recombinants by red – white screening for an altered phenotype caused by the Red Fluorescent Protein (imaged using an UV transilluminator).



**Figure S16.** Restriction patterns of 16S rRNA genes of *E. coli* (line 1) and *S. Typhimurium* (lanes 2-5) digested with Sall. Lane M, size marker 1Kb Plus DNA Ladder (Thermo Scientific™, No: 10787018). Restriction enzyme Sall does not digest *S. Typhimurium* 16S rRNA (1520 bp); however 16S rRNA of *E. coli* was digested in 3 parts (1542 bp = 53\*, 668, 821 bp) where \* stands for an invisible band due to low molecular weight.

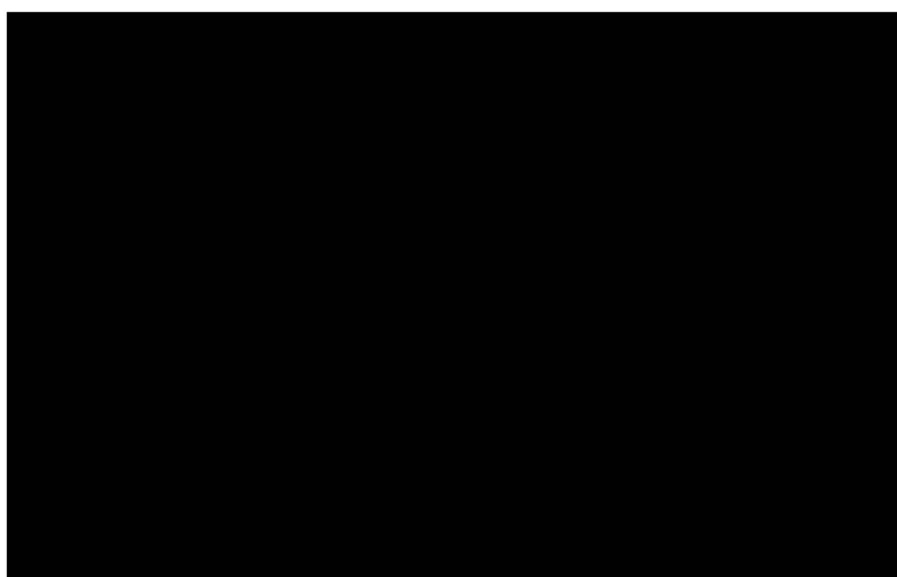
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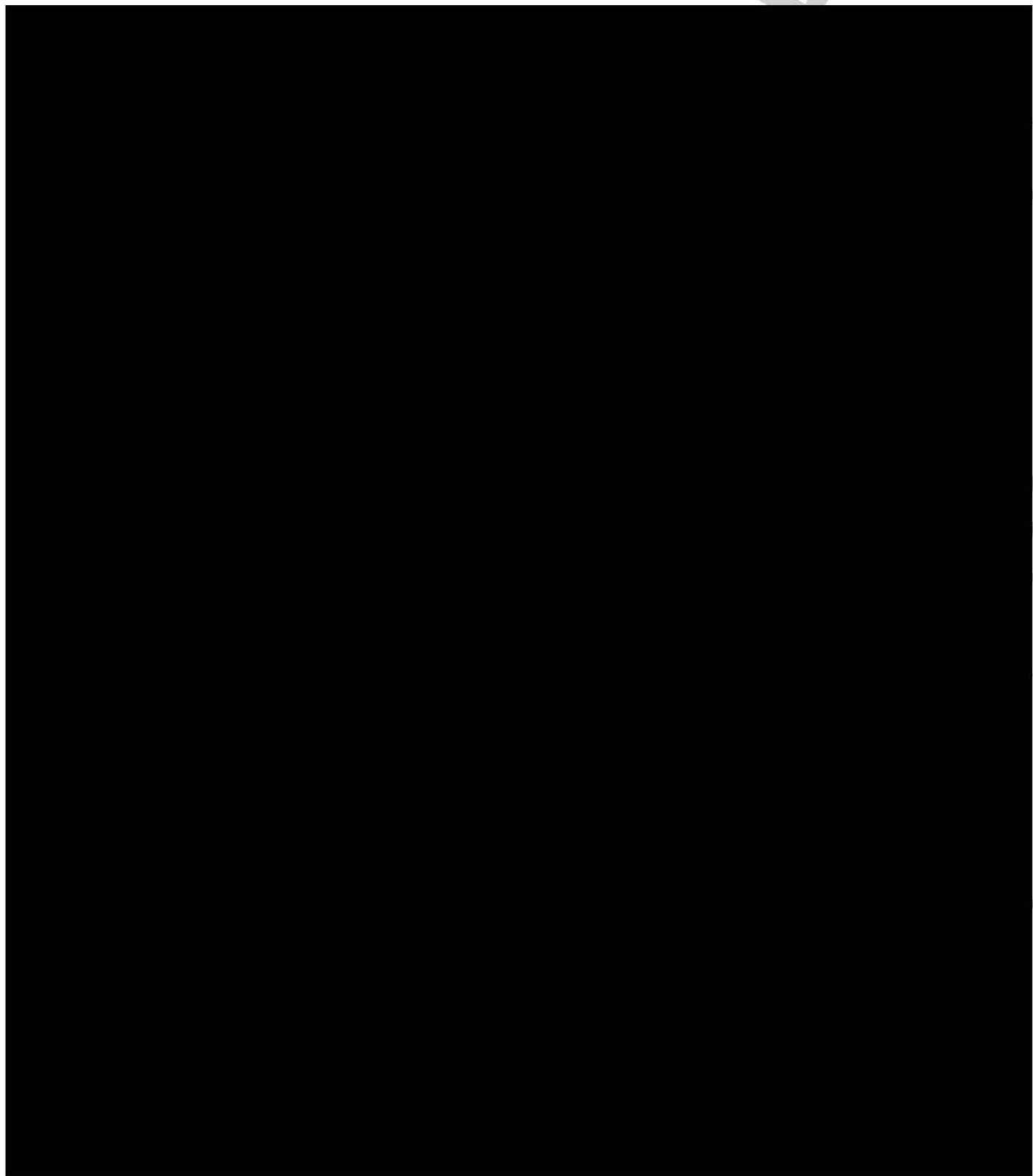
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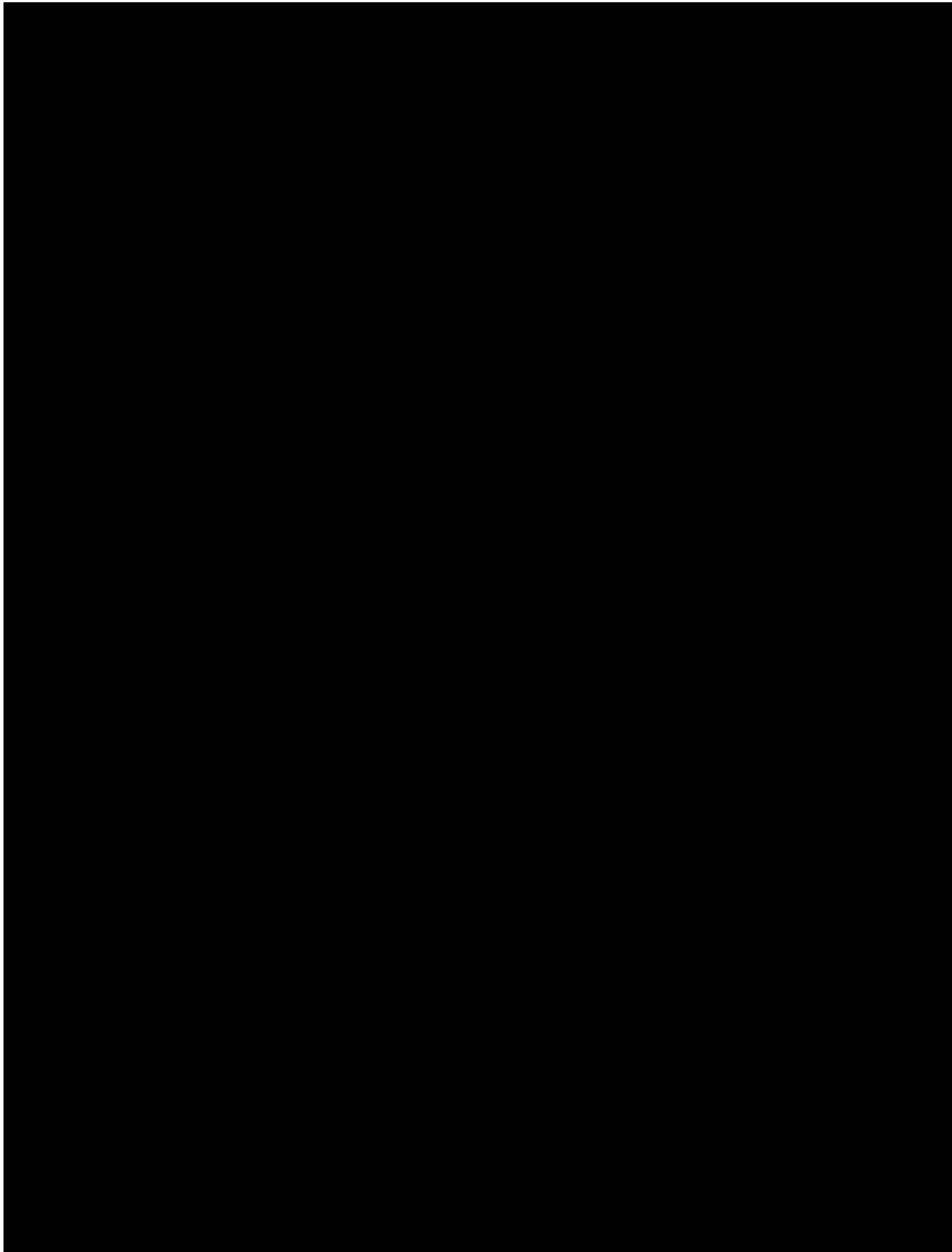


## Does a conjugation site affect transport of vitamin B<sub>12</sub>-PNA conjugates into bacterial cells?

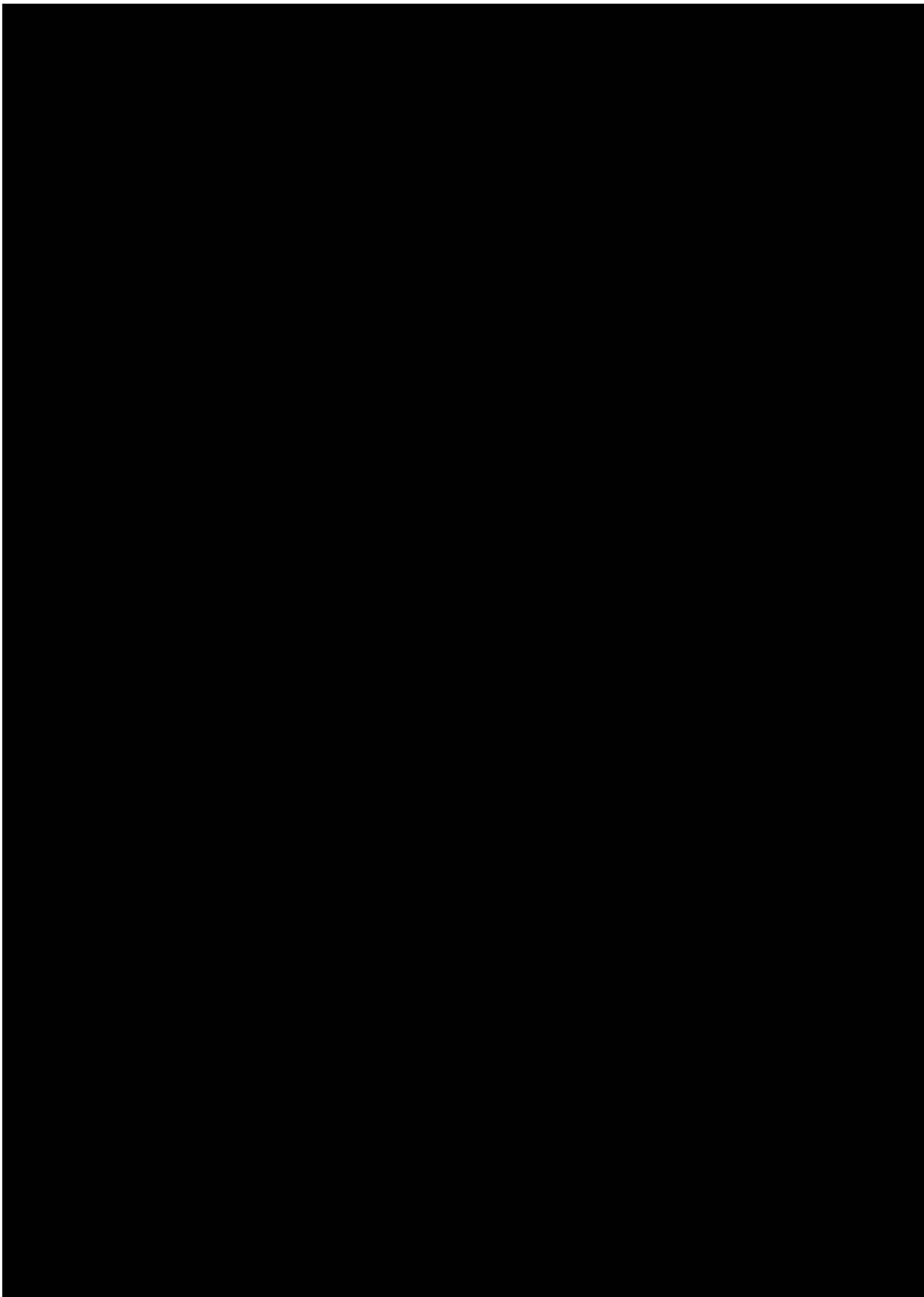
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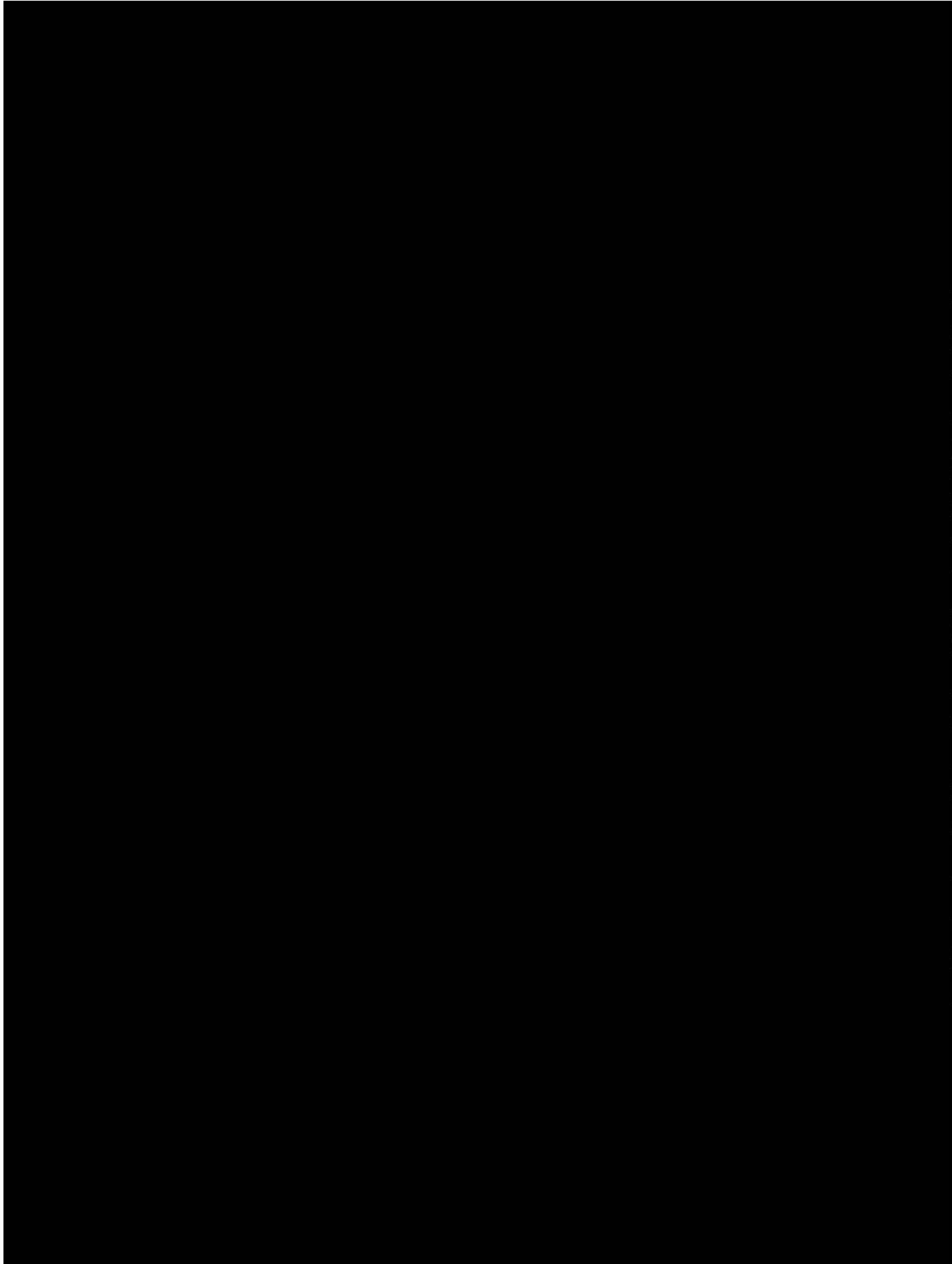
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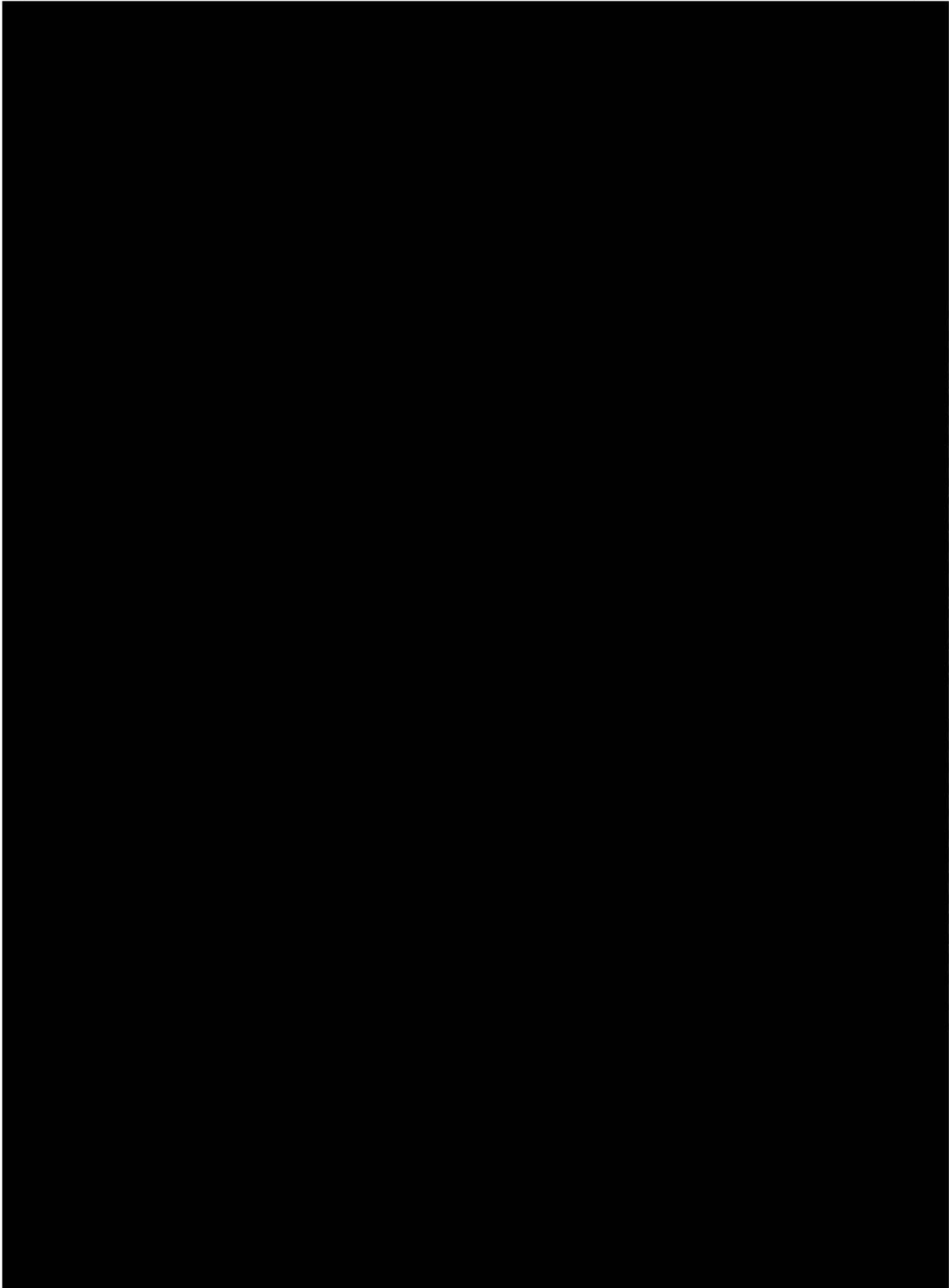
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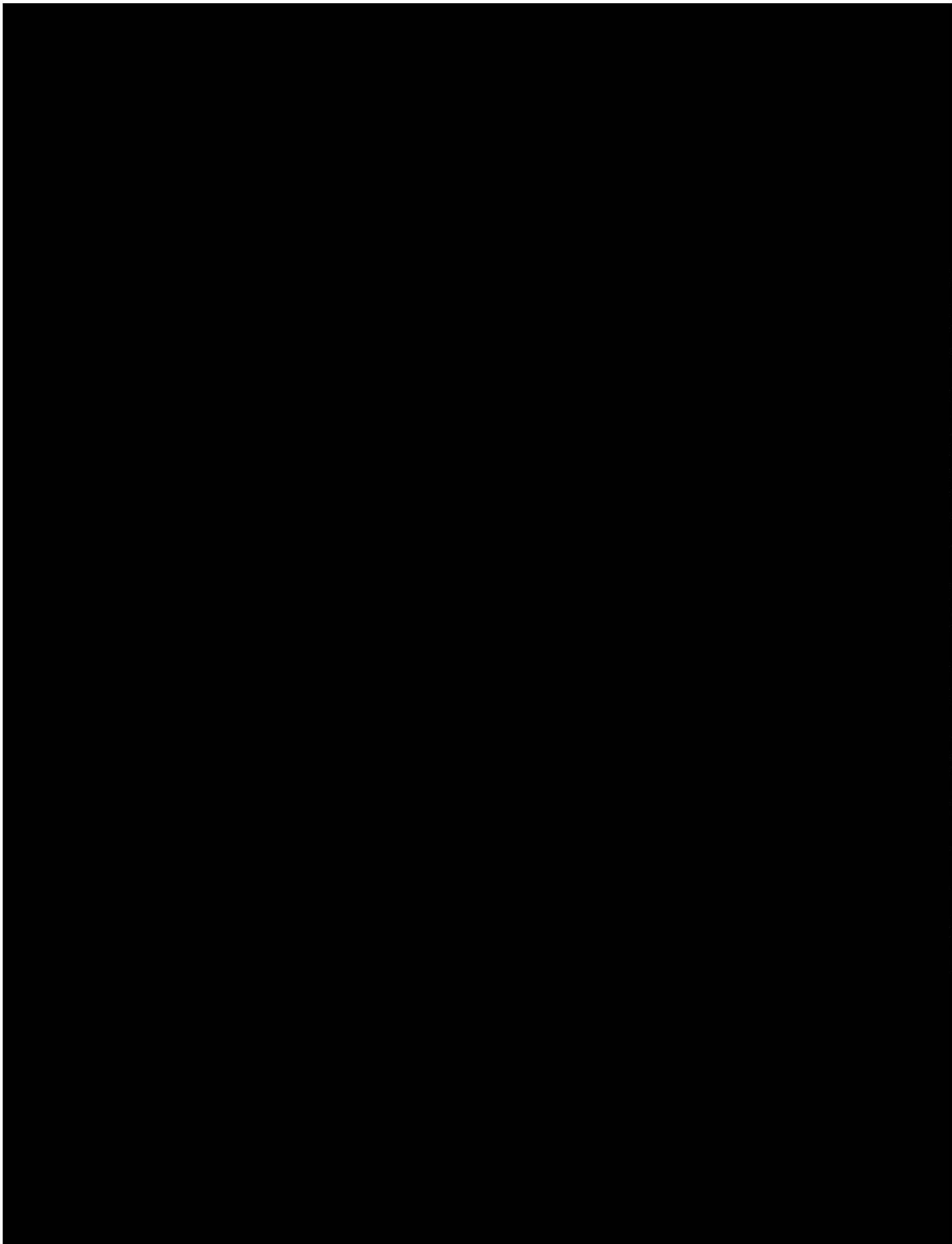
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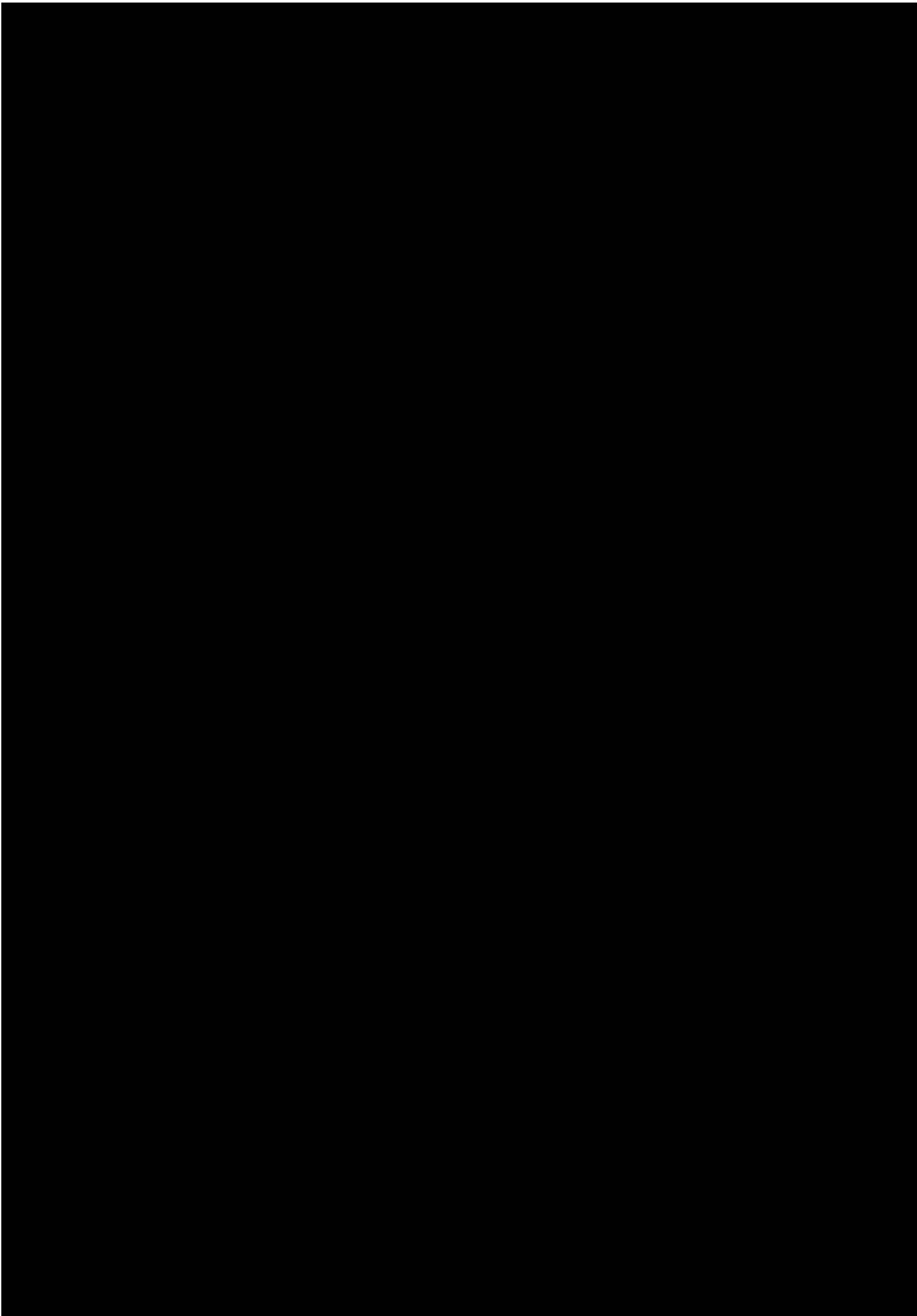
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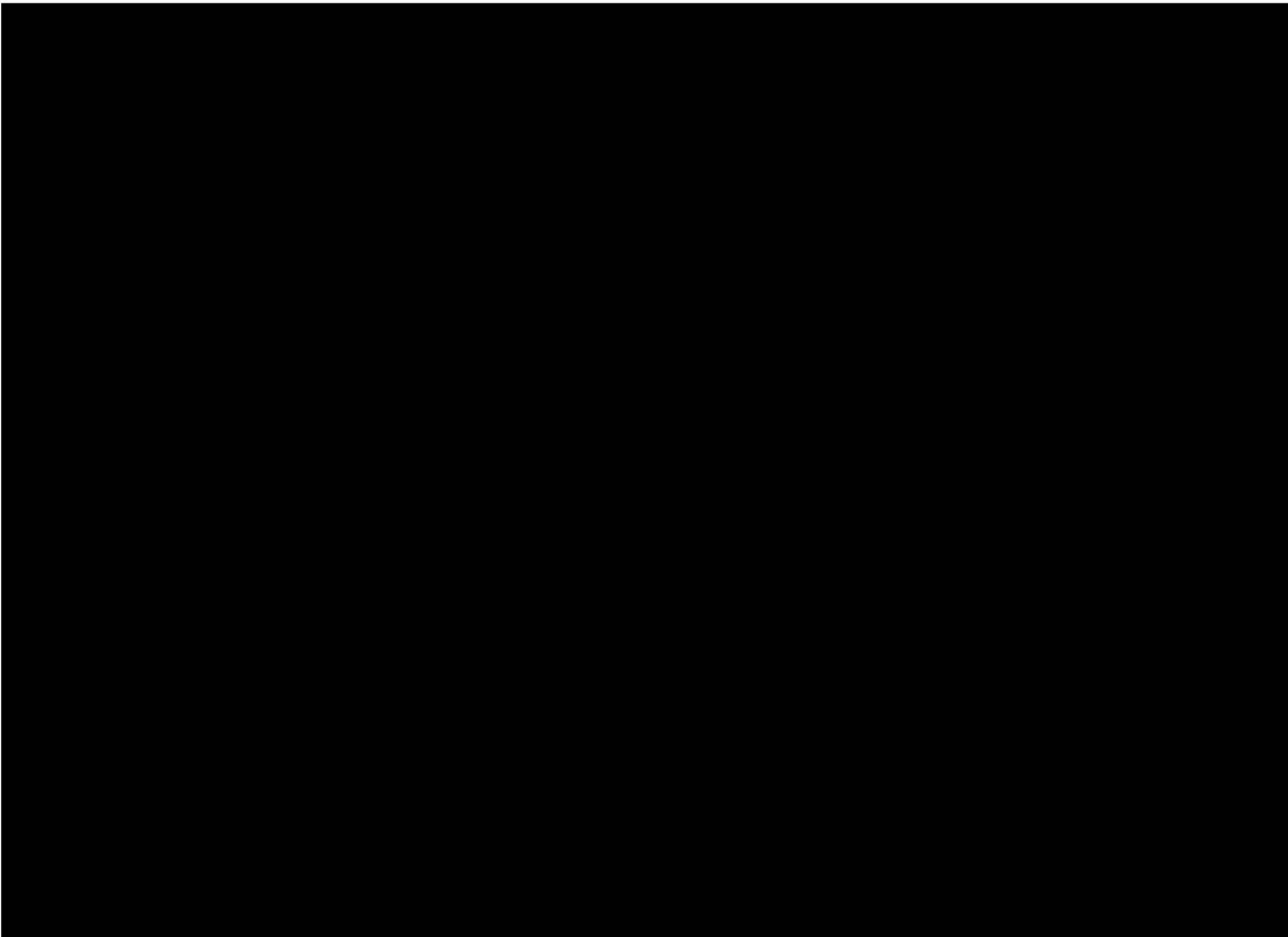


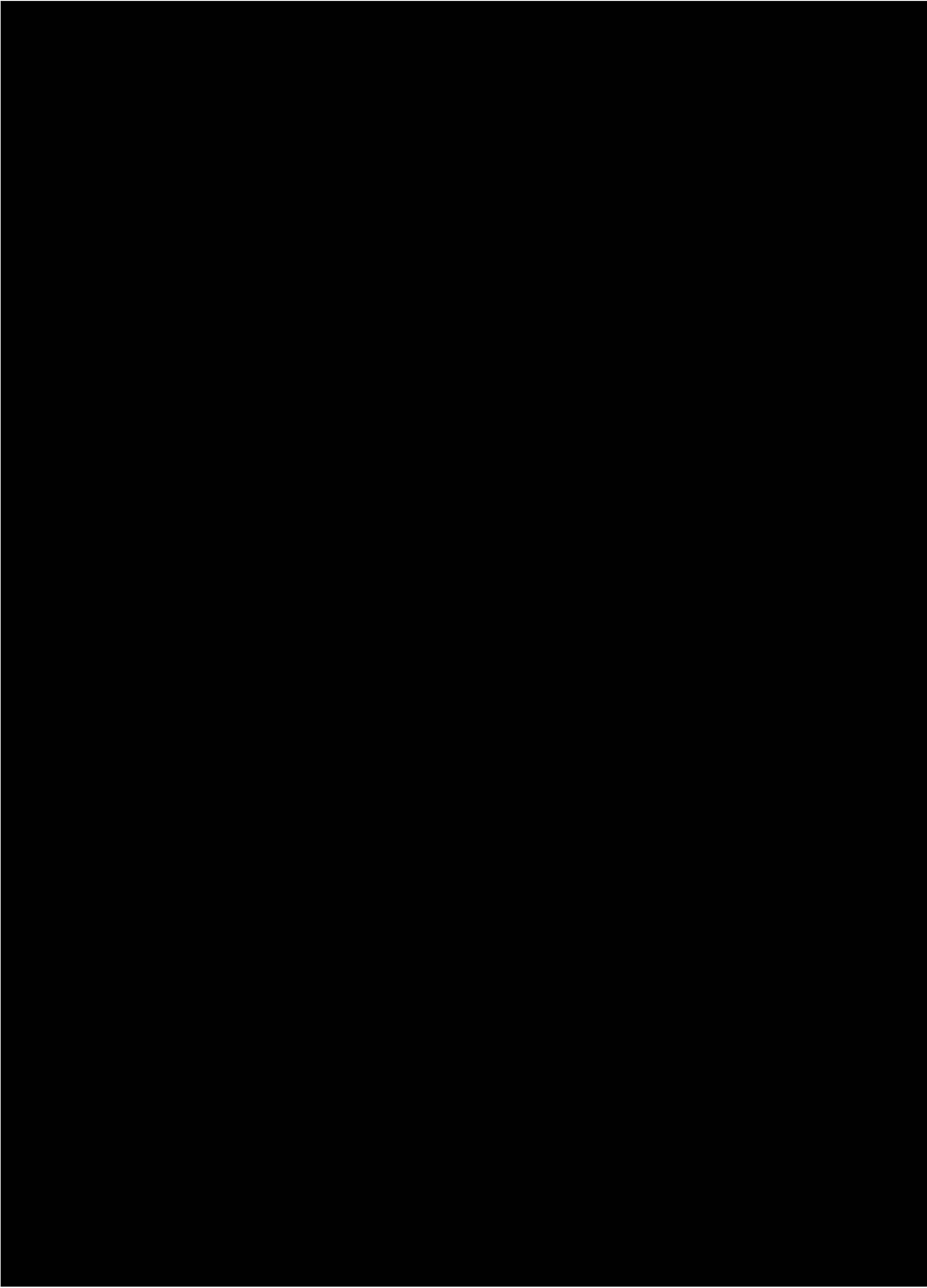


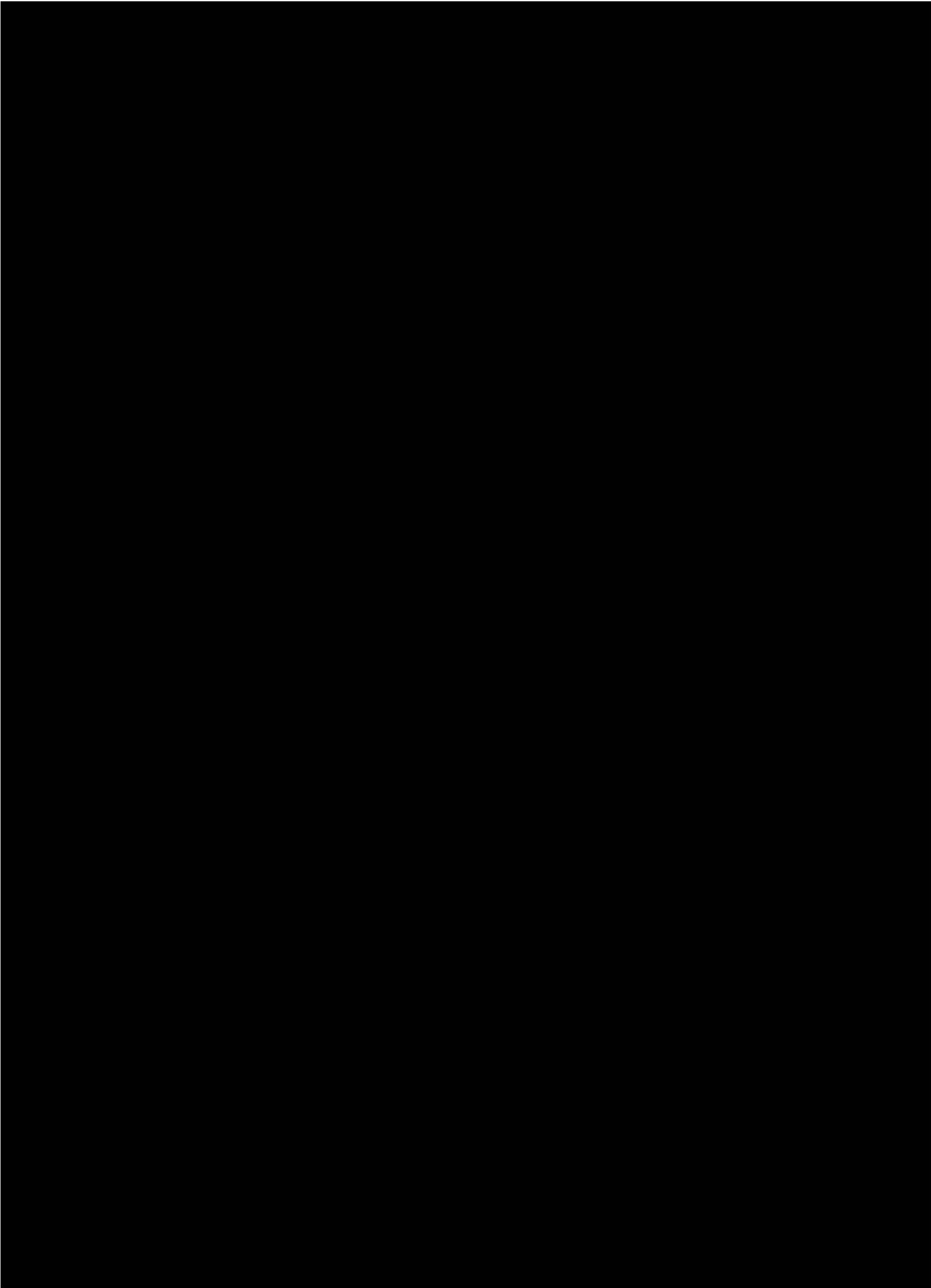
















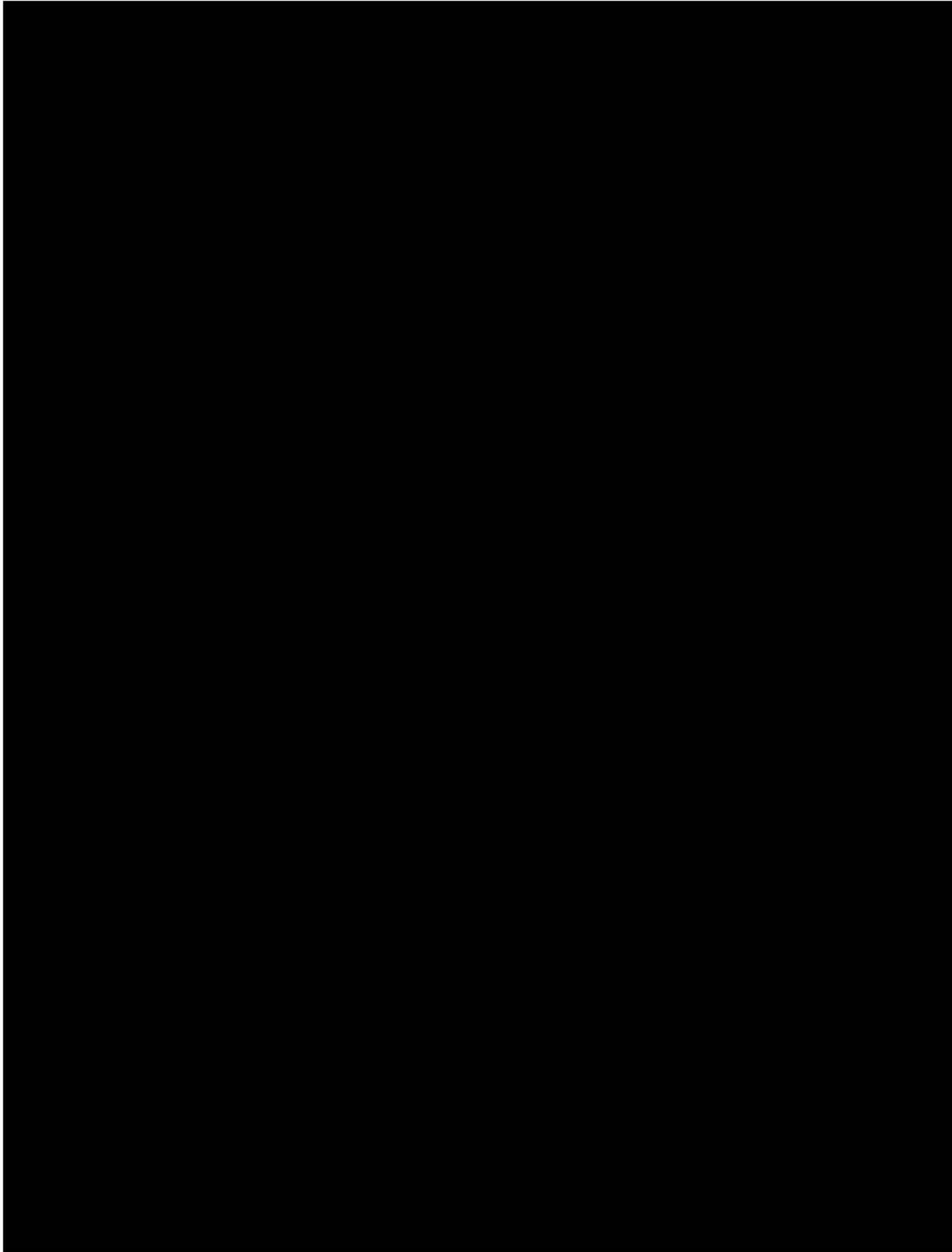


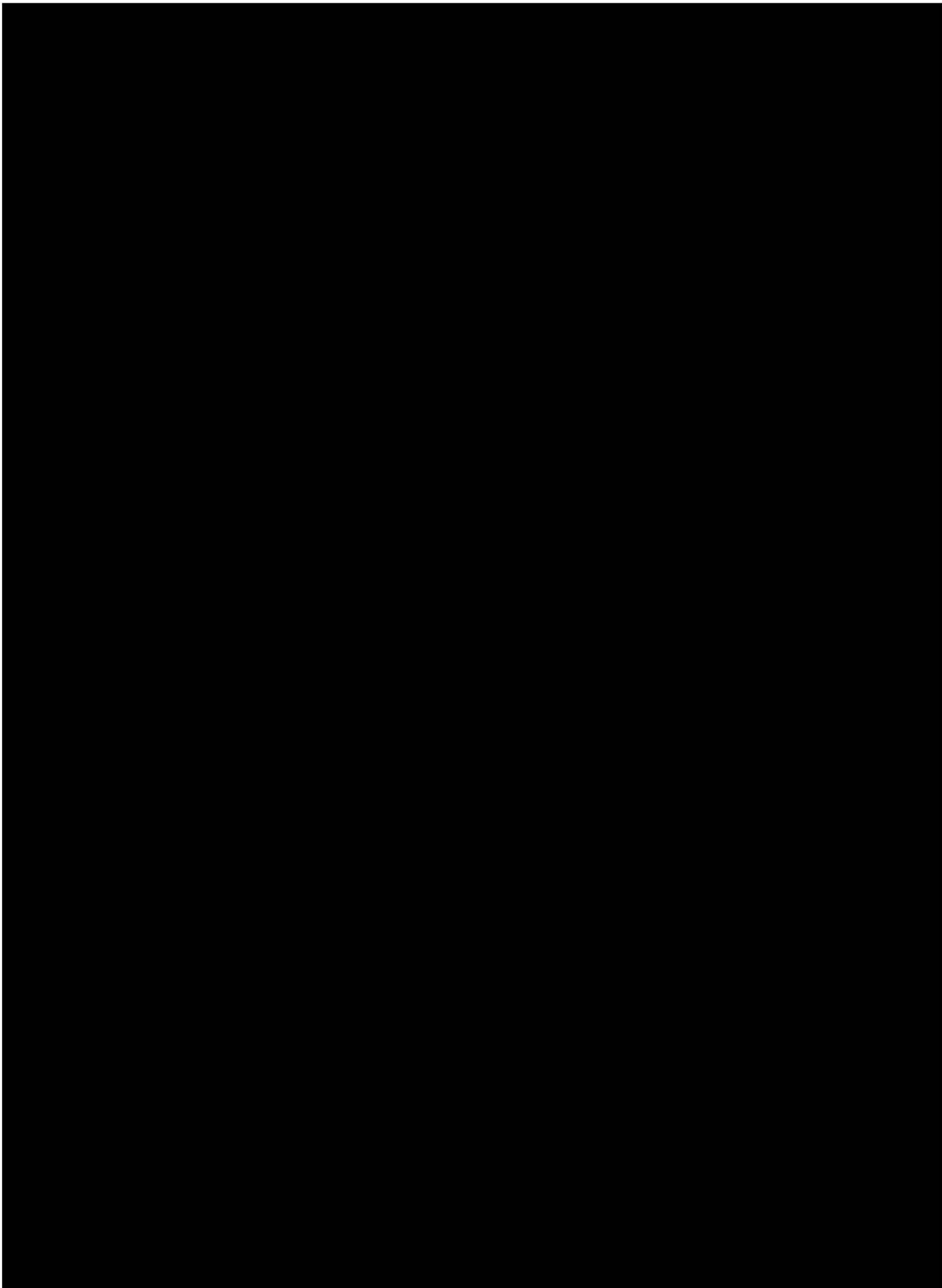


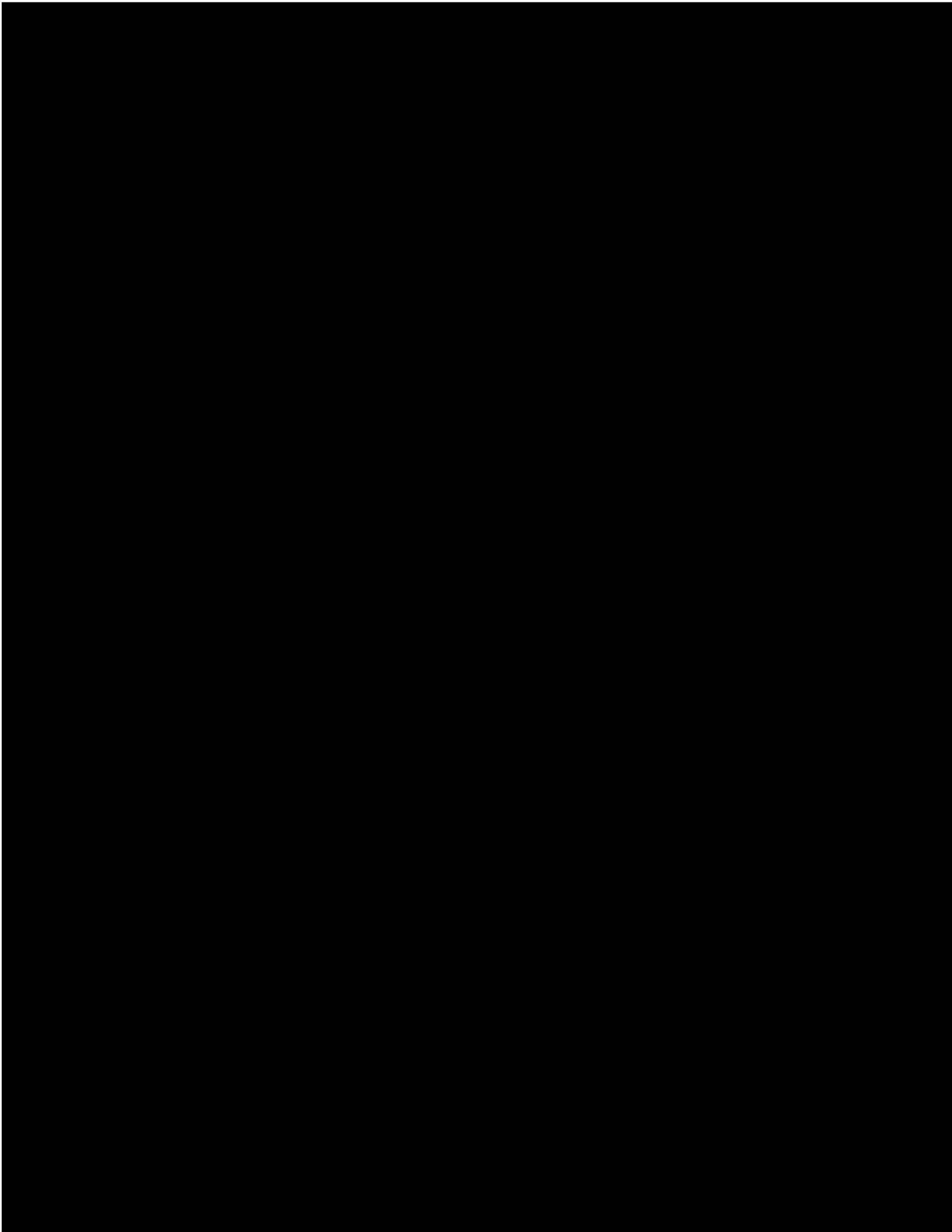


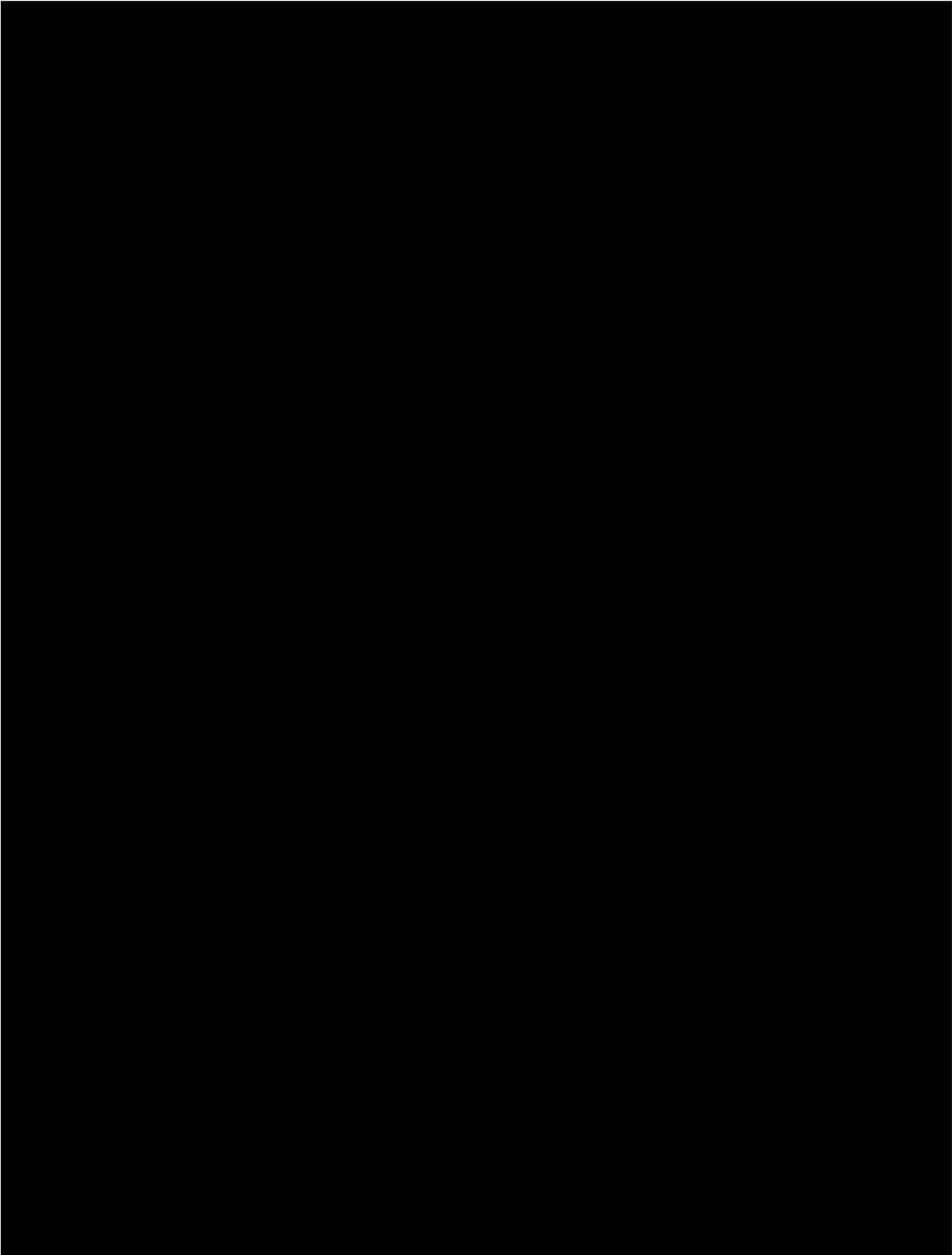


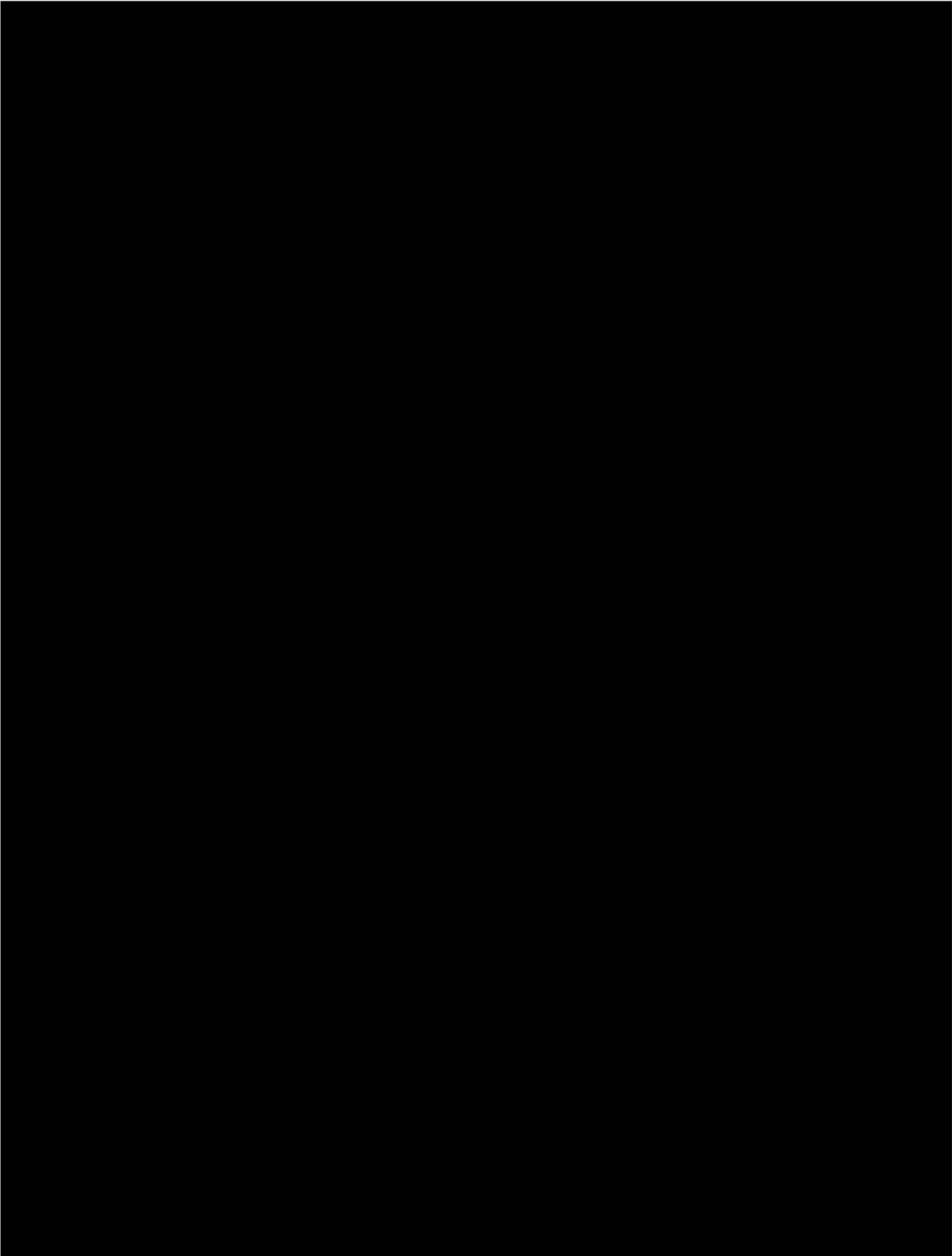










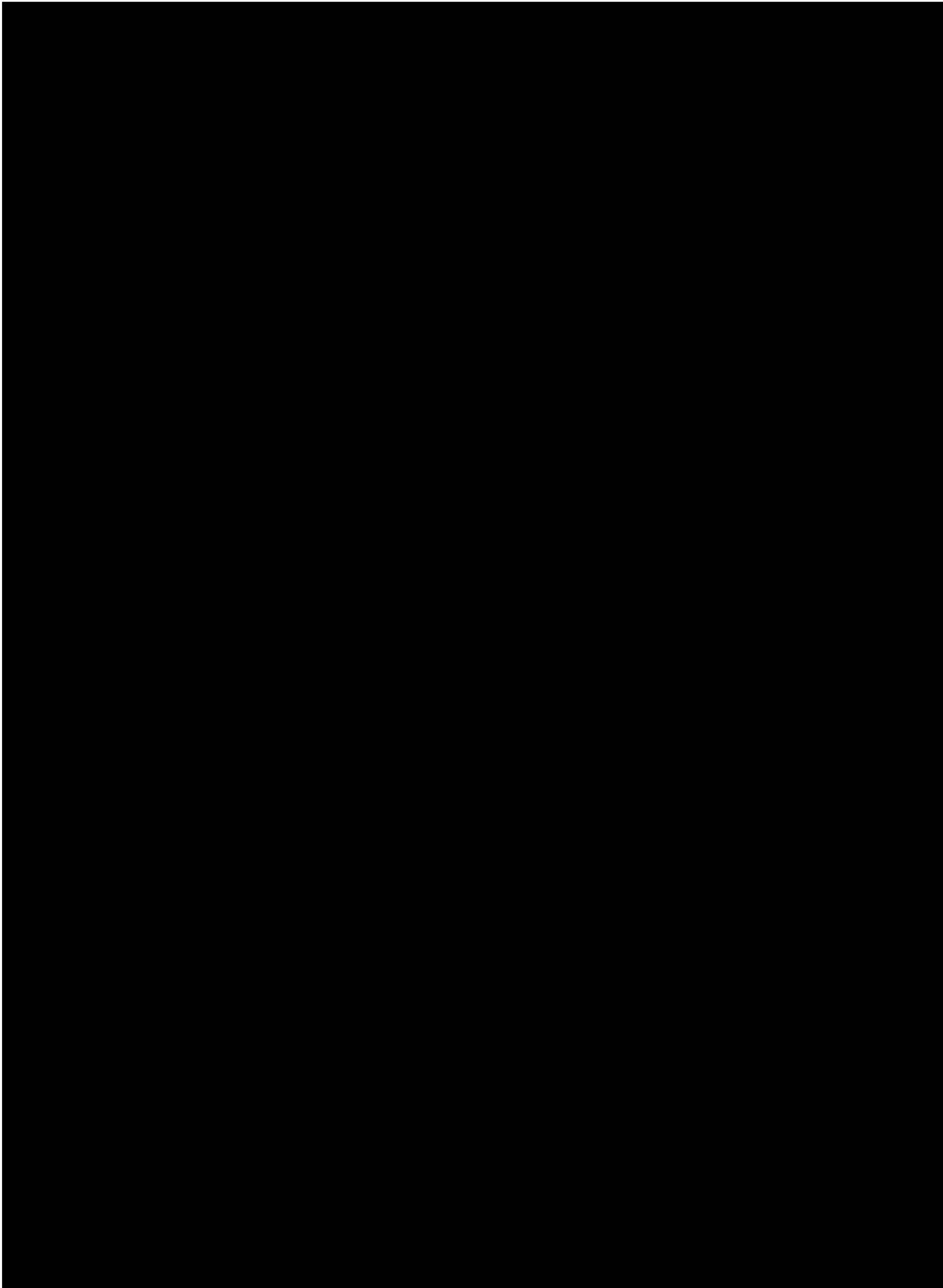


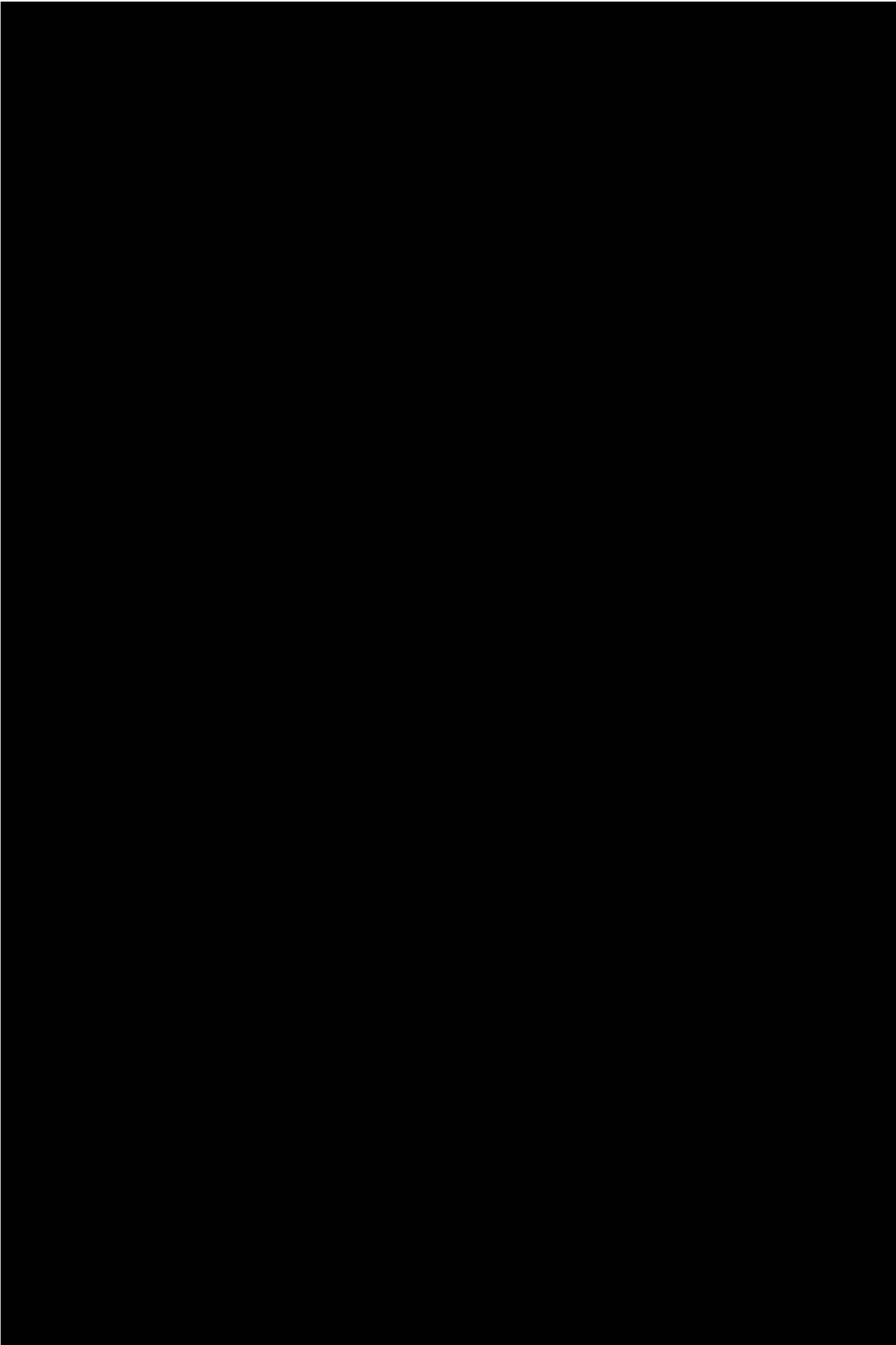




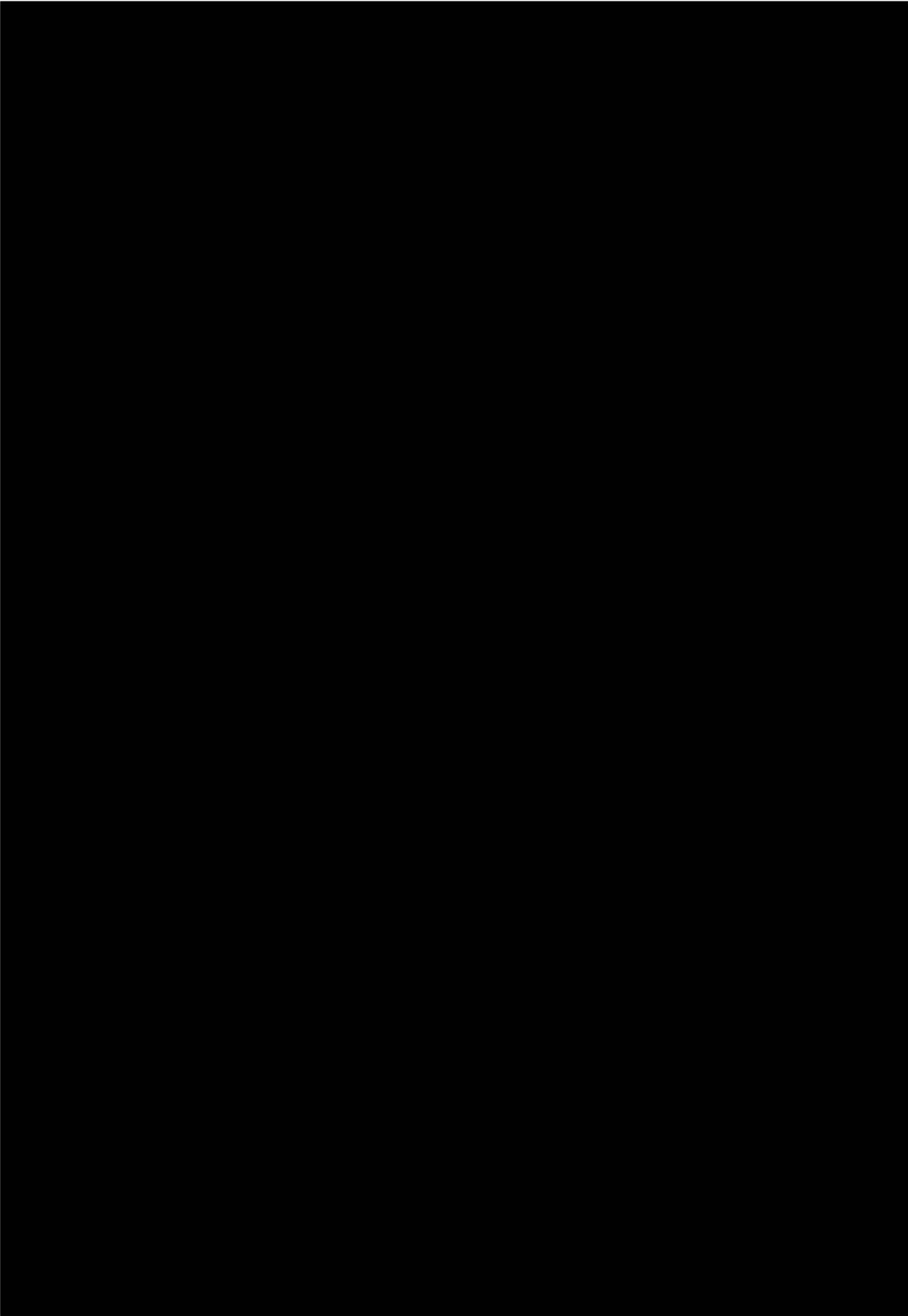


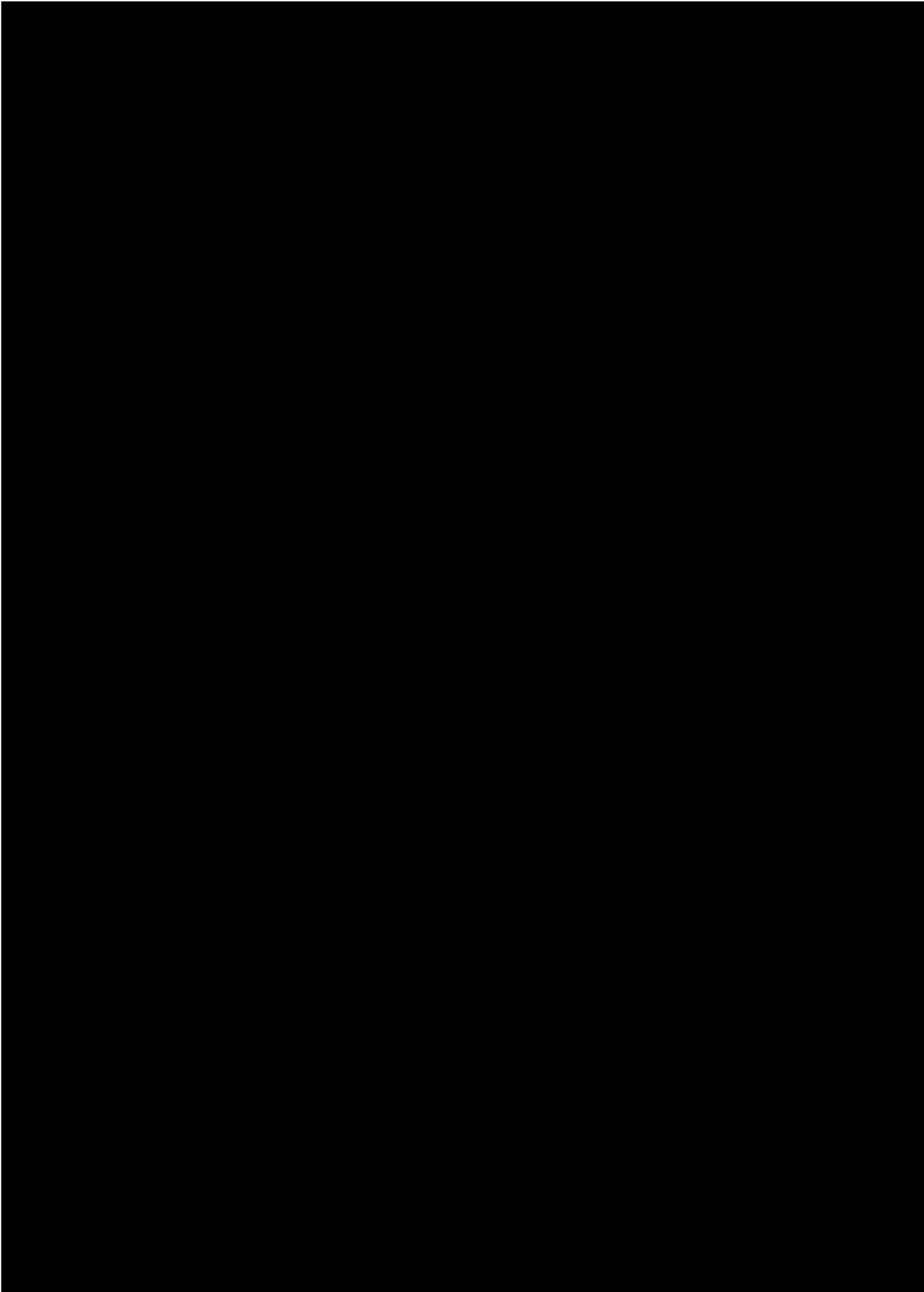


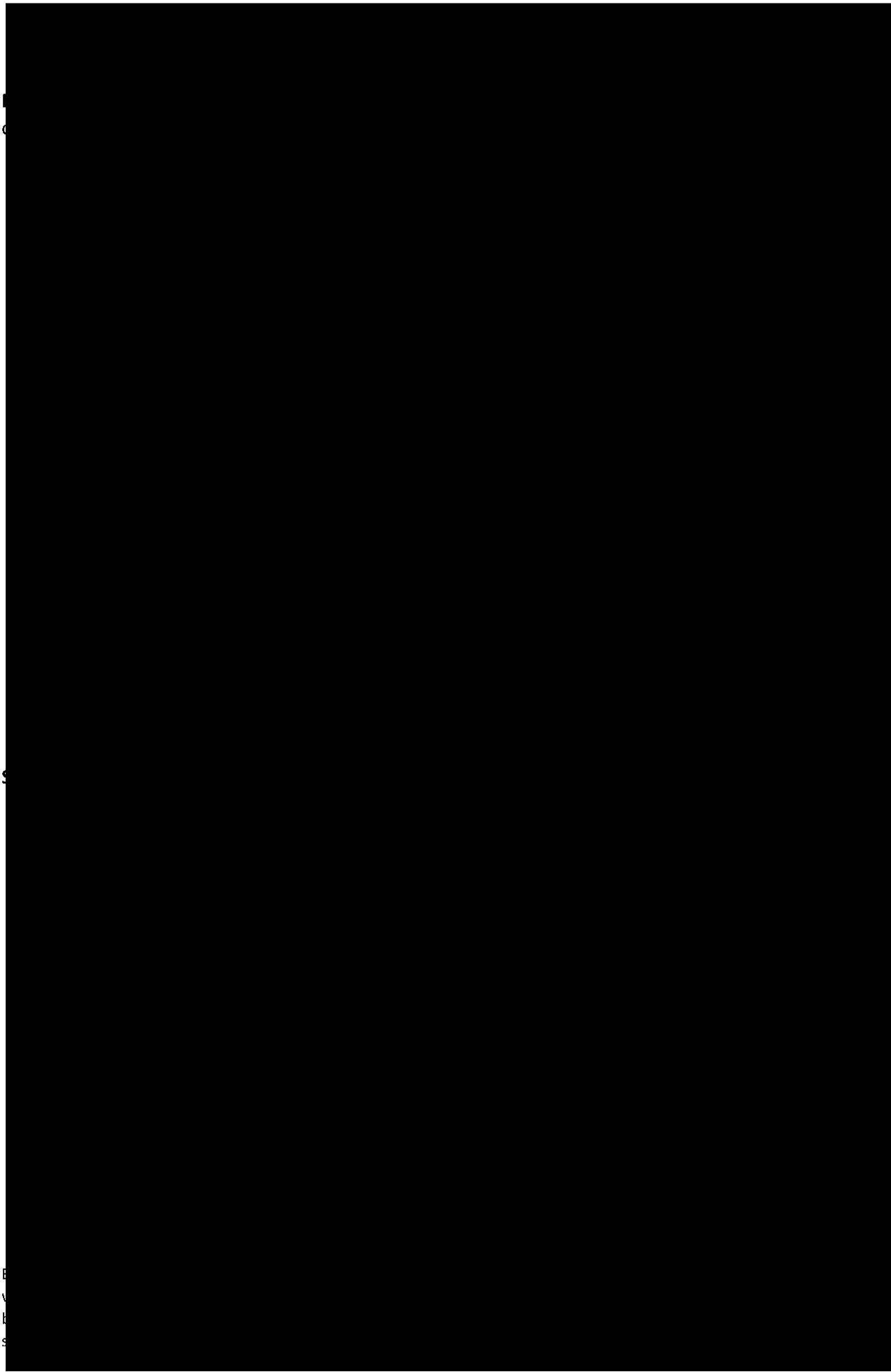






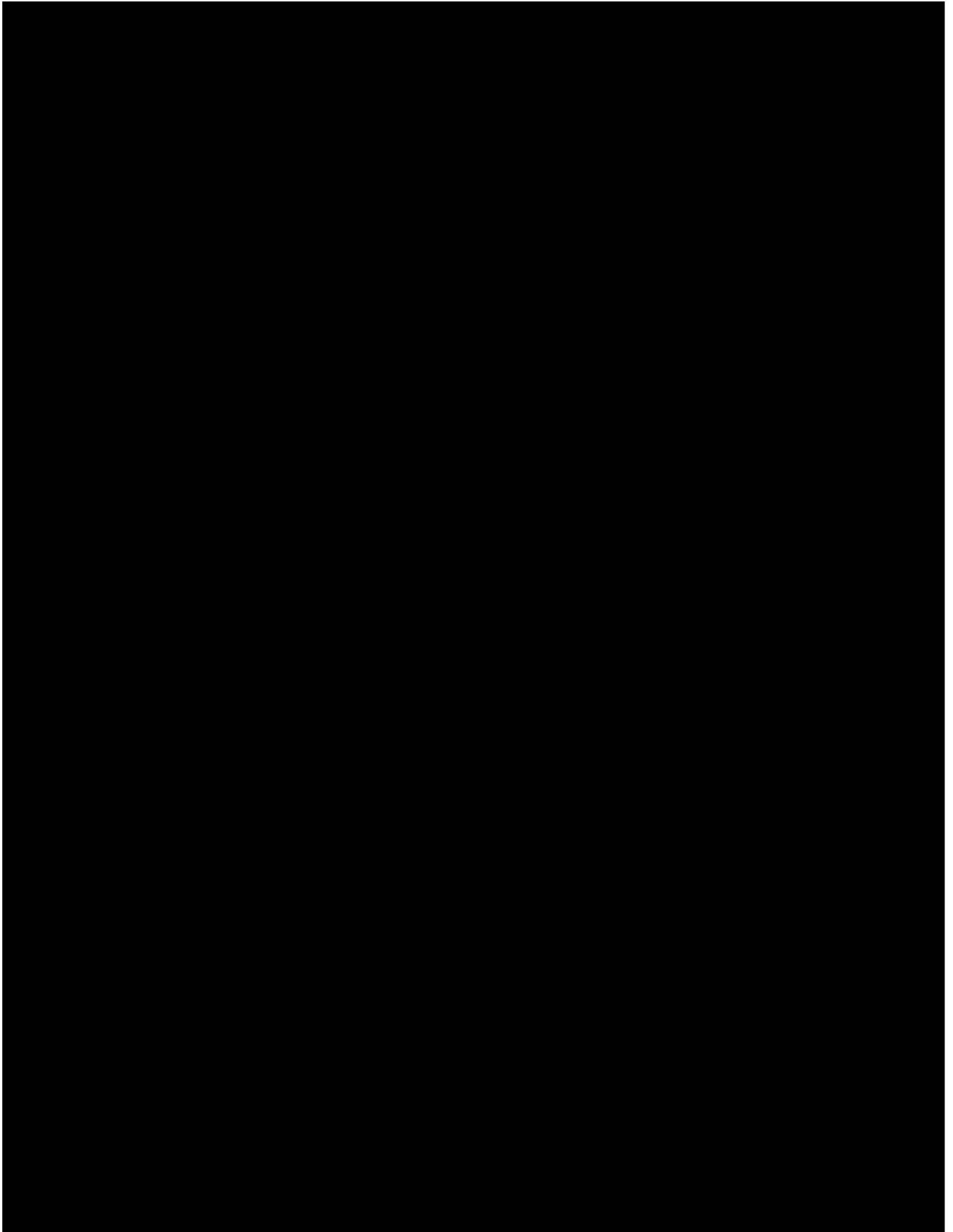




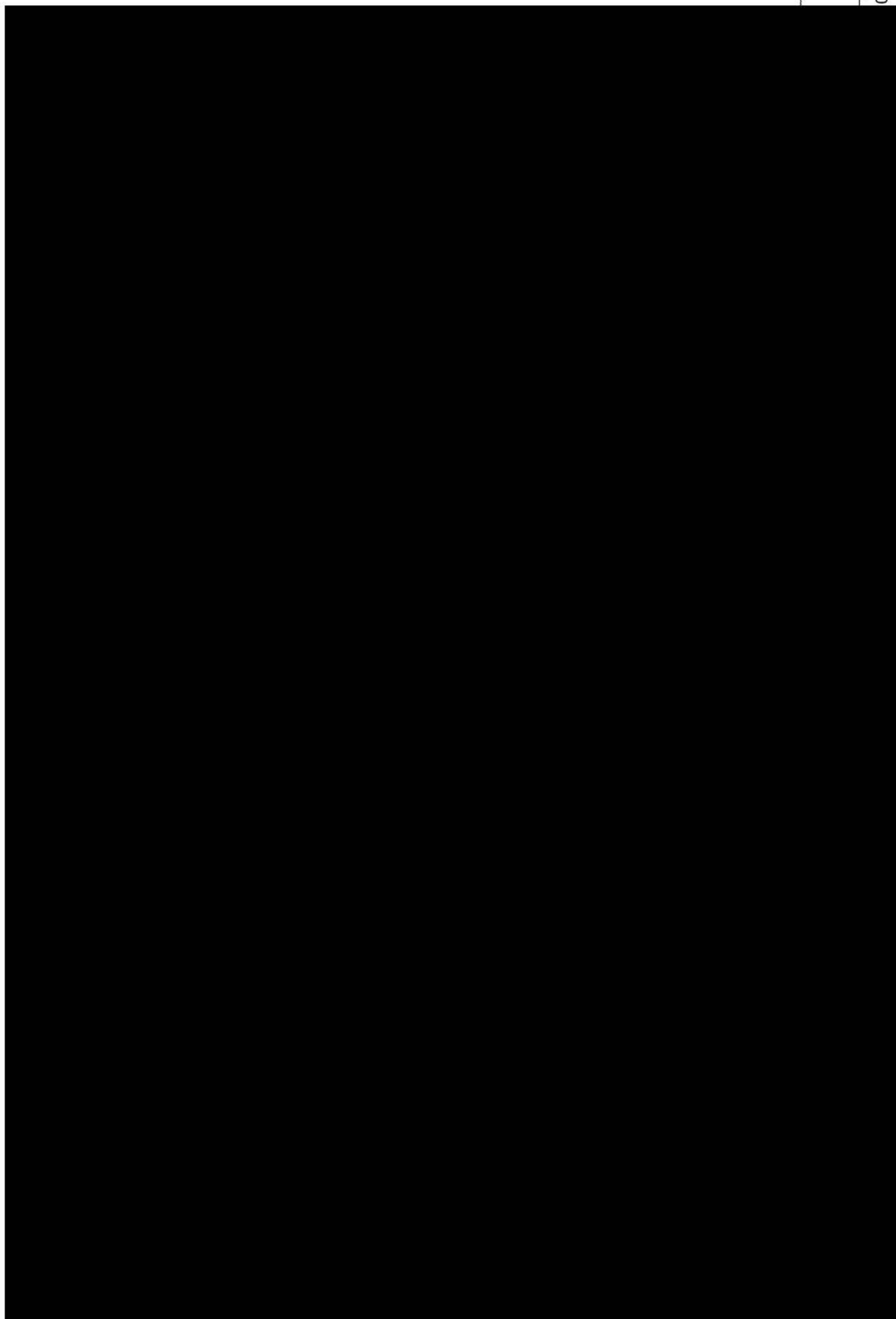




## S6. Binding assays of *meso*-modified cobalamins

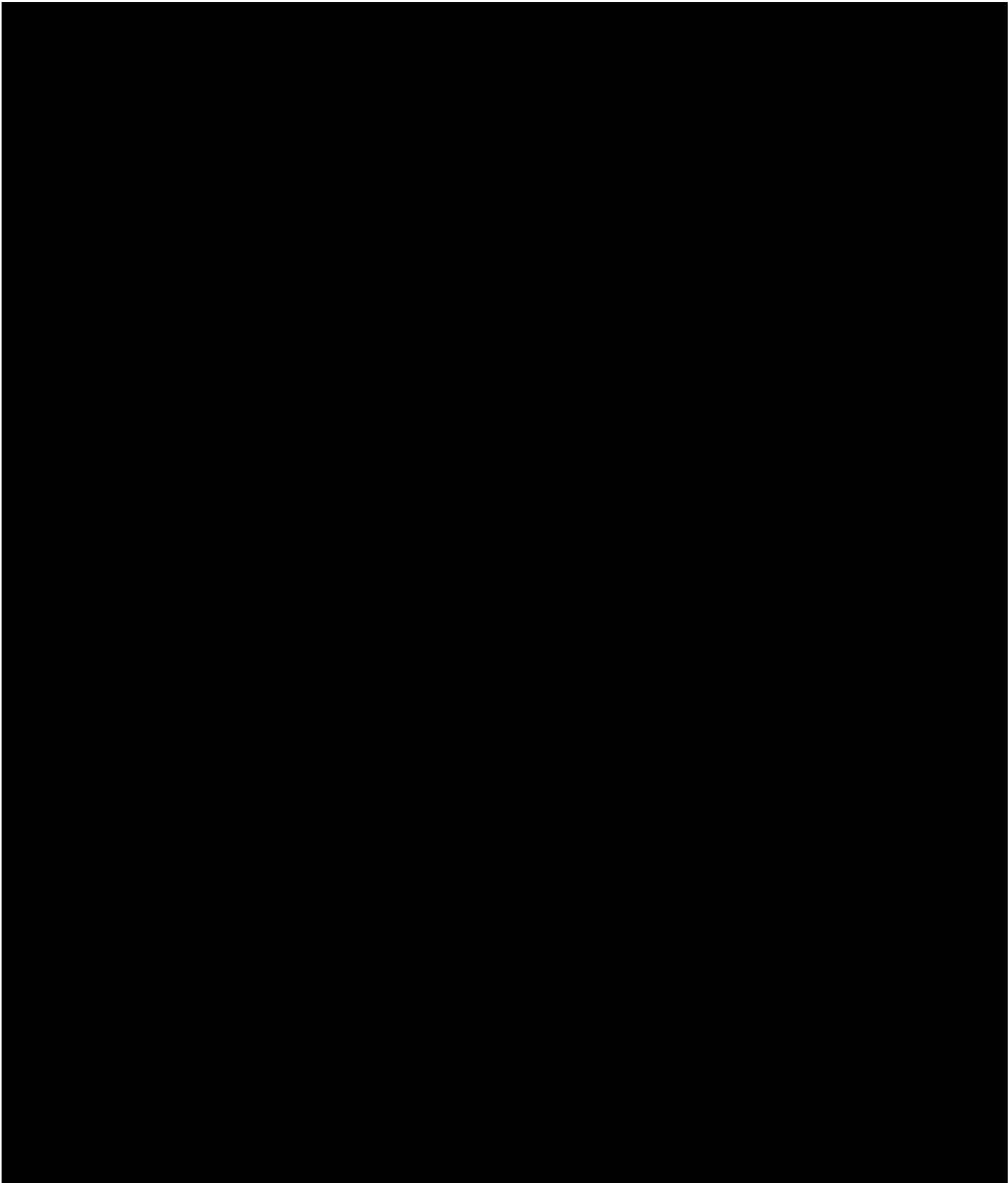


S7.7. Compound 18



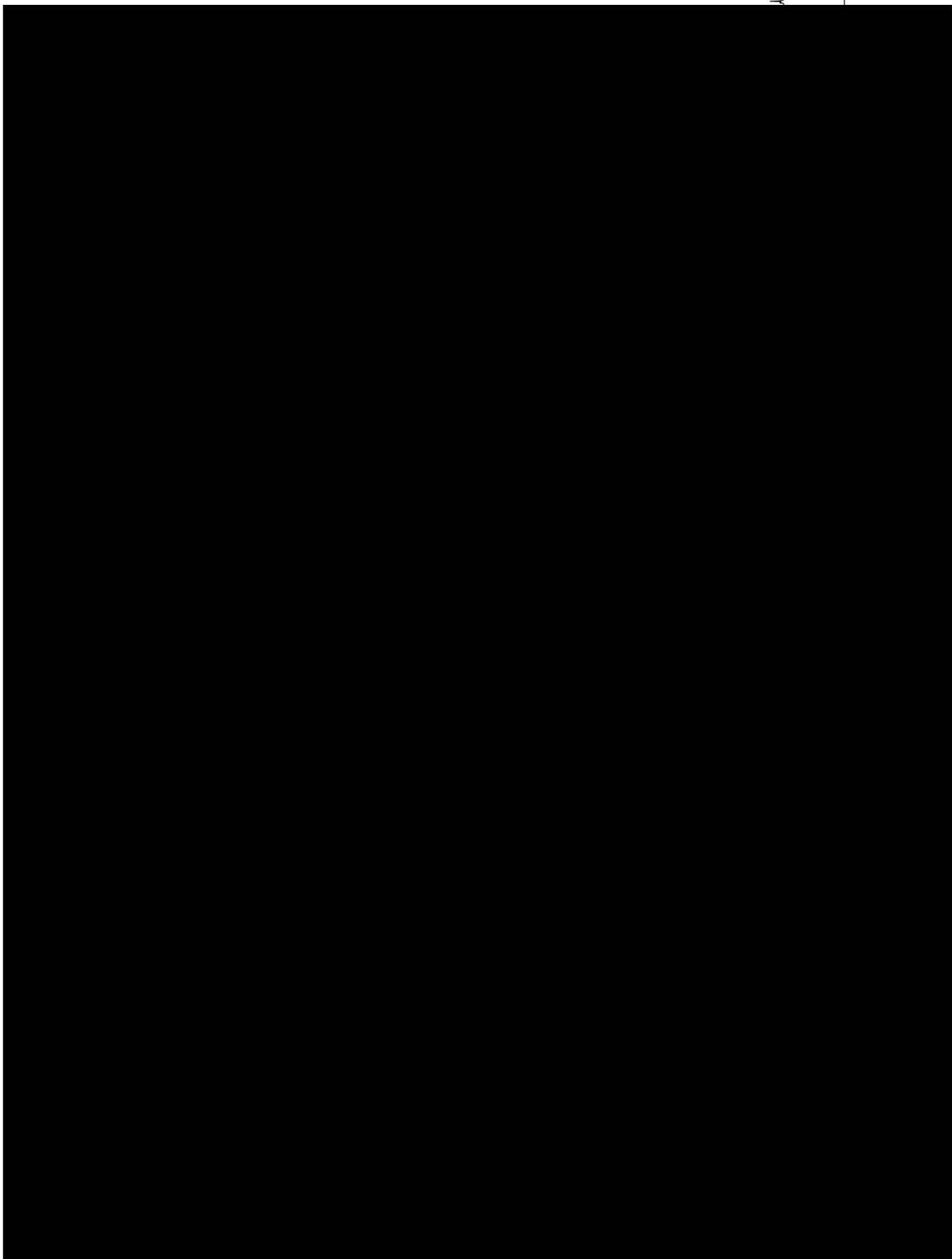
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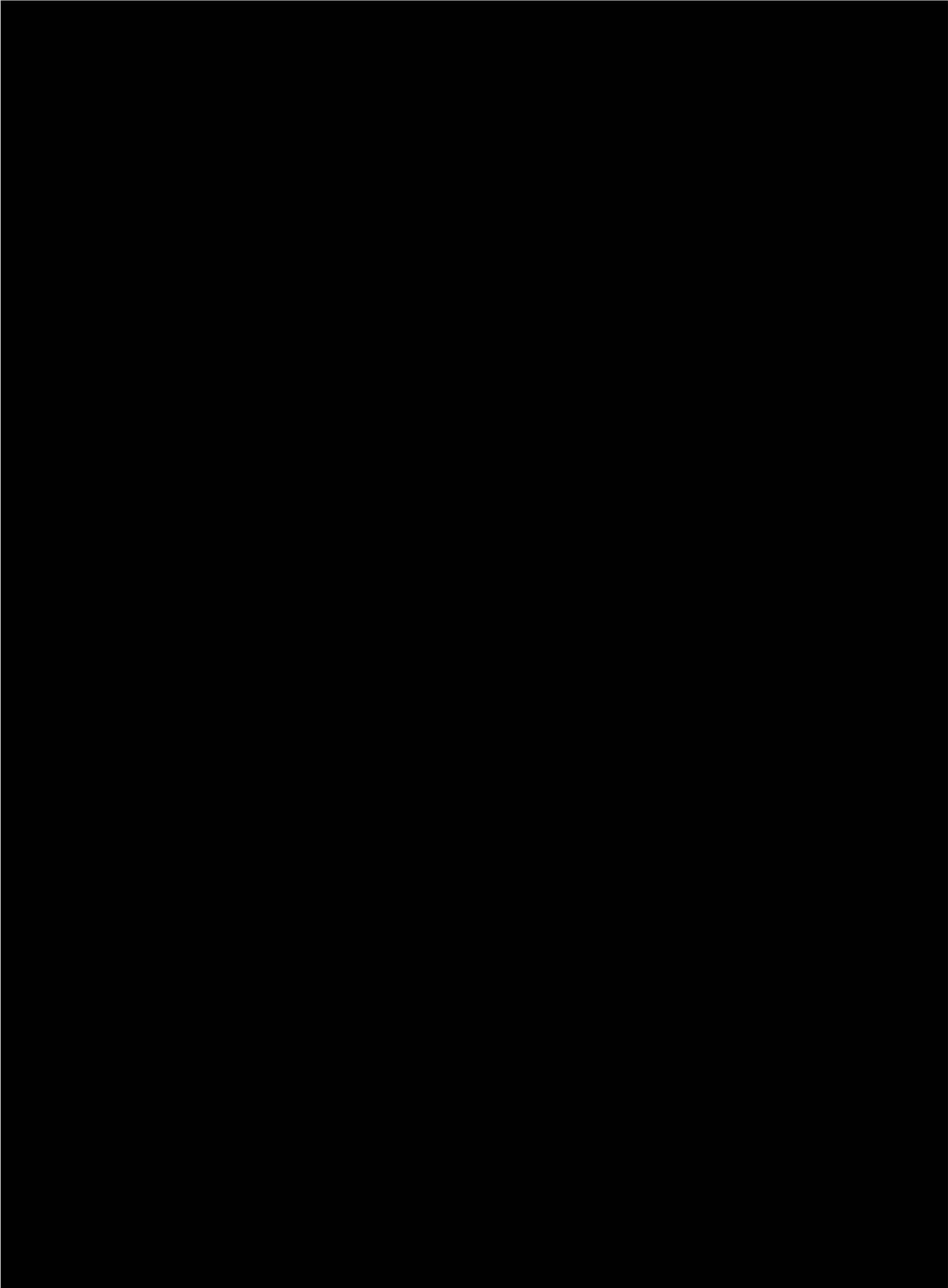
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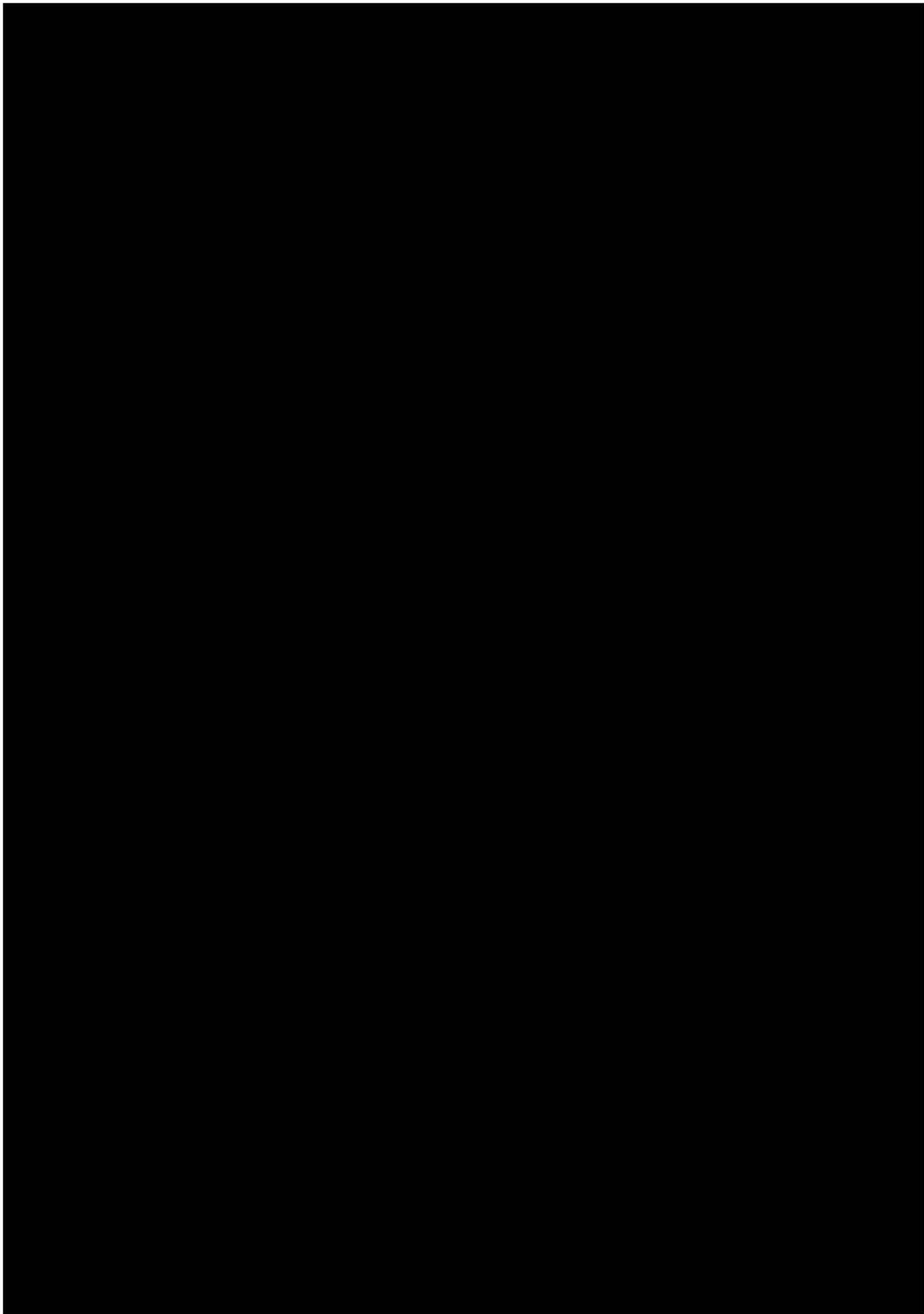
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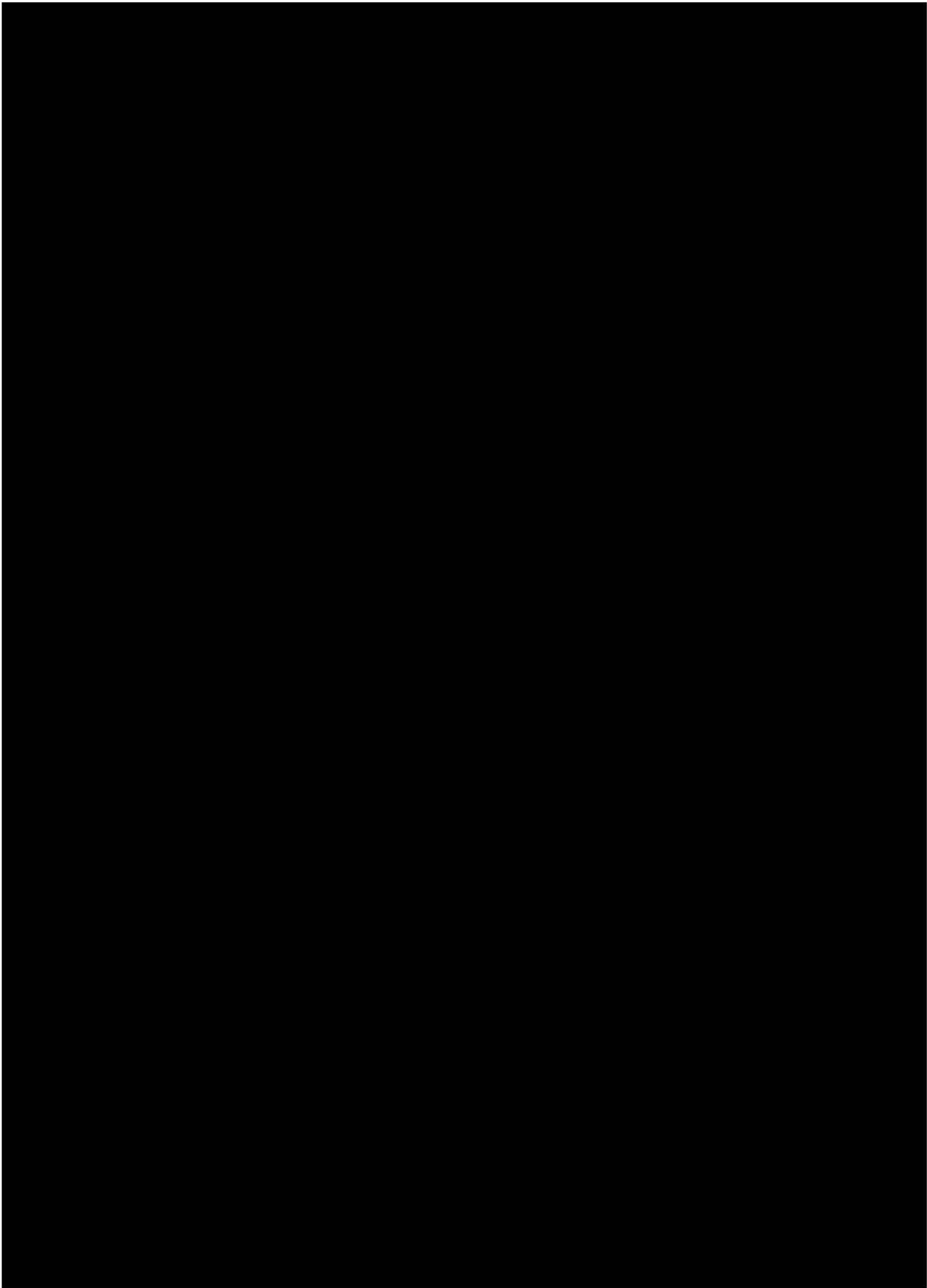






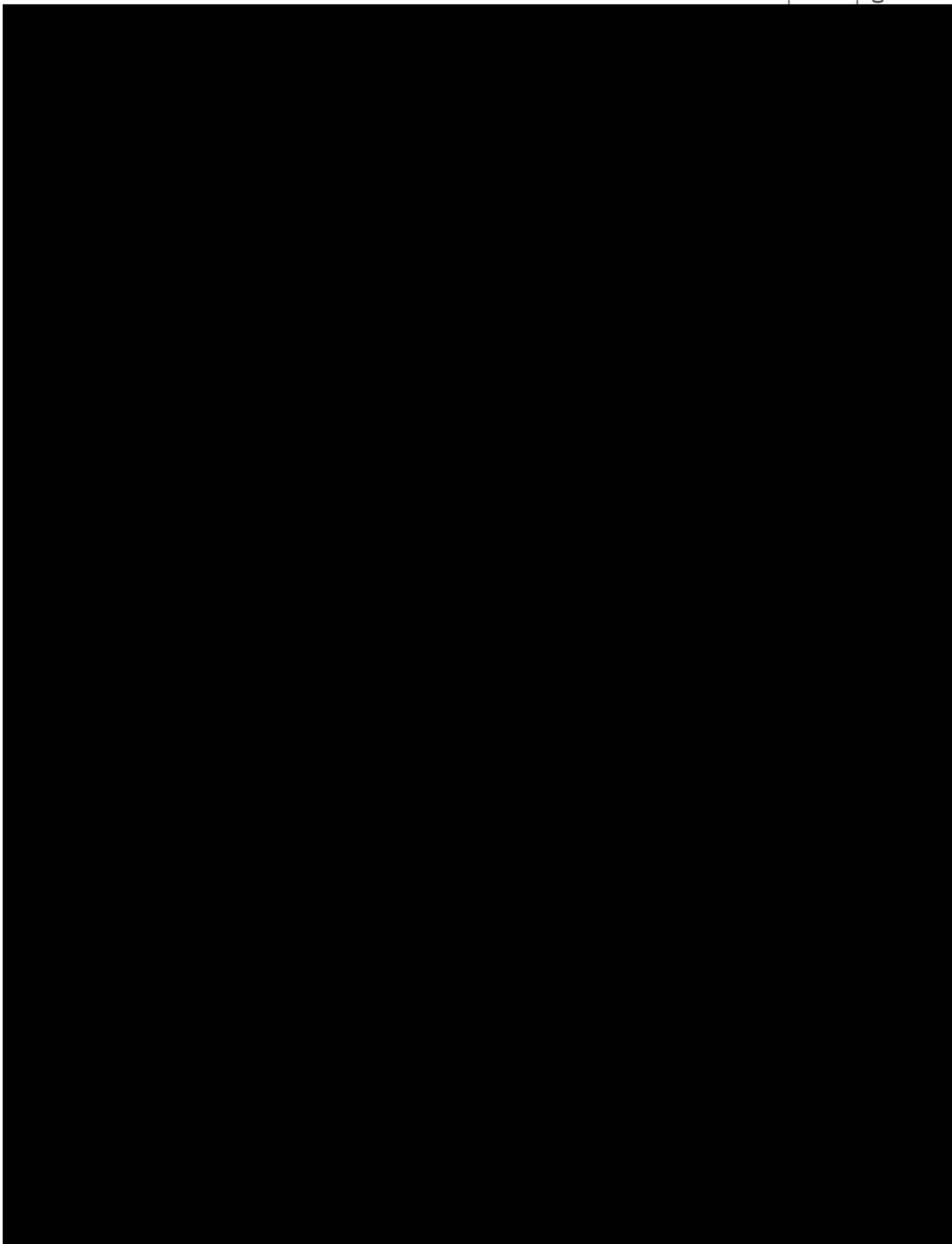


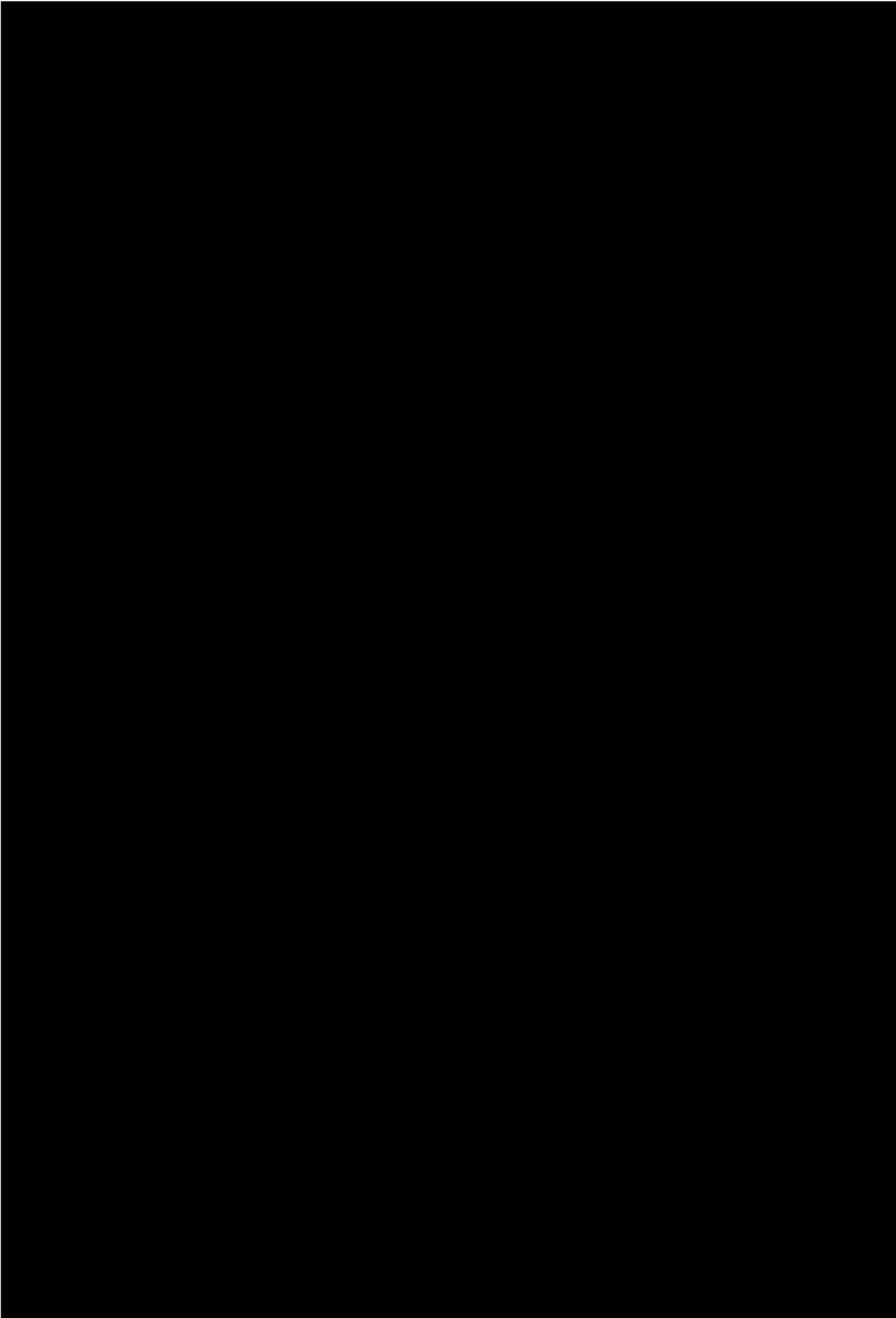




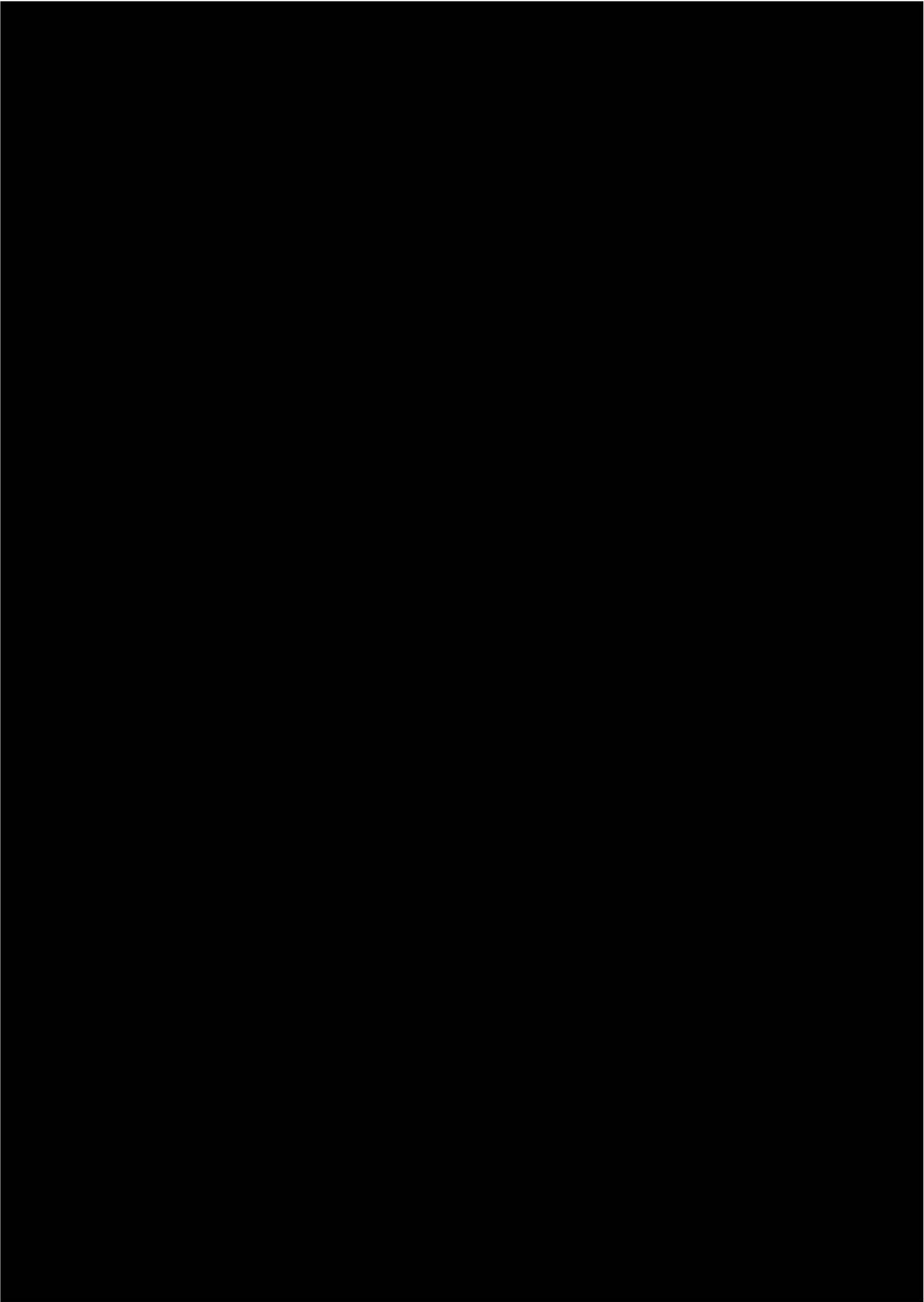
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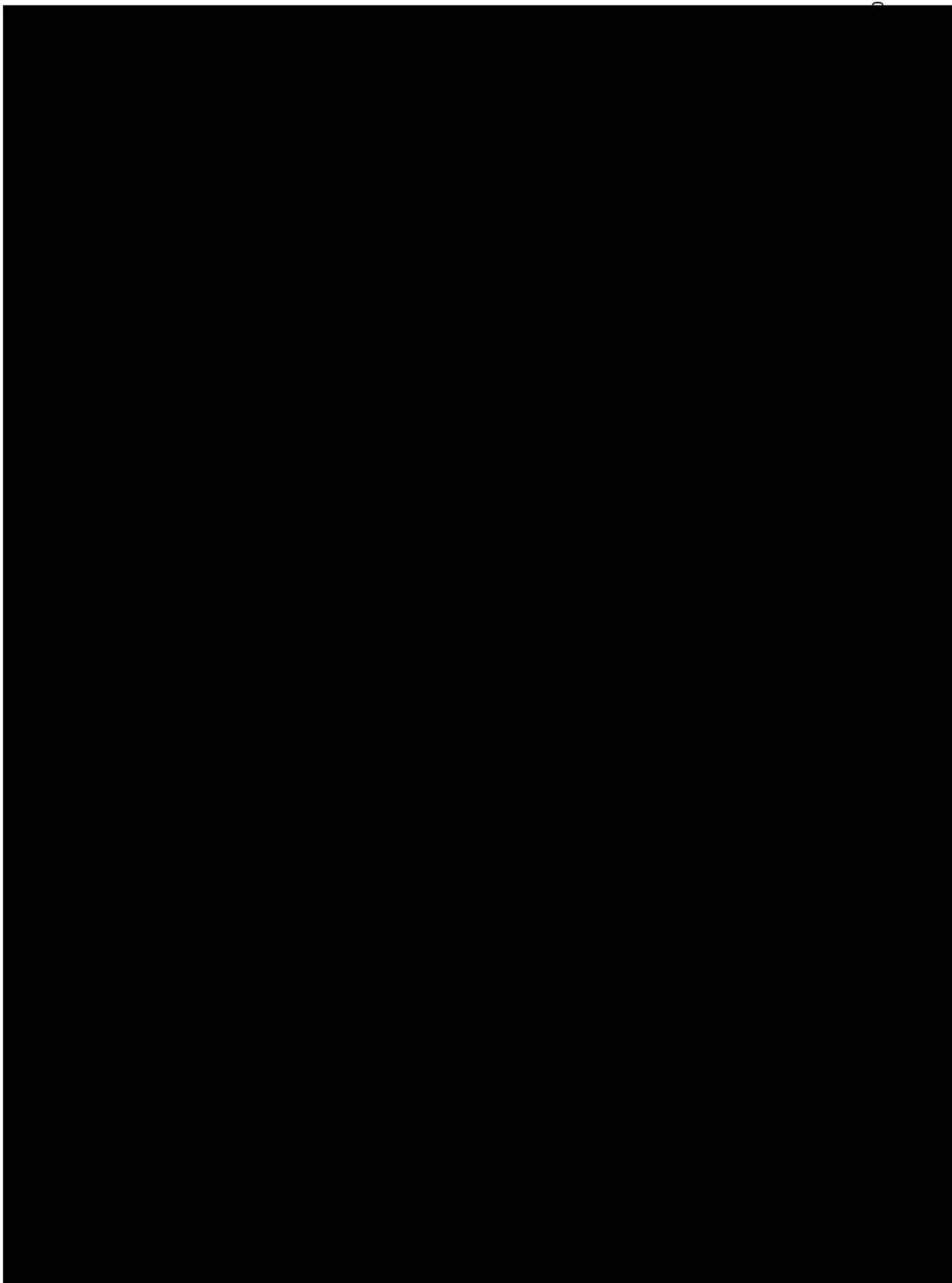








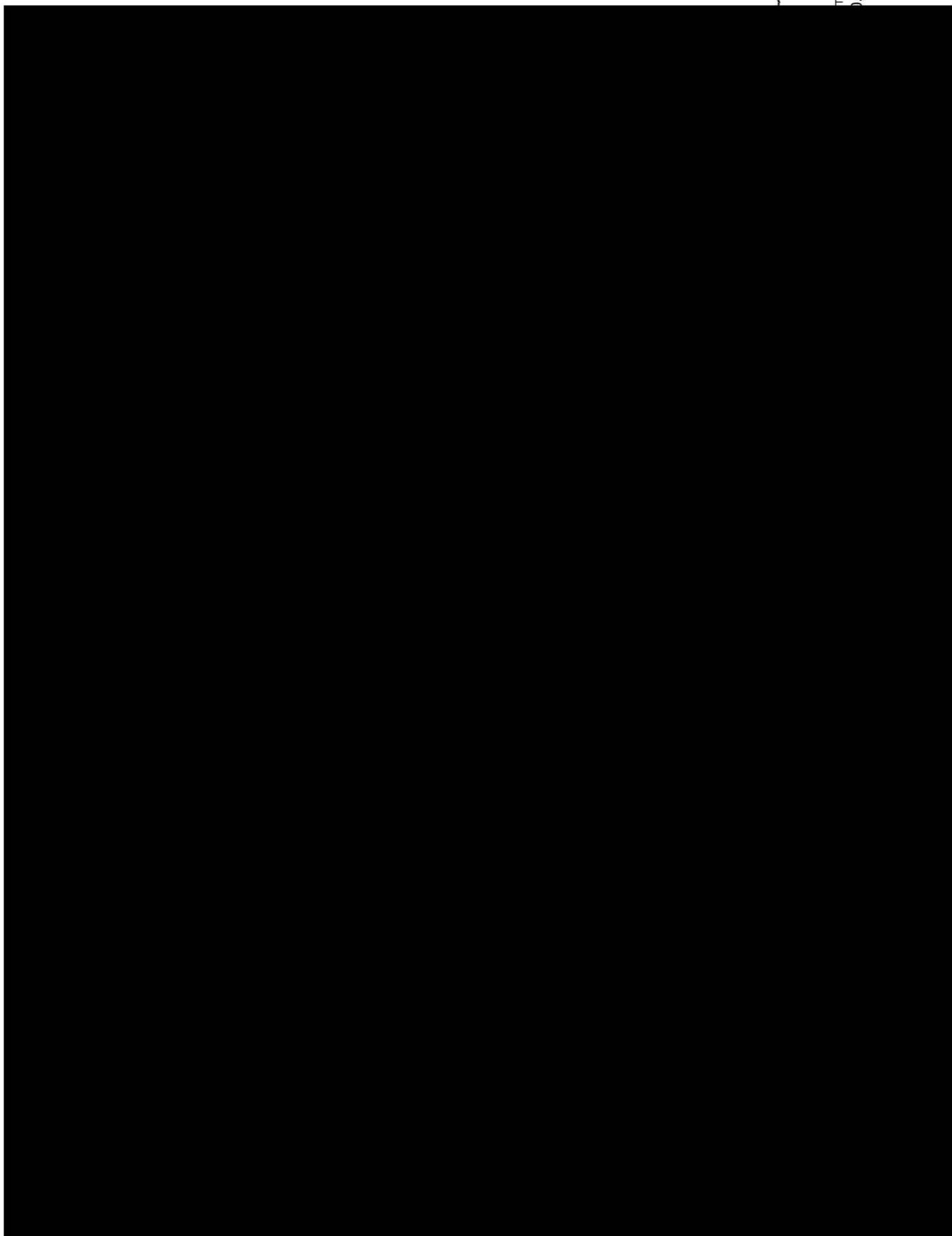
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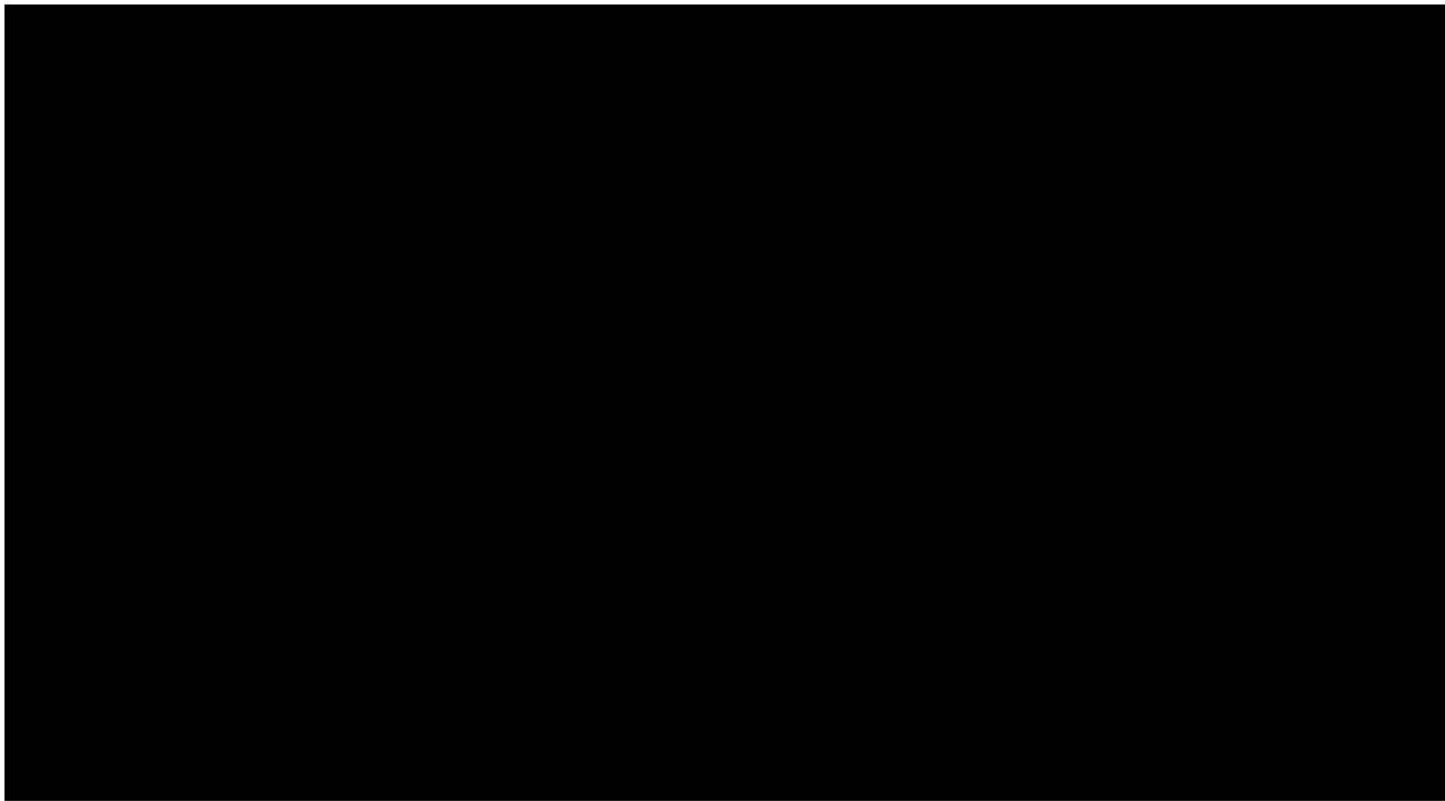
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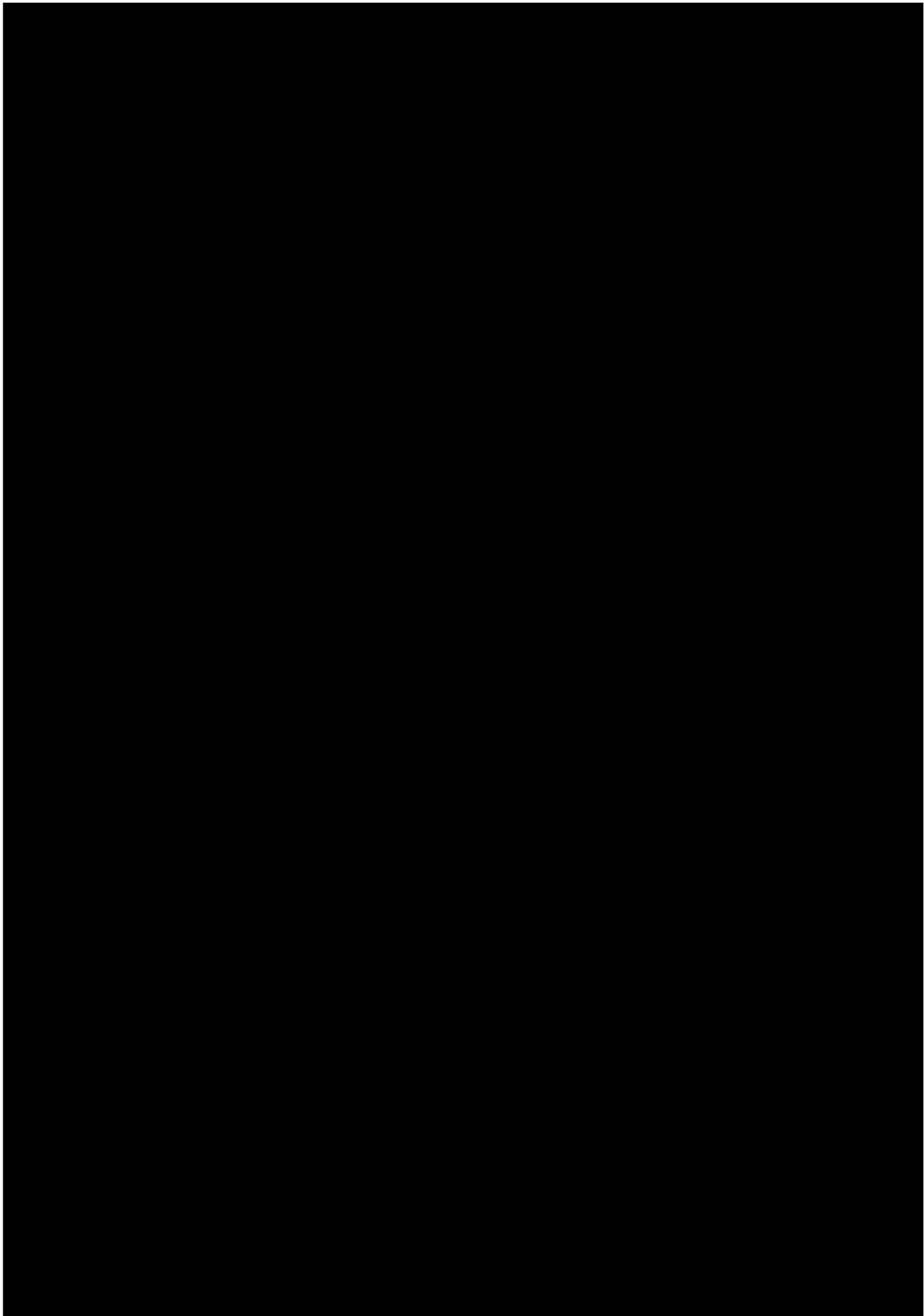


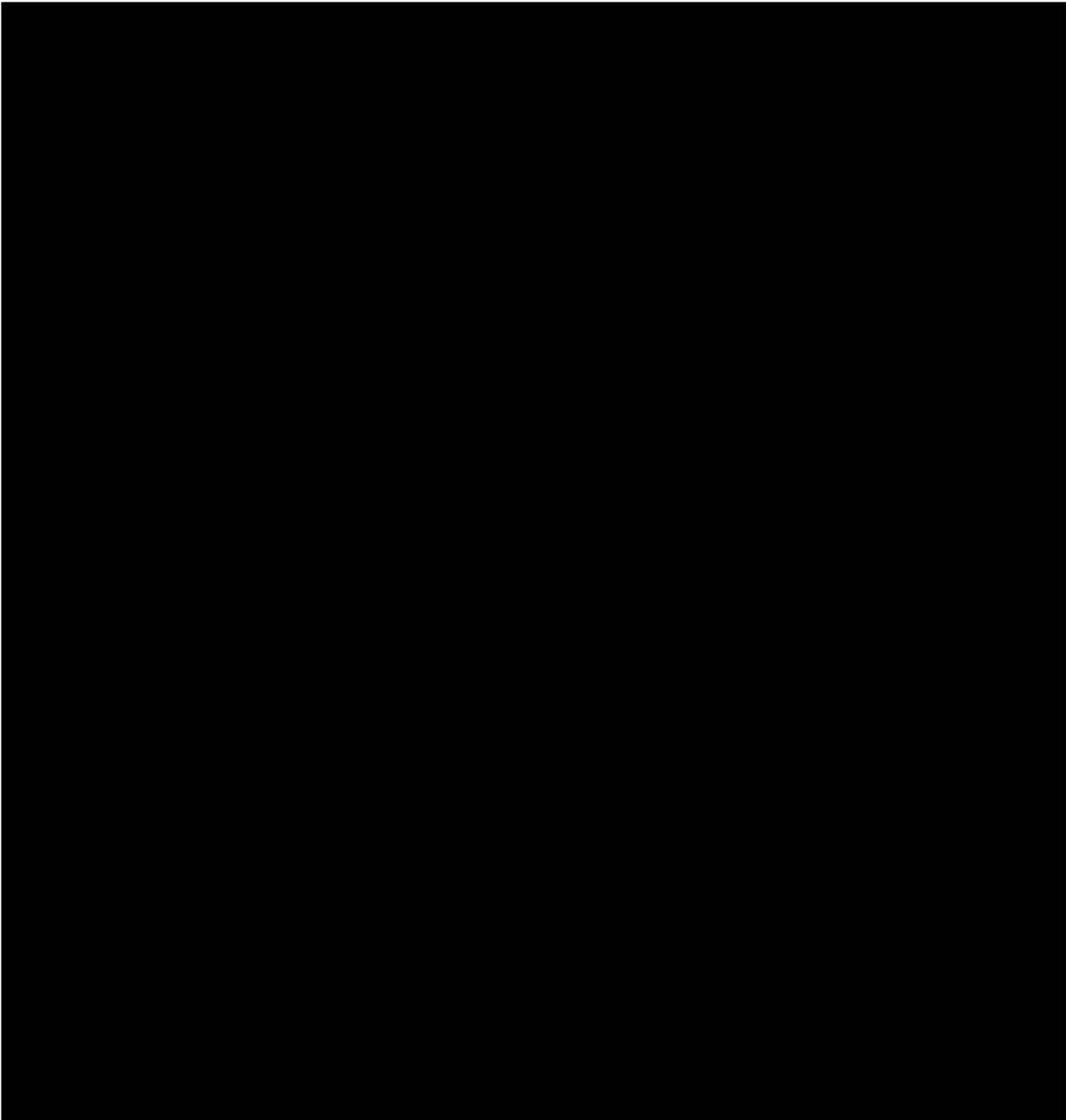


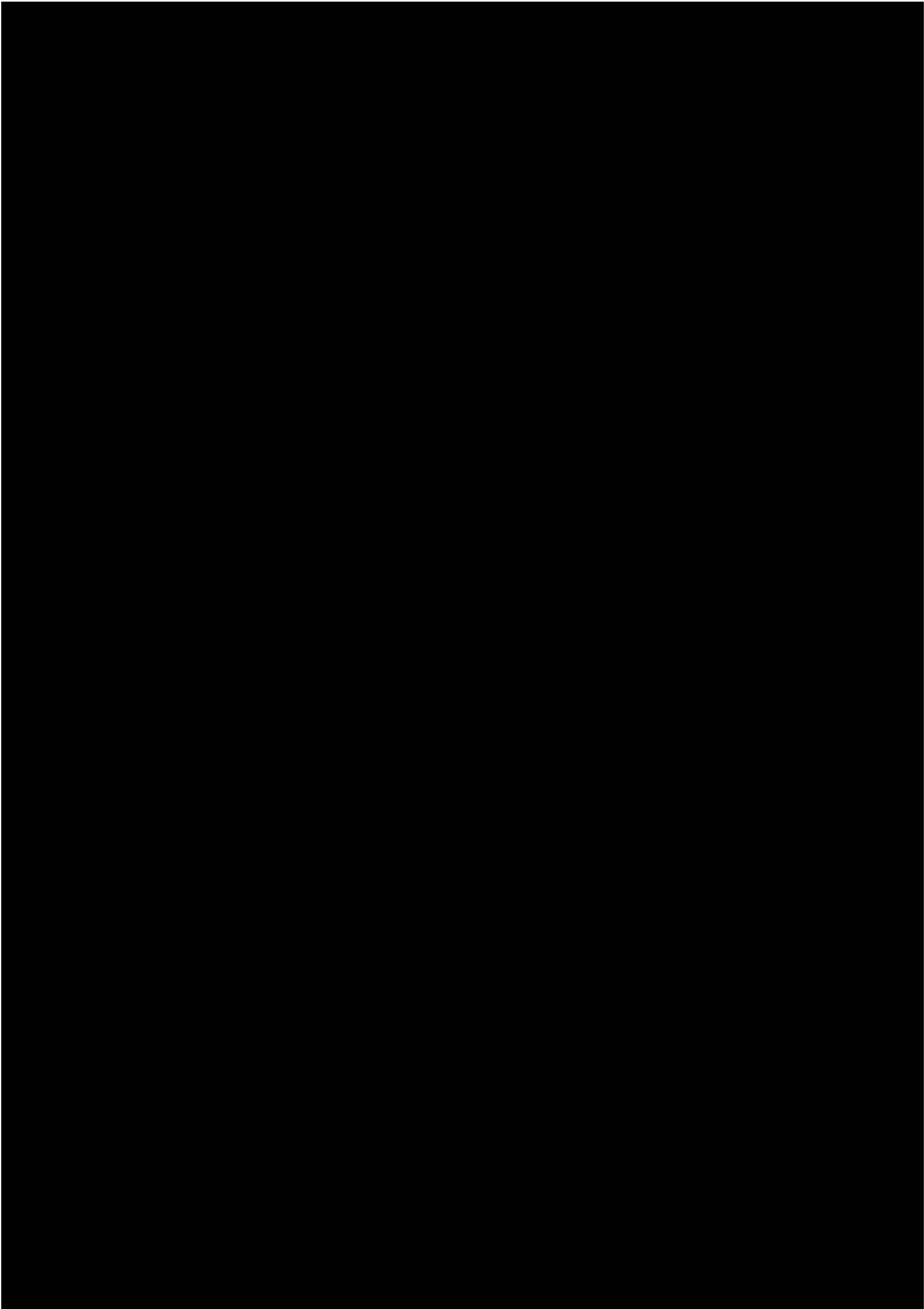


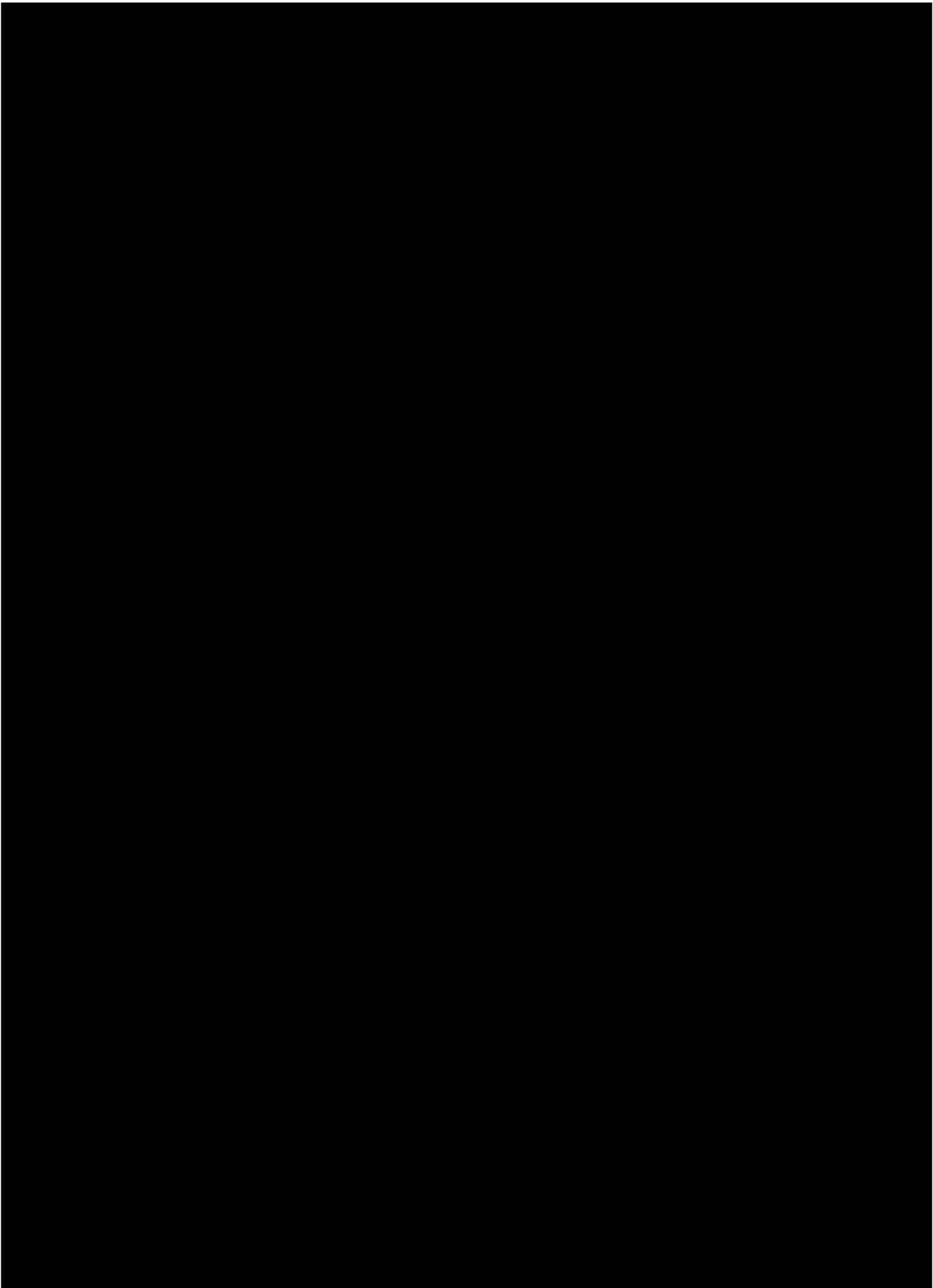















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# Conformational Dynamics of Cyanocobalamin and Its Conjugates with Peptide Nucleic Acids

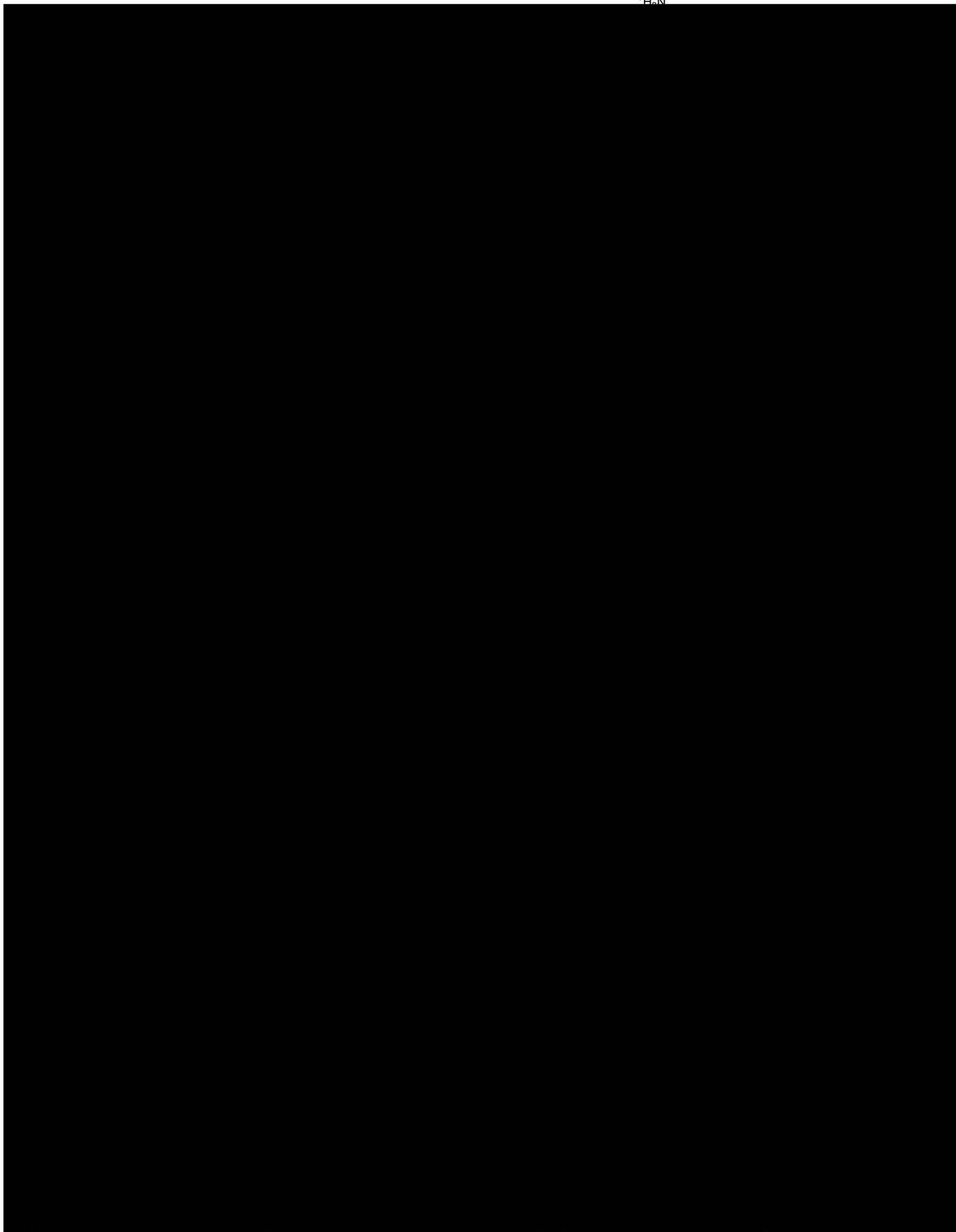
Tomasz Pieńko,<sup>†,‡</sup> Aleksandra J. Wierzbą,<sup>§</sup> Monika Wojciechowska,<sup>†</sup> Dorota Gryko,<sup>\*,§</sup>  
and Joanna Trylska<sup>\*,†</sup> 

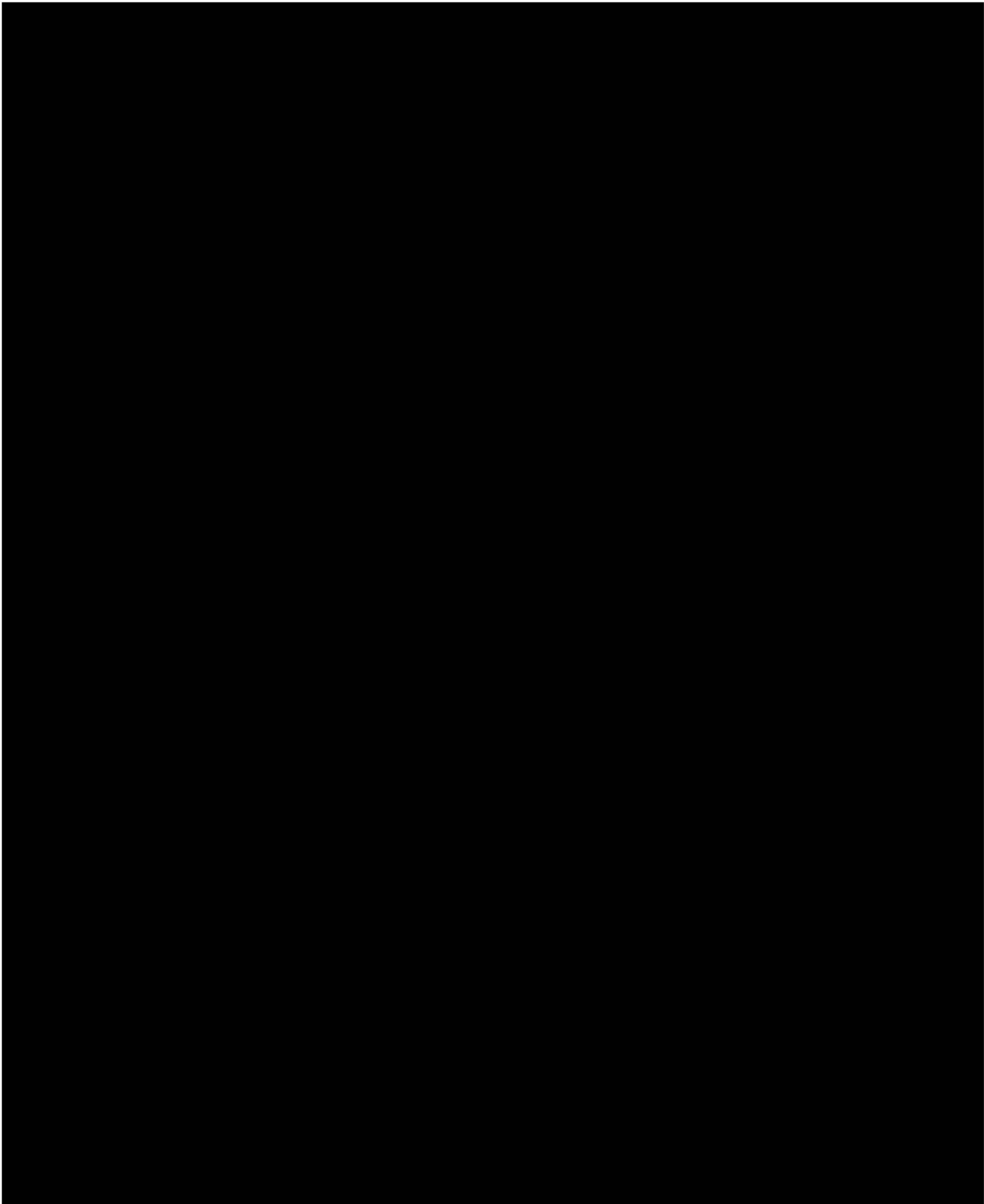
<sup>†</sup>Centre of New Technologies, University of Warsaw, S. Banacha 2c, 02-097 Warsaw, Poland

<sup>‡</sup>Department of Drug Chemistry, Faculty of Pharmacy with the Laboratory Medicine Division, Medical University of Warsaw, S. Banacha 1a, 02-097 Warsaw, Poland

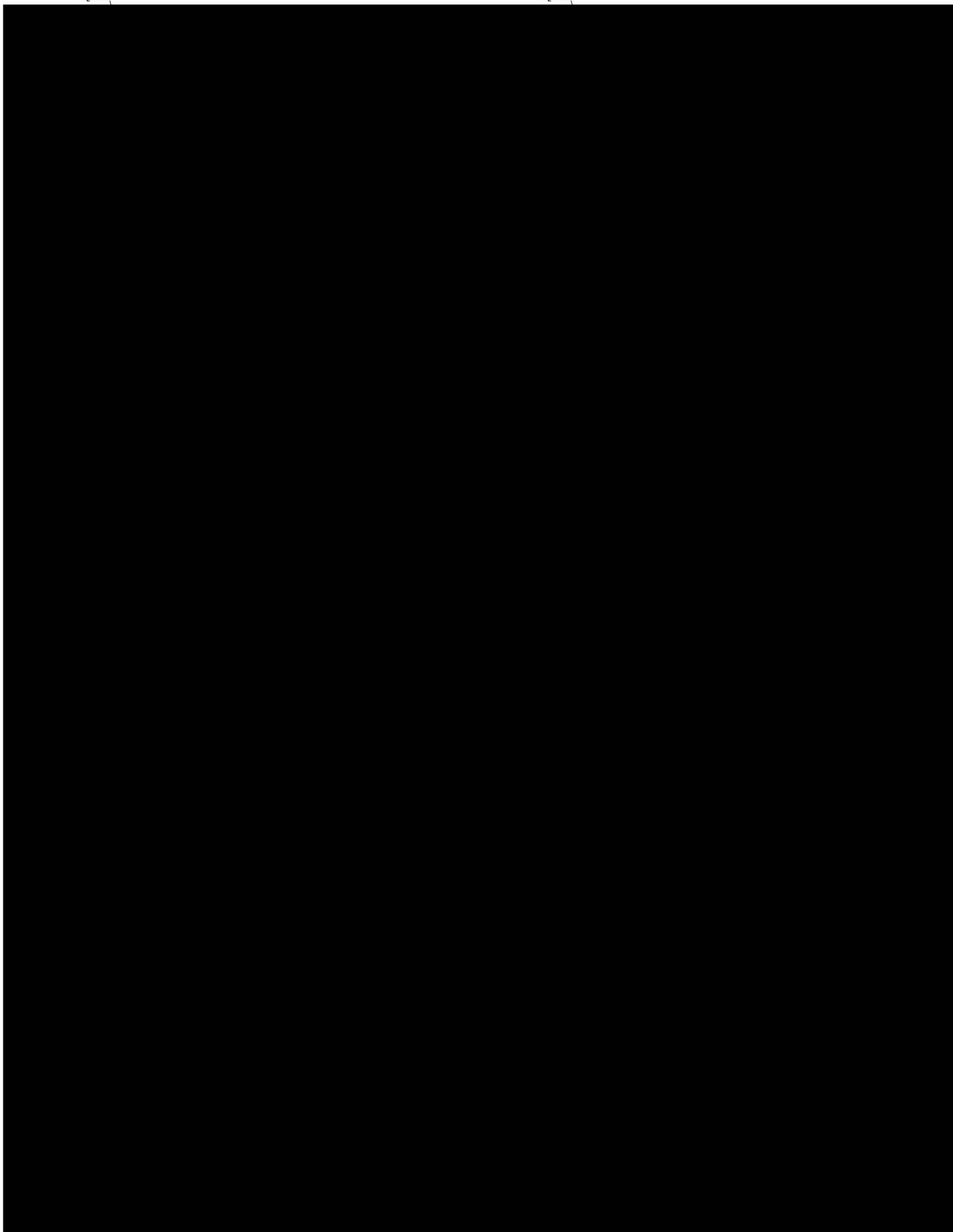
<sup>§</sup>Institute of Organic Chemistry, Polish Academy of Sciences, M. Kasprzaka 44/52, 01-224 Warsaw, Poland

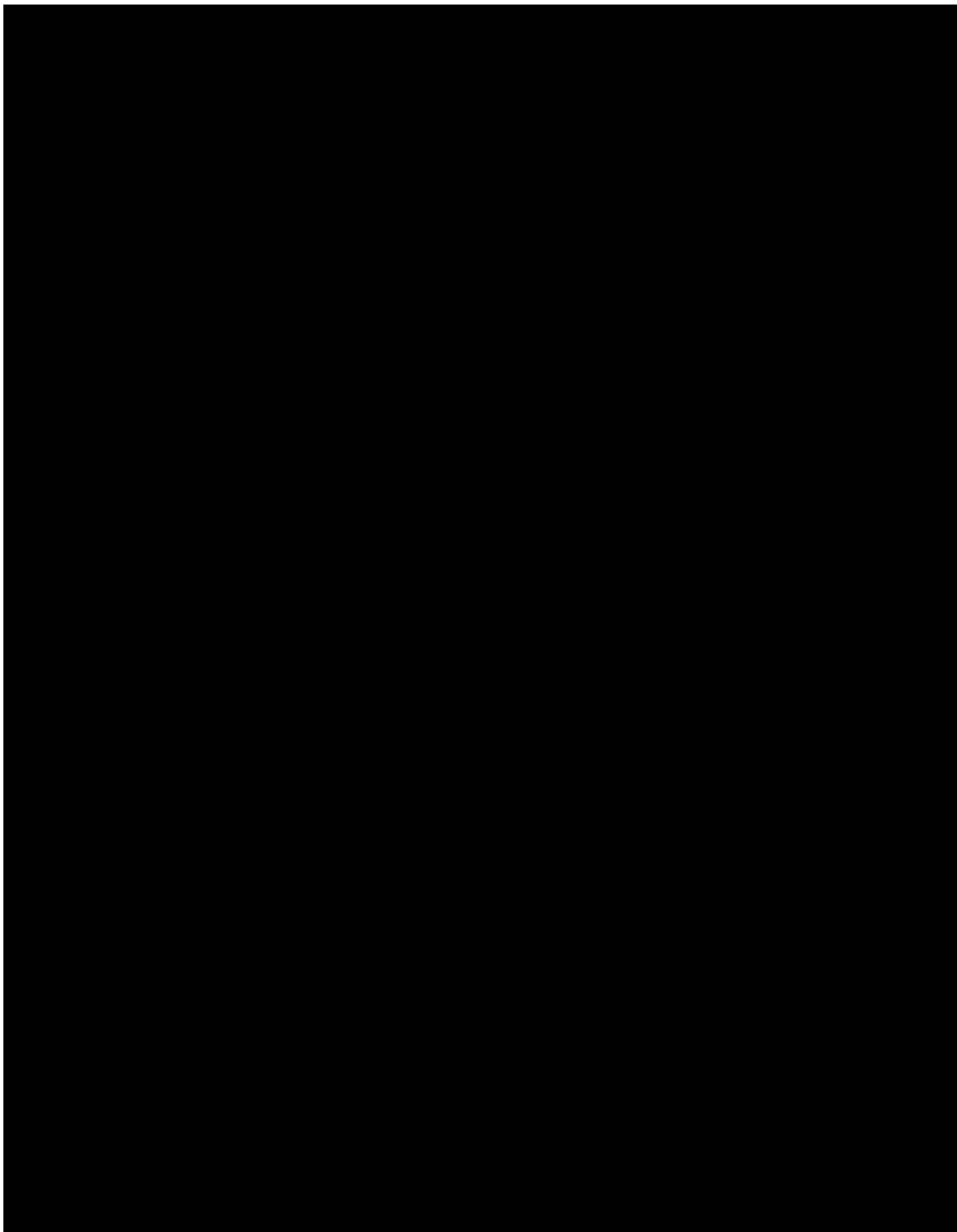
<sup>+</sup>H<sub>2</sub>N

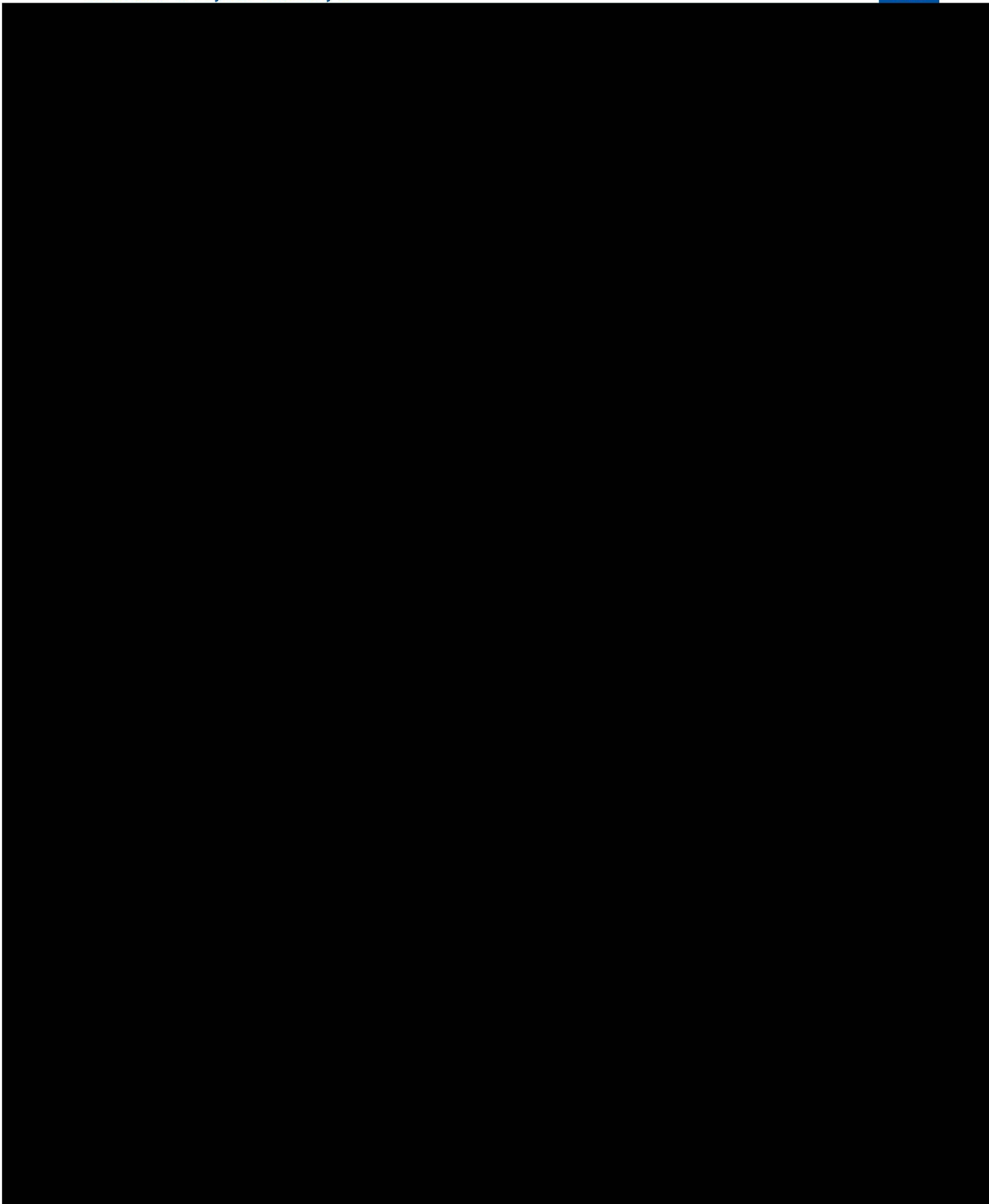


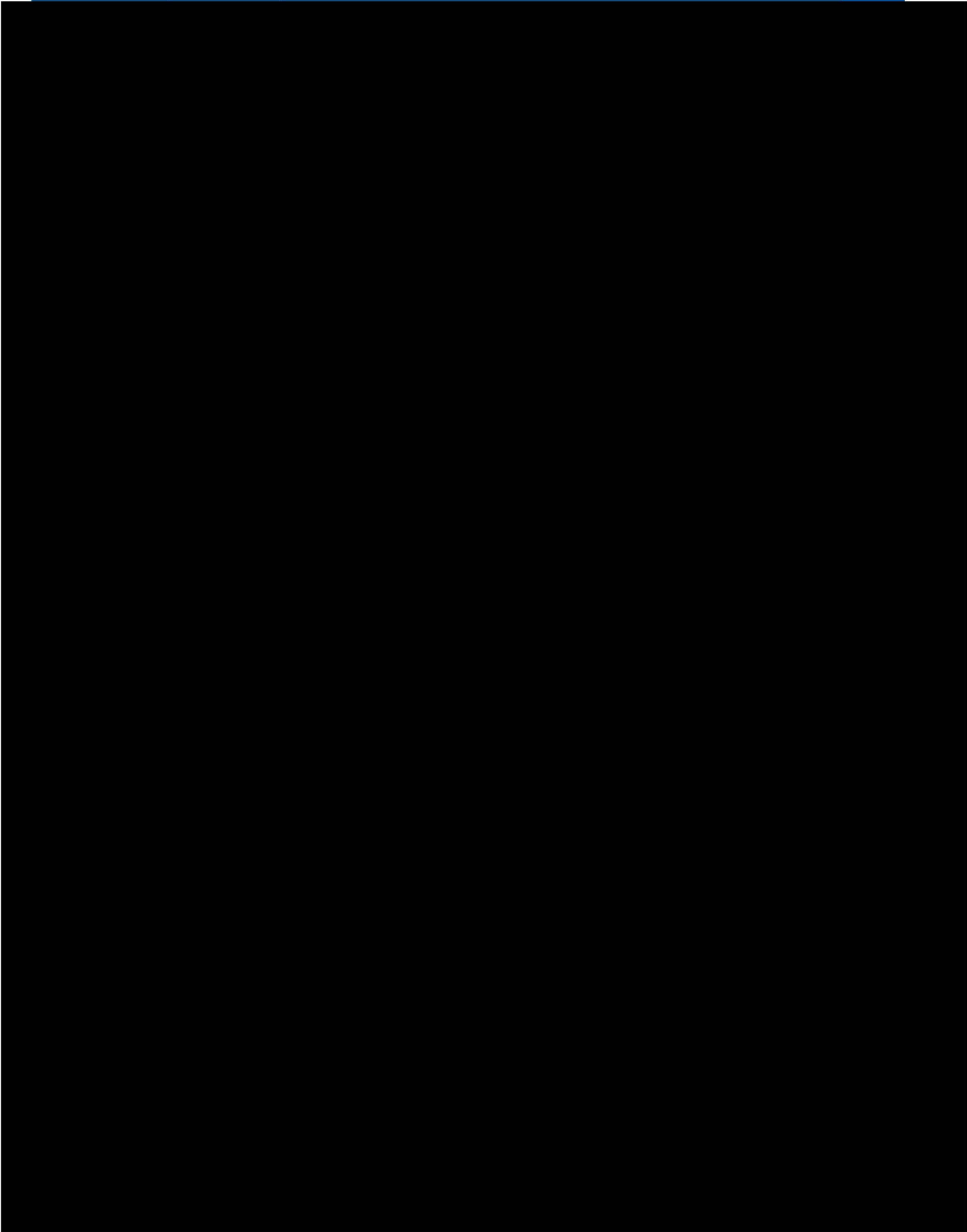


## Scheme 2. Synthesis of Conjugate 7 via 1,3-Dipolar Cycloaddition

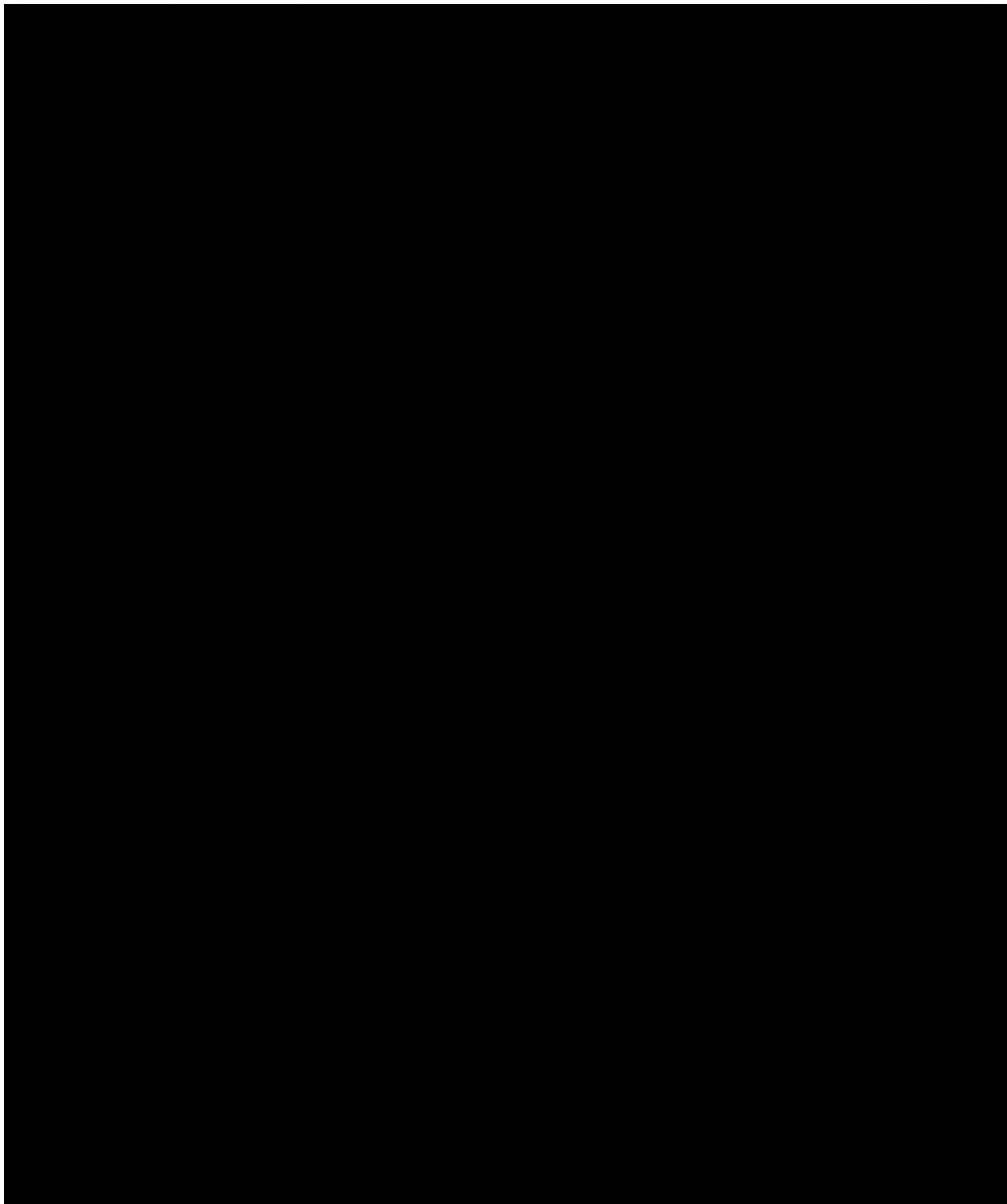
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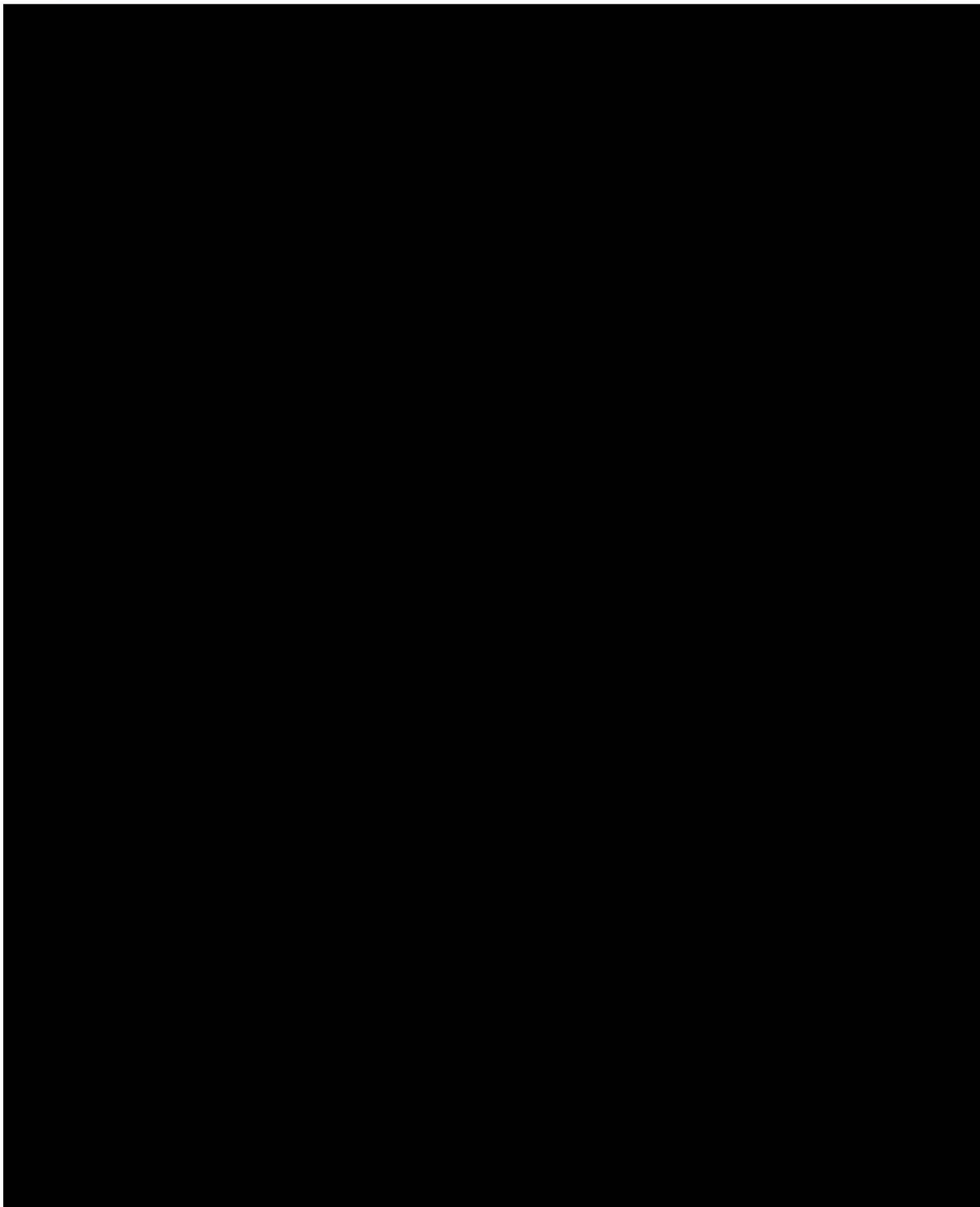


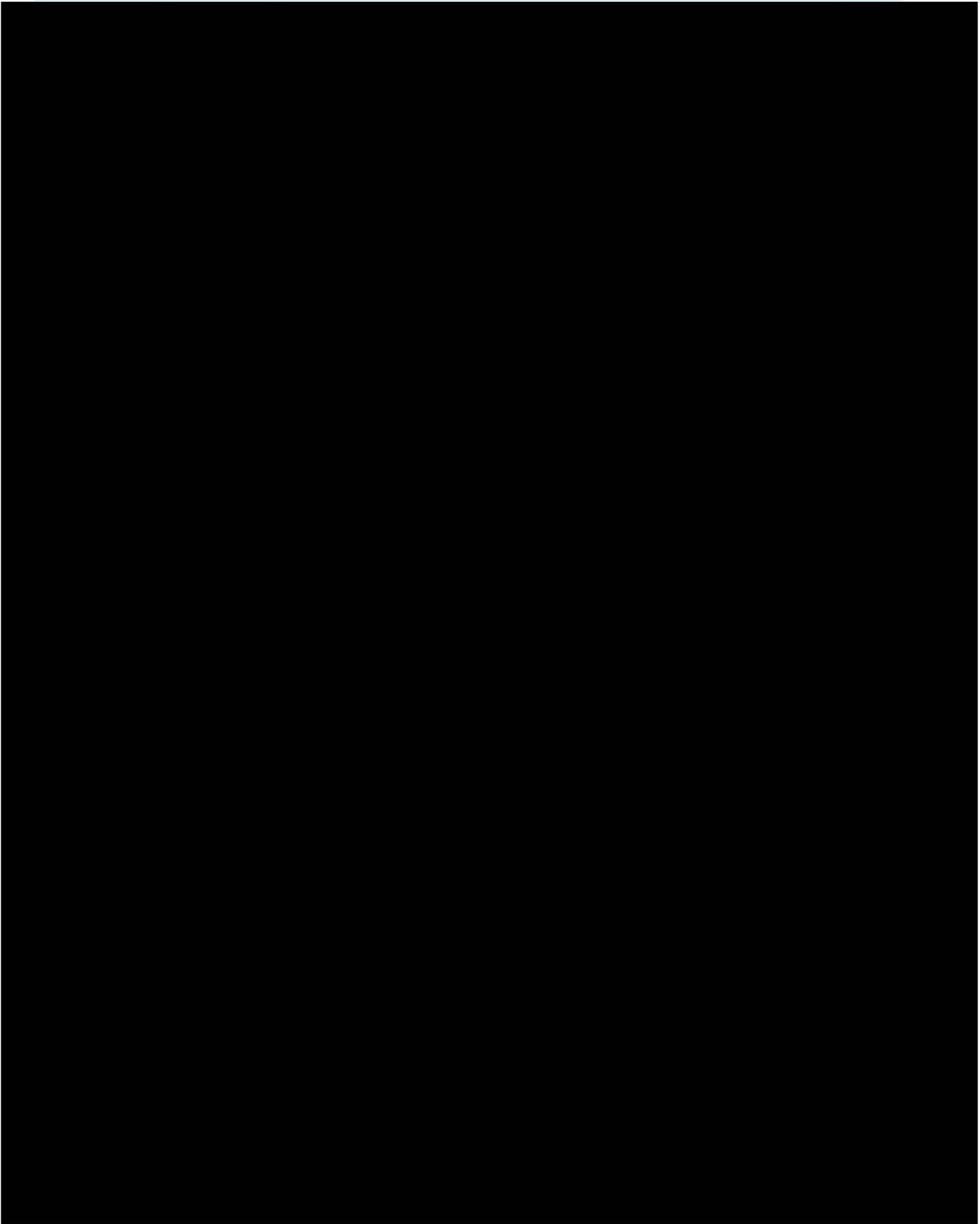


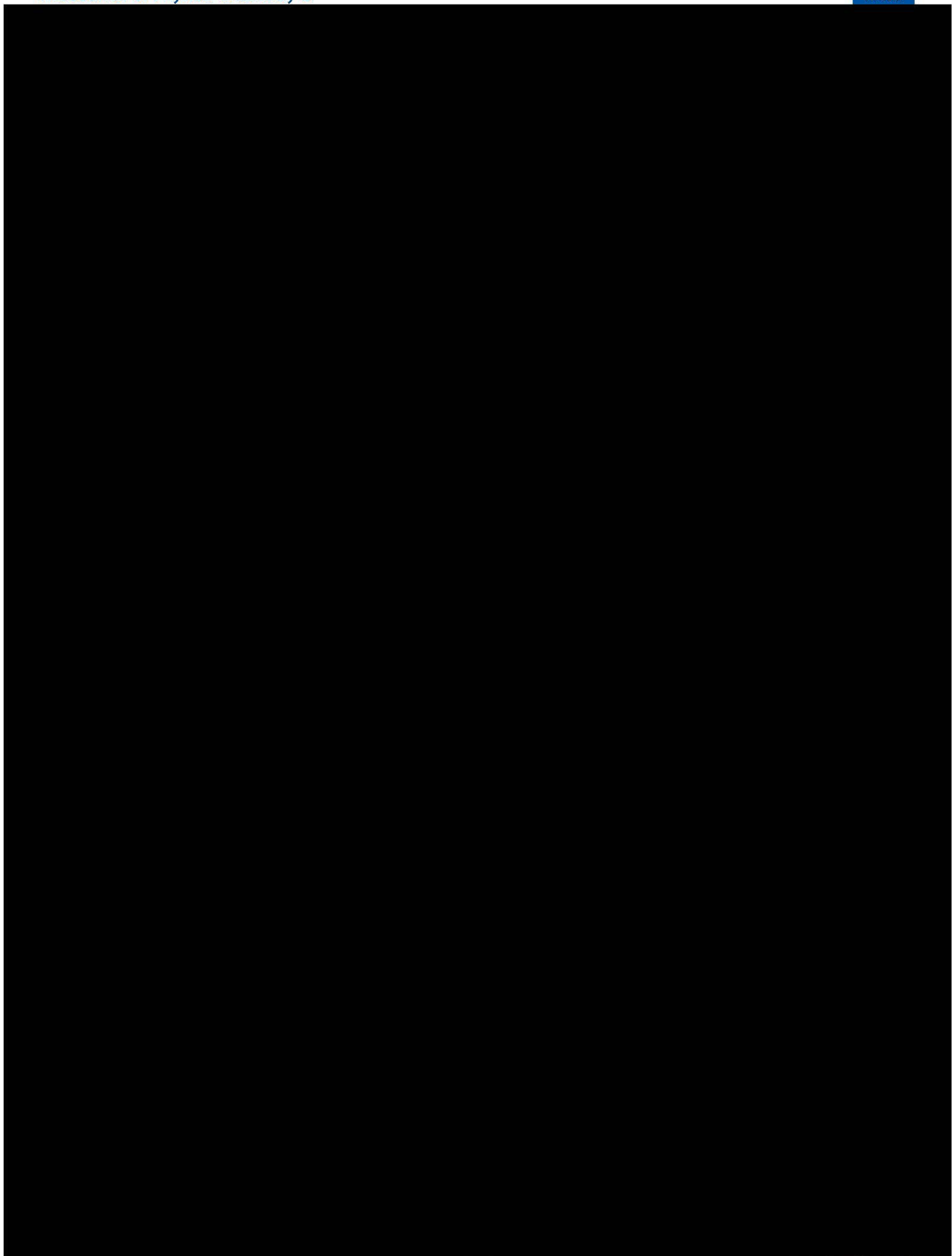
GC > CC > AA in Cbl-PNA. This order suggests that rather the relative position of the base in the PNA sequence not its type determines if the bases stack. The lowest stacking occurrence is observed for AA, CC, and GC; in the simulated PNA sequence (CATCTAGTATTCT) these bases are not close in the sequence. In contrast, close positioning of the C, A and T bases

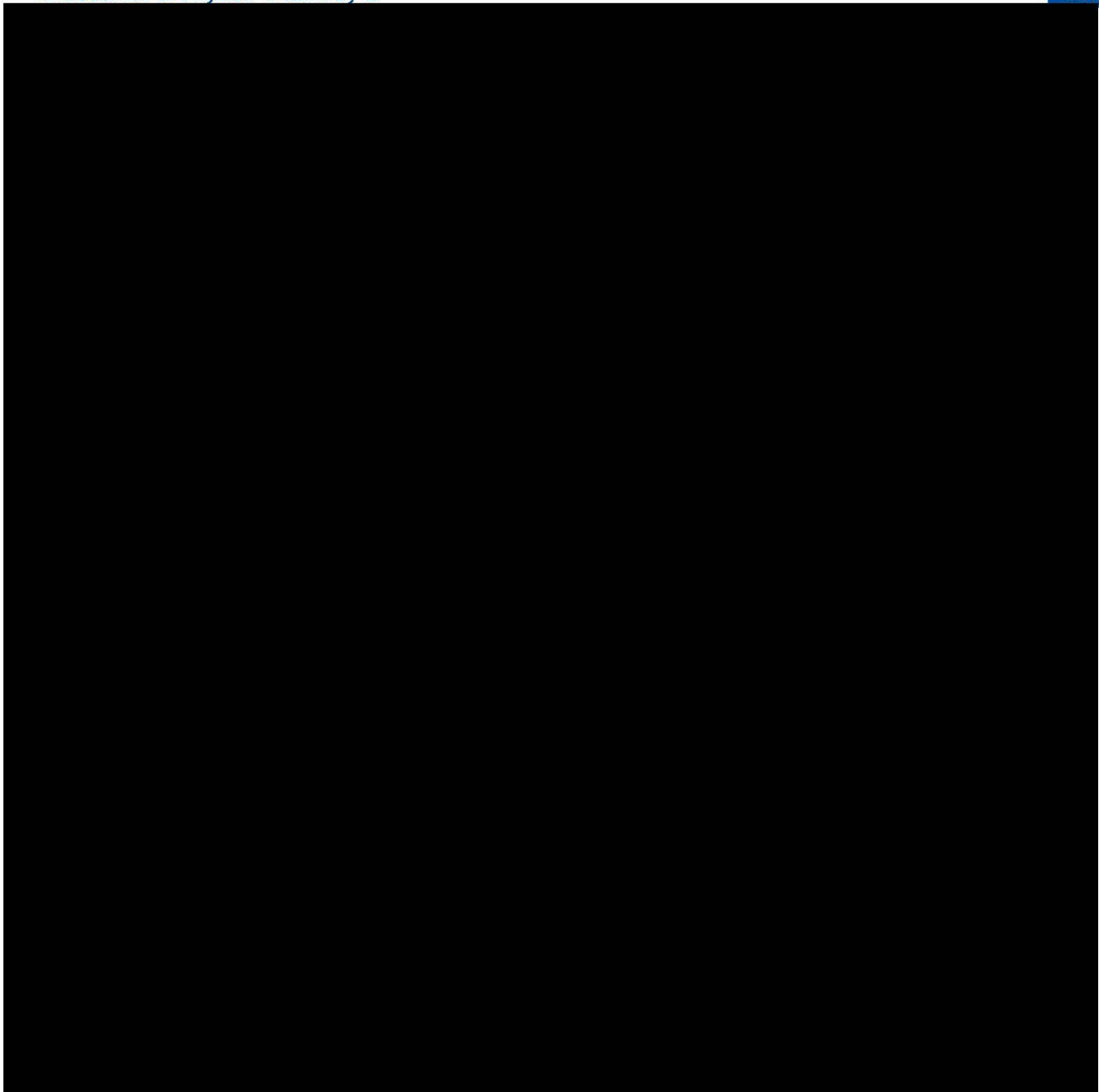
in the PNA sequence increases the occurrence of TT, AT, and CT pairs in accord with Figure 11.

We also examined intramolecular hydrogen bonds. The maximal time that a single hydrogen bond is present does not exceed 24% for Cbl-PNA and 20% for free PNA. However, the total number of detected intramolecular hydrogen bonds in all trajectories within PNA is 1209 in Cbl-PNA and 1573 in free









## Supporting Information

### Conformational Dynamics of Cyanocobalamin and Its Conjugates with Peptide Nucleic Acids

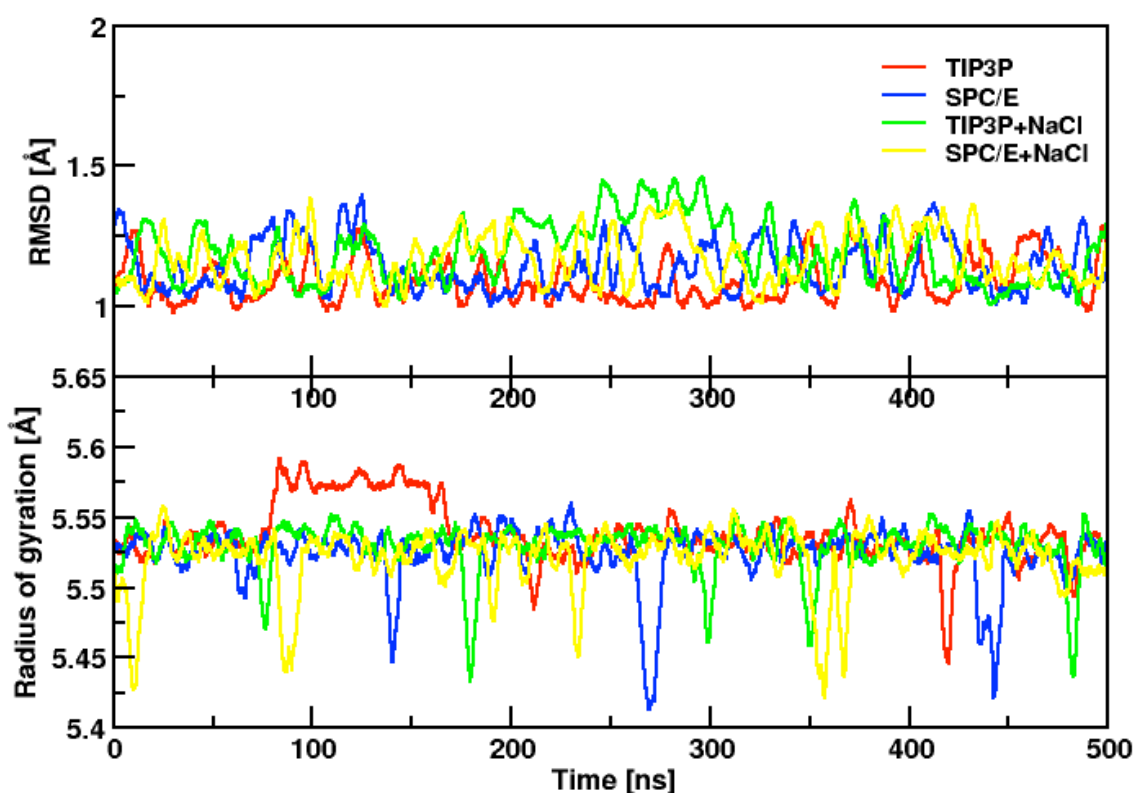
Tomasz Pieńko,<sup>†,‡</sup> Aleksandra J. Wierzbą,<sup>¶</sup> Monika Wojciechowska,<sup>†</sup>  
Dorota Gryko,<sup>¶</sup> Joanna Trylska<sup>†</sup>

<sup>†</sup>Centre of New Technologies, University of Warsaw, S. Banacha 2c, 02-097 Warsaw, Poland,

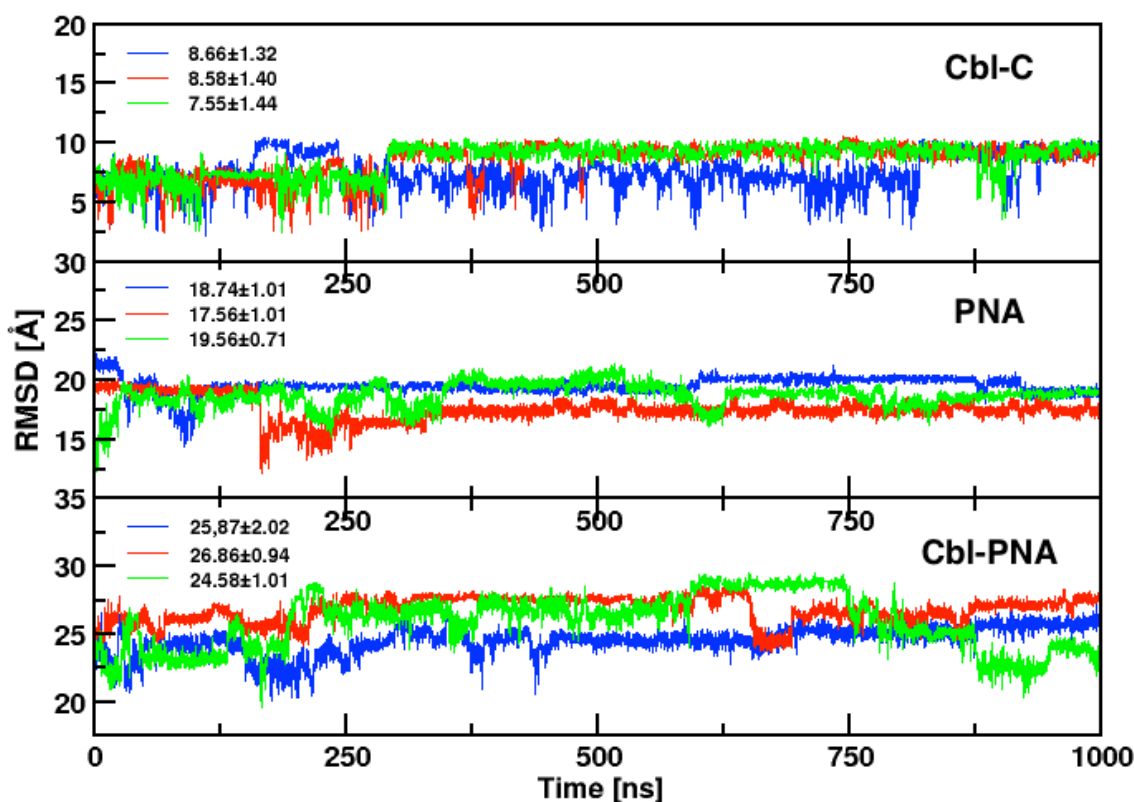
<sup>‡</sup>Department of Drug Chemistry, Faculty of Pharmacy with the Laboratory Medicine Division, Medical University of Warsaw, S. Banacha 1a 02-097 Warsaw, Poland,

<sup>¶</sup>Institute of Organic Chemistry, Polish Academy of Sciences, M. Kasprzaka 44/52, 01-224 Warsaw, Poland

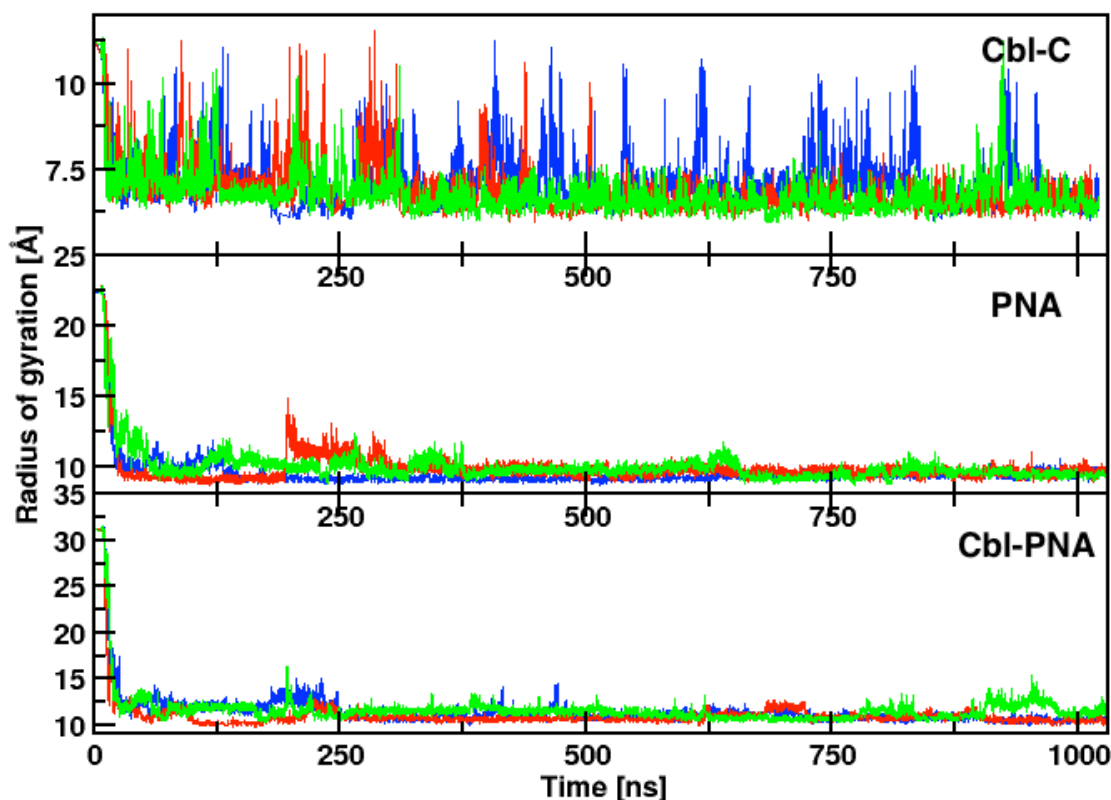
e-mail: joanna@cent.uw.edu.pl, dorota.gryko@icho.edu.pl



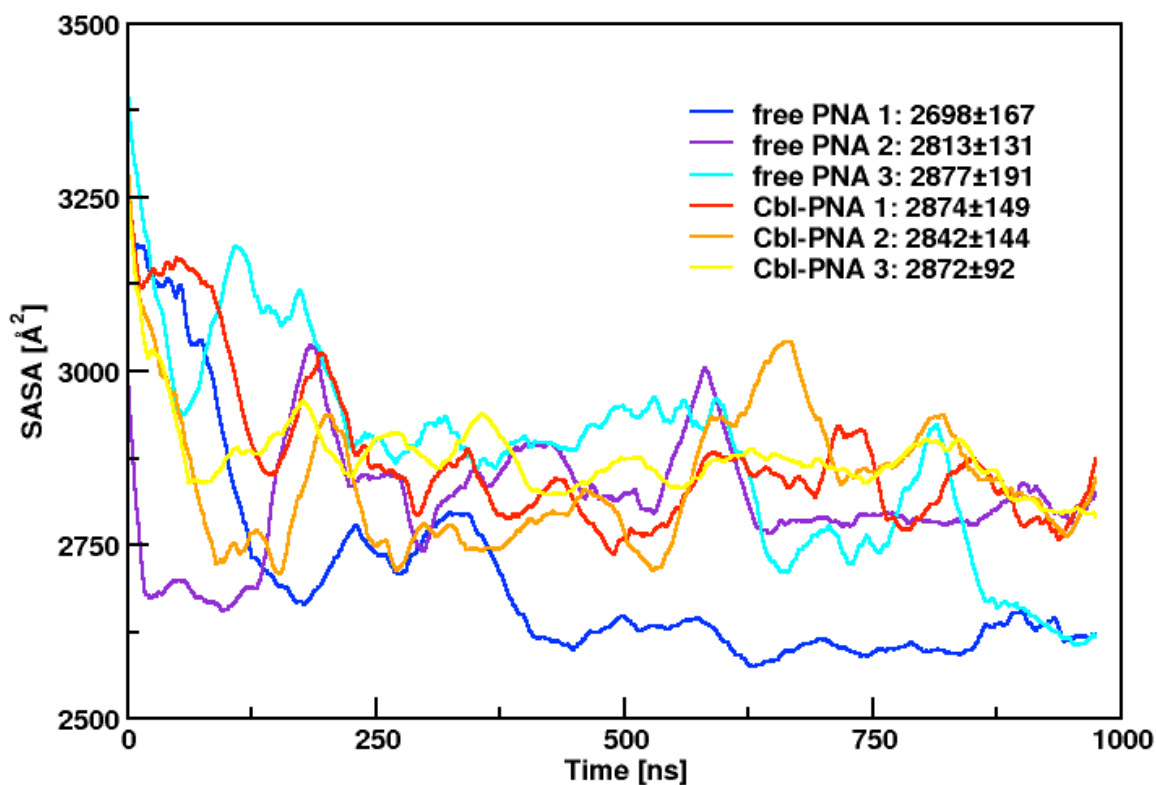
**Figure S1.** RMSD and radius of gyration of heavy atoms of Cbl calculated with respect to the crystal structure in molecular dynamics production simulations in different water models and with or without 100 mM NaCl. Data are shown as running averages computed over 1000 points.



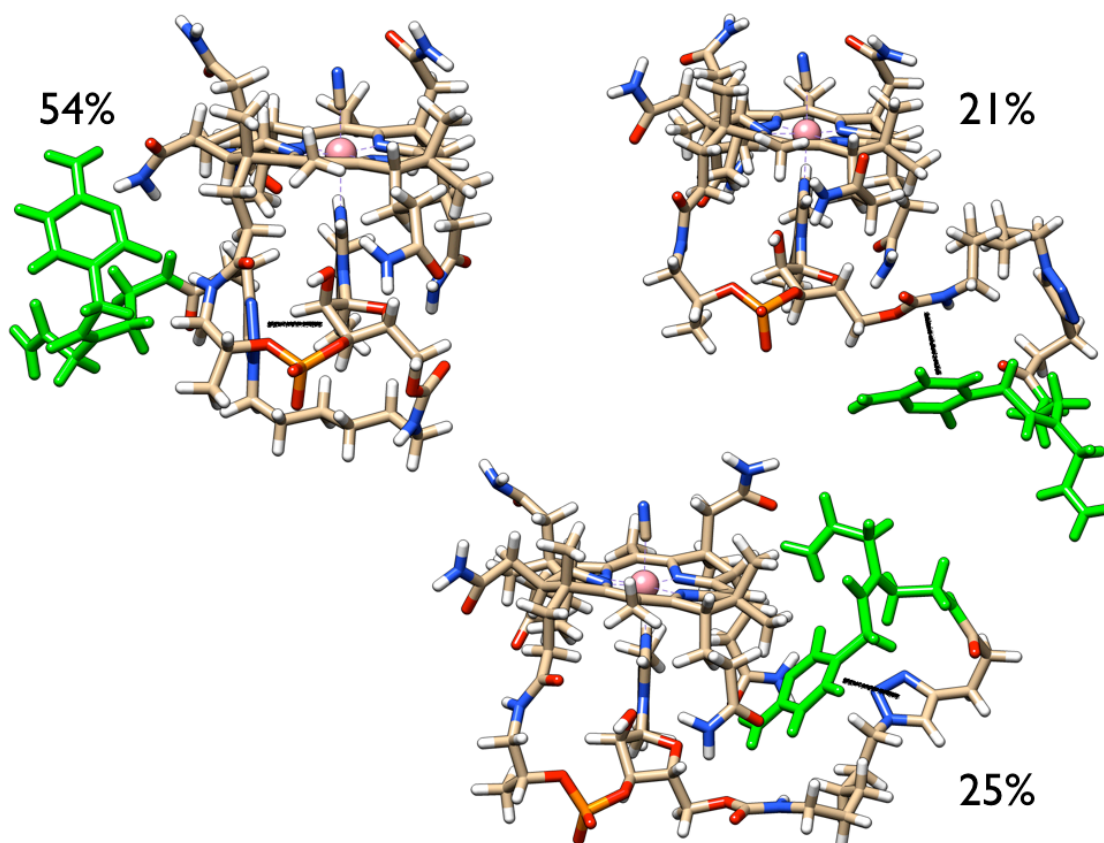
**Figure S2.** RMSD of heavy atoms in the production trajectories of Cbl-C, free PNA oligomer and Cbl-PNA with the starting structure as a reference. Note that PNA was initially built in an extended form not to bias toward any particular conformation, hence high RMSD from the starting structure.



**Figure S3.** Radius of gyration of heavy atoms in the simulations of Cbl-C, free PNA oligomer and Cbl-PNA including the solute equilibration phase with the starting structure as a reference. PNA was initially built in an extended form not to bias toward any particular conformation so in the simulation it acquires a compact form and radius of gyration decreases (middle and bottom plots).

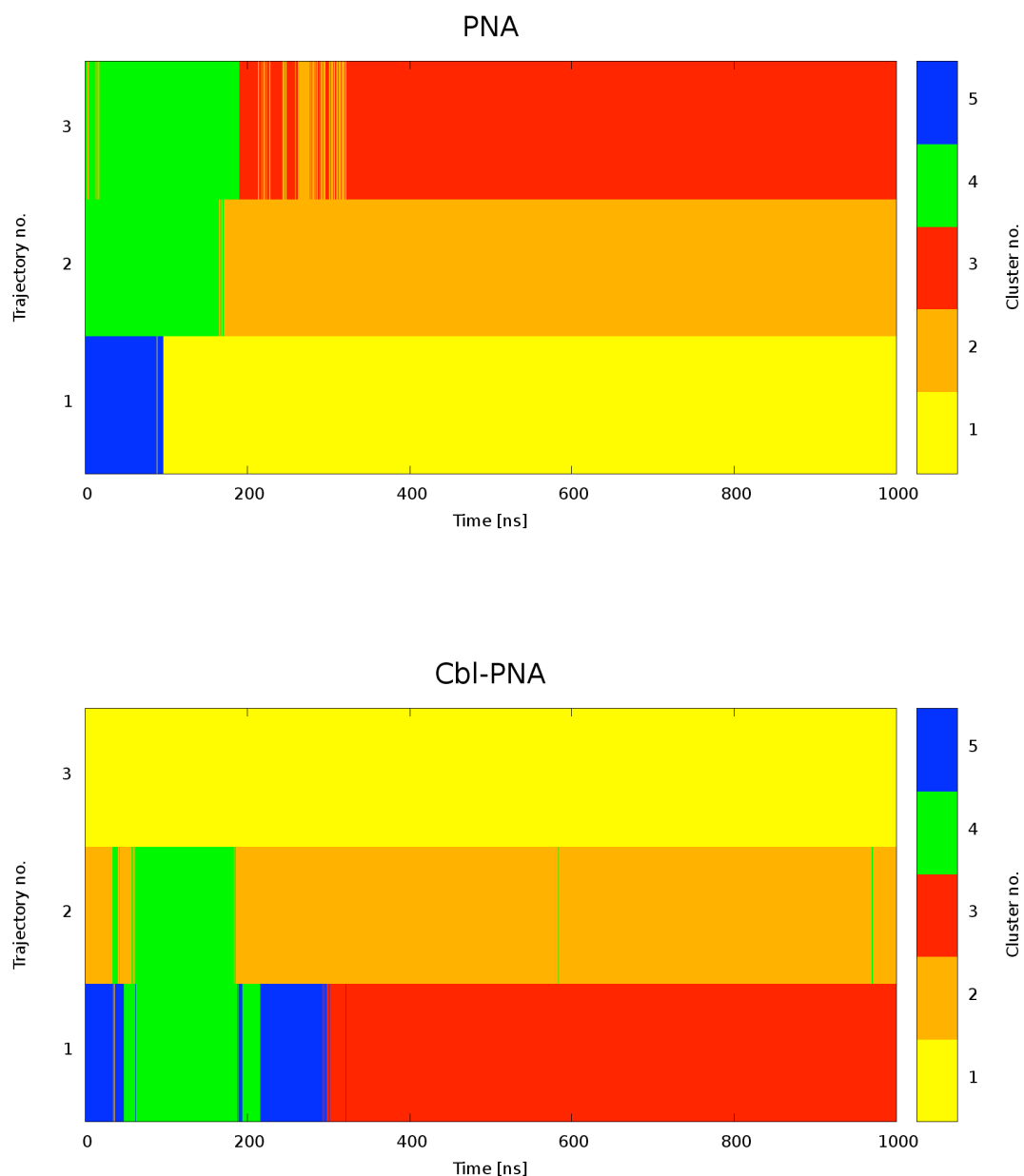


**Figure S4.** Solvent accessible surface area (SASA) of the PNA oligomer as a function of the simulation time from six MD production runs. SASA was calculated for a similar PNA moiety in Cbl-PNA and free PNA. Average SASA values of PNA (in  $\text{\AA}^2$ ) from various trajectories are shown in the legend. For clarity the plot shows running averages over 10 000 simulation points.



**Figure S5.** Representative conformations and occupancies of the clusters derived from the Cbl-C trajectories. PNA monomer C is in green. Characteristic stabilizing stacking interactions in each cluster are marked with black line.



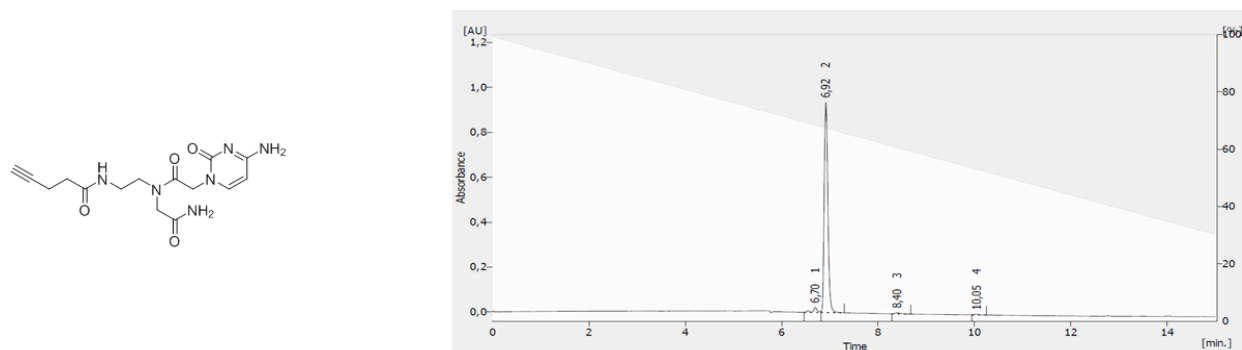


**Figure S6.** The occurrence of clusters as a function of the simulation time shown in three trajectories of PNA (top) and Cbl-PNA(bottom). The cluster numbers have the following occupancies averaged over three trajectories for PNA (no. 1 - 30%, no. 2 - 29%, no. 3 - 25%, no. 4 - 9%, no. 5 - 7%, representative conformations are in Figure 8 in the main text) and for Cbl-PNA (no. 1 -33%, no. 2 - 26%, no. 3 - 21%, no. 4 - 13%, no. 5 - 7%, representative conformations are shown in Figure 9 of the main text.)

## Section S1. Copies of HPLC chromatograms

**Table S1.** HPLC method 1.

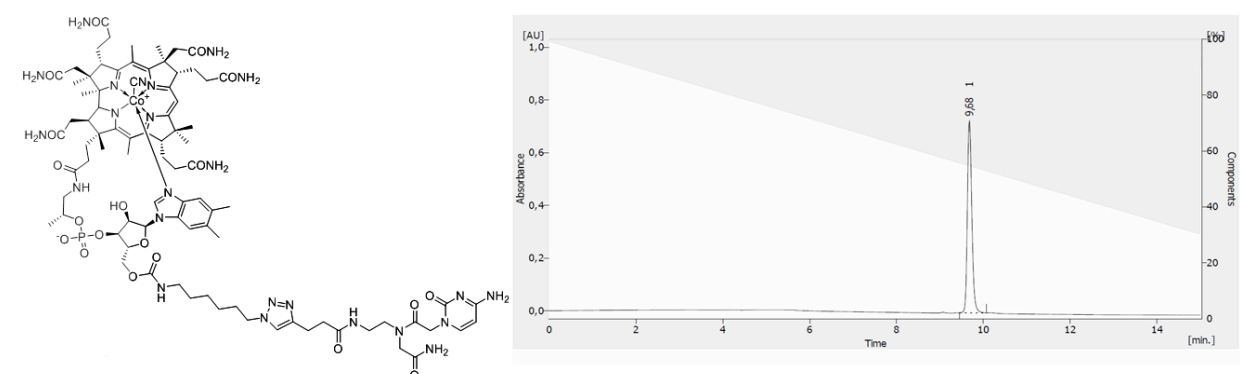
Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN [%]	λ [nm]
Initial	99	1	254
15	30	70	



**Figure S7.** Chromatogram of C-pentynoic acid derivative.

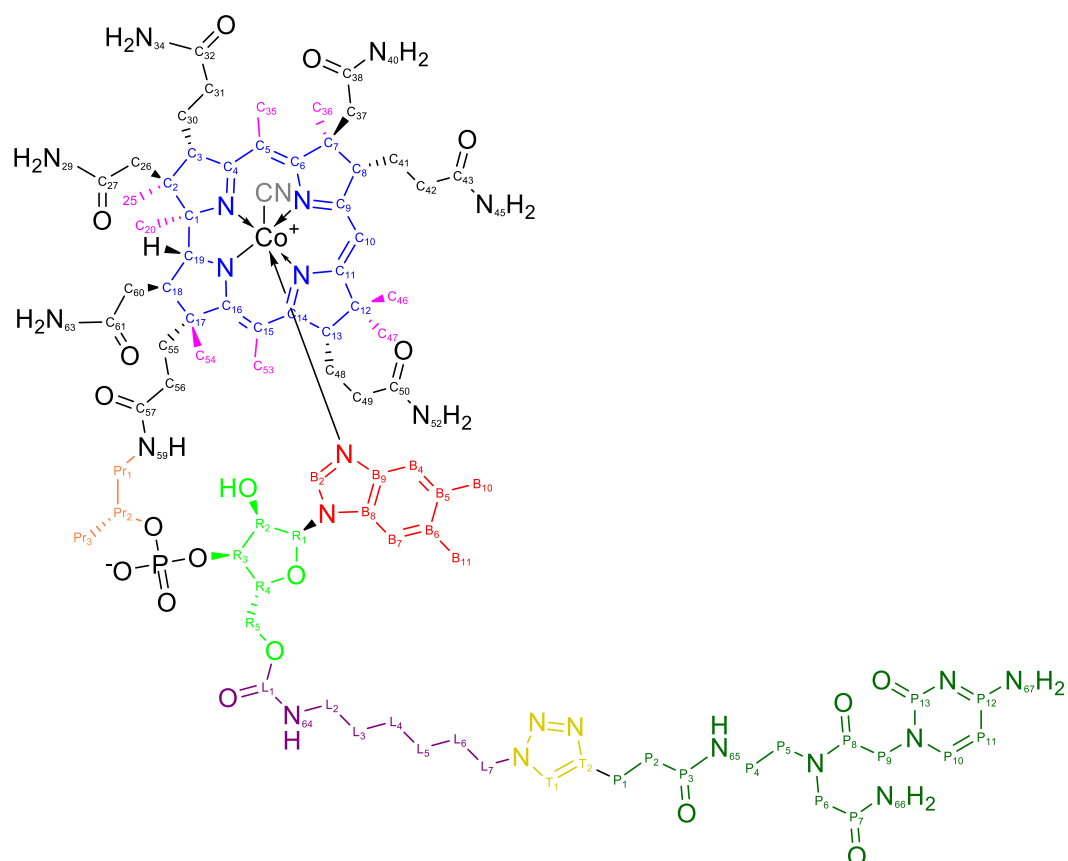
**Table S2.** HPLC method 2.

Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN [%]	λ [nm]
Initial	99	1	361
15	30	70	



**Figure S8.** Chromatogram of Cbl-C conjugate.

## Section S2. NMR assignments of Cbl-C in DMSO-*d*<sub>6</sub> (600 MHz)



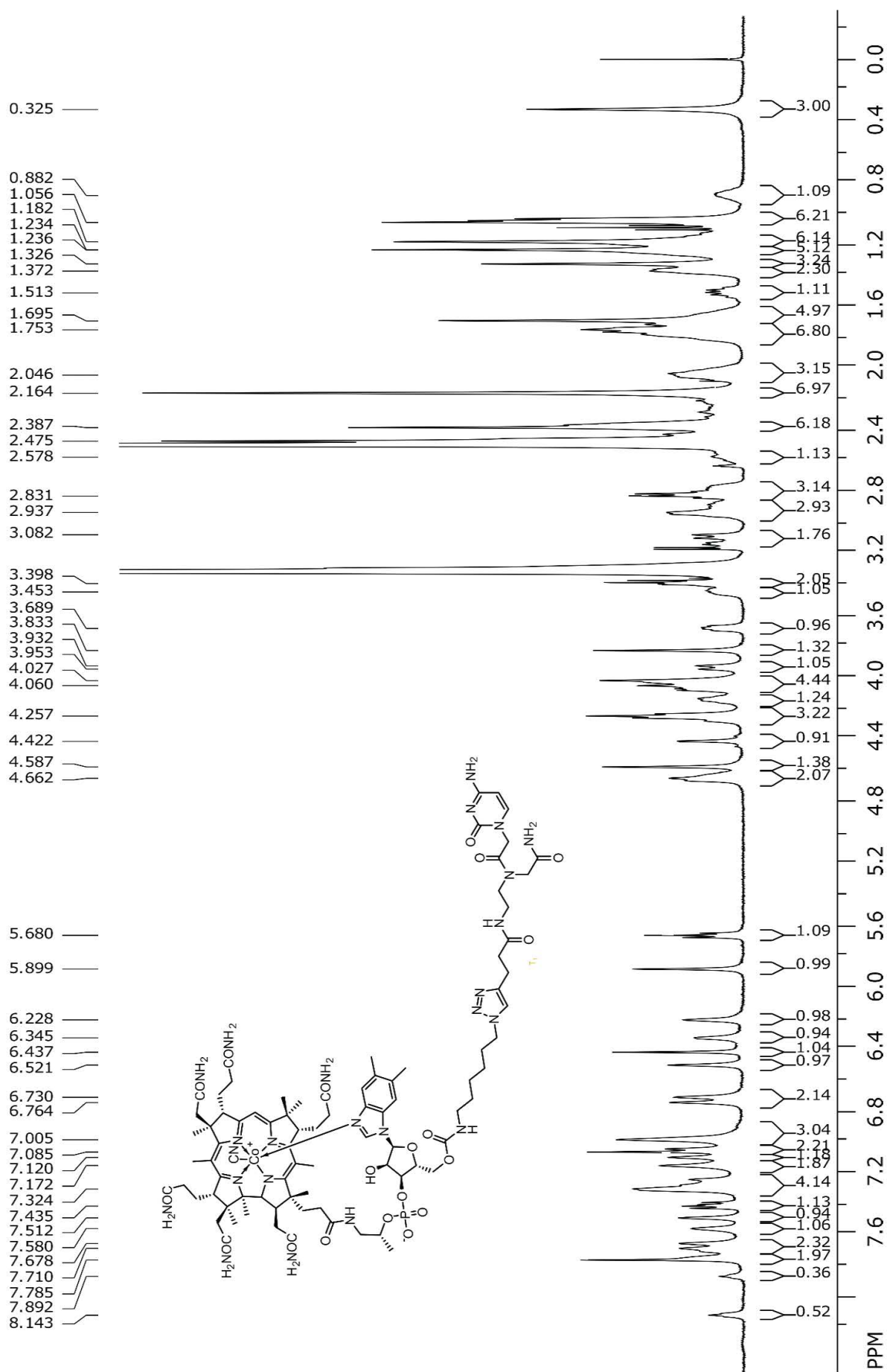
**Figure S9.** Vitamin B<sub>12</sub> labeling. Labels C, N, Pr, L, T, and P relate to protons (C for cobalamin corrin ring, N for amines and amides, Pr for propyl chain in Cbl, L for alkyl linker, T for triazole ring, P for PNA monomer).

**Table S3.** NMR assignments of Cbl-C in DMSO-*d*<sub>6</sub> (600 MHz)

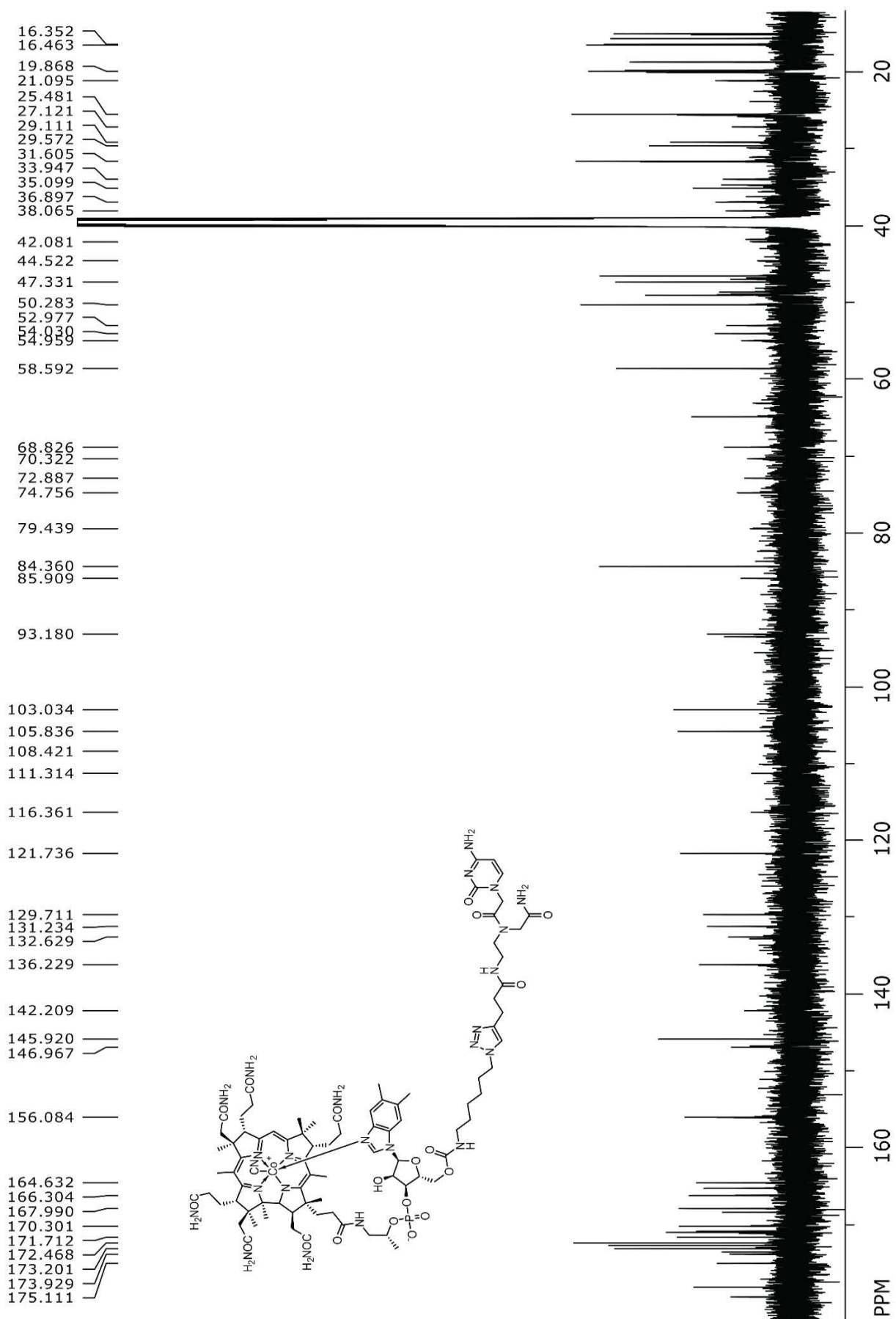
No	Assignment	<sup>13</sup> C Shift [ppm]	HSQC [ppm]	HMBC	COSY	ROESY
1	C4	179.50		C3, C35		
2	C16	178.22		C53, C54		
3	C11	175.11		C10, C13, C46, C47		
4	C50	173.93		C49		
5	C9	173.66		C41		
6	C32	173.20				
7	C43	172.79		C42		
8	C38	172.46		C37		
9	C27	172.46				
10	P3	171.70, 171.23		P1, P2		
11	C61	171.08		C60		
12	C57	170.93				
13	P7	170.30, 170.19		P6		
14	P8	168.48, 167.99		P5, P6, P9		
15	P12	166.30		P10		
16	C6	165.33		C8, C35, C37		
17	C14	164.63		C53		
18	L1	156.12		L2		
19	P13	156.08		P9, P10		

20	P10	146.96, 146.85	7.43	P9, P11	P11	L2, L3, L4, L5, P9, P11
21	T2	145.92		T1, P1, P2		
22	B2	142.21	7.08			C18, C20, C48, C53, R2-OH, R4
23	B9	136.23		B2, B7		
24	B6	132.63		B4, B10		
25	B5	131.23		B7, B11		
26	B8	129.71		B2, B4		
27	T1	121.73	7.79	L7, P1		L3, L4, L5, L6, L7, P1, P2
28	B4	116.36	6.44	B10		B10, C20, C25, C36, C41
29	B7	111.31	7.31	B11		B11, L5, R1, R2
30	CN	110.71				
31	C5	105.83		C35		
32	C15	103.03		C53		
33	C10	93.52	5.90			C8, C41, C42, C46, C47, N45
34	P11	93.18	5.68	P10	P10	C46, P9, P10, N63, N52
35	R1	85.90	6.23		R2	B7, L3, L5, R2, R3
36	C1	84.36		C3, C20, C25		
37	R4	79.46	4.09		R3, R5	B2, R3, R5, N59
38	C19	74.78	3.94	C20	C18	C18, C26, C54, C60
39	R3	72.90	4.65	R5	R2, R4	R1, R2, R4
40	Pr2	70.33	4.15		Pr1, Pr3	Pr1, Pr3, L3, N59
41	R2	68.82	4.04		R1, R3	B7, R1, R3, N59
42	R5	63.17	4.03, 4.29		R4	R4
43	C17	58.59		C56, C54		
44	C3	54.95	4.67	C25	C30	C25, C26, C30, C31, C35
45	C8	54.05	3.69	C10, C37, C36	C41	C10, C36, C41, C42
46	C13	52.96	3.09	C46, C47, C49	C48	C46, C47, C48, C53, N52
47	P6	50.20, 48.64	3.83, 4.06			P4, P5, P9, N65, N66
48	C7	50.28		C36		
49	L7	49.06	4.26	T1, L6	L6	T1, L3, L5, L6
50	P9	48.99, 48.90	4.42, 4.59	P6, P10		P2, P4, P5, P6, P10, P11, L4, L5, N65
51	C12	47.33		C10, C46, C47		
52	P5	46.99, 46.80	3.28, 3.40	P6		P4, P6, P9, N65
53	C2	46.52		C20, C25		
54	Pr1	44.61	2.88, 3.45		Pr2, N59	Pr2, Pr3, N59
55	C37	42.08	1.73, 2.44	C36		N40
56	C26	41.62	2.06, 2.16	C25		C3, C19, C30, N29
57	L2	40.05	2.94	L3	L3	L3, L4, L5, L6, N64, P10

58	C18	38.05	2.77	C54	C19	B2, C19, C20, C56, R2-OH
59	P4	36.88, 36.20	3.14, 3.29	P5	N65	P5, P6, P9, N65
60	C31	35.09	2.28, 2.23	C3	C30	C3, C25, C30, N34
61	P2	34.68	2.38, 2.48	P1	P1	P1, P9, T1, N65
62	C49	33.95	2.37	C13		C47, N52
63	C60	31.65	2.47			C19, C54, C55, N63
64	C56	31.65	2.47		C55	C18, C54, C55, N63
65	C42	31.65	1.14, 1.51	C41	C41	C8, C10, C41, N45
66	C46	31.60	1.06	C13, C47		C10, C13, C47, P11, N52
67	C55	31.04	1.79, 2.34	C54	C56	C56, C60
68	L6	29.57	1.75	L7	L7	L2, L3, L4, L5, L7, T1
69	L3	29.11	1.37		L2	L2, L6, L7, N52, N64, P10, Pr2, R1, T1
70	C48	27.12	1.64, 2.03	C13	C13	B2, C13, C47
71	C41	25.79	0.88, 1.75		C42, C48	B4, C8, C10, C36, C42, N45
72	L4, L5	25.58	1.19, 1.25	L3, L6, L7	L6	B7, L2, L6, L7, P9, P10, R1, T1, N64
73	C30	25.48	1.69, 1.75		C31	C3, C26, C25, C31, C35
74	P1	21.16, 21.09	2.82	P2	P2	T1, P2, N65
75	Pr3	20.06	1.04		Pr2	P11, Pr1, Pr2
76	C47	19.97	1.33	C46		C10, C13, C46, C48, C49, C53, N45, N52
77	C20	19.83	0.32			B2, B4, C18, C25, R2-OH
78	B10	19.87	2.16	B4		B4, C41
79	B11	19.87	2.16			B7, N45
80	C36	18.67	1.69			B4, C8, C35, C41, N40
81	C54	16.46	1.23			C19, C53, C56, C60, P11, N63
82	C25	16.35	1.18			B4, C3, C20, C30, C31, N29
83	C35	15.60	2.48			B4, C3, C30, C36
84	C53	14.99	2.39			B2, C13, C47, C54, N52, R2-OH



**Figure S10.** <sup>1</sup>H NMR spectrum of Cbl-C recorded in DMSO-d<sub>6</sub>



**Figure S11.**  $^{13}\text{C}$  NMR spectrum of Cbl-C recorded in  $\text{DMSO-}d_6$

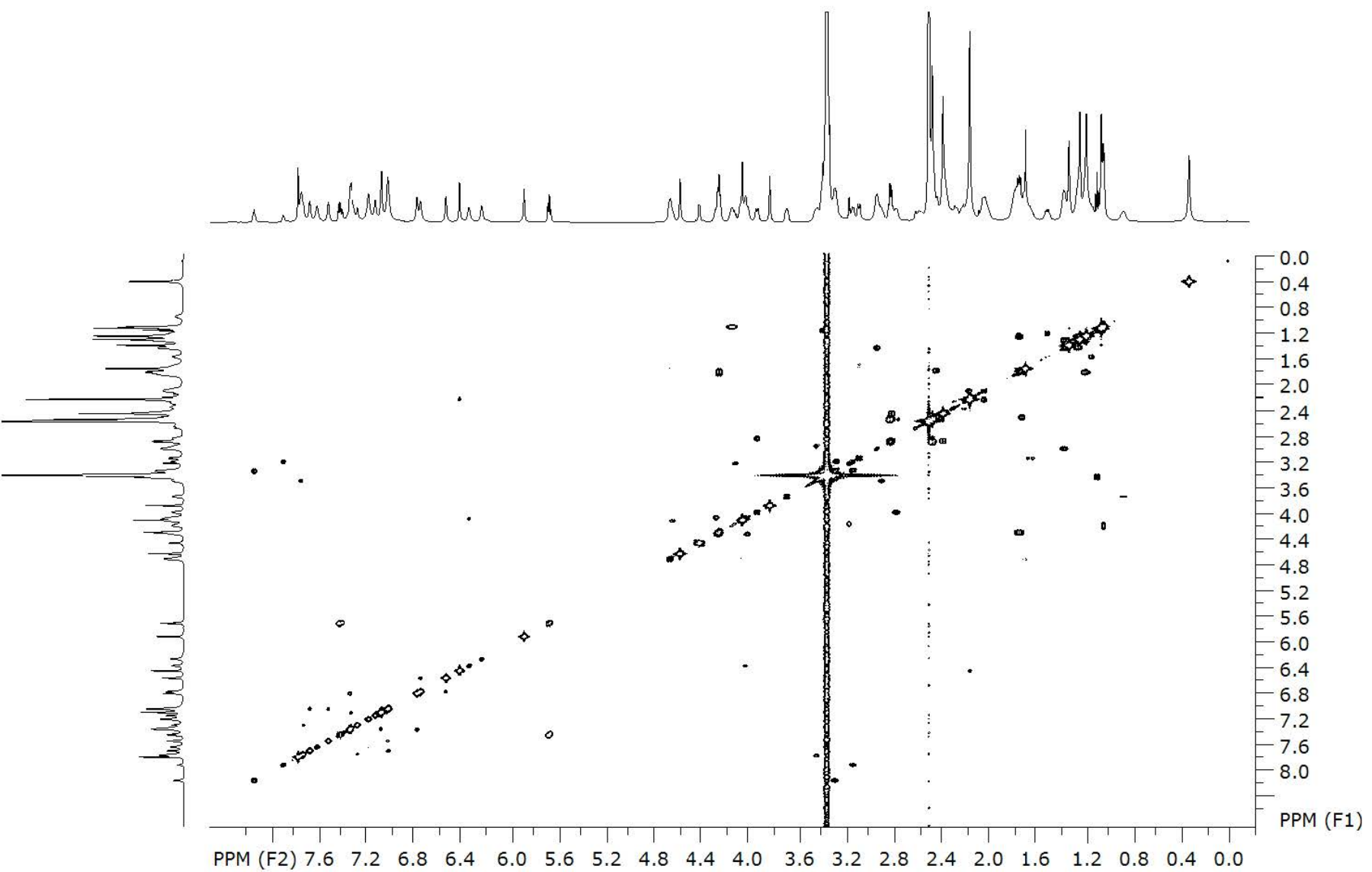
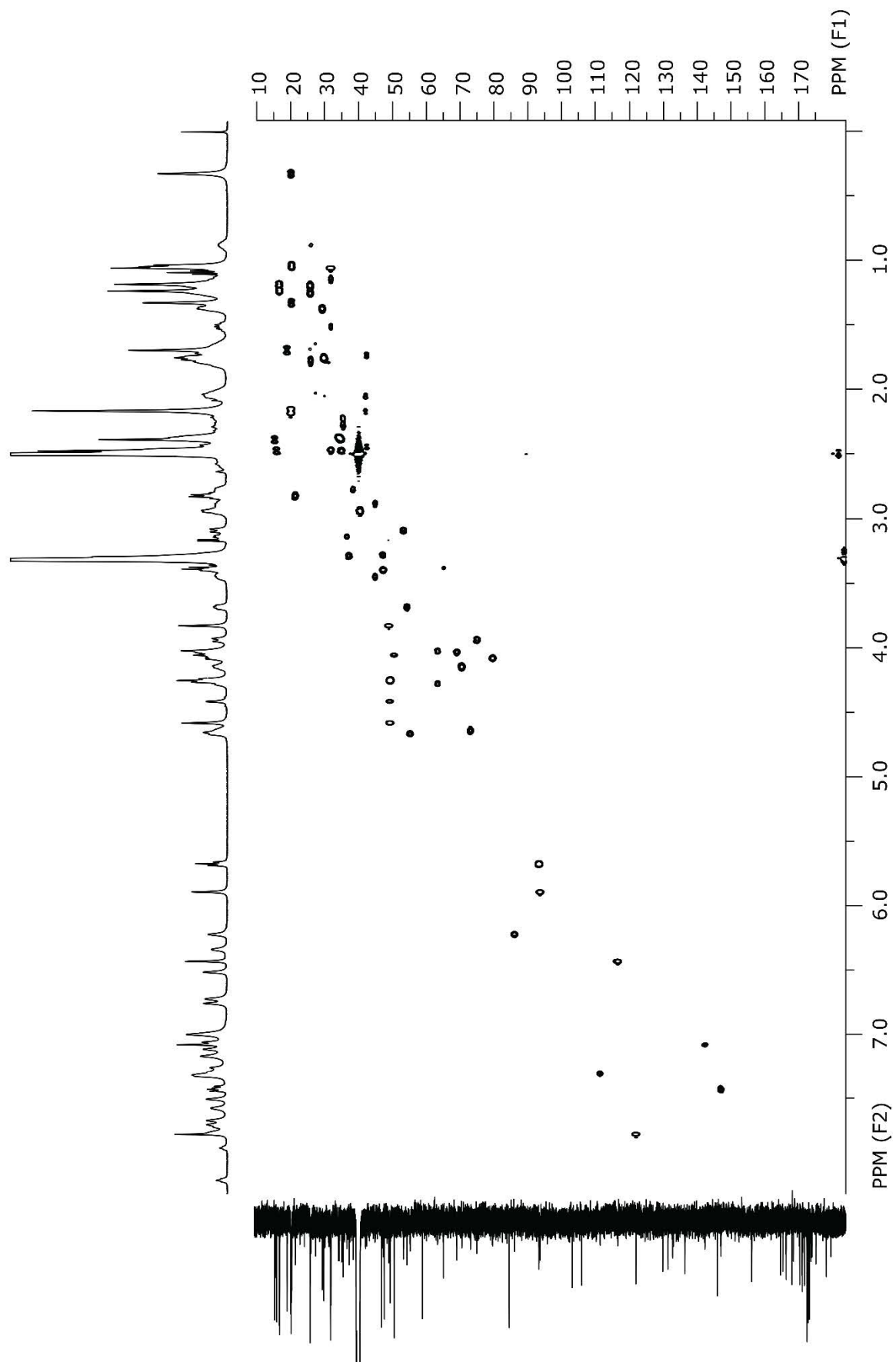
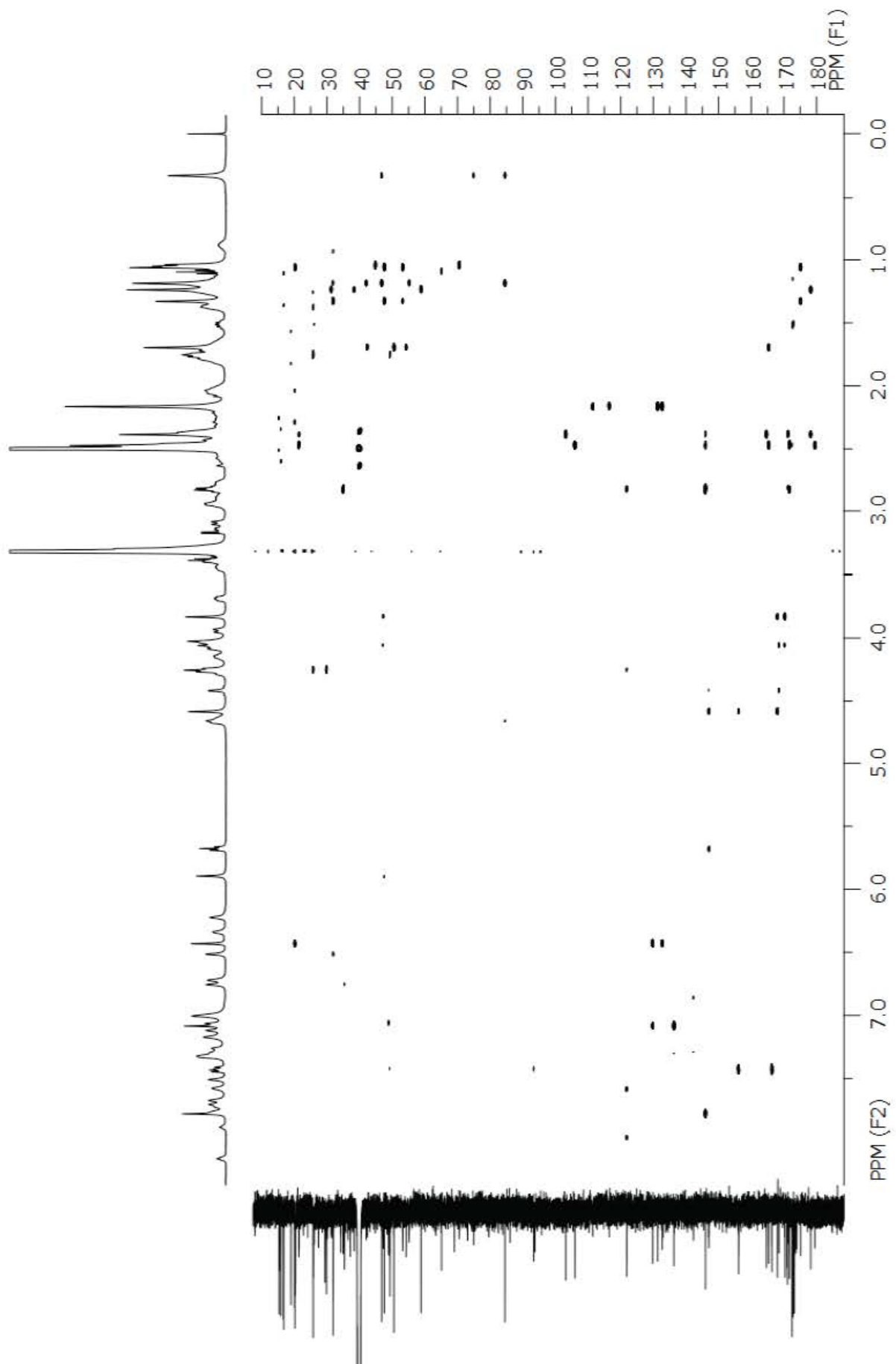


Figure S12.  $^1\text{H}$ - $^1\text{H}$  COSY NMR spectrum of Cbl-C recorded in  $\text{DMSO-}d_6$

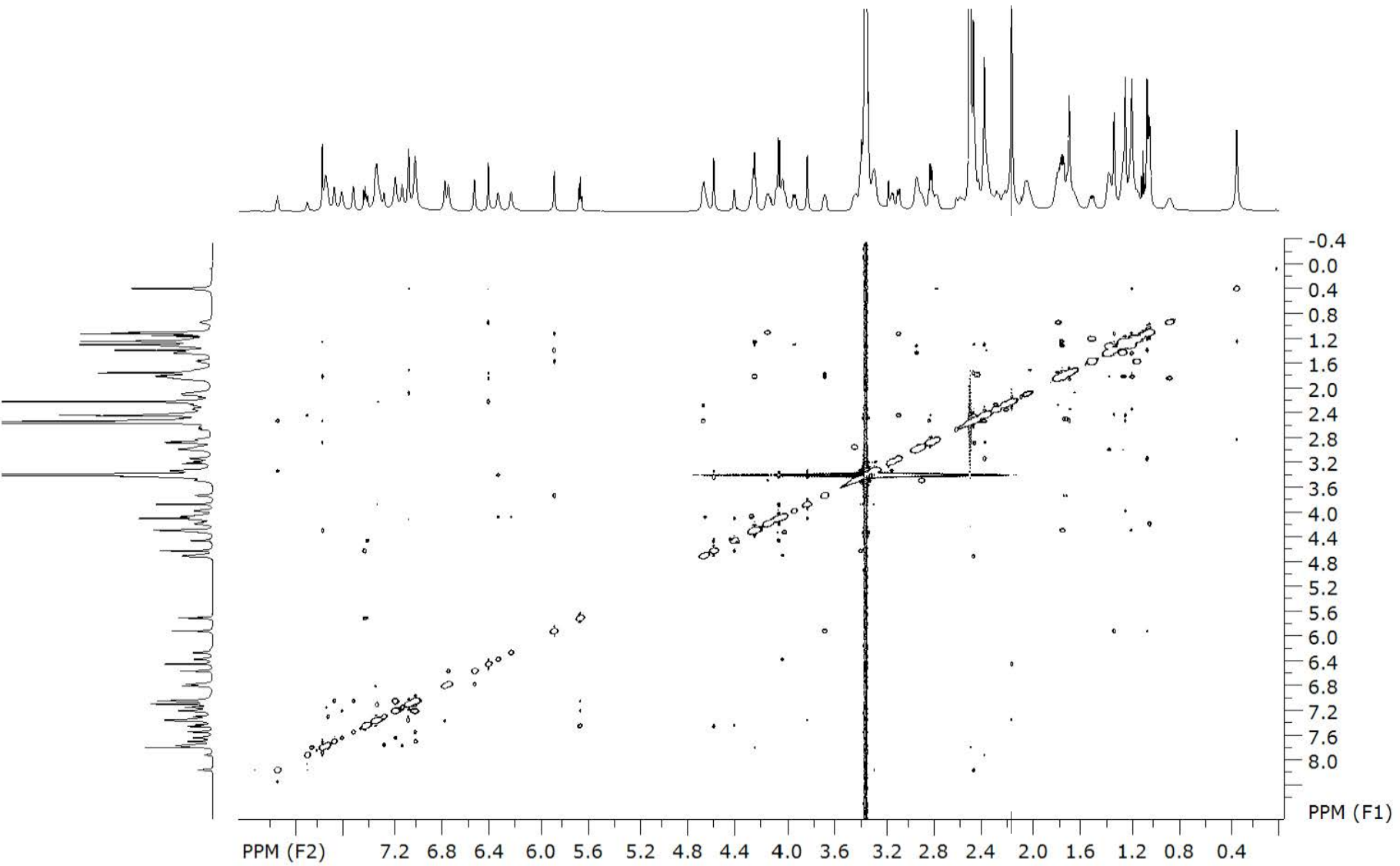


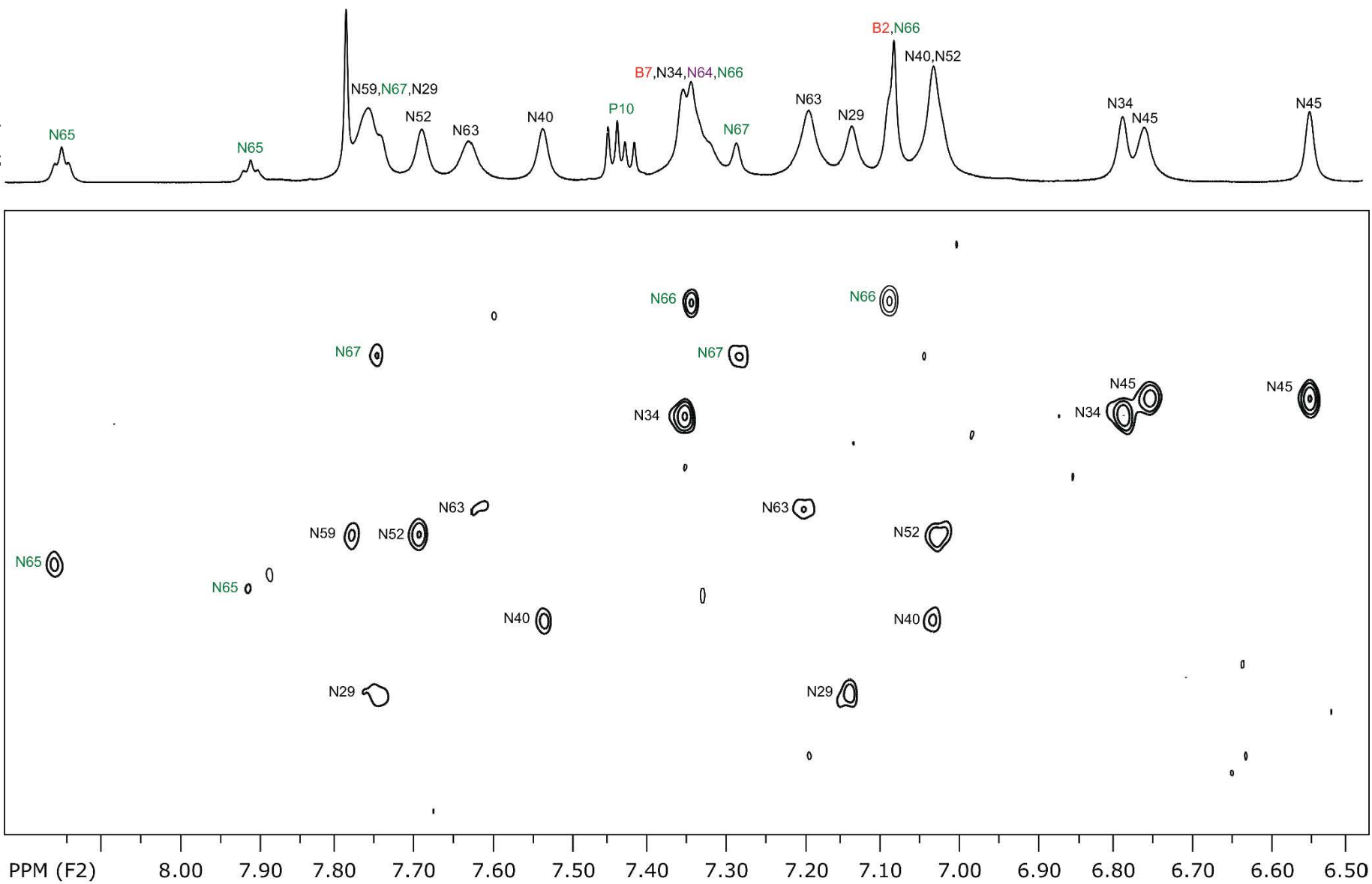


**Figure S13.**  $^1\text{H}$ - $^{13}\text{C}$  HSQC NMR spectrum of Cbl-C recorded in  $\text{DMSO-}d_6$



**Figure S14.**  $^1\text{H}$ - $^{13}\text{C}$  HMBC NMR spectrum of Cbl-C recorded in  $\text{DMSO-}d_6$

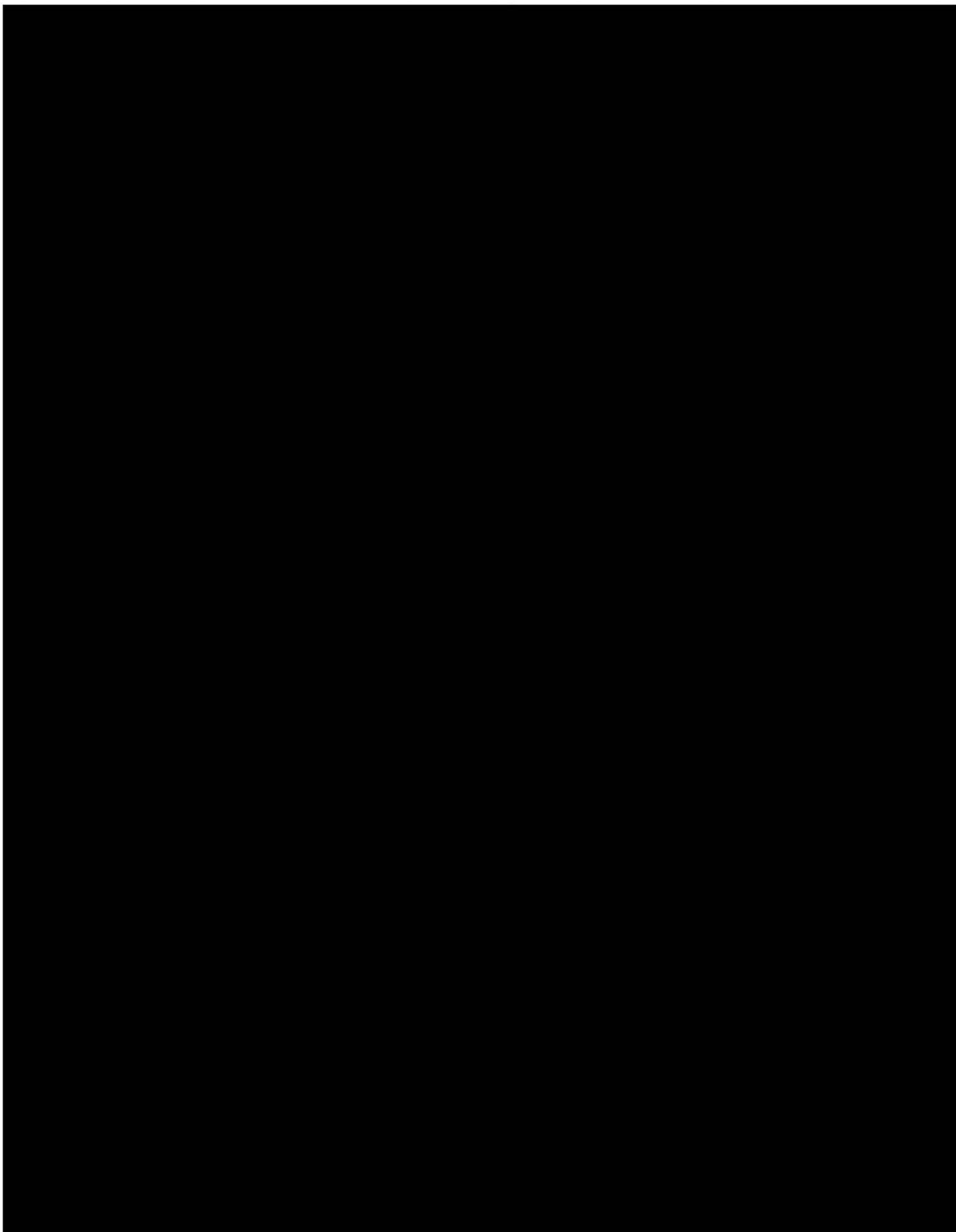


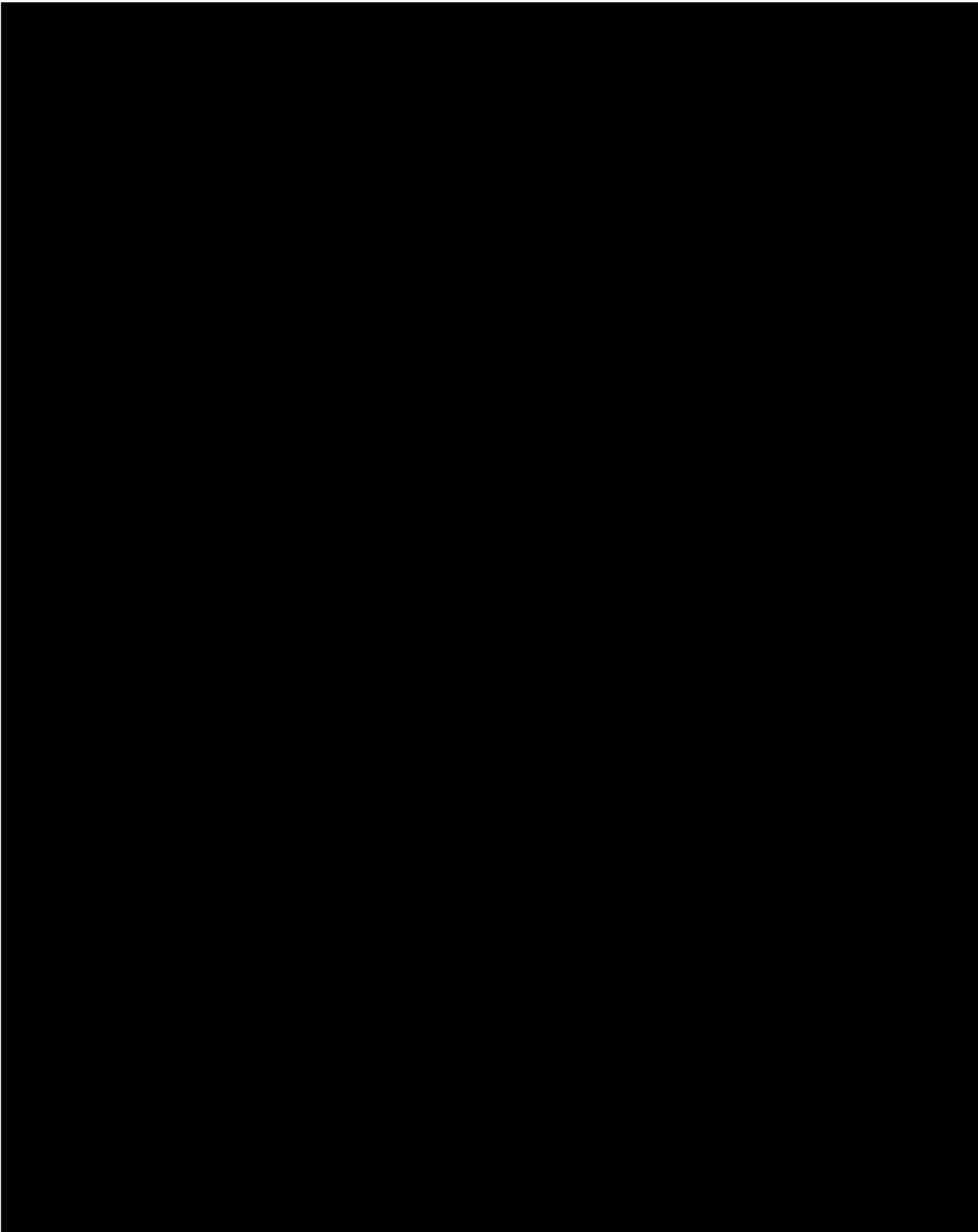
**Figure S16.**  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectrum of Cbl-C recorded in  $\text{DMSO-}d_6$ 

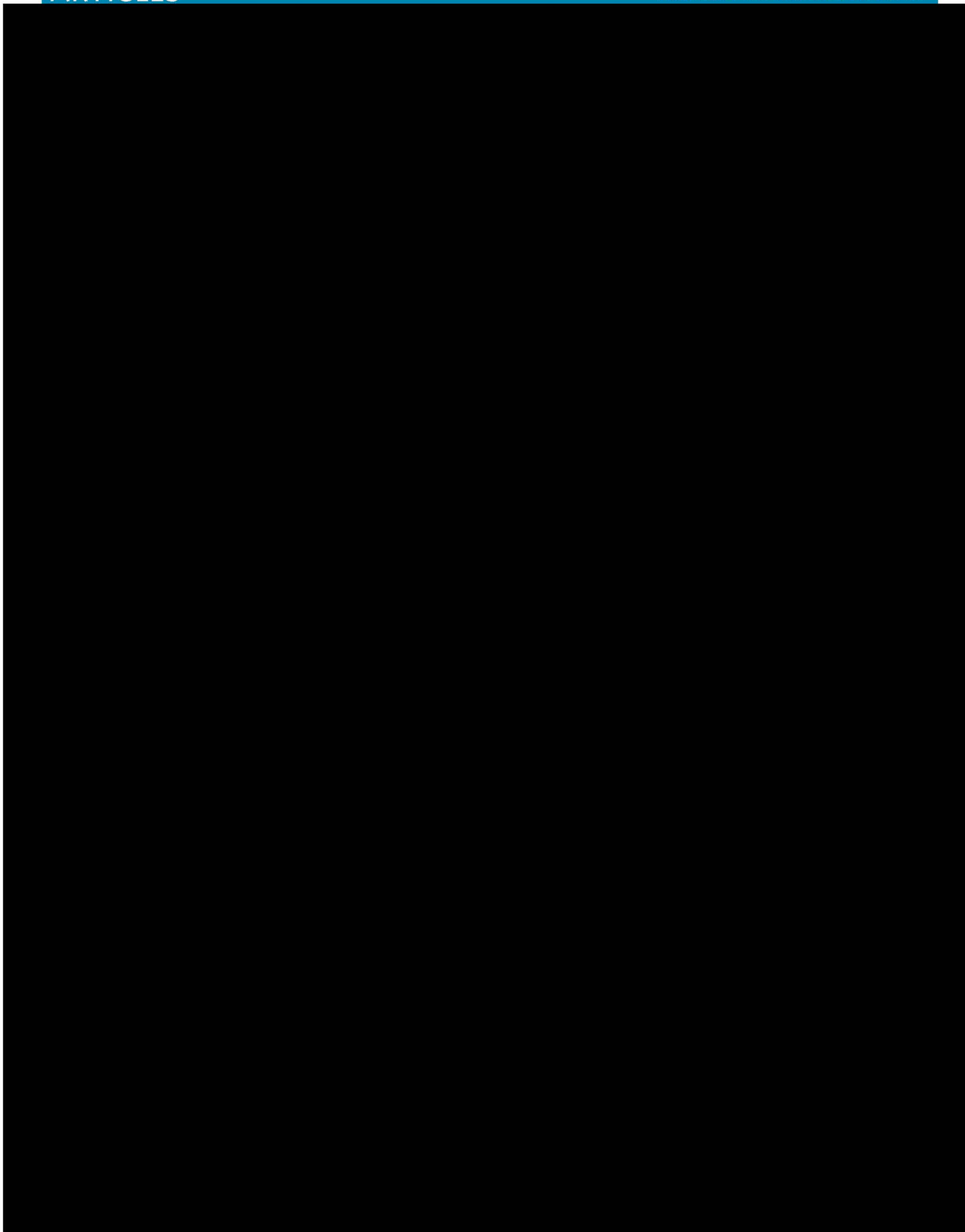
# A multicolor riboswitch-based platform for imaging of RNA in live mammalian cells

Esther Braselmann<sup>1,2</sup>, Aleksandra J. Wierzba<sup>3,7</sup>, Jacob T. Polaski<sup>1,6,7</sup>, Mikołaj Chromiński<sup>3</sup>, Zachariah E. Holmes<sup>1</sup>, Sheng-Ting Hung<sup>4</sup>, Dilara Batan<sup>1,2</sup>, Joshua R Wheeler<sup>1</sup>, Roy Parker<sup>1,5</sup>, Ralph Jimenez<sup>1,4</sup>, Dorota Gryko<sup>3</sup>, Robert T. Batey<sup>1</sup> and Amy E. Palmer<sup>1,2\*</sup>

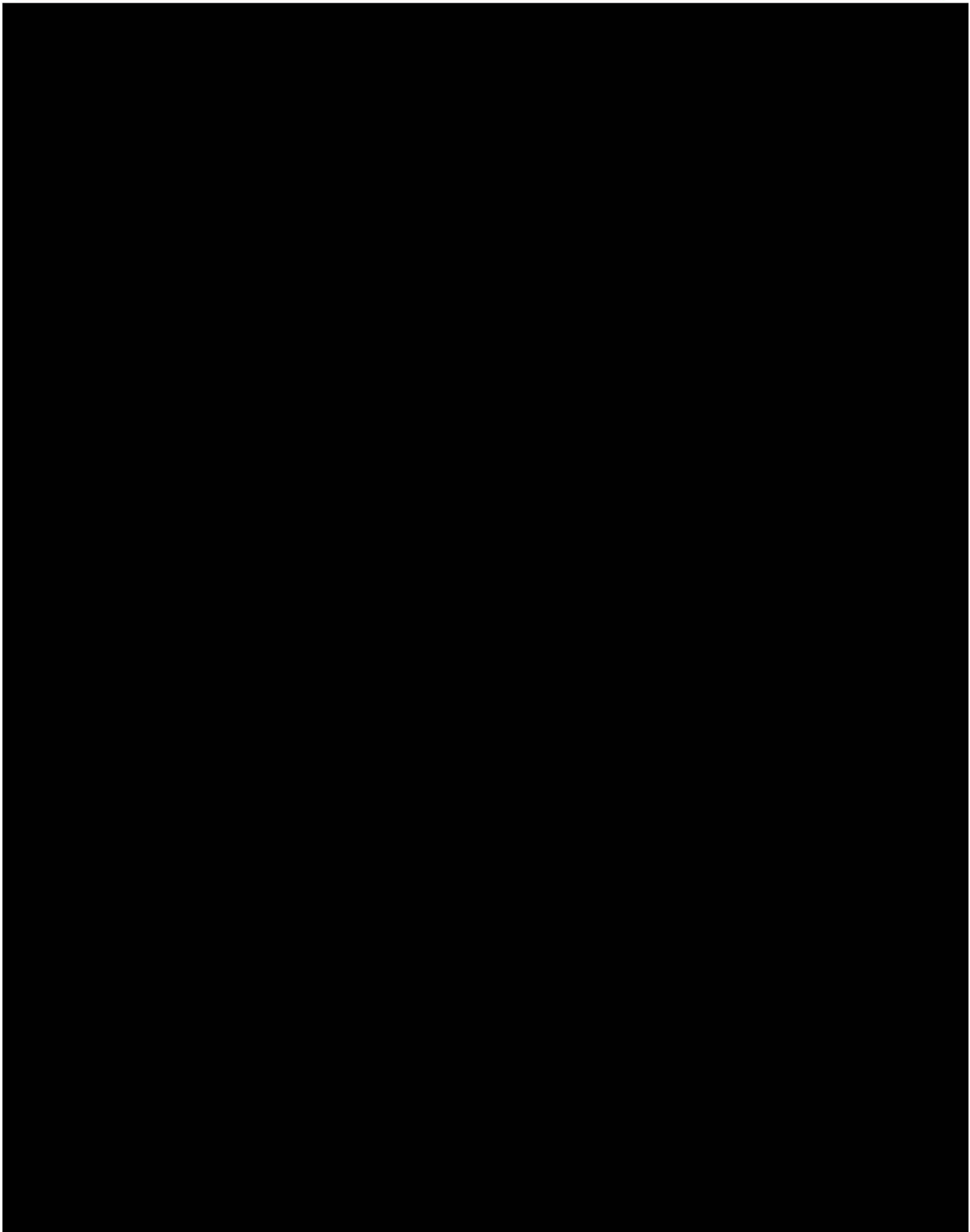
<sup>1</sup>Department of Chemistry and Biochemistry, University of Colorado Boulder, Boulder, CO, USA. <sup>2</sup>BioFrontiers Institute, University of Colorado Boulder, Boulder, CO, USA. <sup>3</sup>Institute of Organic Chemistry, Polish Academy of Sciences, Warsaw, Poland. <sup>4</sup>JILA, University of Colorado and NIST, Boulder, CO, USA. <sup>5</sup>Howard Hughes Medical Institute, Chevy Chase, MD, USA. <sup>6</sup>Present address: Computational Biology Program, Public Health Sciences Division, Basic Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA. <sup>7</sup>These authors contributed equally: Aleksandra J. Wierzba, Jacob T. Polaski. \*e-mail: amy.palmer@colorado.edu







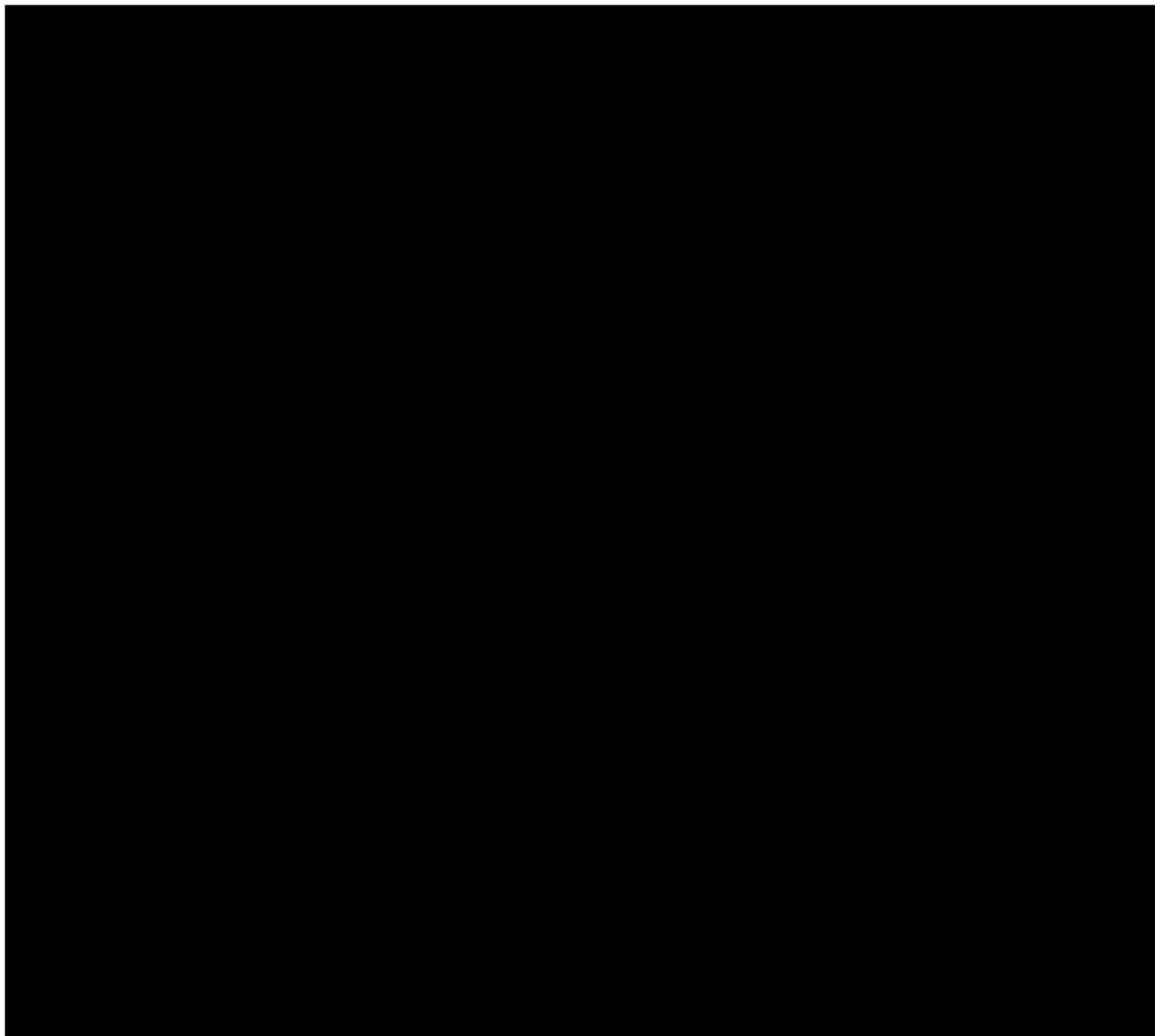




to MS2-GFP (Supplementary Fig. 27). Localization of mRNA-tagged experiments). Scale bars, 10  $\mu$ m. White arrowheads indicate SGS.

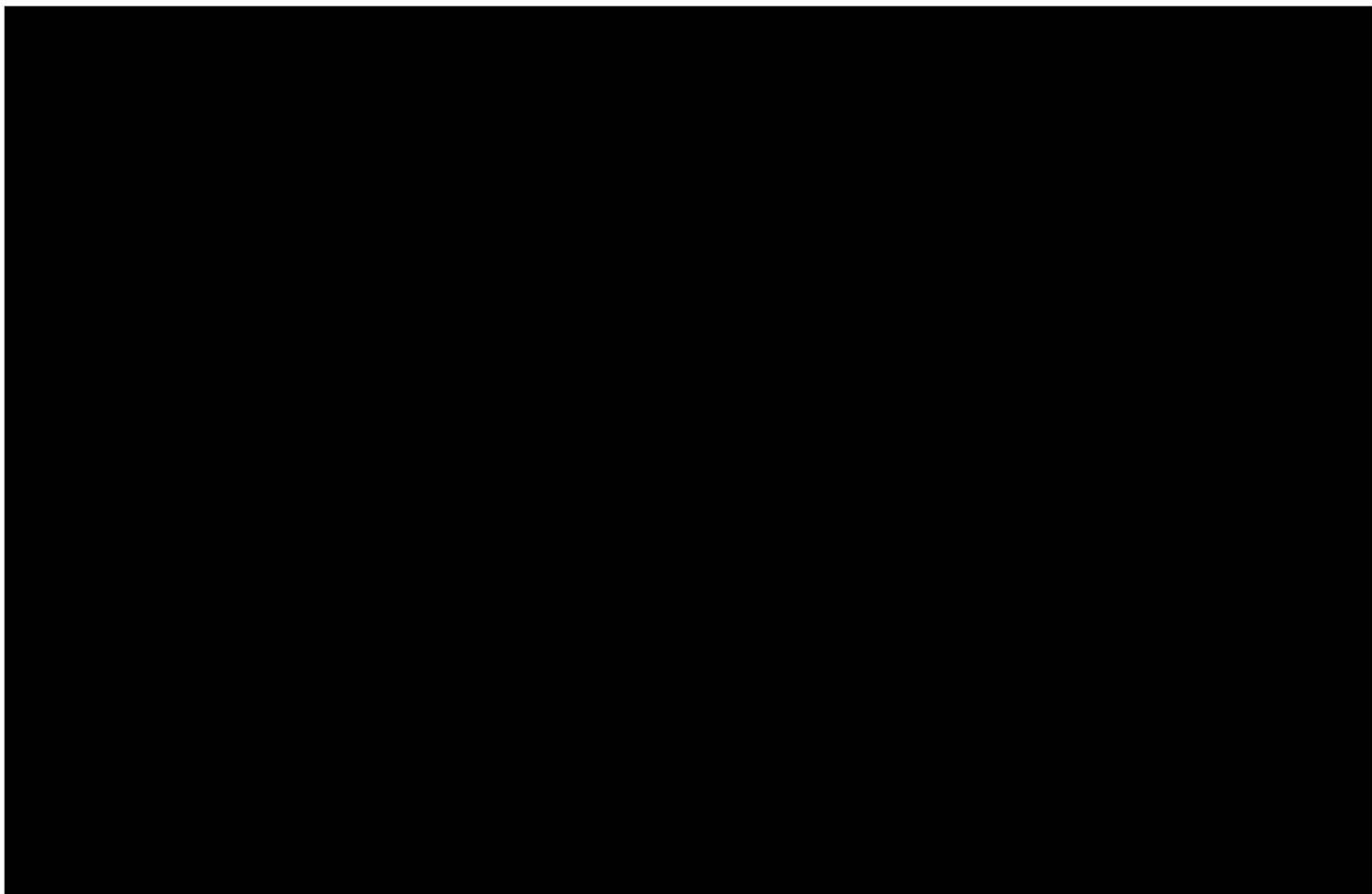




















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## **Supplementary Tables**

### **Supplementary Table 1**

Comparison of length and fluorophore properties for RNA tags used for live cell RNA fluorescence microscopy in this study.

<b>Name of tag</b>	<b>Length of RNA tag (nucleotides)</b>	<b>number of dye / FP molecules per tag</b>	<b>Molecular weight (kDa) of fluorophore</b>
(A <sub>T</sub> )1x	81	1	2.3 (Cbl-5xPEG-ATTO 590)
(A)1x	103	1	2.3 (Cbl-5xPEG-ATTO 590)
(A)2x	212	2	2.3 (Cbl-5xPEG-ATTO 590)
(A)3x		3	2.3 (Cbl-5xPEG-ATTO 590)
(A)4x	430	4	2.3 (Cbl-5xPEG-ATTO 590)
1x MS2 SL	61	2	~88 (MS2-GFP homo dimer)
2x MS2 SL	102	4	~176
4x MS2 SL	210	8	~352
24x MS2 SL	1354	48	~2,112
2xdBroccoli	234	4	0.3 (DFHBI-1T)

## Supplementary Table 2

Sequences of riboswitch RNA tags used in this study. Listed are the RNA sequences that were purified for *in vitro* work (see also Supplementary Fig. 1).

Name	Sequence
A	5'-GGC CUA AAA GCG UAG UGG GAA AGU GAC GUG AAA UUC GUC CAG AUU ACU UGA UAC GGU UAU ACU CCG AAU GCC ACC UAG GCC AUA CAA CGA GCA AGG AGA CUC
A <sub>T</sub>	5'-GGC CUA AAA GCG UAG UGG GAA AGU GAC GUG AAA UUC GUC CAG AUU ACU UGA UAC GGU UAU ACU CCG AAU GCC ACC UAG GCC
A <sub>T,MUT</sub>	5'-GGC CUA AAA GCG UAG UGG GAA AGU GAC GUG AAA UUC GUC CAG AUU ACU UGA UAC GGU UAU ACU CCG UUU UCC ACC UAG GCC
B	5'-GGU ACU GAA AGC GUG GUG GGA AAC AAU GUG AAA UUC AUU GAC UGU UCC UGC AAC GGU AAA AGU AAA AUU GAG UCC GAA UGC CAC CCA GUA AAG UCC GCU GUC GAG UGA AGG CCA GGA AAA GUC UAA CUC UGC AAU AUU AAA
C	5'-GGA CAU CGG UUU UAG UGG GGA ACA GCC ACU AAA AUA AUG GGG AAA GUU UGG UGC AAG UCC AAC ACU GUC CCG CAG CUG UAA GCA GAC UAU CUC UGU GAG UCA GAA CGC CCA CCG AUG UCC CCC GUA AAC ACU UCU GCG AGG UAC AGA AA
D	5'-UAC UGA AAG CGU GGU GGG AAA CAA UGU GAA AGU CAU UGA CUG UUC CUG CAA CGG UAA GCG CUU CGG CGC GAG UCC GAA UGC CAC CCA GUA AAG UCC GCU GUC GAG UGA AGG CCA GGA AAA GUC UAA CUC A

### Supplementary Table 3

Estimates of linker lengths in Cbl-fluorophore probes.

<b>Name of linker</b>	<b>Estimated length (values for PEG linkers published by ThermoFisher Scientific)</b>
C6	10.5 Å (estimated to be similar to 3xPEG)
1xPEG	3.5 Å
2xPEG	7.0 Å
3xPEG	10.5 Å
5xPEG	17.5 Å
4xGly	21.4 Å (Berg, Tymoczko & Stryer, Biochemistry, 2002)

**Supplementary Table 4**

Summary of fold turn-on for Cbl-fluorophore probes in the presence of RNA (Fig. 2)

<b>Name of Cbl-fluorophore probe</b>	<b>Name of RNA</b>	<b>Fold fluorescence turn-on</b>
Cbl-FAM	A <sub>T,MUT</sub>	0.5x
	A <sub>T</sub>	2.5x
Cbl-C6-FAM	A <sub>T,MUT</sub>	1.1x
	A <sub>T</sub>	4.1x
Cbl-3xPEG-FAM	A <sub>T,MUT</sub>	1.1x
	A <sub>T</sub>	2.8x
		7.4x
Cbl-C6-ATTO 488	A <sub>T,MUT</sub>	1.1x
	A <sub>T</sub>	1.3x
Cbl-C6-ATTO 590	A <sub>T,MUT</sub>	1.2x
	A <sub>T</sub>	2.9x
	B	3.9x
Cbl-5xPEG-ATTO 590	A <sub>T,MUT</sub>	1.1x
	A <sub>T</sub>	4.0x
	A	4.9x
	B	3.7x
	C	4.0x
	D	2.9x
Cbl-4xGly-ATTO 590	A <sub>T</sub>	7.3x
	A	5.0x
	D	3.9x

Cbl-C6-ATTO 633	$A_{T,MUT}$	0.9x
	$A_T$	1.9x
	B	2.5x
Cbl-Cy5	$A_{T,MUT}$	1.1x
	$A_T$	2.1x
	A	2.7x
	B	2.5x
	D	2.5x

### Supplementary Table 5

Comparison of maximal distance between the corrin ring in Cbl and the fluorophore in probes. Values are based on structural estimates and the Förster distance  $R_0$  was estimated from spectral properties.

Name	Distance estimate between corrin ring and click linkage to fluorophore	Förster distance $R_0$
Cbl-FAM	9 Å	
Cbl-C6-FAM	12.5 Å	
Cbl-1xPEG-FAM	12.5 Å	35 Å
Cbl-2xPEG-FAM	16 Å	
Cbl-3xPEG-FAM	19.5 Å	
Cbl-C6-ATTO488	12.5 Å	35 Å
Cbl-ATTO 590	9 Å	
Cbl-C6-ATTO 590		
Cbl-5xPEG-ATTO 590	26.5 Å	20 Å
Cbl-4xGly-ATTO 590	30.4 Å	
Cbl-ATTO 633	9 Å	
Cbl-C6-ATTO 633	12.5 Å	15 Å
Cbl-Cy5	9 Å	12 Å

### Supplementary Table 6

Theoretical estimates of parameters for energy transfer between Cbl absorbance and fluorescence emission of each fluorophore.

<b>Fluorophore</b>	<b>Overlap integral <math>J(\lambda)</math> between fluorescence emission and Cbl absorbance</b>	<b>Quantum yield Q of fluorophore (source)</b>	<b>Förster distance <math>R_0</math></b>
<b>FAM</b>	$1.374 \times 10^{14} \text{ nm}^4 \text{ M}^{-1} \text{ cm}^{-1}$	0.93 (Lumiprobe)	35 Å
<b>ATTO 488</b>	$1.424 \times 10^{14} \text{ nm}^4 \text{ M}^{-1} \text{ cm}^{-1}$	0.80 (Atto tec)	35 Å
<b>ATTO 590</b>	$5.266 \times 10^{12} \text{ nm}^4 \text{ M}^{-1} \text{ cm}^{-1}$	0.80 (Atto tec)	20 Å
<b>ATTO 633</b>	$1.026 \times 10^{12} \text{ nm}^4 \text{ M}^{-1} \text{ cm}^{-1}$	0.64 (Atto tec)	15 Å
<b>Cy5</b>	$7.638 \times 10^{11} \text{ nm}^4 \text{ M}^{-1} \text{ cm}^{-1}$	0.28 (Lumiprobe)	12 Å

### Supplementary Table 7

Summary of ITC titrations for different RNAs in the presence of Cbl or the Cbl-5xPEG-ATTO 590 probe.

Name of riboswitch RNA	Name of conjugate	K <sub>D</sub>
A	Cbl	37 ± 1 nM
A	Cbl-5xPEG-ATTO 590	34 ± 9 nM
A <sub>T</sub>	Cbl	290 ± 100 nM
A <sub>T</sub>	Cbl-5xPEG-ATTO 590	1.3 ± 0.56 μM
D	Cbl	2.2 ± 1.6 nM
D	Cbl-5xPEG-ATTO 590	3.0 ± 0.6 nM

All measurements were done in triplicates and values are reported as the mean ± STDEV.



### Supplementary Table 8

Quantum yield of probes in the presence and absence of different RNAs.

<b>Name</b>	<b>Quantum yield (Q)</b>
Cy5	0.28 (Lumiprobe)
Cbl-Cy5	0.09
Cbl-Cy5 + A	0.26
Cbl-Cy5 + D	0.25
ATTO 590	0.8 (Atto Tec)
Cbl-5xPEG-ATTO 590	0.06
Cbl-5xPEG-ATTO 590 + A	0.31
Cbl-5xPEG-ATTO 590 + D	0.31
Cbl-4xGly-ATTO 590	0.09
Cbl-4xGly-ATTO 590 + A	0.55
Cbl-4xGly-ATTO 590 + D	0.45

### Supplementary Table 9

Summary of fluorescence lifetime measurements for Cbl-fluorophore probes in the presence and absence of indicated RNAs. The lifetime decay data presented in Supplementary Fig. 9 was fit and exponential components and their weight are indicated together with the overall fluorescence lifetime.

	<b>t<sub>1</sub> (ns)</b>	<b>t<sub>1</sub> %</b>	<b>t<sub>2</sub> (ns)</b>	<b>t<sub>2</sub> %</b>	<b>t<sub>3</sub> (ns)</b>	<b>t<sub>3</sub> %</b>	<b>lifetime (ns)</b>
Cy5	1.04	100					<b>1.04</b>
Cbl-Cy5	0.33	25		75			<b>0.61</b>
Cbl-Cy5 + D	1	100					<b>1</b>
Cbl-Cy5 + A	1.05	100					<b>1.05</b>
ATTO 590	3.99	100					<b>3.99</b>
Cbl-4xGly-ATTO 590	0.2	9	0.89	32	1.94	59	<b>1.44</b>
Cbl-4xGly-ATTO 590 + D	0.4	6	1.75	46	3.3	48	<b>2.42</b>
Cbl-4xGly-ATTO 590 + A	0.43	5	1.84	33	3.79	62	<b>2.98</b>
Cbl-5xPEG-ATTO 590	0.25	28	1.56	72			<b>1.19</b>
Cbl-5xPEG-ATTO 590 + D	0.4	12	1.61	60	3.22	28	<b>1.93</b>
Cbl-5xPEG-ATTO 590 + A	0.41	13	1.42	43	3.34	44	<b>2.14</b>

### Supplementary Table 10

Experimental conditions for RNA / probe photobleaching experiments.

<b>Probe</b>	<b>RNA</b>	<b>Excitation wavelength (nm)</b>	<b>Irradiance (W/cm<sup>2</sup>)</b>
55 $\mu$ M Cbl-Cy5	550 $\mu$ M A	640	0.27
48 $\mu$ M Cbl-5xPEG-ATTO 590	480 $\mu$ M A	555	1.31
7 $\mu$ M DFHBI-1T	80 $\mu$ M Broccoli	470	3.16

**Supplementary Table 11**

Photophysical properties of fluorophores, probes and Cbl.

<b>Name</b>	<b>Extinction coefficient <math>\epsilon</math> [L mol<sup>-1</sup> cm<sup>-1</sup>] (source)</b>	<b>Excitation <math>\lambda</math></b>	<b>Emission range</b>
FAM	80,000 (490 nm) (Lumiprobe)	488 nm	503 - 660 nm
Cbl-FAM	80,000 (490 nm) (Lumiprobe)	488 nm	503 - 660 nm
Cbl-C6-FAM	80,000 (490 nm) (Lumiprobe)	488 nm	503 - 660 nm
Cbl-1xPEG-FAM	80,000 (490 nm) (Lumiprobe)	488 nm	503 - 660 nm
Cbl-2xPEG-FAM	80,000 (490 nm) (Lumiprobe)	488 nm	503 - 660 nm
Cbl-3xPEG-FAM	80,000 (490 nm) (Lumiprobe)	488 nm	503 - 660 nm
ATTO 488	90,000 (501 nm) (Atto tec)	501 nm	511 - 700 nm
Cbl-C6-ATTO488	90,000 (501 nm) (Atto tec)	501 nm	511 - 700 nm
ATTO 590	120,000 (594 nm) (Atto tec)	594 nm	604 - 820 nm
Cbl-ATTO 590		594 nm	604 - 820 nm
Cbl-C6-ATTO 590	120,000 (594 nm) (Atto tec)	594 nm	604 - 820 nm
Cbl-5xPEG-ATTO 590	120,000 (594 nm) (Atto tec)	594 nm	604 - 820 nm
Cbl-4xGly-ATTO 590	120,000 (594 nm) (Atto tec)	594 nm	604 - 820 nm
ATTO 633	130,000 (629 nm) (Atto tec)	629 nm	639 - 850 nm
Cbl-ATTO 633	130,000 (629 nm) (Atto tec)	629 nm	639 - 850 nm
Cbl-C6-ATTO 633	130,000 (629 nm) (Atto tec)	629 nm	639 - 850 nm
Cy5	271,000 (646 nm) (Lumiprobe)	646 nm	656 - 800 nm
Cbl-Cy5	271,000 (646 nm) (Lumiprobe)	646 nm	656 - 800 nm
Cbl	27,642.26 (361 nm)		

### Supplementary Table 12

Microscope settings for live and fixed fluorescence microscopy. Images were integrated once unless otherwise noted below.

Experiment	pinhole	Laser settings	Notes
Live imaging of A-tagged mRNA, Cbl-Cy5 probe (U2-OS cells)	67.7 $\mu\text{m}$	DAPI: 1.0 (HV = 100) TRITC: 0.4 (HV = 20) Cy5: 12.0 (HV = 100)	4x integration for all channels
Live imaging of A-tagged mRNA, Cbl-4xGly-ATTO 590 probe (U2-OS cells)	67.7 $\mu\text{m}$	DAPI: 1.0 (HV = 100) TRITC: 1.0 (HV = 80) Cy5: 5.0 (HV = 100)	
Correlative imaging (U2-OS cells, live)	67.7 $\mu\text{m}$	GFP: 1.0 (HV = 40) Cy5: 7.0 (HV = 110)	
Correlative imaging (fixed)	26.8 $\mu\text{m}$	GFP: 2.0 (HV = 40) TRITC: 1.0 (HV = 40)	40x Plan Apo Air objective, Nyquist sampling at 0.16 $\mu\text{m}$ per pixel
Live imaging of 2xBroccoli-tagged mRNA (U2-OS cells)	67.7 $\mu\text{m}$	DAPI: 5.0 (HV = 110) GFP: 1.0 (HV = 110) TRITC: 1.0 (HV = 40)	
	67.7 $\mu\text{m}$	DAPI: 5.0 (HV = 110) GFP: 1.0-2.5 (HV = 60-100) TRITC: 0.9 (HV = 40)	GFP settings adjusted for optimal contrast without detector saturation
FISH on fixed cells, exogenously produced RNA	63.9 $\mu\text{m}$	GFP: 1.5 (HV = 80) TRITC: 2.0 (HV = 110) Cy5: 6.0 (HV = 110)	

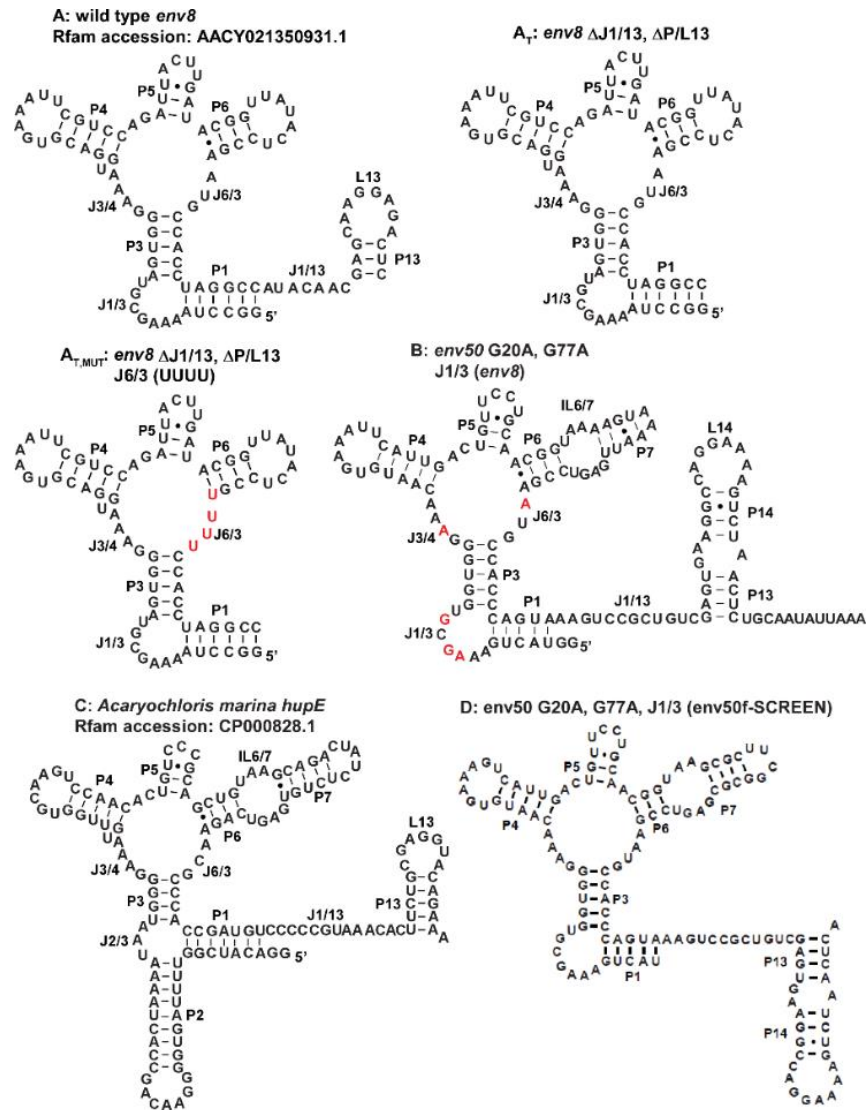
FISH on fixed cells, endogenous ACTB mRNA	58.7 $\mu\text{m}$	GFP: 0.4 (HV = 40) Cy5: 12.0 (HV = 120)	16x integration for Cy5 channel
Immunofluorescence / FISH on fixed cells, endogenous SMN / DDX20 and U1 RNA	49.8 $\mu\text{m}$	DAPI: 2.0 (HV = 90) TRITC: 0.3 (HV = 40) for SMN / 1.0 (HV = 40) for DDX20 Cy5: 10.0 (HV = 120)	
Immunofluorescence / FISH on fixed cells, cotransfected GFP-SMN and A <sub>T</sub> -U1 RNA	49.8 $\mu\text{m}$	GFP: 0.3 (HV = 25) Cy5: 10.0 (HV = 120)	
Immunofluorescence / FISH on fixed cells, transfected EGFP-Coilin and U1 RNA	0.8 AU	DAPI: 1.0 (HV = 100) GFP: 2.0 (HV = 40) Cy5: 4.0 (HV = 110)	
Live imaging of A <sub>T</sub> - tagged U1 RNA, (HeLa cells)	51.1 $\mu\text{m}$	GFP: 0.5 (HV = 50) TRITC: 2-5 (HV = 50-80)	TRITC settings adjusted for optimal contrast without detector saturation
Live imaging of GFP- SMN, with or without cotransfected A <sub>T</sub> -tagged U1 RNA, (HeLa cells)	51.1 $\mu\text{m}$	GFP: 0.5 (HV = 30-50) TRITC: 2 (HV = 50)	GFP settings adjusted for optimal contrast without detector saturation

**Supplementary Table 13**

Sequence and properties of DNA oligos used in FISH.

<b>Name</b>	<b>Sequence</b>	<b>Melting temperature (IDT)</b>	
ACTB-FISH-Cy5	5'-Cy5-CAC AGC TTC TCC TTA ATG TCA CGC ACG ATT TCC CGC TCG GCC GTG- 3'	71.1°C	300 ng
A <sub>T</sub> -FISH-Cy5	5'-Cy5-CCT AGG TGG CAT TCG GAG TAT AAC CGT ATC AAG TAA TCT G-3'	63.3°C	200-300 ng
A <sub>T</sub> -FISH- Alexa546	5'-Alexa546-CCT AGG TGG CAT TCG GAG TAT AAC CGT ATC AAG TAA TCT G-3'	63.3°C	300 ng
Broccoli-FISH- Cy5	5'-Cy5-TTG CCA TGA ATG ATC CAG CCC ACA CTC-3'	62.8°C	300 ng
MS2SL-FISH-Cy5	5'-Cy5-GTT TAA ACG AAT TCG CCC TTA GAT CTG ATG AAC CCT GG-3'	63.5°C	300 ng
U1-FISH-Cy5	5'-Cy5-TCA GCA CAT CCG GAG TGC AAT GGA TAA GCC TCG CCC TGG GAA AA-3'	71.3°C	200-300 ng

## Supplementary Figures

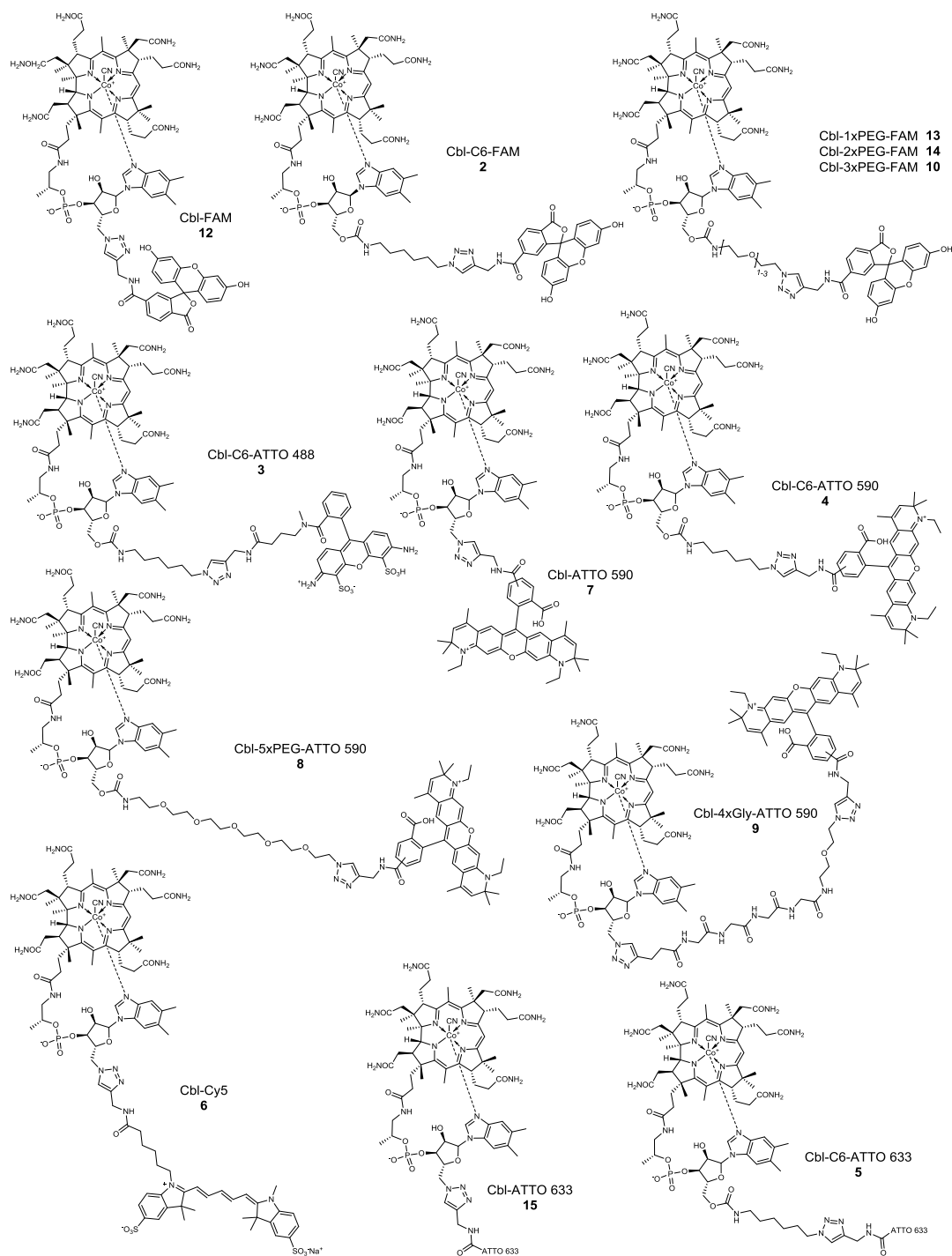


Secondary structures of RNAs used in this study with key structural regions denoted as P (paired), J (junction), L (loop), and IL (internal loop). Naturally derived sequences are shown with accompanying Rfam accession numbers, and the secondary structure of wild type *env8* (variant A) is based on crystallographic data (Johnson *et al.*, *Nature* 492, 133–7, 2012).

Nucleotides that are colored red in variant A<sub>T,MUT</sub> represent point mutations made to the binding core of wild type *env8* that abrogate cobalamin binding. Nucleotides that are colored red in variant B represent point mutations derived from wild type *env8* that have been shown to

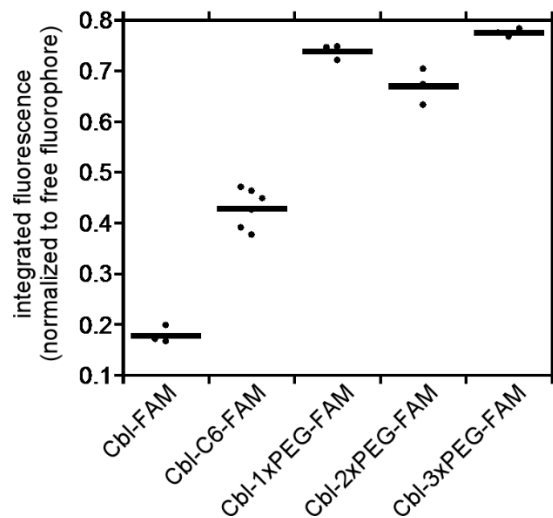


increase the affinity of this RNA (Polaski *et al.*, *J. Biol. Chem.* 292, 11650–11658, 2017) for forms of cobalamin similar to the conjugates used in this study. Features that induce bulkiness of the RNA include P13 for variant A, P7, P13, P14 for variant B and P7, P2, P13 for variant C. Variant D is an L4 and P7-optimized variant B that improved cobalamin affinity (data not published prior to this study, see also Supplementary Fig. 7, Supplementary Table 7).



## Supplementary Figure 2

Chemical structures of Cbl-fluorophore probes used in this study. Note that ATTO 590 is a mixture of the para and meta isomer and that the chemical structure of the ATTO 633 dye is proprietary and unknown.



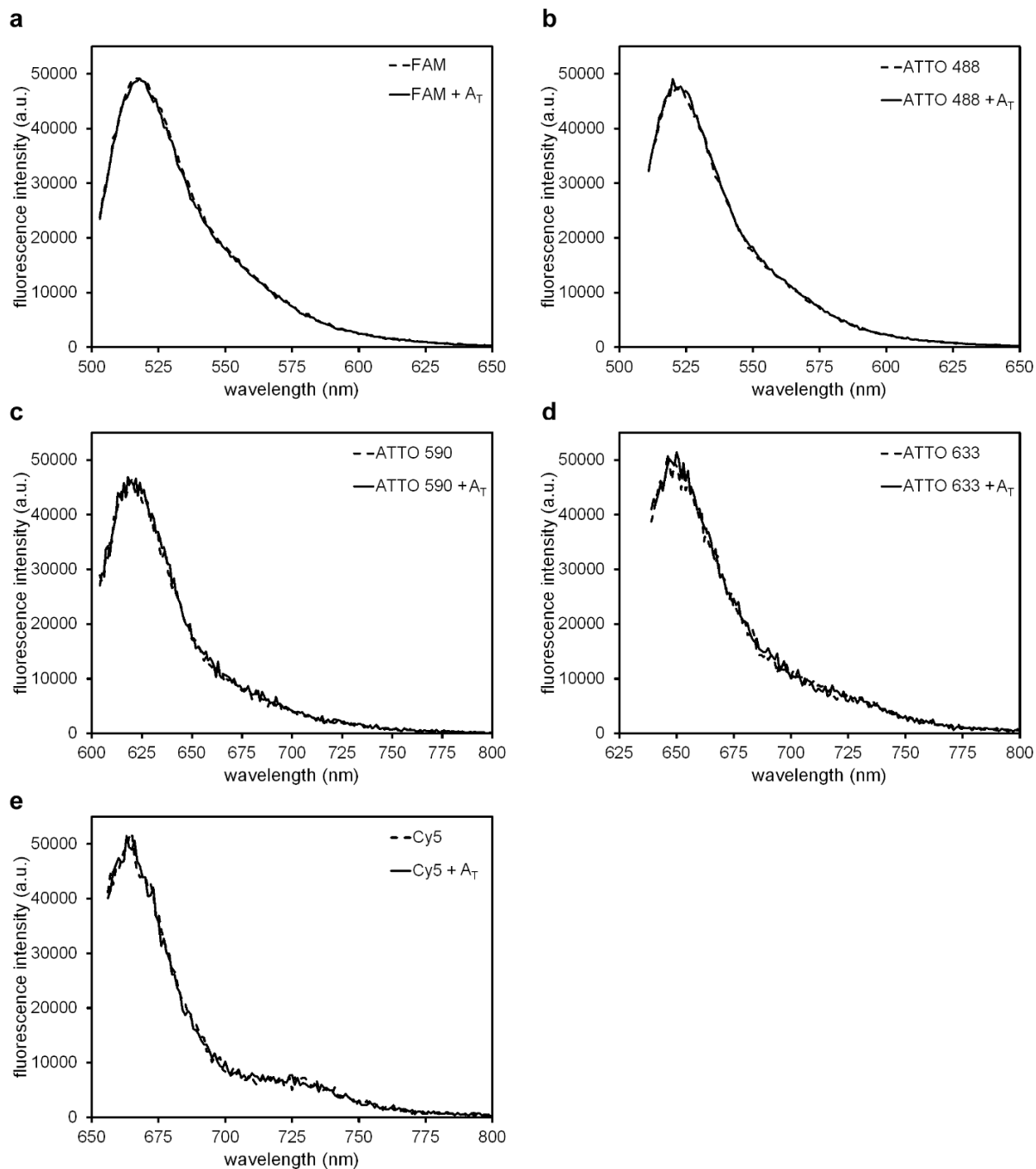
### Supplementary Figure 3

Comparison of residual fluorescence for Cbl-FAM probes with varied linkers. The fluorescence was quantified and compared to the signal of the free fluorophore at the same concentration as in Figure 1d. The mean for n=3 independent measurements for all probes (except n=6 for Cbl-C6-FAM) is shown.

#### **Supplementary Figure 4**

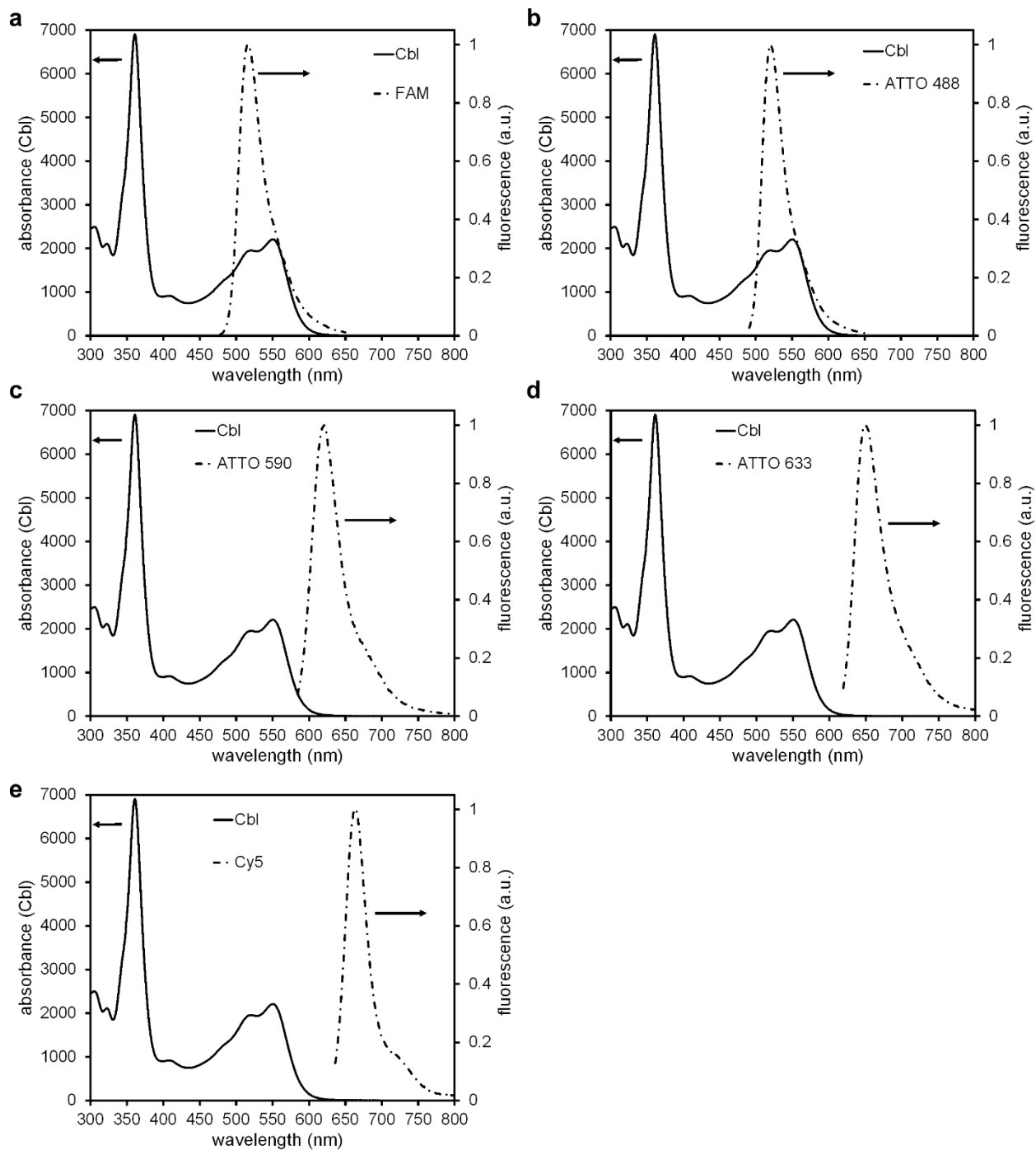
Representative fluorescence spectra of Cbl-fluorophore probes in the presence and absence of RNAs used in this study (see also Supplementary Table 11 for photophysical properties).

Spectra show an increase in fluorescence intensity upon binding RNAs A, A<sub>T</sub>, B, or C but not the non-binding variant A<sub>T,MUT</sub>. Repeated measurements of spectra shown here were used to generate the bar graphs presented in Fig. 2 (see legend for Fig. 2 for n values).



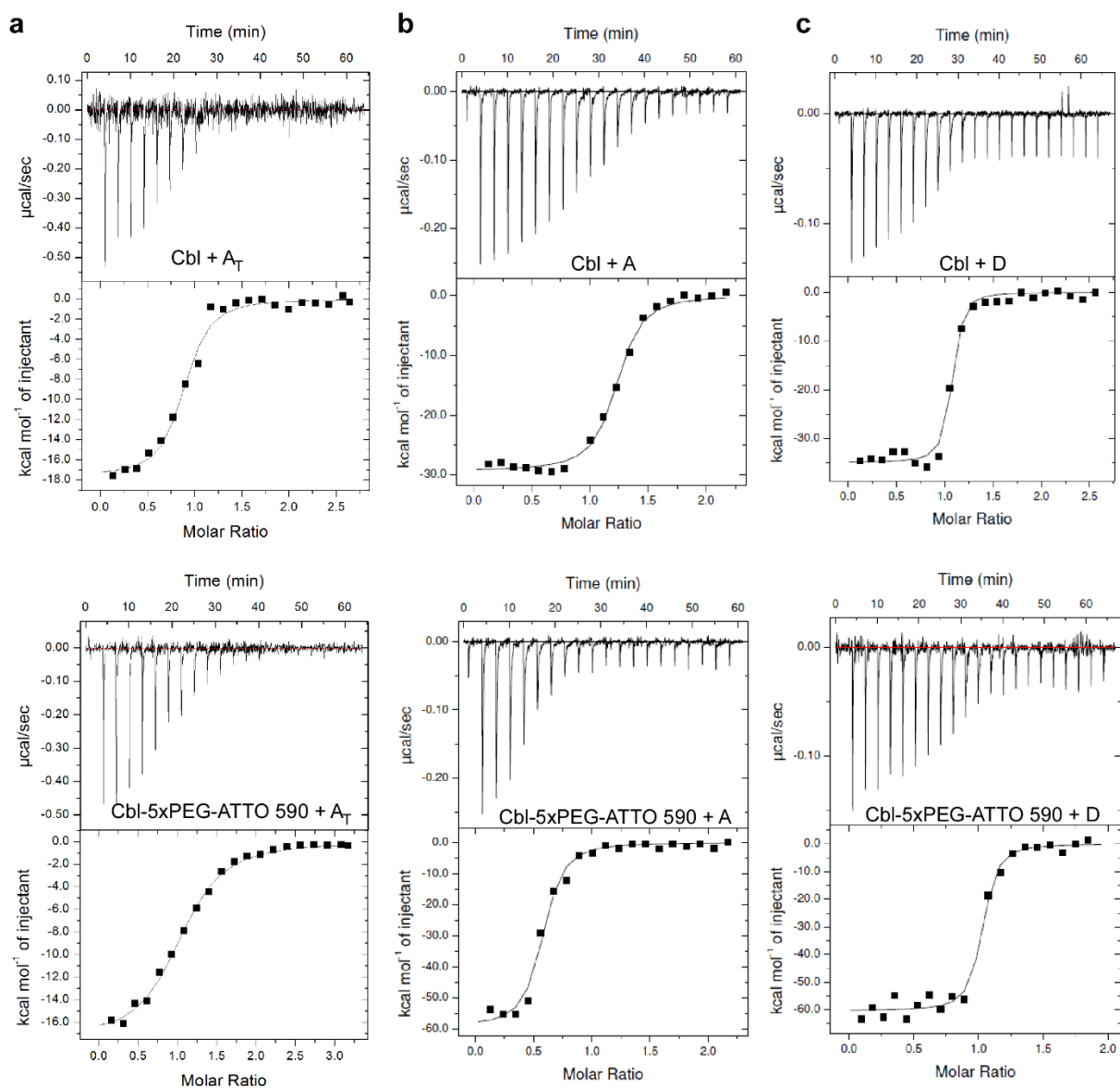
### Supplementary Figure 5

Representative fluorescence spectra of free fluorophores used in this study in the presence and absence of RNA variant  $A_T$ . Spectra reveal no change in fluorescence intensity of free fluorophores in the presence of  $A_T$ . Repeated measurements of spectra shown here were used to generate the bar graphs presented in Fig. 1d (see legend for Fig. 1d for n values).



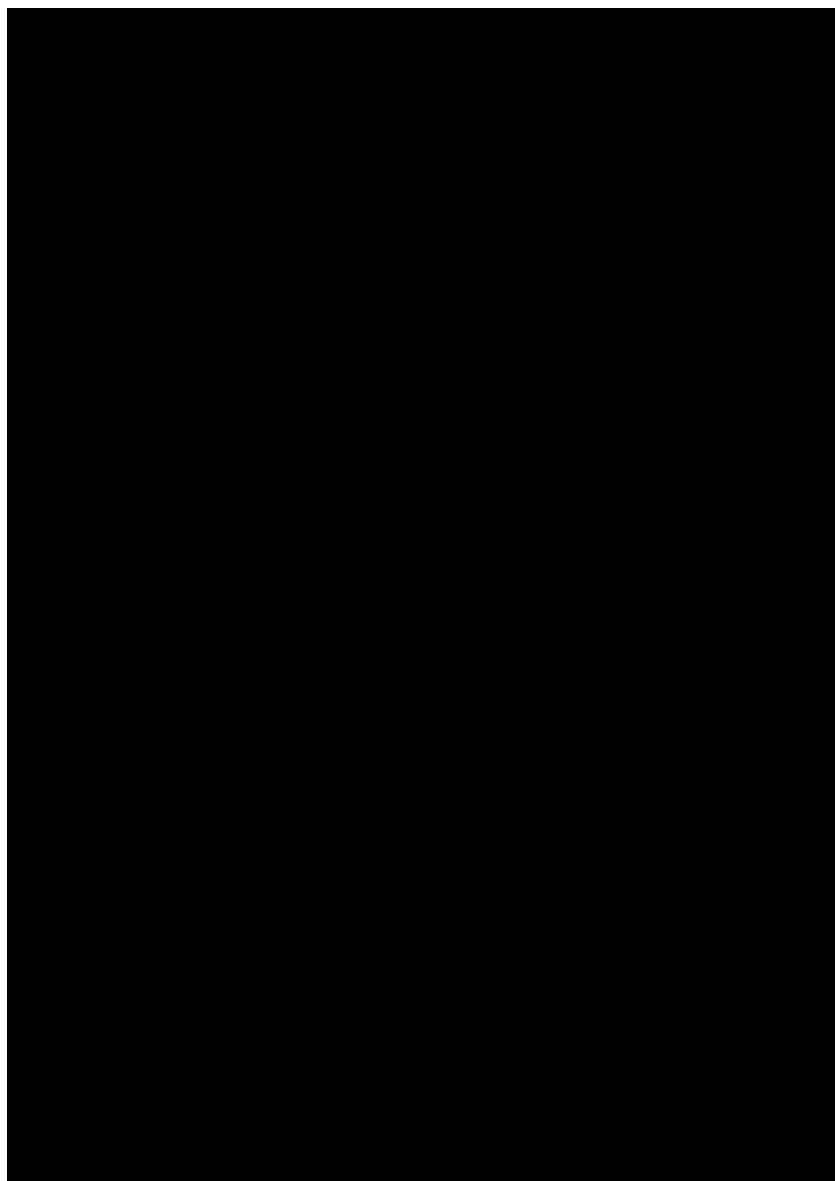
### Supplementary Figure 6

Cbl absorbance spectra and fluorescence emission spectra of fluorophores to calculate the overlap integral  $J(\lambda)$ . Arrows assign y axis for the indicated spectra.



### Supplementary Figure 7

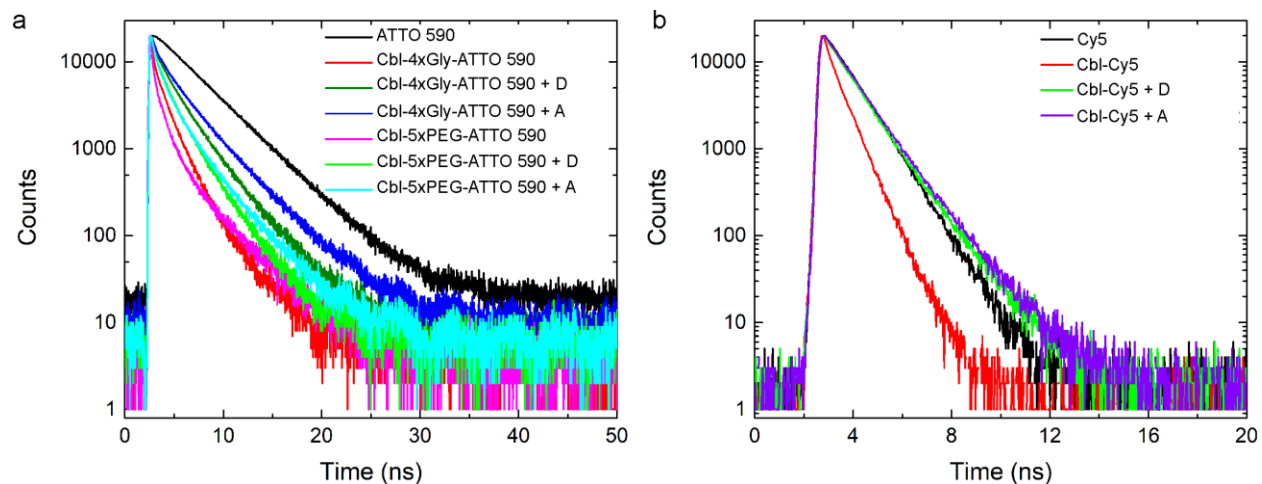
RNA variants A and D (panels b and c), but not  $A_T$  (panel a), bind to the probe Cbl-5xPEG-ATTO 590 with a dissociation constant ( $K_D$ ) in the nM range. Representative isothermal titration calorimetry thermograms of the RNA binding to Cbl (top) and Cbl-5xPEG-ATTO 590 (bottom) are presented.  $K_D$  values as the mean of  $n=3$  independent experiments  $\pm$  STDEV are listed in Supplementary Table 7.



### **Supplementary Figure 8**

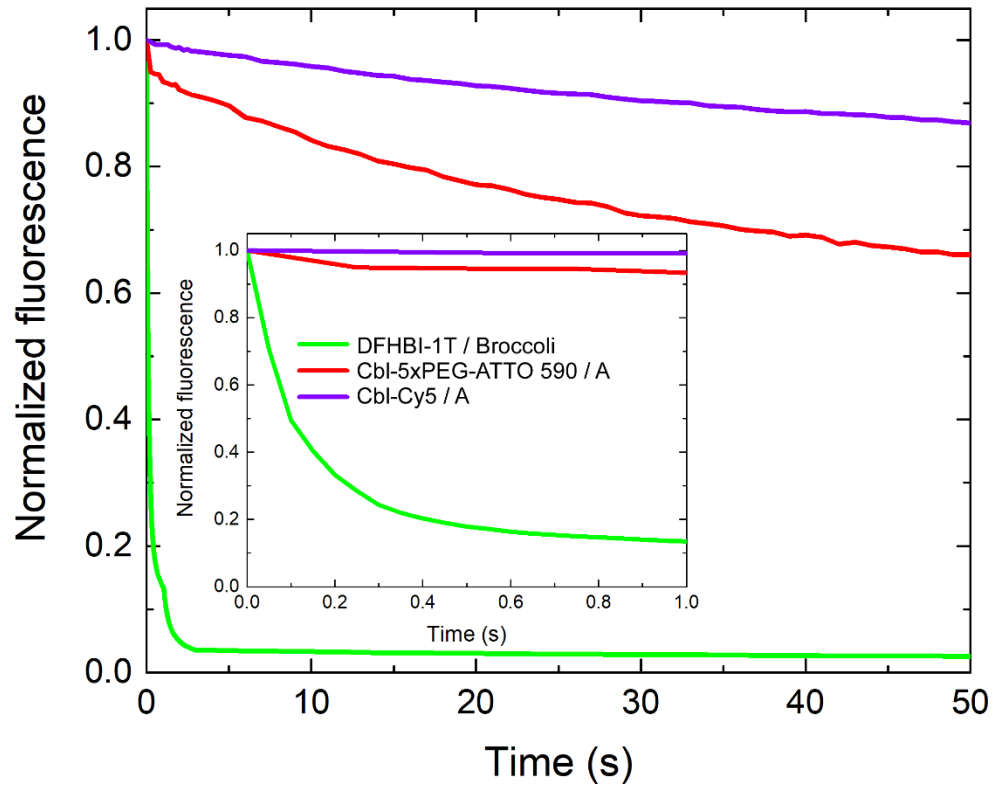
Determination of the quantum yield for different Cbl-fluorophore probes in the presence and absence of various RNAs. (a) The quantum yield for ATTO 590 conjugates Cbl-5xPEG-ATTO 590 and Cbl-4xGly-ATTO590 in the presence and absence of variants A and D was measured using ATTO 590 as a reference. (b) The quantum yield for Cbl-Cy5 in the presence and absence of variants A and D was measured using Cy5 as a reference. The absorbance and integrated fluorescence sample was measured for samples of a dilution series of the indicated samples. See Supplementary Table 8 for a summary of quantum yield results.



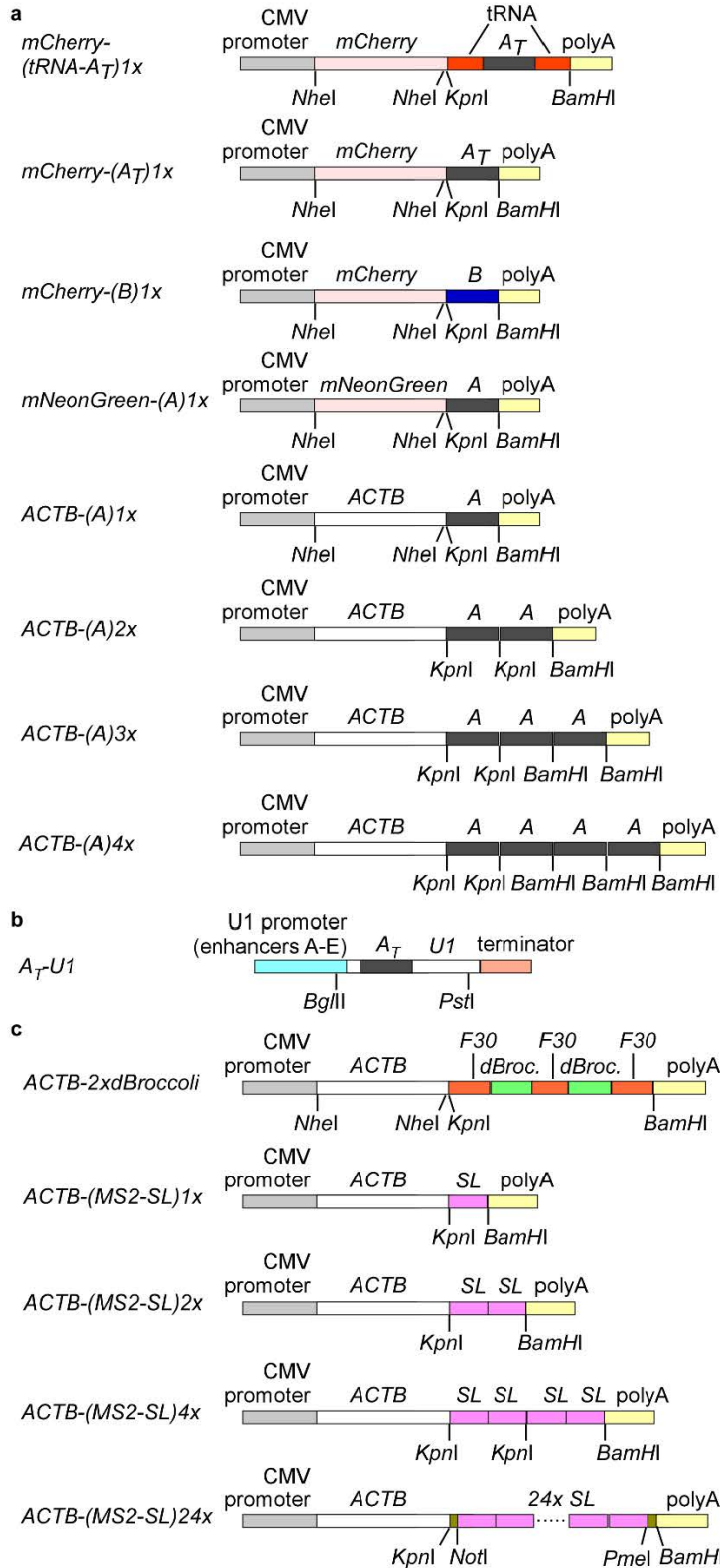


### Supplementary Figure 9

Measurement of fluorescence lifetime of Cbl-fluorophore probes in the presence and absence of RNA (n=1 experiment per condition). (a) The fluorescence lifetime of 0.5  $\mu\text{M}$  Cbl-4xGly-ATTO 590 and Cbl-5xPEG-ATTO 590 in the presence and absence of 5  $\mu\text{M}$  of RNA variants A or D was measured in comparison to 0.5  $\mu\text{M}$  free ATTO 590. (b) The fluorescence lifetime of 0.5  $\mu\text{M}$  Cbl-Cy5 in the presence and absence of 5  $\mu\text{M}$  of RNA variant A or D was measured in comparison to 0.5  $\mu\text{M}$  free Cy5. See Supplementary Table 9 for a summary of lifetime results.

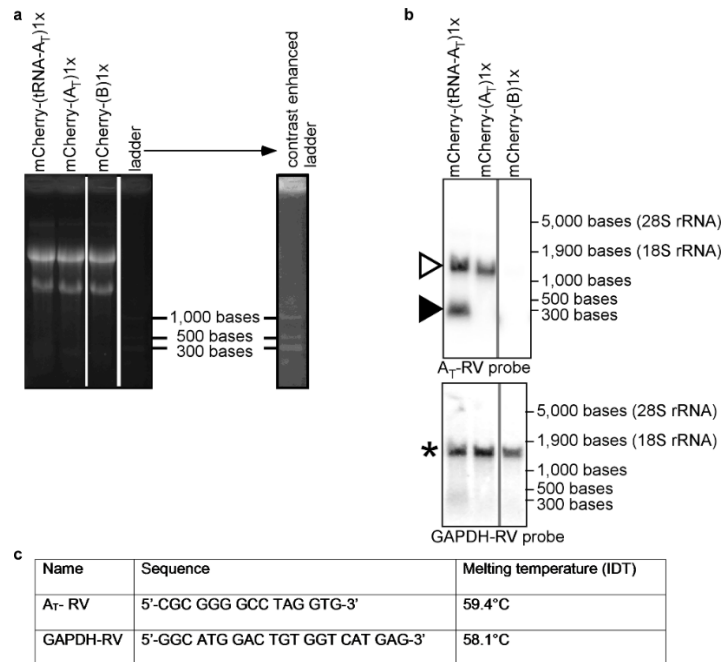


Supplementary Figure 10



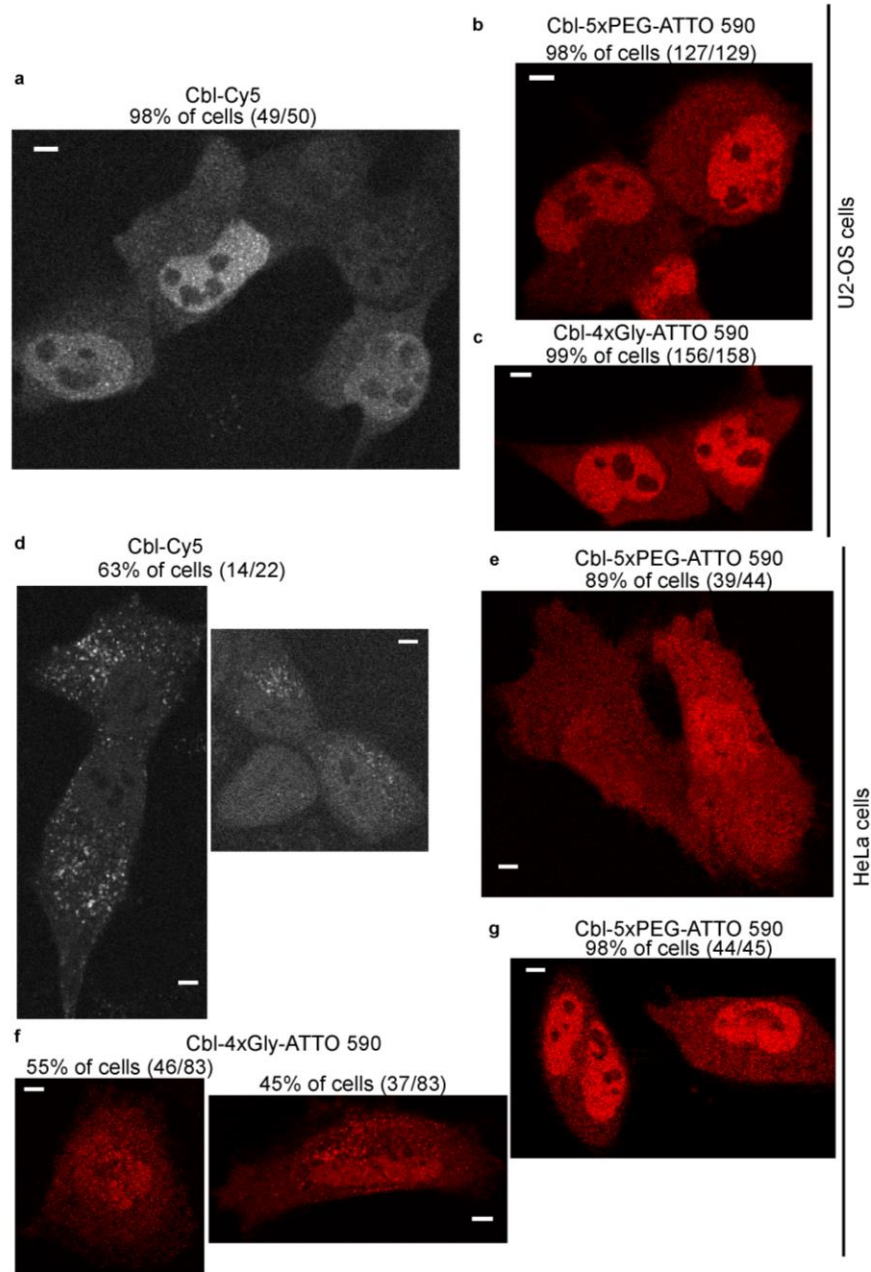
### Supplementary Figure 11

Plasmid maps of RNA fusion constructs used in this study.



### Supplementary Figure 12

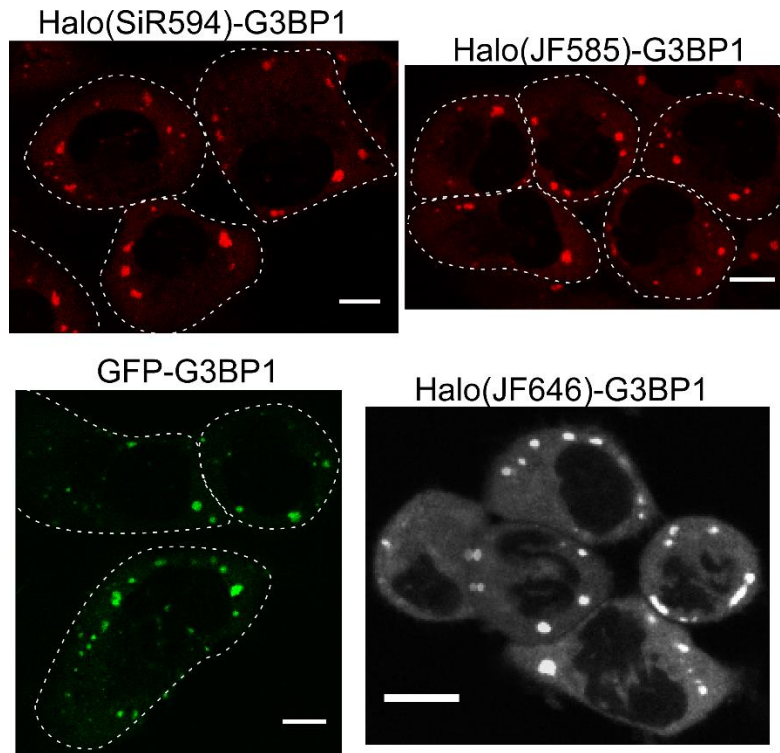
mRNA can be tagged with RNA variant A<sub>T</sub> without unwanted processing. HEK 293T cells were transiently transfected with plasmid DNA where the A<sub>T</sub> or B tag was genetically fused to a reporter mRNA (encoding mCherry). The tag A<sub>T</sub> was produced with or without the tRNA folding scaffold. (a) Total RNA was separated by agarose gel electrophoresis. The 28S and 18S rRNA bands across samples serve as loading controls and indicate that no unwanted RNA processing occurred during RNA preparation. Non-consecutive lanes of the same gel are indicated by vertical lines. Contrast settings were identical for all parts of the gel. A contrast enhanced version of the lane with the RNA ladder is shown as a reference. (b) Northern blot probed against A<sub>T</sub> (top panel) indicates that the full length mRNA (open triangle) is processed when produced with the tRNA folding scaffold (filled triangle). The blot was stripped and probed for GAPDH mRNA (star in bottom panel). Non-consecutive lanes of the same blot are indicated by vertical lines. No changes were made to contrast settings after cropping lanes. (c) Properties of oligonucleotides from (b) (purchased as DNA oligos from IDT). The tRNA processing phenotype (panels a and b) was reproducible for two independent experiments.



### Supplementary Figure 13

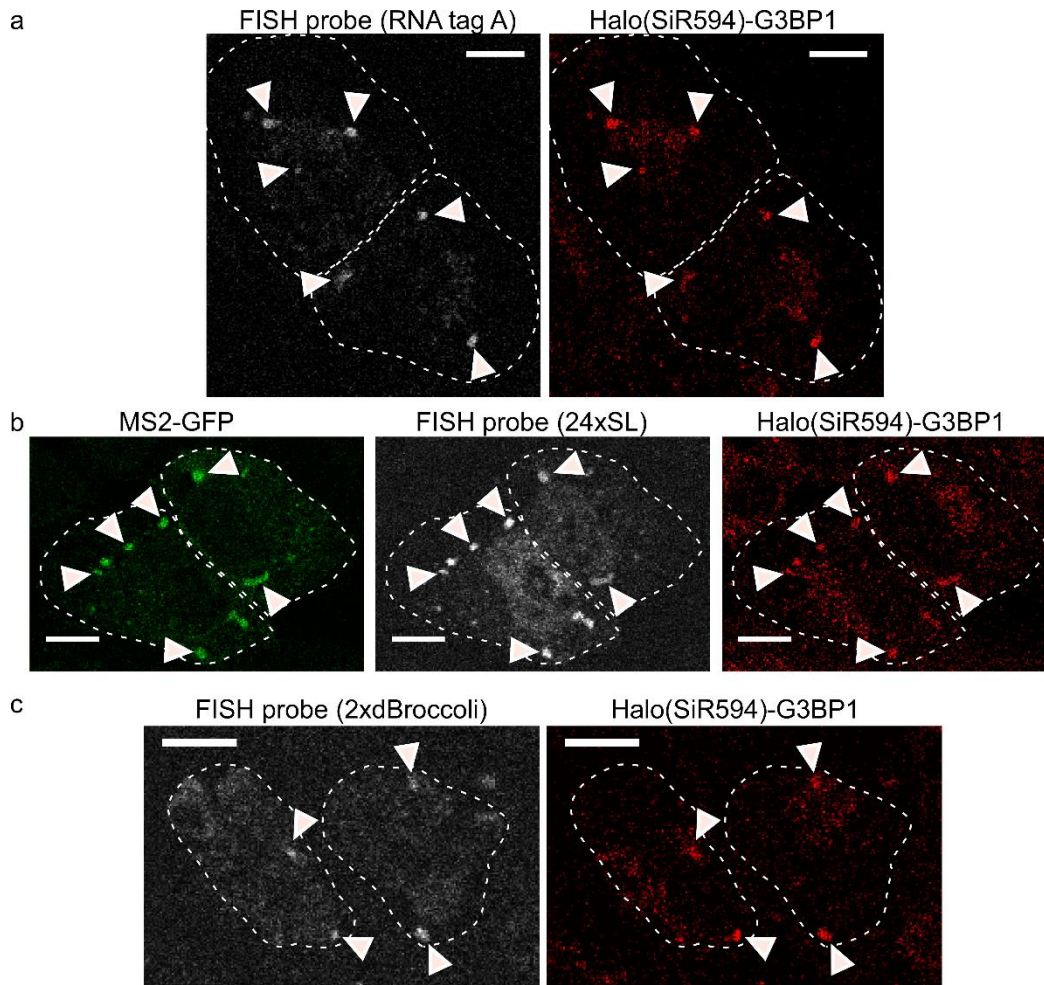
Residual fluorescence of Cbl-fluorophore probes reports on probe localization in live cells upon bead loading. (a) Bead loading of Cbl-Cy5 in U2-OS Halo-G3BP1 cells results in diffuse cytosolic and nuclear probe localization with negligible probe aggregation (1 experiment, 50 cells). (b) Cbl-5xPEG-ATTO 590 loaded in U2-OS Halo-G3BP1 cells localizes diffusely in the cytosol and nucleus (1 experiment, 129 cells). (c) Cbl-4xGly-ATTO 590 localizes diffusely in the

cytosol and nucleus of U2-OS Halo-G3BP1 cells (1 experiment, 158 cells). (d) Bead loading of Cbl-Cy5 in HeLa cells results in substantial localization of the Cbl-Cy5 probe in puncta (63% of cells, 1 experiment, 22 cells). (e) Cbl-5xPEG-ATTO 590 loaded in HeLa cells is largely localized diffusely in the nucleus and cytosol (89% of cells, 1 experiment, 44 cells). (f) In about half of HeLa cells loaded with Cbl-4xGly-ATTO 590 the probe localized in puncta in the cytosol (45% of cells, 1 experiment, 83 cells). (g) Increasing the concentration of Cbl-5xPEG-ATTO 590 when loading HeLa cells does not alter diffuse cytosolic and nuclear localization of the probe (1 experiment, 45 cells). Compare localization of loading 50  $\mu$ M probe in (g) versus 0.5  $\mu$ M probe in (e). Scale bar = 5  $\mu$ m.



#### Supplementary Figure 14

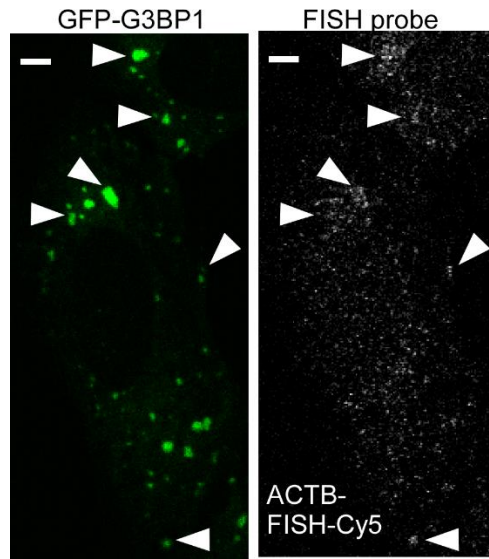
Stress granule (SG) visualization by Halo-tag or GFP in U2-OS cells. Top panel: U2-OS cells producing Halo-tagged G3BP1 from the chromosome were treated with two different red fluorescent Halo dyes (SiR594, 1 experiment, 234 cells; and JF585, 1 experiment, 253 cells). Arsenite stress induced recruitment of Halo-G3BP1 to SGs. Bottom panel, left: U2-OS cells stably producing GFP-G3BP1 were treated with arsenite to induce SGs (1 experiment, 14 cells). Bottom panel, right: U2-OS cells producing Halo-tagged G3BP1 from the chromosome were treated with a far red fluorescent Halo dye (JF646, 1 experiment, 294 cells). Scale bar = 10  $\mu$ m.



### Supplementary Figure 15

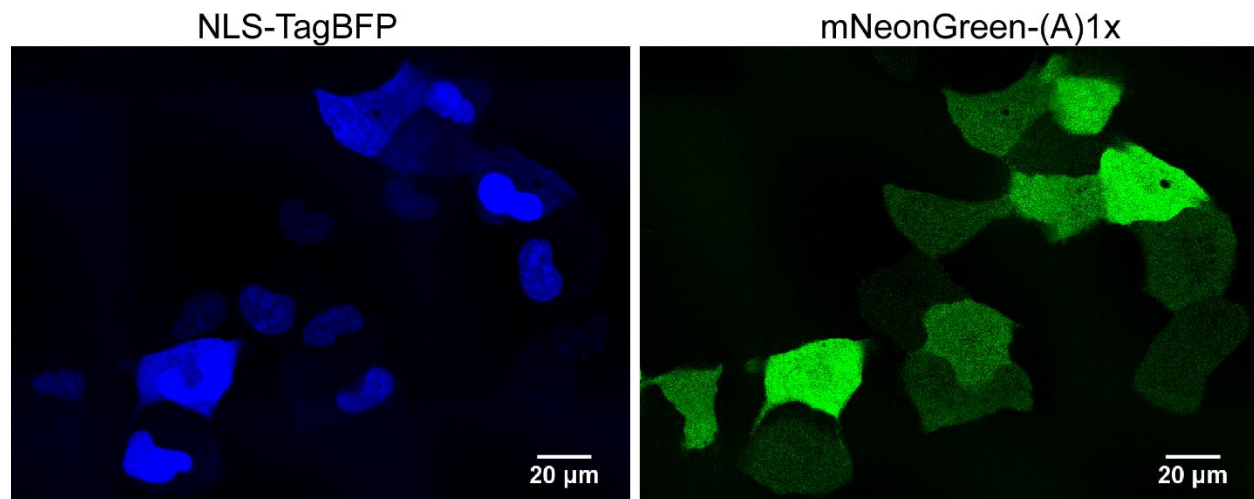
ACTB mRNA fused with different RNA visualization tags localizes to stress granules (SG). Plasmids encoding for ACTB tagged with RNA visualization tags at the 3' end were transfected in U2-OS Halo-G3BP1 cells. G3BP1 was labeled with red fluorescent SiR594 dye 24 h post transfection and SGs were induced by treatment with arsenite for 30-45 min. Cells were fixed, permeabilized and the RNA tag was probed with Cy5-fluorescently labeled oligos against the indicated RNA tag. (a) Transfection with ACTB-(A)4x (1 experiment, 15 cells) (FISH probe: A<sub>T</sub>-FISH-Cy5); (b) transfection with ACTB-(MS2-SL)24x (1 experiment, 8 cells) (FISH probe: MS2SL-FISH-Cy5); (c) transfection with ACTB-2xdBroccoli (1 experiment, 13 cells) (FISH probe: Broccoli-FISH-Cy5). Scale bar = 10 μm.





### Supplementary Figure 16

Endogenous ACTB mRNA colocalizes with GFP-G3BP1, a marker protein for SGs in U2-OS cells. Detection of endogenous ACTB mRNA in U2-OS cells that stably produce GFP-G3BP1, a SG marker protein. Cells were fixed, permeabilized and ACTB fusion mRNA was visualized by FISH using a Cy5-conjugated probe (FISH probe: ACTB-FISH-Cy5). Representative cells show localization of ACTB mRNA to SGs (1 experiment, 15 cells). Scale bar = 5  $\mu$ m.

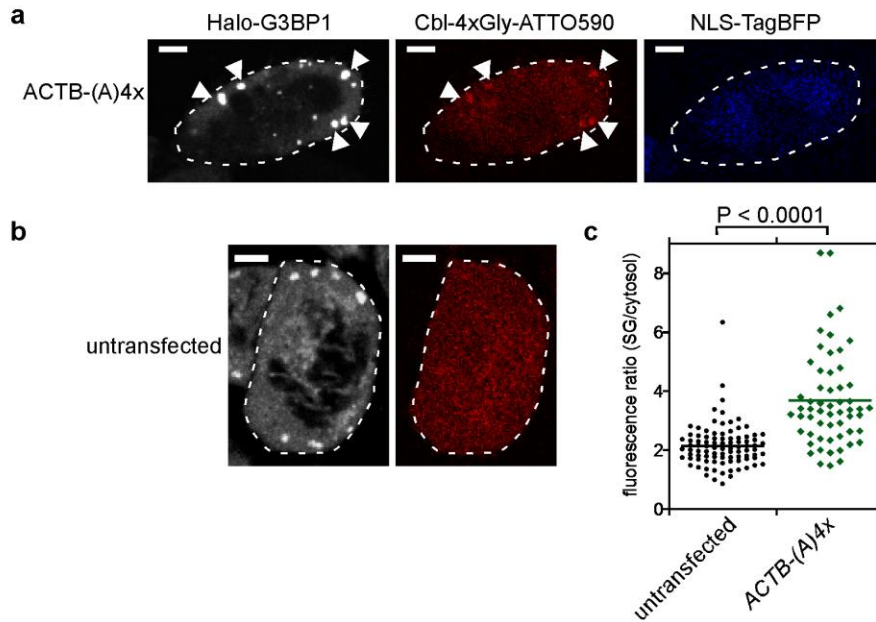


### Supplementary Figure 17

Transfecting U2-OS cells with a plasmid encoding a nuclear localized blue fluorescent protein (NLS-TagBFP) serves as a transfection marker for mRNA-tagged plasmids. U2-OS cells were transfected with a 1:1 mixture of NLS-TagBFP and mNeonGreen-(A)1x and the fluorescence signal was analyzed 24 h post transfection. Each cell harboring the blue NLS-TagBFP transfection marker was assessed for presence of green fluorescence as a measure for successful transfection of mNeonGreen mRNA tagged with riboswitch variant A. 94% of cells with NLS-TagBFP also have mNeonGreen signal (2 independent experiments, 561 cells).

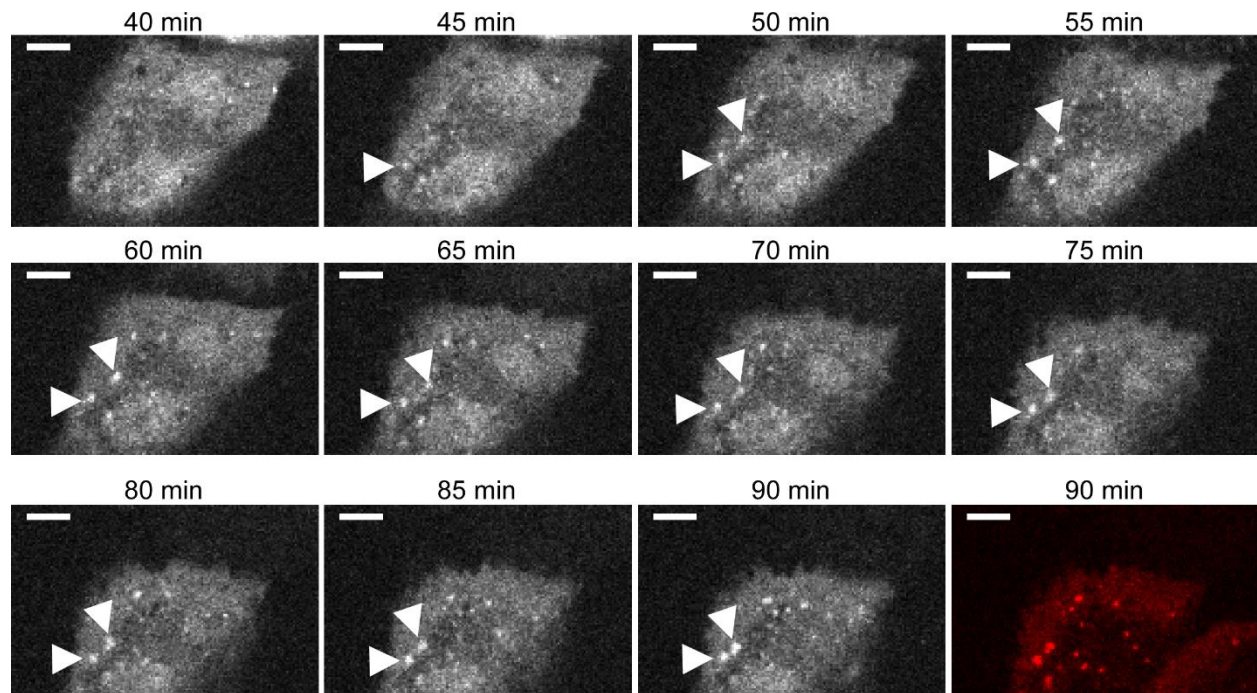
### **Supplementary Figure 18**

Analysis of live U2-OS cells to quantify Cbl-fluorophore probe fluorescence in stress granules (SG). (a) SGs were identified in the Halo-G3BP1 channel via labeling of G3BP1 with JF585 and a line trace was drawn through the SG including cytosolic fluorescence near the SG. The same signal trace was recorded in the probe fluorescence channel (shown here for Cbl-Cy5). (b) After background subtraction, the Cbl-fluorophore probe fluorescence trace as well as the control Halo-G3BP1 trace were plotted. The maximum fluorescence signal for the Cbl-fluorophore probe was determined and divided by the average probe fluorescence in the cytosol. Shown is the analysis to calculate the ratio for SG/cytosol for one data point for condition ACTB-(A)4x presented in Fig. 3b and 3e (3 independent experiments, 16 cells, 74 SGs).



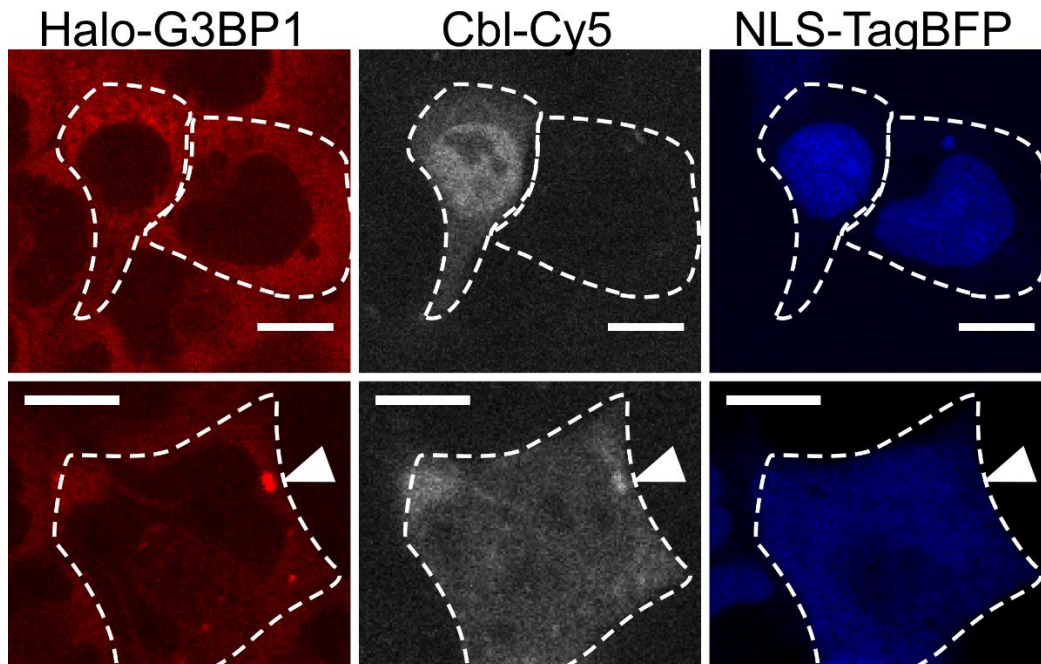
### Supplementary Figure 19

Cbl-4xGly-ATTO590 fluorescence signal increased in SGs in cells that were transiently transfected with ACTB-(A)4x. (a) U2-OS cells producing Halo-G3BP1 were transfected with ACTB-(A)4x and the transfection marker TagBFP. 24 h post transfection, cells were stained with the JF646 Halo dye. The probe Cbl-4xGly-ATTO590 was introduced into cells by bead loading, SG formation was induced by treatment with 0.5 mM arsenite for 45 min, followed by live cell microscopy (2 experiments, 22 cells, 59 SGs). In 64% of the cells at least one SG was detectable and 58% of all SGs were detected in the Cbl-4xGly-ATTO 590 channel. (b) The same experiment as in (a) was performed, except that ACTB-(A)4x was not transfected (2 experiments, 37 cells, 92 SGs). In 0% of the cells at least one SG was detectable and 0% of all SGs were detected in the Cbl-4xGly-ATTO 590 channel. (c) Fluorescence increase for Cbl-4xGly-ATTO590 in SGs was quantified by collecting a line trace through each SG (identified in the JF646 channel) and calculating the ratio of the highest signal in the SG over the average signal in the cytosol for datasets from panels a and b (see Supplementary Fig. 18 for details on ratio calculation, presented is the mean). One way ANOVA (95% confidence limit), post hoc test (Tukey HSD), scale bar = 10  $\mu$ m.



### Supplementary Figure 20

Time course of ACTB mRNA recruitment to stress granules (SG) and dynamics of SGs. U2-OS cells constitutively producing Halo-G3BP1 were transfected with a plasmid to produce ACTB-(A)4x mRNA and a transfection marker (NLS-TagBFP) as in Fig. 3. 24 h post transfection, cells were stained with the JF585 Halo dye, bead loaded with Cbl-Cy5 and treated with 0.5 mM arsenite to induce SGs. Shown are fluorescence images in the Cbl-Cy5 channel (collected in 5 min intervals). Cbl-Cy5 labels ACTB-(A)4x mRNA and formation of SGs is visible at about 45 min post arsenite treatment (white arrows). SGs move throughout the cell and are closer together by 90 min post arsenite treatment (white arrows). Red panel: 90 min time point collected in the red fluorescence channel to visualize Halo-G3BP1 via the JF585 dye (2 experiments, 6 cells). Scale bar = 5  $\mu$ m.



### Supplementary Figure 21

Localization phenotypes for U2-OS cells transfected with tagged mRNA and loaded with Cbl-Cy5 probe in the absence of arsenite stress. U2-OS cells producing Halo-G3BP1 from the genome were transfected with the plasmid ACTB-(A)4x and the transfection marker plasmid NLS-TagBFP as in Fig. 3. Halo-G3BP1 was labeled with JF585 24 h post transfection and Cbl-Cy5 was bead loaded. Shown are representative cells demonstrating two different phenotypes. Top panel: Halo-G3BP1 was diffusely localized in the cytosol and Cbl-Cy5 displayed nuclear and cytosolic localization as in the untransfected control Fig. 3d). Bottom panel: In some cells, stress granules (SGs) formed in the absence of arsenite treatment, as indicated by the signal in the red Halo-G3BP1 channel. The process of transfection has been shown to lead to formation of SGs in a small number of cells. In the cell shown here, Cbl-Cy5 fluorescence colocalized with SGs (white arrow), as expected due to recruitment of ACTB tagged with the RNA tag variant A to SGs. 2 experiments, 20 cells positive for Cbl-Cy5 and NLS-TagBFP (12 cells without SGs in red channel, 8 cells with SGs in red channel). Scale bar = 10  $\mu$ m.

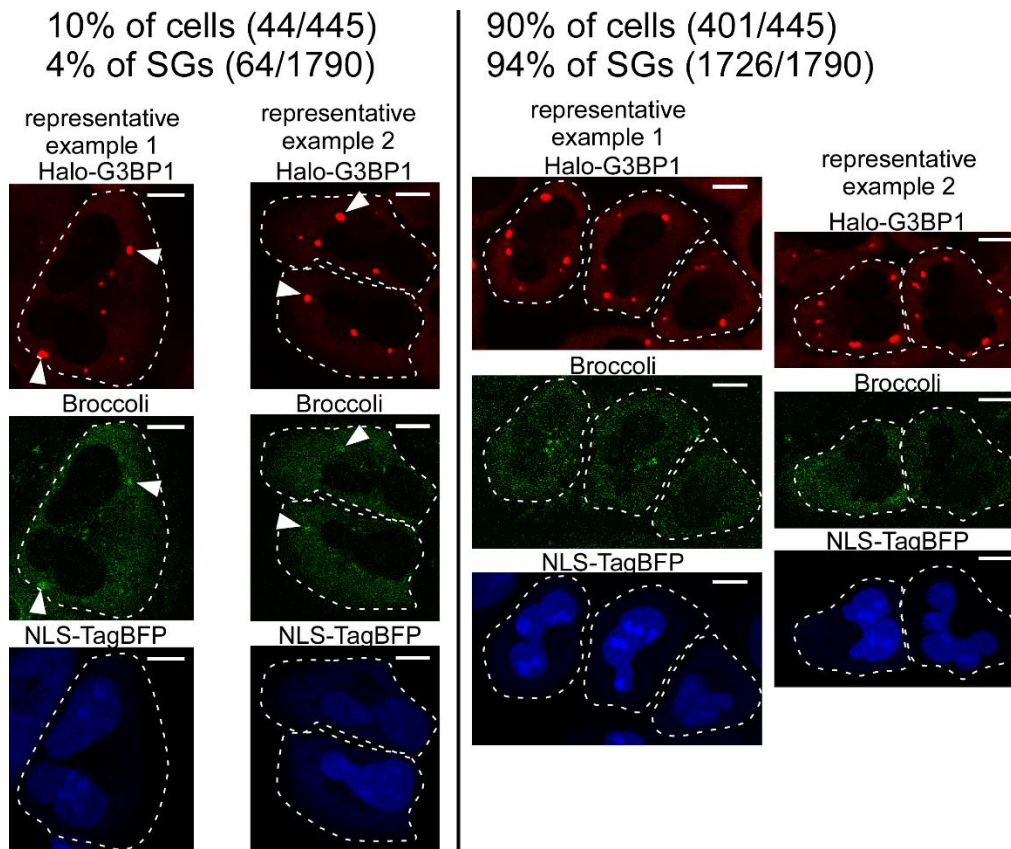
### **Supplementary Figure 22**

Correlative fluorescence microscopy of live (a) and fixed cells (b) confirms colocalization of ACTB mRNA to SGs. U2-OS cells that stably produce GFP-G3BP1 were transfected with a plasmid encoding for ACTB-(A<sub>T</sub>)<sub>4x</sub>. 24 h post transfection, the probe Cbl-Cy5 was loaded and SGs were induced by arsenite treatment. After live imaging, cells were fixed, permeabilized and probed with a FISH probe against the A<sub>T</sub> tag. (a) Fluorescence of Cbl-Cy5 colocalized with SGs. (b) After fixation, localization of the A<sub>T</sub> tag to SGs was directly assessed by a red fluorescent (Alexa546) FISH probe (A<sub>T</sub>-FISH-Alexa546) (2 experiments, 4 cells). Scale bar = 5 μm.

### Supplementary Figure 23

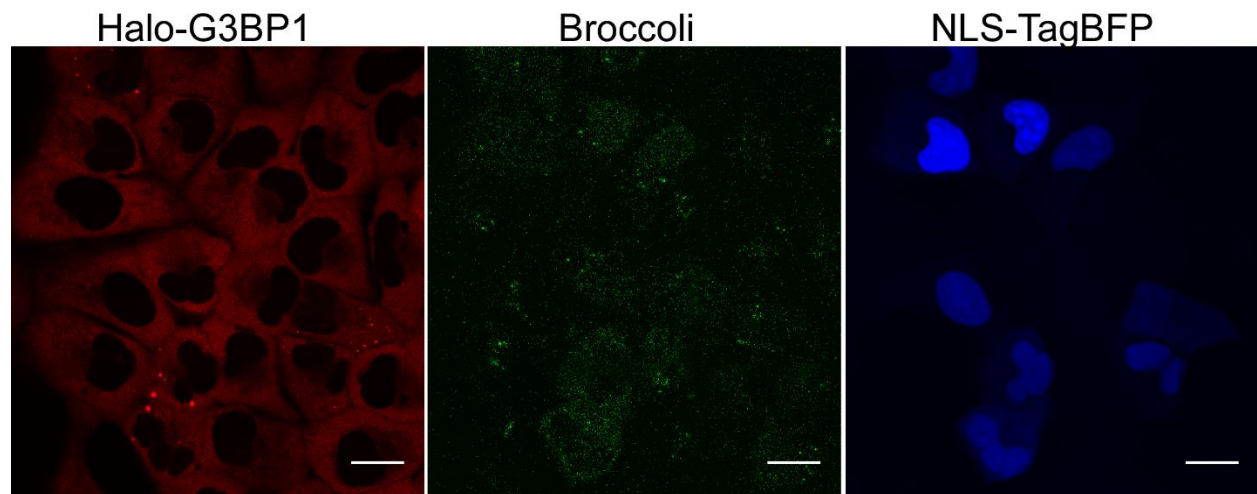
Visualization of Broccoli RNA in HEK 293T cells using published expression platforms and published imaging conditions. Plasmids pAVU6+27-F30-2xdBroccoli and pAV5S-F30-2xdBroccoli were transfected in HEK 293T cells and split into imaging dishes 48 h post transfection following published protocol (Filonov & Jaffrey, *Curr. Prot. Chem. Bio.*, 8, 2016). 24 h later, DFHBI-1T was added at a final concentration of 40  $\mu$ M and cells were imaged under widefield illumination conditions as recommended (Filonov & Jaffrey, *Curr. Prot. Chem. Bio.*, 8, 2016). (a) pAV5S-F30-2xdBroccoli (2 experiments, 14 cells). (b) pAVU6+27-F30-2xdBroccoli. Shown are two representative phenotypes where green fluorescence was either observed diffusely throughout the cell (top) or localized to cytosolic puncta (bottom) (2 experiments, 23 cells). (c) Untransfected control (2 experiments, 32 cells). The brightness and contrast was adjusted to be constant for all images shown. Scale bar = 5  $\mu$ m.





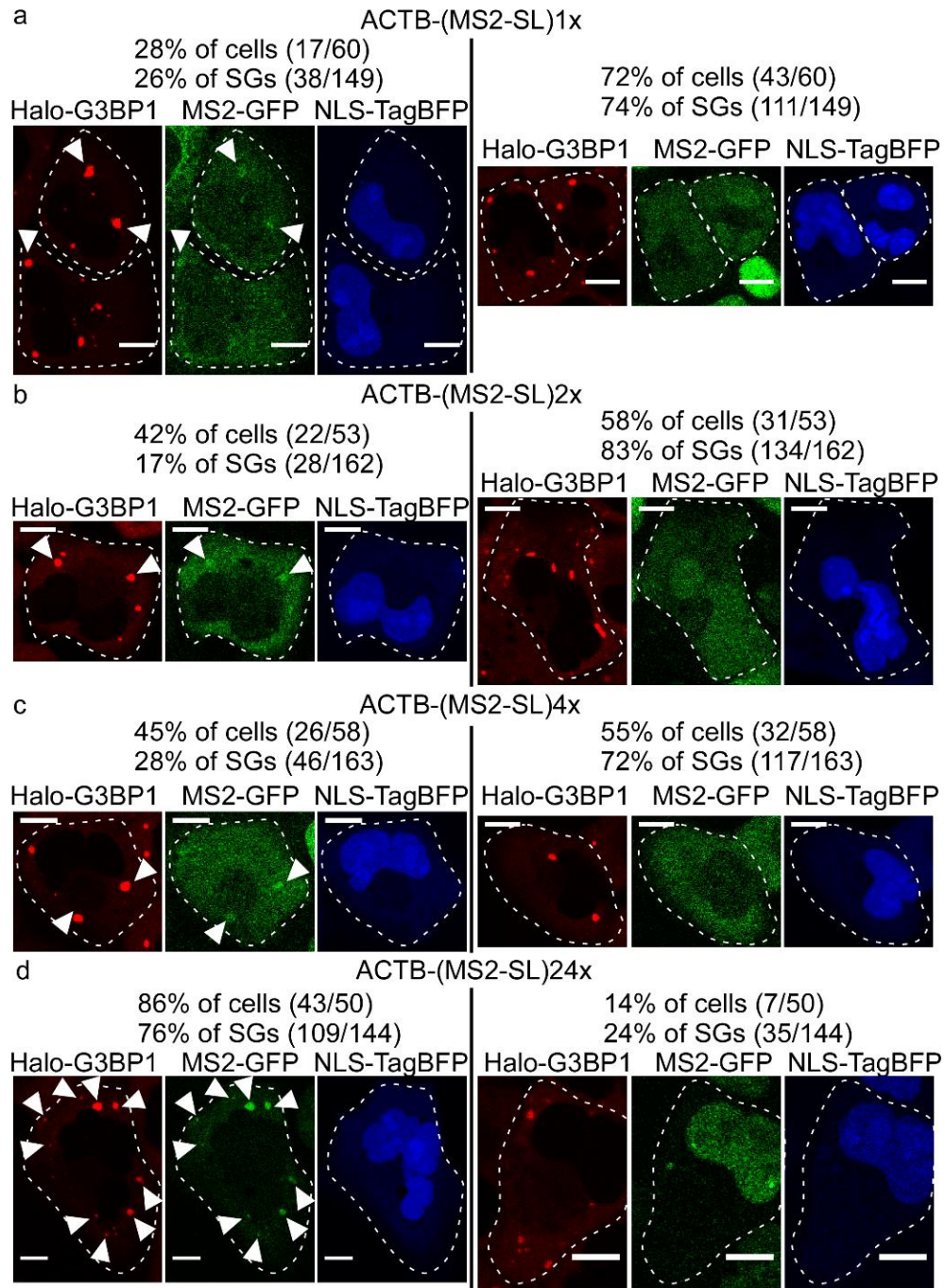
### Supplementary Figure 24

Visualization of ACTB mRNA in stress granules (SG) by RNA tagging with Broccoli. ACTB-(2xdBroccoli) was transfected in U2-OS Halo-G3BP1 cells together with the NLS-TagBFP transfection marker. Halo-G3BP1 was labeled with the red fluorescent JF585 dye and SGs were induced by incubation with arsenite for 30-45 min. SGs were identified via the red fluorescent JF585 signal. After addition of the Broccoli probe DFHBI-1T, all cells positive for the blue transfection marker (445 cells, 3 experiments), were assessed for visible SGs in the green Broccoli channel. In 10% of all cells with TagBFP signal, at least one SG was detected in the green channel and overall, 4% of all SGs identified in red were also detected in the green Broccoli channel (1790 SGs total in all 445 cells). Representative SGs visualized by Broccoli are indicated with white arrows. Scale bar = 10  $\mu$ m.



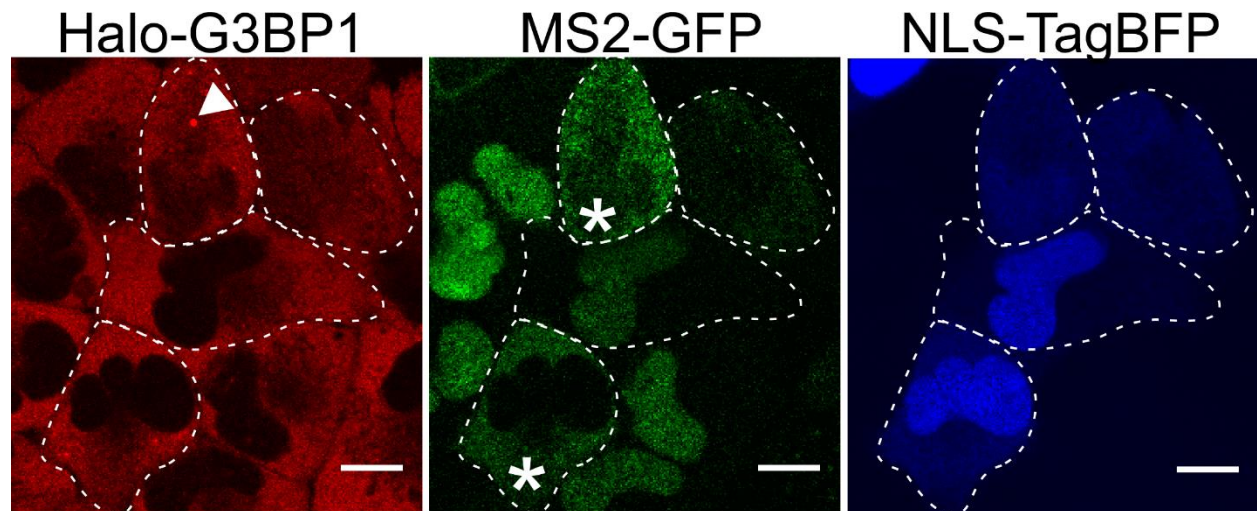
### Supplementary Figure 25

Visualization of Broccoli-tagged ACTB mRNA in the absence of arsenite treatment. Plasmids encoding for ACTB-(2xdBroccoli) and the transfection marker NLS-TagBFP were transfected in U2-OS cells. 24 h after transfection, G3BP1 was labeled with the red fluorophore JF585 and the Broccoli dye DFHBI-1T was added. Shown are representative cells (2 experiments, 798 cells). Scale bar = 20  $\mu\text{m}$ .



Visualization of ACTB mRNA in stress granules (SG) by RNA tagging with MS2 stem-loop (SL) repeats. A plasmid encoding for ACTB tagged with MS2 SL repeats was transfected in U2-OS Halo-G3BP1 cells that stably produce MS2-GFP with the NLS-TagBFP transfection marker. Halo-G3BP1 was labeled with the red fluorescent JF585 dye for SG identification and SGs were

induced by arsenite. (a) ACTB-(MS2-SL)1x was transfected (in 28% of all transfected cells at least one SG was detected in the green channel; 38/149 SGs) (2 experiments, 60 cells). (b) ACTB-(MS2-SL)2x was transfected (in 42% of all transfected cells at least one SG was detected in the green channel; 28/162 SGs) (2 experiments, 53 cells). (c) ACTB-(MS2-SL)4x was transfected (in 45% of all transfected cells at least one SG was detected in the green channel; 46/163 SGs) (2 experiments, 58 cells). (d) ACTB-(MS2-SL)24x was transfected (in 86% of all transfected cells at least one SG was detected in the green channel; 109/144 SGs) (2 experiments, 50 cells). Representative SGs visualized by MS2-GFP are indicated with white arrows. Scale bar = 10  $\mu$ m.

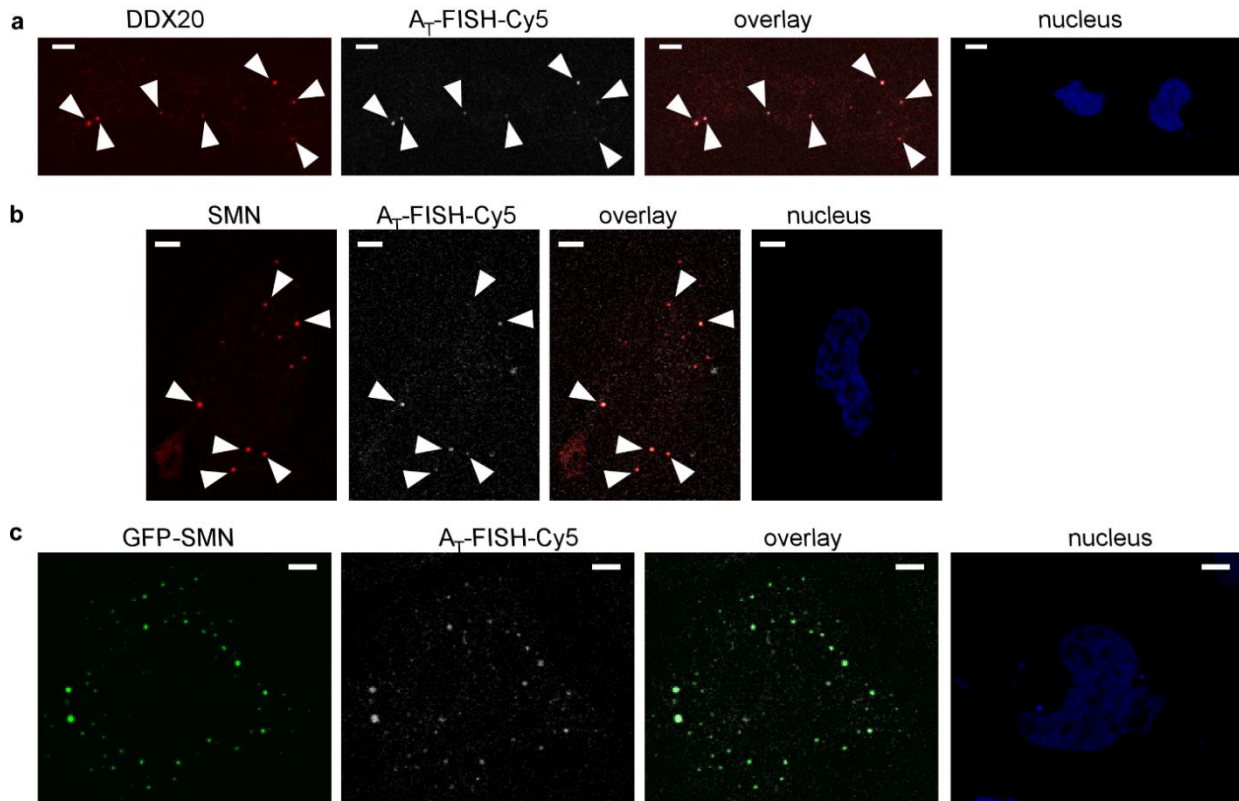


### Supplementary Figure 27

Cotransfection of NLS-TagBFP and ACTB-(MS2-SL)24x in U2-OS induces different phenotypes. U2-OS cells that stably produce Halo-G3BP1 and MS2-GFP were cotransfected with plasmid encoding for NLS-TagBFP and ACTB(MS2-SL)24x (1 experiment, 188 cells). 24 h post transfection, G3BP1 was labeled by the red JF585 fluorophore. Cells positive for the NLS-TagBFP transfection marker are indicated by white dashed lines. Untransfected cells as well as some NLS-TagBFP positive cells displayed nuclear MS2-GFP (see for example oval shaped cell in the middle of the field of view). In other cases, MS2-GFP was localized to the cytosol (see cells marked with white stars). Occasionally, stress granules were observed in the red JF585 channel in transfected cells (white arrow). Scale bar = 10  $\mu$ m.

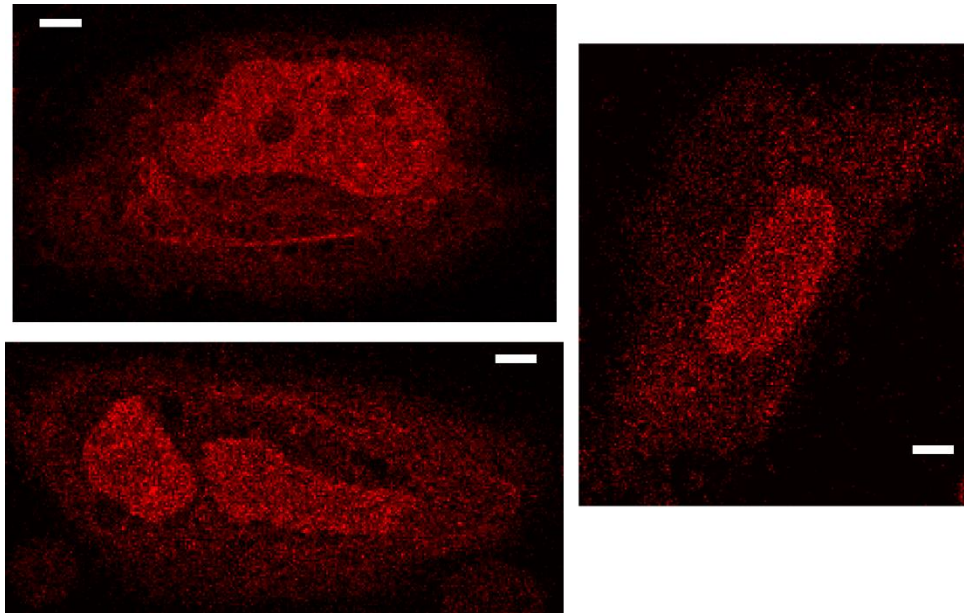
### **Supplementary Figure 28**

Localization phenotypes of U1 snRNA in normal and thapsigargin-stressed HeLa cells. (a) Endogenous U1 snRNA colocalizes with nuclear Coilin-containing foci. HeLa cells were transiently transfected with a plasmid to produce EGFP-Coilin, fixed and permeabilized. U1 snRNA FISH probe: U1-FISH-Cy5 (1 experiment, 6 cells). (b) A<sub>T</sub>-U1 RNA can localize to Coilin-containing nuclear foci. HeLa cells were transiently transfected with two plasmids to produce GFP-Coilin and A<sub>T</sub>-U1 snRNA, fixed and permeabilized (FISH probe against A<sub>T</sub>-U1 snRNA: A<sub>T</sub>-FISH-Cy5, 2 experiments, 3 cells). (c) Endogenous U1 snRNA colocalizes with two marker proteins for U-bodies, endogenous SMN (1 experiment, 5 cells) and endogenous DDX20 (1 experiment, 9 cells), upon thapsigargin treatment. HeLa cells were fixed and permeabilized. U1 snRNA FISH probe: U1-FISH-Cy5. DDX20 and SMN were visualized by immunofluorescence. Scale bar = 5 μm.



### Supplementary Figure 29

Transiently transfected U1 snRNA tagged with A<sub>T</sub> can localize to U-body marker proteins DDX20 and SMN. (a) A<sub>T</sub>-U1 snRNA can colocalize with the U-body marker protein DDX20 after treatment of cells with thapsigargin. A<sub>T</sub>-U1 snRNA was visualized via FISH probe A<sub>T</sub>-FISH-Cy5 and endogenous DDX20 was detected by immunofluorescence (1 experiment, 12 cells). (b) A<sub>T</sub>-U1 snRNA can colocalize with the U-body marker protein SMN after treatment of cells with thapsigargin. A<sub>T</sub>-U1 snRNA was visualized via FISH probe A<sub>T</sub>-FISH-Cy5 and endogenous SMN was detected by immunofluorescence (1 experiment, 8 cells). (c) A<sub>T</sub>-U1 snRNA colocalizes with transiently transfected GFP-SMN after treatment of cells with thapsigargin. A<sub>T</sub>-U1 snRNA was visualized via FISH probe A<sub>T</sub>-FISH-Cy5 and SMN was detected by GFP fluorescence (1 experiment, 57 cells). Scale bar = 5 μm.

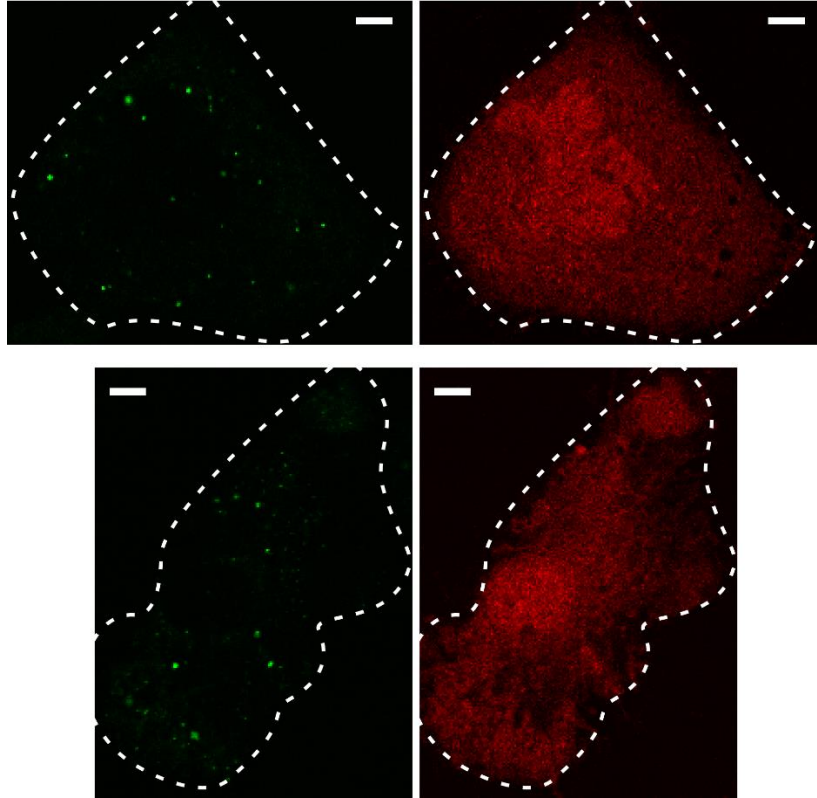


### Supplementary Figure 30

In the absence of thapsigargin-treatment, HeLa cells transfected with A<sub>T</sub>-U1 and bead loaded with Cbl-5xPEG-ATTO 590 do not display cytosolic granules resembling U-bodies. HeLa cells were transfected with A<sub>T</sub>-U1, bead loaded with Cbl-5xPEG-ATTO 590 24 h later and live microscopy was performed as described in Fig. 5, except that no prior treatment with thapsigargin was done. Out of 86 cells (1 experiment), 2 cells (2%) displayed cytosolic puncta.

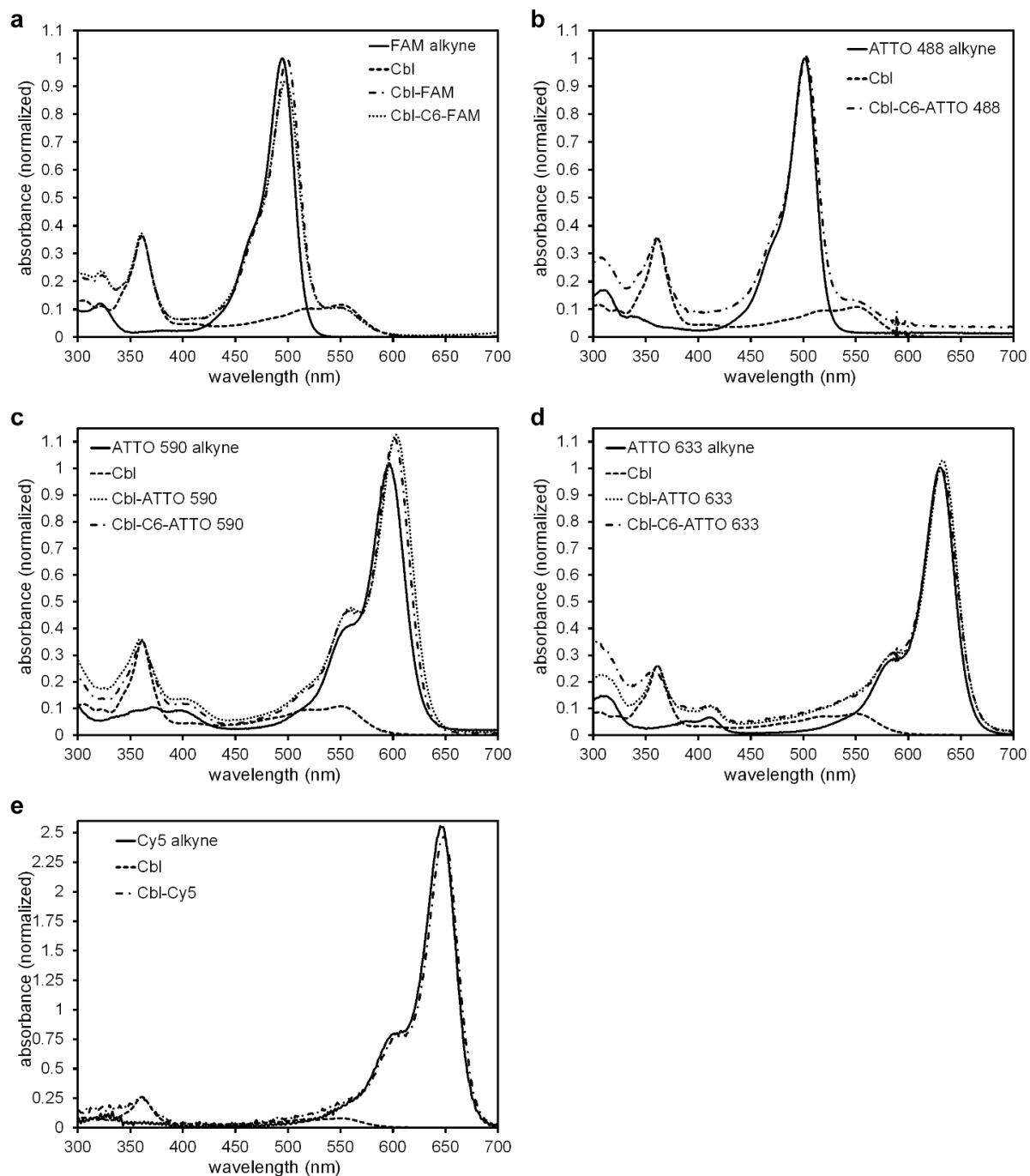
Scale bar = 5 μm.





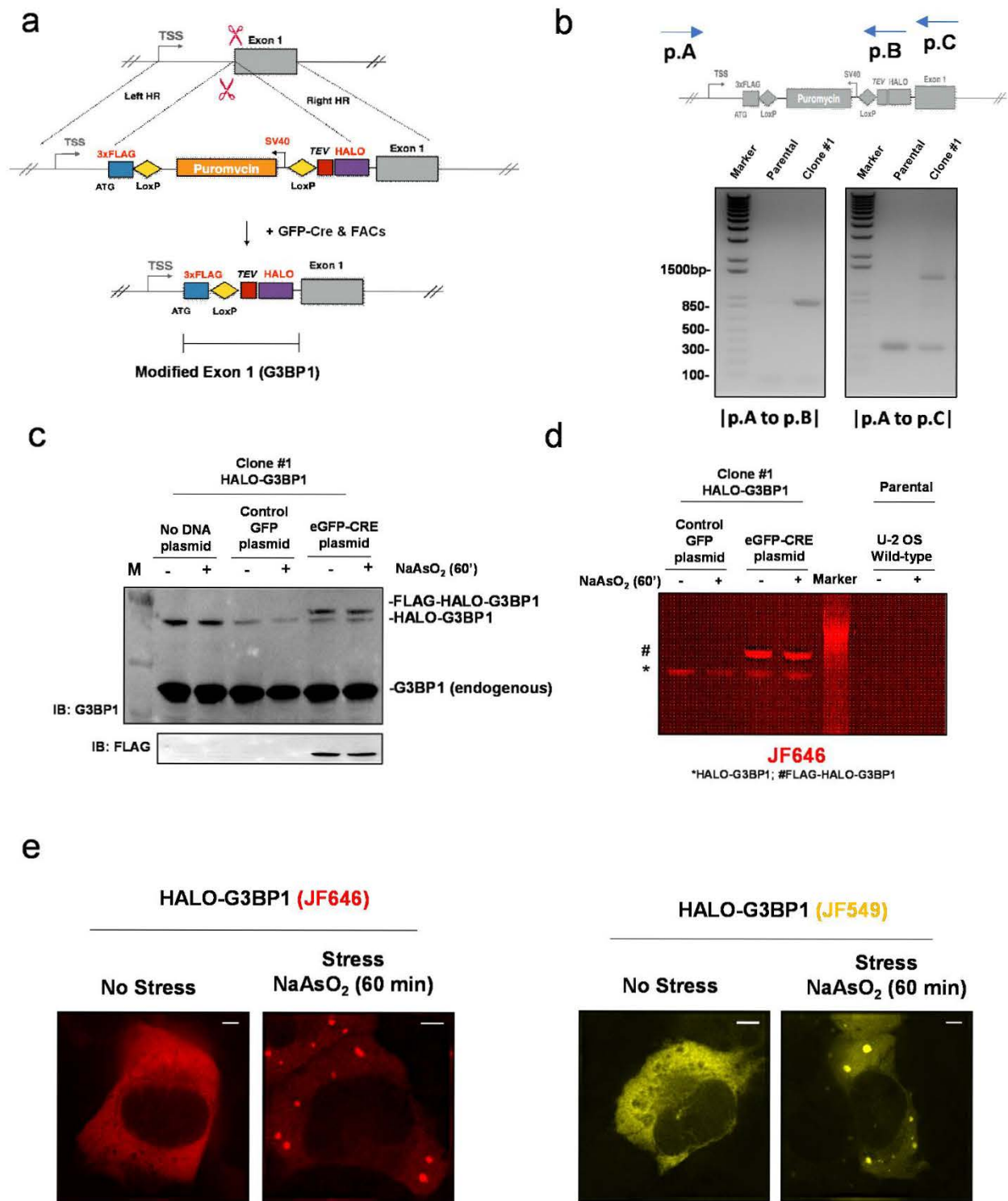
### Supplementary Figure 31

Cbl-5xPEG-ATTO 590 does not colocalize to GFP-SMN puncta in the absence of A<sub>T</sub>-U1 snRNA. HeLa cells were transiently transfected with a plasmid to produce GFP-SMN, treated with thapsigargin and loaded with Cbl-5xPEG-ATTO 590. In the absence of a co-transfected plasmid to produce A<sub>T</sub>-U1 snRNA, the probe does not accumulate in the puncta marked with GFP (compare with Fig. 5c) (3 experiments, 6 cells). Scale bar = 5 μm.



### Supplementary Figure 32

Absorbance spectra of representative Cbl-fluorophore probes in comparison to the spectra of free Cbl and each fluorophore. The absorbance intensities were normalized to the maximum absorbance peak of Cbl at 361 nm to allow for evaluation of changes in absorbance peak shapes. Spectra for each compound were collected once.



Generation of endogenous Halo-G3BP1 cell line. (a) Schematic for CRISPR/Cas9-mediated 3xFlag-HALO-tag knockin into endogenous G3BP1 locus. (b) Genotyping results reveals

proper integration in mixed U2-OS population. (c) Transient transfection of eGFP-Cre, but not control plasmids, in edited cell reveals correct expression of 3xFlag-HALO-tagged G3BP1 resolved by Western blotting. (d) Same as (c) expect 3xFlag-Halo-tag integration at the G3BP1 locus resolved by fluorescent imaging of protein gels. (e) 3xFlag-Halo-tagged G3BP1, as resolved by different fluorescent dyes, redistributes from the cytoplasm to concentrate into stress granules during sodium arsenite stress. (b-e): Shown are representative data from n=3 independent experiments. Scale bar = 5  $\mu$ m.

# Supplementary Note

## Compound synthesis protocols and characterization

### A multicolor riboswitch-based platform for imaging of RNA in live mammalian cells

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## 1. General Information

Commercially available reagents and solvents were used as received. 6-FAM alkyne and sulfo-Cyanine5 alkyne were purchased from Lumiprobe and ATTO propargylamides were obtained from ATTO-TEC. As supplied ATTO 590 consists of a mixture of two isomers with practically identical absorption and fluorescence (para and meta isomer).

alkyne was not provided by the producer, hence it is not included on schemes.

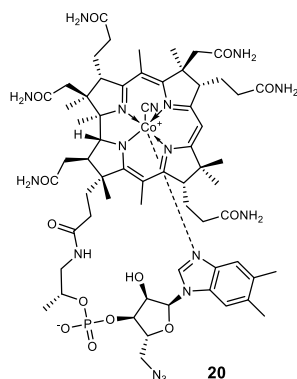
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker 500 MHz or Varian 500 MHz spectrometer with the residual solvent peak used as an internal standard. Data are reported as follows: chemical shift [ppm], multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant and integration. HRMS spectra were recorded on a spectrometer with TOF mass analyzer.

The scale of the reactions with ATTO and Cyanine dyes did not provide sufficient amount of products for NMR analyses thus the HPLC and MS analyses were performed to characterize those compounds. During the synthesis of Cbl conjugates, the conversion of a substrate to a product was estimated based on a dye as the vitamin B<sub>12</sub> derivative was used in excess and was calculated using the integration of a signal coming from the dye in RP-HPLC analysis. All reactions described in Section 3 proceeded with the conversion >99% (on the HPLC chromatograms only signals corresponding to the desired conjugate and the remaining azide were observed. For Cbl-1xPEG-FAM (**13**), Cbl-2xPEG-FAM (**14**) and Cbl-3xPEG-FAM (**10**) signals in  $^1\text{H}$  NMR spectra recorded in CD<sub>3</sub>OD were much broader comparing to Cbl-FAM (**12**) and Cbl-C6-FAM (**2**) and subtle structure of multipletes or integrations could not be fully distinguished. Compounds **4,7-9** were obtained as a mixture of isomers (para and meta of ATTO 590 part).

Preparative chromatography was performed using LiChroprep® RP-18 gel (Merck) with redistilled water and HPLC grade MeCN as eluents. Progress of the reactions was monitored using RP-HPLC techniques. HPLC measurement conditions: column, Eurospher II 100-5, C18, 250 mm × 4.6 mm with a precolumn or Kromasil C18 5 μm 250 mm × 4.0 mm; detection, UV/vis; pressure, 10 MPa; temperature, 30°C; flow rate, 1mL/min; wavelengths and HPLC methods are listed for each compound. Abbreviations: CDT – 1,1'-Carbonyl-di-(1,2,4-triazole); RP HPLC – Reverse-phase high-performance liquid chromatography; TBTA – Tris[(1-benzyl-1H-1,2,3-triazol-4 yl)methyl]amine; TEA – Triethylamine

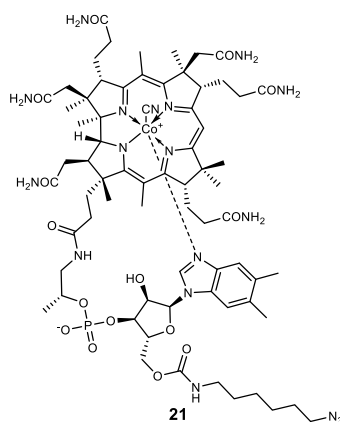
## 2. Synthesis of cobalamin azide

### 2.1 Cbl-N<sub>3</sub> (**20**)



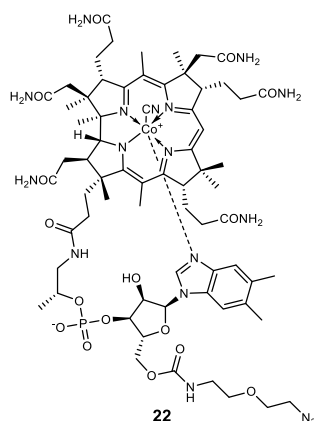
Compound **20** was synthesized according to the procedure described in *Chem. Eur. J.*,**19**, 5141 – 5148 (2013). All spectra matched that reported in the literature.

## 2.2 Cbl-C6-N<sub>3</sub> (**21**)



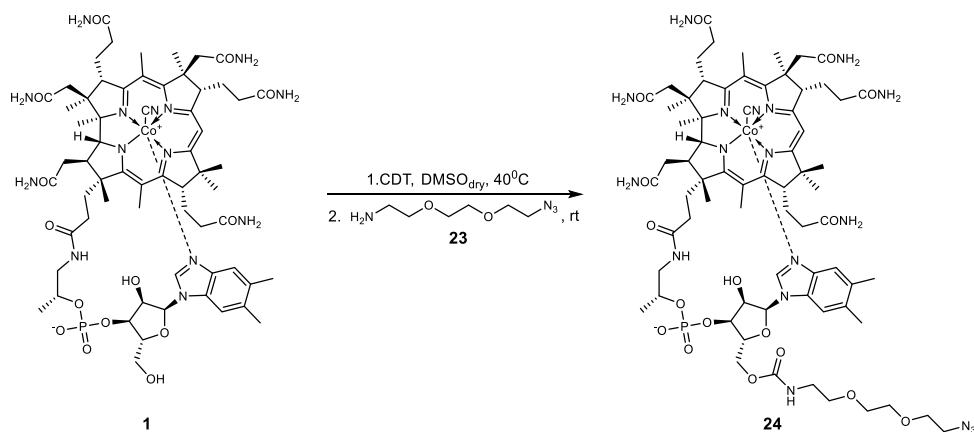
Compound **21** was synthesized according to the procedure described in *J. Porphyrins Phthalocyanines*, **17**,110-117 (2013). All spectra matched those reported in the literature.

## 2.3 Cbl-1xPEG-N<sub>3</sub> (**22**)



Compound **22** was synthesized according to the procedure described in *J. Porphyrins Phthalocyanines*,**17**, 110-117 (2013). All spectra matched those reported in the literature.

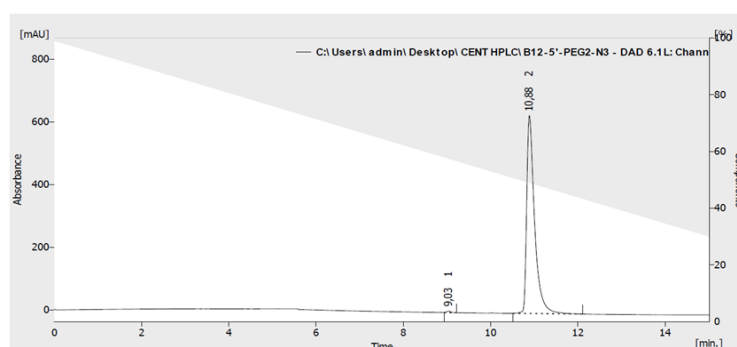
## 2.4 Cbl-2xPEG-N<sub>3</sub> (**24**)



**Cbl-2xPEG-N<sub>3</sub>**: Cobalamin (**1**) (0.075 mmol, 100 mg) was dissolved in dry DMSO (2.5 mL) at 40 °C under an argon atmosphere. To a stirring solution under argon solid CDT (50 mg, 0.30 mmol) was added. When full consumption of the substrate (monitored by the RP HPLC) was observed (approx. 1.5h), heating bath was removed and 2-[2-(2-azidoethoxy)ethoxy]-ethanamine (**23**) (100  $\mu$ L) was added in one portion. The resulting solution was stirred overnight, then the reaction mixture was poured into AcOEt (50 mL), and centrifuged. The precipitate was washed twice with Et<sub>2</sub>O (2 x 15mL). After drying it was dissolved in water and purified by RP column chromatography (80 mL) with a mixture of MeCN and H<sub>2</sub>O as eluents (10% v/v). The desired compound **24** was obtained as a red powder; yield: 66% (0.0495 mmol, 77 mg). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD)  $\delta$  7.25 (s, 1H), 7.15 (s, 1H), 6.58 (s, 1H), 6.23 (d, *J* = 2.6 Hz, 1H), 6.04 (s, 1H) 4.66 (d, *J* = 9.9 Hz, 1H), 4.51 (d, *J* = 8.2 Hz, 1H), 4.40 – 4.32 (m, 1H), 4.24 – 4.20 (m, 2H), 4.17 (dd, *J* = 12.2, 2.4 Hz, 1H), 4.13 (d, *J* = 11.5 Hz, 1H), 3.60 – 3.67 (m, 7H), 3.54 (t, *J* = 5.6 Hz, 2H), 3.36 (t, *J* = 5.6 Hz, 2H), 2.93 – 2.85 (m, 2H), 2.59 (s, 3H), 2.58 (s, 3H), 2.67 – 2.42 (m, 12H), 2.41 – 2.34 (m, 2H), 2.29 (s, 3H), 2.28 (s, 3H), 2.21 – 2.14 (m, 1H), 2.12 – 1.96 (m, 4H), 1.94 – 1.82 (m, 3H), 1.89 (s, 3H), 1.77 – 1.70 (m, 1H), 1.47 (m, 3H), 1.39 (s, 3H), 1.39 – 1.37 (m, 2H), 1.37 (s, 3H), 1.30 – *J* = 6.3 Hz, 3H), 1.19 (s, 3H), 1.16 – 1.08 (m, 1H), 0.47 (s, 3H). <sup>13</sup>C NMR (126 MHz, CD<sub>3</sub>OD)  $\delta$  180.1, 178.7, 176.1, 176.0, 175.9, 175.1, 174.1, 174.1, 173.8, 173.2, 172.6, 165.7, 165.5, 157.2, 141.9, 136.8, 134.2, 132.5, 129.9, 116.5, 111.0, 107.3, 103.8, 94.2, 86.8, 85.0, 79.9, 74.9, 73.7, 72.0, 72.0, 70.1, 70.0, 69.7, 69.6, 69.1, 62.8, 58.9, 56.2, 55.5, 53.6, 51.1, 50.3, 45.2, 42.5, 41.6, 40.4, 38.7, 34.8, 33.7, 31.8, 31.5, 31.2, 30.9, 30.9, 28.1, 26.0, 25.9, 19.5, 19.1, 19.0, 18.9, 18.7, 18.7, 18.5, 16.1, 15.7, 14.9, 14.7. UV/vis (H<sub>2</sub>O)  $\lambda_{\text{max}}$  (nm) ( $\epsilon$ , L mol<sup>-1</sup> cm<sup>-1</sup>) 551 (7.8  $\times$  10<sup>3</sup>), 522 (6.8  $\times$  10<sup>3</sup>), 361 (2.4  $\times$  10<sup>4</sup>), 278 (1.3  $\times$  10<sup>4</sup>), 222 (4.2  $\times$  10<sup>4</sup>). HRMS (ESI) *m/z* [M + Na]<sup>+</sup> calcd for C<sub>70</sub>H<sub>100</sub>N<sub>18</sub>O<sub>17</sub>PCoNa 1577.6481, found 1577.6455. Anal. calcd for C<sub>70</sub>H<sub>100</sub>N<sub>18</sub>O<sub>17</sub>PCo·6H<sub>2</sub>O: C, 50.54; H, 6.79; N, 15.15. Found: C, 50.62; H, 7.03; N, 14.95.

#### HPLC Method:

Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN[%]	$\lambda$ [nm]	R <sub>t</sub> [min]
Initial	99	1	361	10.88
15	30	70		

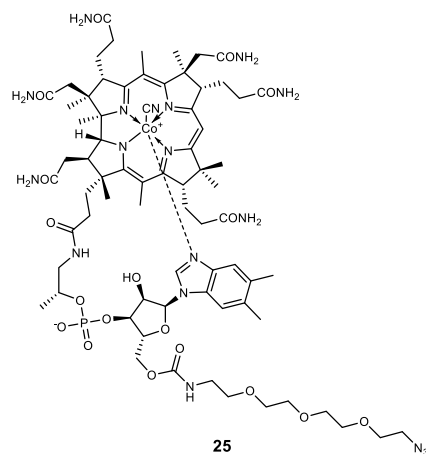


Result Table (Uncal - C:\Users\admin\Desktop\CENT HPLC\B12-5'-PEG2-N3 - DAD 6.1L: Channel 2)

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	9,033	31,715	5,208	0,4	0,8	779
2	10,883	7950,937	631,253	99,6	99,2	573
Total		7982,652	636,501	100,0	100,0	

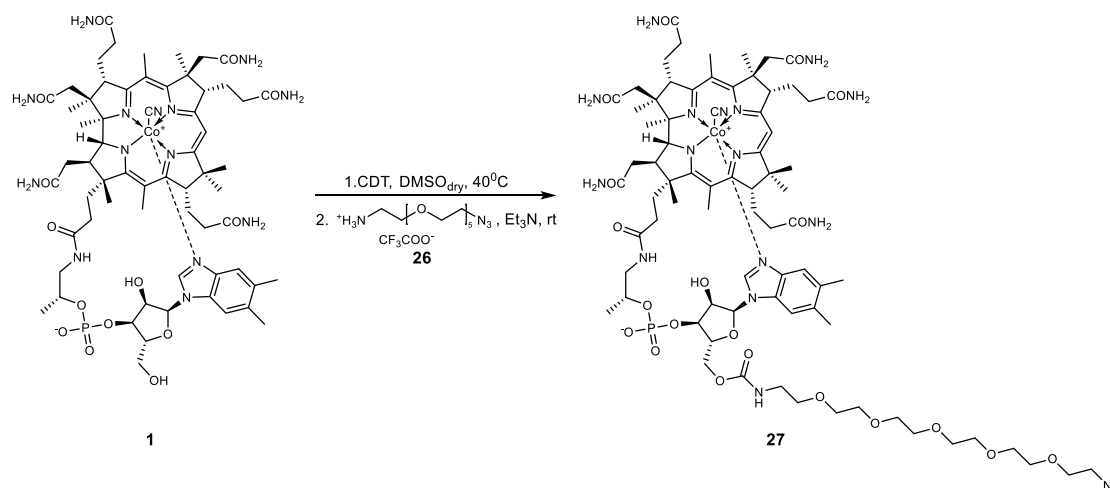


## 2.5 Cbi-3xPEG-N<sub>3</sub> (25)



Compound **25** was synthesized according to the procedure described in *J. Porphyrins Phthalocyanines*, **17**, 110-117 (2013). All spectra matched those reported in the literature.

## 2.6 Cbi-5xPEG-N<sub>3</sub> (27)

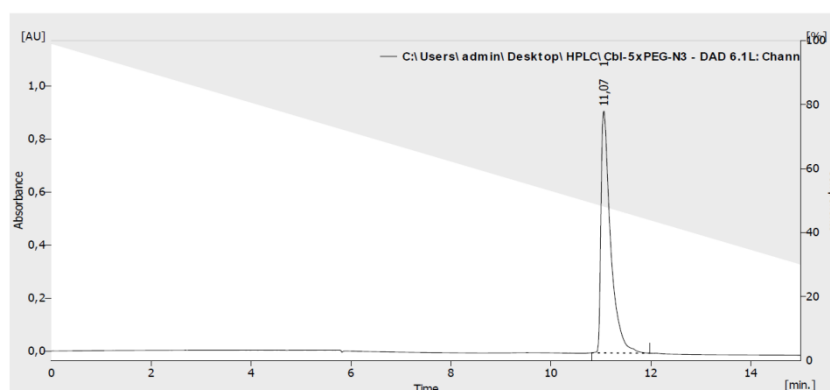


**Cbi-5xPEG-N<sub>3</sub>**: Cobalamin (**1**) (0.146 mmol, 200 mg) was dissolved in dry DMSO (5 mL) at 40 °C under an argon atmosphere. To a stirring solution under argon solid CDT (100 mg, 0.609 mmol) was added. When full consumption of the substrate (monitored by the RP HPLC) was observed (approx. 1.5h), heating bath was removed and 2-[2-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethanamine in the form of TFA salt (**26**) (100 mg) was added in one portion. Subsequently TEA (80 µL) was added and the resulting solution was stirred overnight. Then the reaction mixture was poured into AcOEt (50 mL) and centrifuged. The precipitate was then washed twice with Et<sub>2</sub>O (2 x 15mL). After drying it was dissolved in water and purified by RP column chromatography (80 mL) with a mixture of MeCN and H<sub>2</sub>O as eluents (gradually from 10 to 15% v/v). The desired compound **27** was obtained as a red powder; yield: 43% (0.063 mmol, 106 mg). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ 7.24 (s, 1H), 7.14 (s, 1H), 6.57 (s, 1H), 6.22 (d, *J* = 2.6 Hz, 1H), 6.03 (s, 1H), 4.92 (m, 1H), 4.65 (d, *J* = 10.5 Hz, 1H), 4.49 (d, *J* = 8.8 Hz, 1H), 4.41 – 4.30 (m, 1H), 4.26 – 4.08 (m, 4H), 3.67 – 3.56 (m, 23H), 3.52 (t, *J* = 5.4, 2H), 3.37 – 3.32 (m, 2H), 3.27 (m, 1H), 2.95 – 2.81 (m, 2H), 2.70 – 2.31 (m, 8H), 2.58 (s, 3H), 2.57 (s, 3H), 2.28 (s, 3H), 2.27 (s, 3H), 2.22 – 1.93 (m, 6H), 1.92 – 1.80 (m, 3H), 1.88 (s, 3H), 1.80 – 1.57 (m, 1H), 1.46 (s, 3H), 1.38 (s, 3H), 1.36 (s, 3H), 1.31 –

1.20 (m, 2H), 1.24 (d,  $J = 6.3$  Hz, 3H), 1.18 (s, 3H), 1.14 – 1.05 (m, 1H), 0.46 (s, 3H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  181.6, 180.2, 177.6, 177.426, 177.4, 176.6, 176.4, 175.6, 175.5, 175.3, 174.6, 167.2, 166.9, 143.4, 138.3, 135.7, 134.0, 131.4, 117.9, 112.5, 108.8, 105.3, 95.7, 88.3, 86.4, 76.4, 73.4, 71.6, 71.6, 71.6, 71.3, 71.1, 71.0, 70.6, 60.3, 57.7, 57.0, 55.1, 52.6, 51.8, 49.6, 48.4, 46.6, 44.0, 43.0, 41.8, 40.1, 36.2, 35.1, 33.3, 33.0, 32.6, 32.4, 32.3, 29.5, 27.4, 27.4, 20.3, 20.6, 20.5, 20.3, 20.2, 20.0, 17.5, 17.1, 16.4, 16.2. UV/vis ( $\text{H}_2\text{O}$ )  $\lambda_{\text{max}}$  (nm) ( $\epsilon$ ,  $\text{L mol}^{-1} \text{cm}^{-1}$ ) 549 ( $5.8 \times 10^3$ ), 520 ( $5.2 \times 10^4$ ), 361 ( $1.8 \times 10^4$ ), 277 ( $1.1 \times 10^4$ ), 220 ( $3.3 \times 10^4$ ). HRMS (ESI)  $m/z$   $[\text{M} + \text{Na}]^+$  calcd for  $\text{C}_{76}\text{H}_{112}\text{N}_{18}\text{O}_{20}\text{PCoNa}$  1709.7268, found 1709.7219. Anal. calcd for  $\text{C}_{76}\text{H}_{112}\text{N}_{18}\text{O}_{20}\text{PCo} \cdot 7\text{H}_2\text{O}$ : C, 50.33; H, 7.00; N, 13.90. Found: C, 50.22; H, 6.76; N, 14.22.

#### HPLC Method:

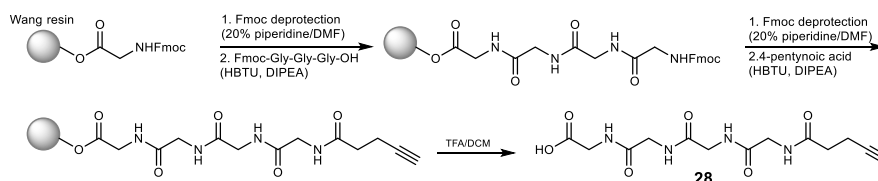
	$\text{H}_2\text{O} + 0.5\% \text{TFA}$ [%]	MeCN [%]	$\lambda$ [nm]	$R_t$ [min]
Initial	99	1	361	11.07
15	30	70		



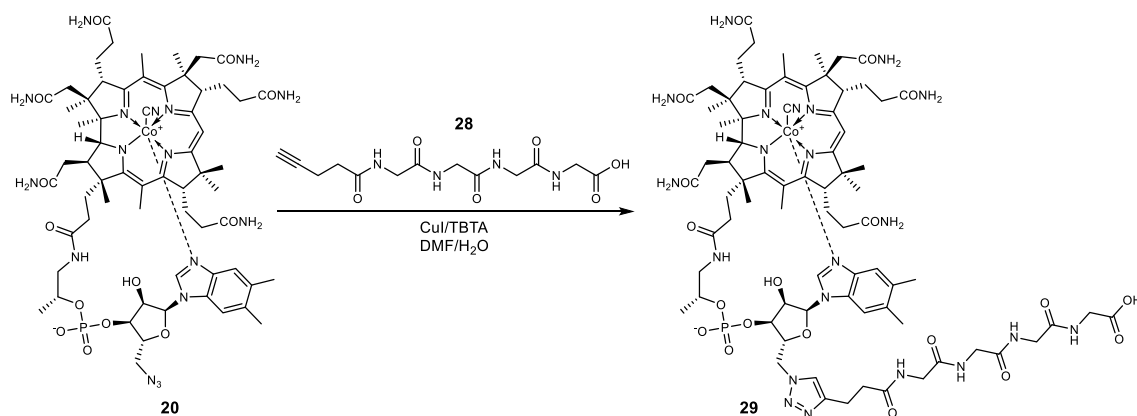
Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-5xPEG-N3 - DAD 6.1L: Channel 2)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	11.067	12246,454	911,440	100,0	100,0	0,20	616
	Total	12246,454	911,440	100,0	100,0		

## 2.7 Cbl-4xGly-N<sub>3</sub> (31)



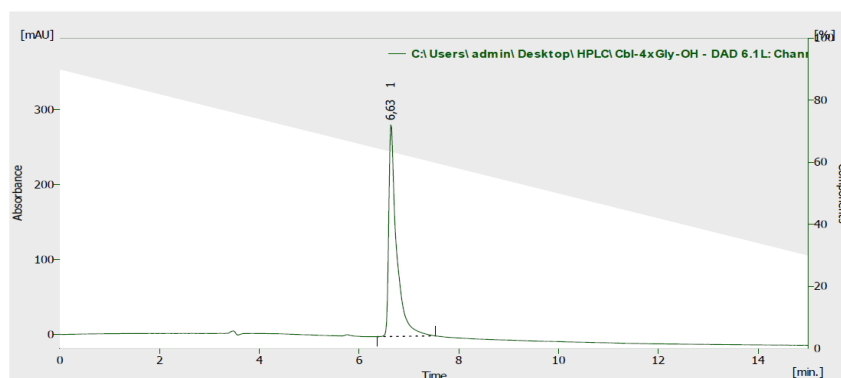
**STEP 1:** HO-4xGly-alkyne (**28**) was synthesized manually by Fmoc chemistry on a 0.124 mmol scale of Fmoc-Gly attached to the Wang resin (Fmoc-Gly-Wang resin), 4-fold molar excess of the Fmoc-Gly-Gly-Gly-OH and 5-fold molar excess of 4-pentynoic acid. Fmoc deprotection was performed with 20% piperidine in DMF (1.5 mL, 1-2 h) and coupling with the use of HBTU (6 equiv.) and DIPEA (6 equiv.) in DMF (2 mL). After final coupling the resin was washed with DMF (5 x 1 mL), DCM (5 x 1 mL) and dried. Cleavage from the resin was carried with the use of a TFA/DCM (25%, v/v) with the catalytic amount of anisole for 2.5 h. Obtained product **28** was precipitated with  $\text{Et}_2\text{O}$  and centrifuged. LRMS (ESI)  $m/z$   $[\text{M} + \text{Na}]^+$  calcd for  $\text{C}_{13}\text{H}_{18}\text{N}_4\text{O}_6\text{Na}$  349.12, found 349.20.



**STEP 2:** Cbl-N<sub>3</sub> (**20**) (0.068 mmol, 94 mg) and HO-4xGly-alkyne (0.062 mmol, 20 mg) was dissolved in DMF/H<sub>2</sub>O mixture (5:3 v/v,  $\Sigma = 4$  mL). Catalyst – CuI (0.032 mmol, 6 mg) and TBTA (0.057 mmol, 30 mg) mixed in 2 mL of DMF for 20 min – was added and the resulting solution was stirred for 16 h at 40°C. The reaction mixture was diluted with MeOH (5 mL) and poured into Et<sub>2</sub>O (60 mL). The resulting precipitate was filtered through a cotton wool, washed with AcOEt (2 x 10 mL) and Et<sub>2</sub>O (2 x 10 mL). After drying, the resulting solid was dissolved in MeOH and concentrated in vacuo. The crude was dissolved in water and purified by RP column chromatography with a mixture of MeCN/H<sub>2</sub>O (10% v/v) as an eluent. The desired compound **29** was obtained as a red powder, yield: 59% (0.073 mmol, 124 mg). <sup>1</sup>H NMR spectra were recorded for D<sub>2</sub>O at rt and at 80°C but the signals were very broad and subtle structure of multipletes or integrations could not be fully distinguished (see part NMR spectra). <sup>13</sup>C NMR (126 MHz, D<sub>2</sub>O)  $\delta$  182.5, 181.4, 180.2, 180.0, 179.4, 179.4, 178.2, 178.1, 177.5, 177.2, 176.0, 175.1, 174.6, 174.2, 168.4, 167.8, 144.3, 139.0, 137.7, 135.6, 132.4, 119.0, 113.8, 110.0, 106.7, 97.4, 89.0, 87.6, 81.3, 77.3, 76.8, 75.6, 71.1, 61.6, 58.7, 58.2, 56.2, 53.9, 50.5, 49.7, 47.6, 45.4, 45.2, 45.1, 41.8, 41.5, 37.4, 37.0, 36.6, 34.4, 34.3, 34.7, 34.0, 33.8, 30.1, 28.9, 28.5, 28.4, 27.1, 26.9, 22.3, 21.9, 21.8, 21.7, 21.5, 21.5, 19.3, 18.3, 17.8, 17.7. UV/vis (H<sub>2</sub>O)  $\lambda_{\max}$  (nm) ( $\epsilon$ , L mol<sup>-1</sup> cm<sup>-1</sup>) 549( $5.8 \times 10^3$ ), 520 ( $5.2 \times 10^3$ ), 361 ( $1.8 \times 10^4$ ), 276 ( $1.0 \times 10^4$ ), 222 ( $3.5 \times 10^4$ ). HRMS (ESI)  $m/z$  [M + Na]<sup>+</sup> calcd for C<sub>76</sub>H<sub>105</sub>N<sub>21</sub>O<sub>19</sub>PCoNa 1728.6863, found 1728.6897.

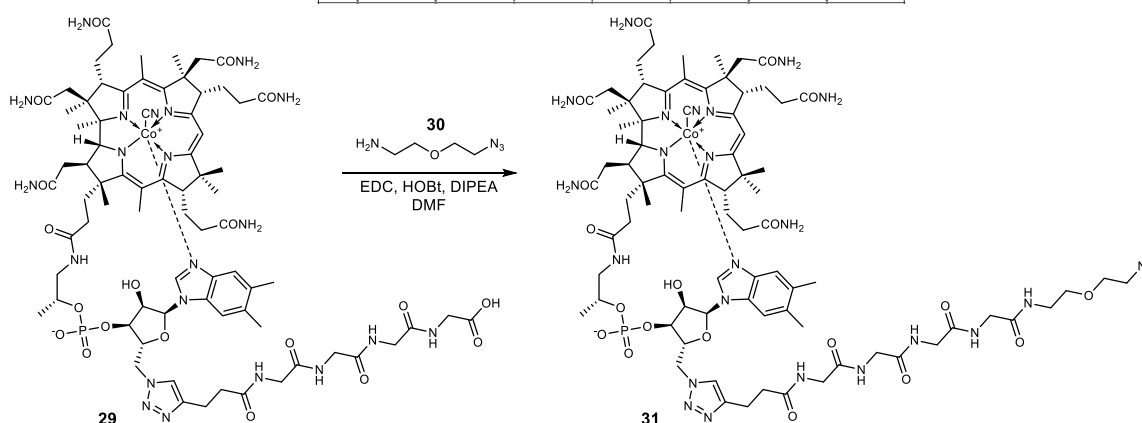
#### HPLC Method:

Time [min]	H <sub>2</sub> O+0.2%TFA [%]	MeCN [%]	$\lambda$ [nm]	R <sub>t</sub> [min]
Initial	90	10	361	6.63
15	30	70		



Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-4xGly-OH - DAD 6.1L- Channel 2)

	Reten. Time [min]	Area [mAU·s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	6.633	3089,950	282,314	100,0	100,0	0,15	823
Total		3089,950	282,314	100,0	100,0		

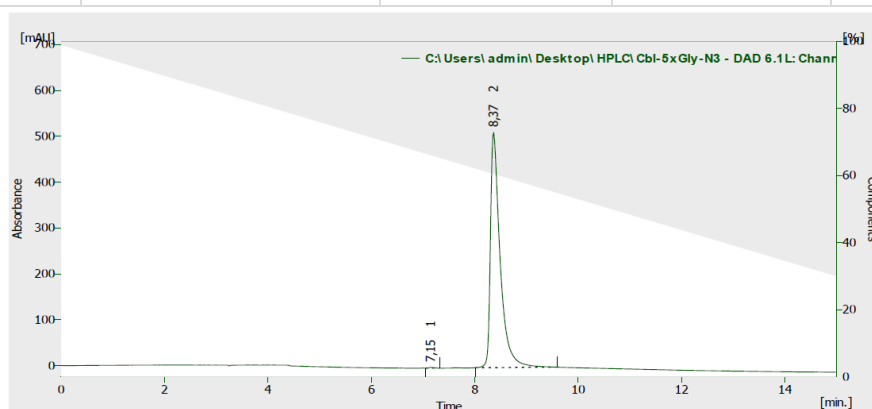


**STEP 3:** Cbl-4xGly-OH (**29**) (80 mg, 0.047 mmol) and 2-(2-azidoethoxy)ethanamine (**30**) (300  $\mu$ L) was dissolved in DMF (5 mL). EDC (90 mg, 0.470 mmol), HOBt (127 mg, 0.940 mmol) and DIPEA (0.470, 82  $\mu$ L) were added. The resulting solution was stirred at rt for 1 h. Desired product, the product lacking CN ligand and the unreacted substrate were present in the reaction mixture in the approx. ratio 2.3 : 2.5 : 1 (according to RP-HPLC). The reaction mixture was diluted with MeOH (5 mL) and poured into Et<sub>2</sub>O (60 mL). The resulting precipitate was filtered through a cotton wool, washed with AcOEt (2 x 10 mL) and Et<sub>2</sub>O (2 x 10 mL). After drying, the resulting solid was dissolved in MeOH and concentrated in vacuo. Desired compound was purified by RP column chromatography with a mixture of MeCN/H<sub>2</sub>O (gradually from 8% to 40% v/v). Order of elution: substrate, desired product, product lacking CN ligand. The solvent was concentrated in vacuo and the product was obtained as a red solid. Yield of desired product **31** (with CN ligand): 41% (0.019 mmol, 35 mg), yield of product **31** without CN ligand (L=H<sub>2</sub>O): 14% (0.007 mmol, 12 mg). <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD)  $\delta$  8.18 (s, 1H), 7.19 (s, 1H), 7.15 (s, 1H), 6.56 (s, 1H), 6.06 (s, 1H), 5.90 (d, J = 2.9 Hz, 1H), 5.02 (dd, J = 14.7, 3.5 Hz, 1H), 4.86 – 4.82 (m, 1H), 4.54 (td, J = 8.4, 4.0 Hz, 1H), 4.49 (d, J = 8.7 Hz, 1H), 4.44 – 4.33 (m, 2H), 4.13 (d, J = 11.3 Hz, 1H), 4.09 – 4.04 (m, 1H), 4.02 – 3.81 (m, 8H), 3.69 (d, J = 13.9 Hz, 1H), 3.66 – 3.60 (m, 3H), 3.55 (t, J = 5.7 Hz, 2H), 3.38 – 3.34 (m, 4H), 3.25 (d, J = 10.5 Hz, 1H), 3.09 – 3.06 (m, 2H), 2.90 – 2.85 (m, 2H), 2.73 – 2.31 (m, 19H), 2.27 (s, 3H), 2.24 (s, 3H), 2.22 – 1.97 (m, 5H), 1.96 – 1.80 (m, 3H), 1.89 (s, 3H), 1.77 – 1.68 (m, 1H), 1.49 (s, 3H), 1.380 (s, 3H), 1.375 (s, 3H), 1.32 – 1.24 (m, 1H), 1.28 (d, J = 6.2 Hz, 3H), 1.20 (s, 3H), 1.15 – 1.07 (m, 1H), 0.44 (s, 3H). <sup>13</sup>C NMR (126 MHz, CD<sub>3</sub>OD)  $\delta$  181.6, 180.1, 177.5, 177.3, 177.2, 176.6, 176.0, 175.6, 175.5, 175.3, 174.6, 174.2, 173.0, 172.7, 172.2, 171.7, 167.8, 166.9, 147.3, 143.3, 138.2, 135.8, 134.0, 131.3, 126.0, 117.8, 112.6, 108.8, 105.2, 95.8, 87.9, 86.4, 80.9, 80.7, 76.4, 75.0, 73.7, 73.6, 70.9, 70.4, 70.3, 66.9, 60.3, 57.7, 57.0, 55.5, 52.6, 51.8, 50.6, 46.8, 44.3, 44.0, 43.9, 43.5, 43.1, 40.4, 40.1, 36.2, 35.9, 35.4, 33.4, 32.9,

32.8, 32.6, 32.4, 32.3, 29.5, 27.4, 22.4, 20.9, 20.5, 20.4, 20.3, 19.9, 17.5, 17.1, 16.4 16.1, 15.4. UV/vis (H<sub>2</sub>O)  $\lambda_{\max}$  (nm) ( $\epsilon$ , L mol<sup>-1</sup> cm<sup>-1</sup>) 548 (8.0 × 10<sup>3</sup>), 520 (7.0 × 10<sup>3</sup>), 361 (2.5 × 10<sup>4</sup>), 279 (1.3 × 10<sup>4</sup>), 222 (4.5 × 10<sup>4</sup>). HRMS (ESI)  $m/z$  [M + Na]<sup>+</sup> calcd for C<sub>80</sub>H<sub>113</sub>N<sub>25</sub>O<sub>19</sub>PCoNa 1840.7612, found 1840.7611.

### HPLC Method:

Time [min]	H <sub>2</sub> O+0.2%TFA [%]	MeCN [%]	$\lambda$ [nm]	R <sub>t</sub> [min]
Initial	90	10	361	8.37
15	30	70		

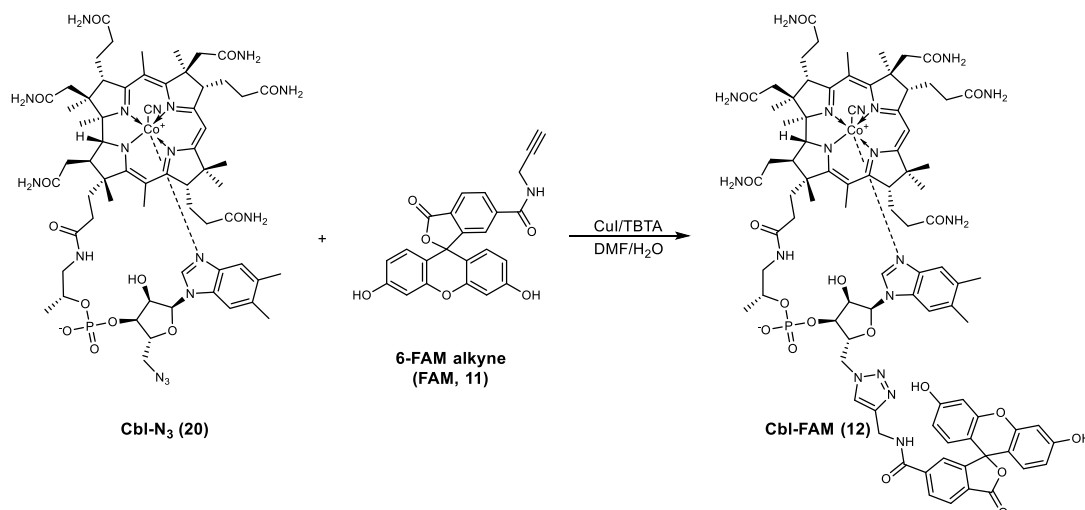


Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-5xGly-N3 - DAD 6.1L: Channel 2)

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	11,134	1,720	0,2	0,3	0,12	91,2
2	6698,033	512,670	99,8	99,7	0,20	56,3
Total	6709,167	514,390	100,0	100,0		

## 3. Synthesis of Cbl conjugates with various dyes

### 3.1 Cbl-FAM (12)



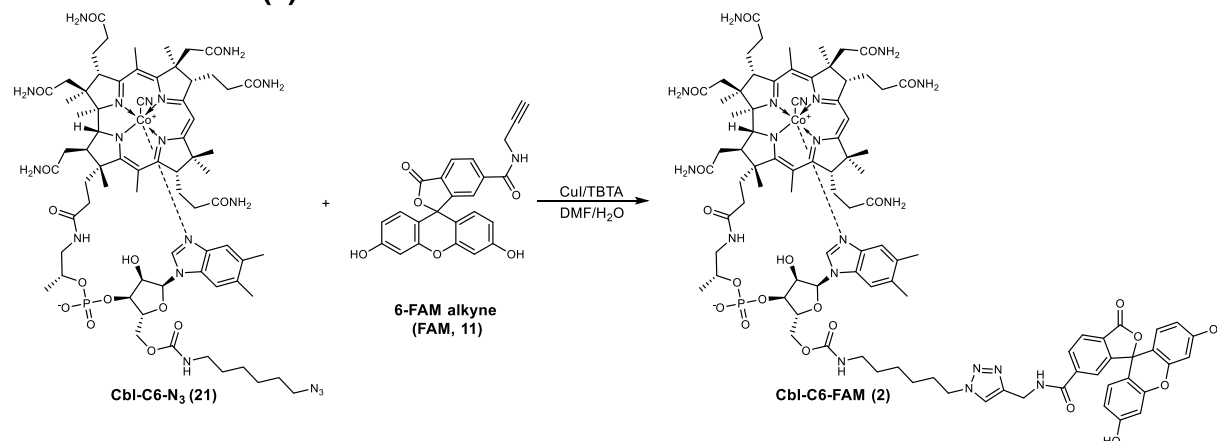
**Cbl-FAM:** CuI (1 mg, 5  $\mu$ mol) and TBTA (5 mg, 10  $\mu$ mol) were dissolved in DMF/H<sub>2</sub>O (1 mL, 3:1 v/v) and stirred for 20 min. Subsequently Cbl-N<sub>3</sub> (20) (24 mg, 17.4  $\mu$ mol) and 6-FAM alkyne (11) (6 mg, 14.5  $\mu$ mol) were added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (15 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (15 mL), and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (30 mL) and purified gradually with MeCN/H<sub>2</sub>O from 10 to 20% v/v yielding 1 as an orange solid. <sup>1</sup>H NMR

(500 MHz, CD<sub>3</sub>OD)  $\delta$  8.21 (s, 1H), 8.20 (s, 1H), 8.11 (d,  $J = 7.6$  Hz, 1H), 7.74 (s, 1H), 7.14 (s, 1H), 7.10 (s, 1H), 6.75 (bs, 2H), 6.71 – 6.64 (m, 2H), 6.59 (m, 2H), 6.56 (s, 1H), 6.03 (s, 1H), 5.95 (d,  $J = 2.3$ , 1H), 4.98 (d,  $J = 13.0$  Hz, 1H), 4.62 (bs, 2H), 4.53 (bs, 1H), 4.49 (d,  $J = 8.6$  Hz, 1H), 4.41 – 4.35 (m, 1H), 4.31 (bs, 1H), 4.13 (d,  $J = 11.4$  Hz, 1H), 4.06 (m, 1H), 3.63 (dd,  $J = 5.1, 10.7$  Hz, 1H), 3.57 (d,  $J = 13.8$  Hz, 1H), 3.21 (d,  $J = 10.1$  Hz, 1H), 2.88 – 2.81 (m, 1H), 2.78 (dd,  $J = 9.2, 13.7$  Hz, 1H), 2.68 – 2.43 (m, 8H), 2.58 (s, 3H), 2.55 (s, 3H), 2.41 – 2.31 (m, 2H), 2.27 (s, 3H), 2.21 (s, 3H), 2.14 – 1.95 (m, 6H), 1.95 – 1.83 (m, 2H), 1.89 (s, 3H), 1.82 – 1.65 (m, 2H), 1.42 (s, 3H), 1.38 (s, 3H), 1.35 (s, 3H), 1.30 – 1.20 (m, 2H), 1.23 (d,  $J = 5.7$  Hz, 3H), 1.20 – 1.07 (m, 1H), 1.18 (s, 3H), 0.42 (s, 3H). <sup>13</sup>C NMR (126 MHz, CD<sub>3</sub>OD)  $\delta$  181.6, 180.1, 177.6, 177.35, 177.2, 176.5, 175.6, 175.5, 175.3, 174.6, 174.2, 167.2, 166.9, 143.2, 138.2, 135.8, 134.0, 131.3, 130.6, 117.9, 114.5, 112.4, 108.8, 105.1, 103.6, 95.7, 87.9, 86.4, 81.2, 76.3, 75.8, 70.4, 60.3, 57.7, 57.0, 55.4, 52.6, 43.9, 43.1, 40.1, 36.2, 35.4, 33.5, 33.1, 32.9, 32.4, 32.3, 29.3, 27.4, 20.9, 20.5, 20.30, 20.26, 20.2, 19.9, 17.5, 17.1, 16.4, 16.2. HRMS (ESI)  $m/z$   $[M + 2Na]^{2+}$  calcd for C<sub>87</sub>H<sub>102</sub>CoN<sub>18</sub>O<sub>19</sub>PNa<sub>2</sub>, 919.3211; found, 919.3182.

### HPLC Method:

Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN [%]	$\lambda$ [nm]	R <sub>t</sub> [min]
Initial	99	1	361	12.08
20	15	85		
40	15	85		

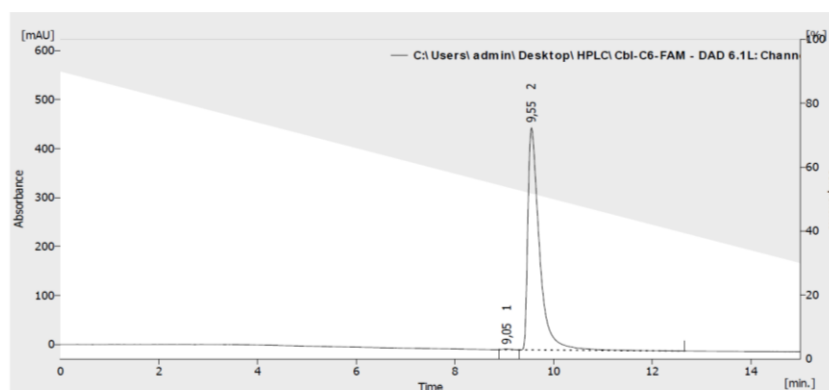
### 3.2 Cbl-C6-FAM (2)



**Cbl-C6-FAM:** Cul (1 mg, 5  $\mu\text{mol}$ ) and TBTA (5 mg, 10  $\mu\text{mol}$ ) were dissolved in DMF/H<sub>2</sub>O (1 mL, 3:1 v/v) and stirred for 20 min. Subsequently Cbl-C6-N<sub>3</sub> (**21**) (27 mg, 17.7  $\mu\text{mol}$ ) and 6-FAM alkyne (**11**) (6 mg, 14.5  $\mu\text{mol}$ ) were added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (15 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (15 mL) and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O (small amount of MeOH was added for better dissolution), loaded onto RP column (30 mL) and purified gradually with MeCN/H<sub>2</sub>O from 15 to 30% v/v yielding **2** as an orange solid. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD)  $\delta$  8.13 (s, 2H), 7.88 (s, 1H), 7.68 (s, 1H), 7.18 (s, 1H), 7.13 (s, 1H), 6.79 (bs, 2H), 6.68 (s, 2H), 6.61 – 6.49 (m, 3H), 6.18 (bs, 1H), 6.03 (s, 1H), 4.92 (m, 1H), 4.66 – 4.47 (m, 6H), 4.42 – 4.28 (m, 1H), 4.33 (t,  $J = 6.8$  Hz, 2H), 4.20 (bs, 2H), 4.16 – 4.07 (m, 2H), 3.67 – 3.57 (m, 2H), 3.29 (m, 1H), 3.06 – 2.92 (m, 2H), 2.94 – 2.82 (m, 2H), 2.68 – 2.42 (m, 8H), 2.58 (s, 6H), 2.41 – 2.31 (m, 2H), 2.28 (s, 3H), 2.23 (s, 3H), 2.20 – 1.78 (m, 8H), 1.88 (s, 3H), 1.77 – 1.68 (m, 1H), 1.48 – 1.40 (m, 2H), 1.44 (s, 3H), 1.38 (s, 3H), 1.36 (s, 3H), 1.33 – 1.20 (m, 8H), 1.24 (d,  $J = 6.1$  Hz, 3H), 1.18 (s, 3H), 1.16 – 1.07 (m, 1H), 0.46 (s, 3H). <sup>13</sup>C NMR (126 MHz, CD<sub>3</sub>OD)  $\delta$  181.6, 180.1, 177.6, 177.5, 177.4, 176.7, 175.5, 175.3, 174.6, 174.1, 168.3, 167.2, 166.9, 143.3, 140.1, 138.2, 136.8, 135.6, 133.9, 131.3, 131.2, 131.1, 130.1, 117.9, 112.4, 108.7, 105.2, 103.8, 95.6, 88.2, 86.4, 81.4, 76.4, 75.2, 73.5, 70.5, 64.2, 60.3, 57.7, 56.9, 55.0, 52.6, 51.3, 49.9, 46.6, 43.9, 43.0, 41.6, 40.1, 36.3, 36.2, 35.2, 33.3, 33.1, 32.7, 32.4, 32.3, 31.0, 30.8, 30.6, 29.5, 27.4, 27.0, 20.9, 20.5, 20.4, 20.2, 19.9, 17.5, 17.1, 16.4, 16.2. HRMS (ESI)  $m/z$  [M + Na]<sup>+</sup> calcd for C<sub>94</sub>H<sub>116</sub>CoN<sub>19</sub>O<sub>21</sub>P, 1959.7560; found, 1959.7555.

#### HPLC Method:

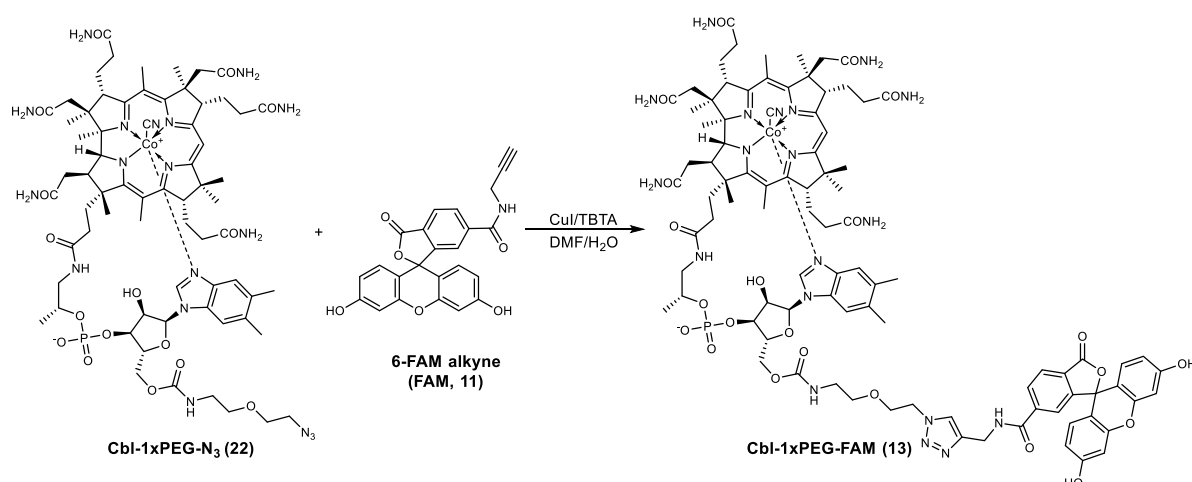
Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN [%]		R <sub>t</sub> [min]
Initial	90	10	361	9.55
15	30	70		



Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-C6-FAM - DAD 6.1L: Channel 2)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	9,050	14,792	1,208	0,2	0,3	0,22	999
2	9,550	7407,348	453,759	99,8	99,7	0,25	460
	Total	7422,140	454,967	100,0	100,0		

### 3.3 Cbi-1xPEG-FAM (13)

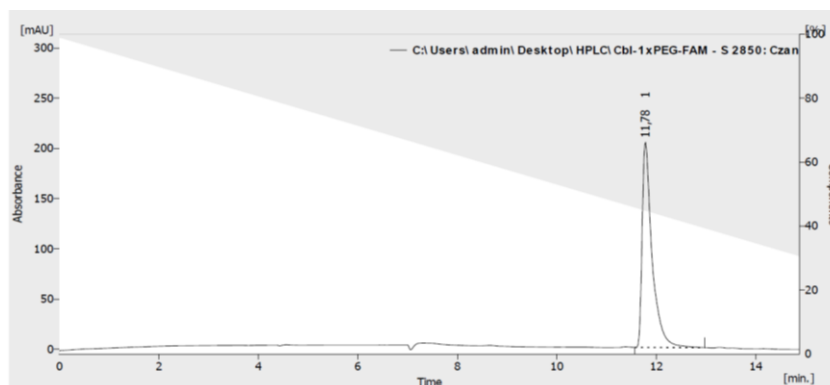


**Cbi-1xPEG-FAM:** CuI (1 mg, 5  $\mu\text{mol}$ ) and TBTA (5 mg, 10  $\mu\text{mol}$ ) were dissolved in DMF/H<sub>2</sub>O (1 mL, 3:1 v/v) and stirred for 20 min. Subsequently Cbi-1xPEG-N<sub>3</sub> (**22**) (27 mg, 17.9  $\mu\text{mol}$ ) and 6-FAM alkyne (**11**) (6 mg, 14.5  $\mu\text{mol}$ ) were added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (15 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (15 mL) and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (30 mL) and purified gradually with MeCN/H<sub>2</sub>O from 10 to 20% v/v yielding **13** as an orange solid. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD)  $\delta$  8.22 (bs, 1H), 8.19 – 8.13 (m, 2H), 7.76 (bs, 1H), 7.11 (s, 1H), 7.13 (s, 1H), 6.87 (d, *J* = 4.0 Hz, 2H), 6.84 – 6.77 (m, 2H), 6.72 (s, 1H), 6.70 (s, 1H), 6.57 (s, 1H), 6.28 (bs, 1H), 6.02 (s, 1H), 4.98 (bs, 1H), 4.60 (bs, 1H), 4.55 – 4.44 (m, 3H), 4.40 (bs, 1H), 4.28 (bs, 1H), 4.22 (bs, 1H), 4.14 (d, *J* = 11.2 Hz, 1H), 4.05 (bs, 1H), 3.78 (bs, 2H), 3.71 – 3.58 (m, 2H), 3.46 – 3.36 (m, 2H), 3.27 (d, *J* = 10.7 Hz, 1H), 3.13 – 3.02 (m, 2H), 2.92 – 2.82 (m, 2H), 2.72 – 2.44 (m, 10H), 2.58 (s, 6H), 2.43 – 2.32 (m, 3H), 2.27 (s, 3H), 2.19 (s, 3H), 2.15–1.67 (m, 8H), 1.89 (s, 3H), 1.40 (s, 3H), 1.38 (s, 3H), 1.37 (s, 3H), 1.33 – 1.22 (m, 2H), 1.25 (bs, 3H), 1.17 (s, 3H), 1.20 – 1.08 (m, 1H), 0.45 (s, 3H). <sup>13</sup>C NMR (126 MHz, CD<sub>3</sub>OD)  $\delta$  181.6, 180.1, 177.6, 177.4, 176.7, 175.6, 175.5, 175.4, 174.6, 174.3, 167.2, 166.9, 158.3, 143.3, 138.3, 135.7, 134.0, 131.3, 131.2, 130.7, 117.9, 115.7, 112.5, 108.7, 105.2, 103.7, 95.6, 88.3, 86.4, 81.3, 76.3, 75.4, 74.7, 70.7, 70.6, 69.9, 64.1, 60.3, 57.7, 57.0, 55.2, 52.5, 46.6, 43.0, 41.6, 40.1, 36.2, 33.5, 33.3, 33.0, 32.3, 29.5, 27.5, 27.4, 20.9, 20.53, 20.45, 20.3, 20.1, 19.9, 17.5, 17.1, 16.4, 16.1. HRMS (ESI) *m/z* [M + H + Na]<sup>2+</sup> calcd for C<sub>92</sub>H<sub>112</sub>CoN<sub>19</sub>O<sub>22</sub>PNa, 973.8593; found, 973.8585.

#### HPLC Method:

Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN [%]	$\lambda$ [nm]	R <sub>t</sub> [min]
Initial	99	1	361	11.78
15	30	70		

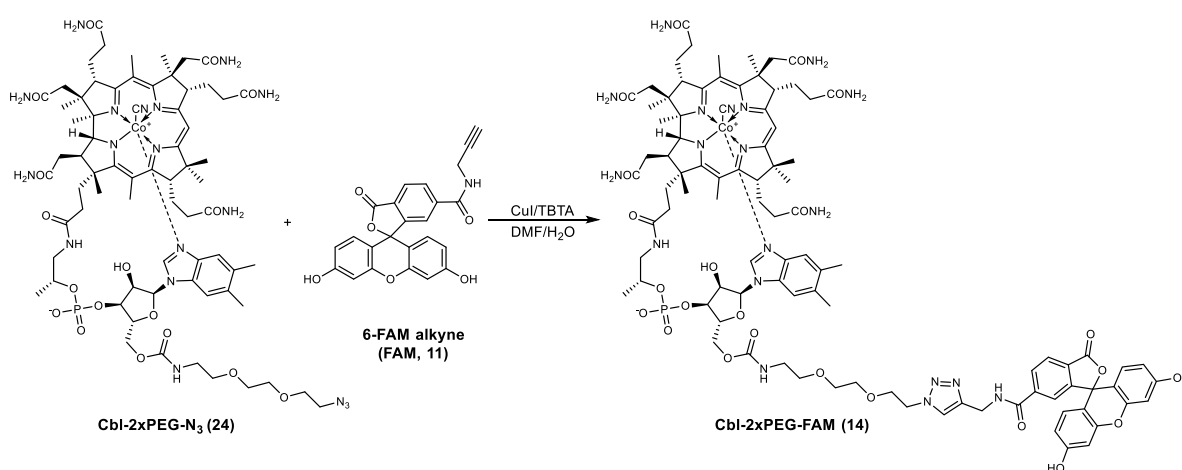




Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-1xPEG-FAM - S 2850: Czan 1)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	11.783	2832,624	204,294	100,0	100,0	0,20	664
	Total	2832,624	204,294	100,0	100,0		

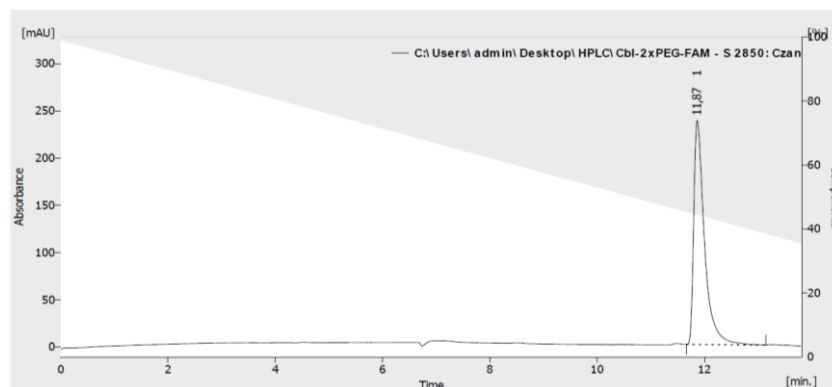
### 3.4 Cbl-2xPEG-FAM (14)



**Cbl-2xPEG-FAM:** CuI (1 mg, 5  $\mu\text{mol}$ ) and TBTA (5 mg, 10  $\mu\text{mol}$ ) were dissolved in DMF/H<sub>2</sub>O (1 mL, 3:1 v/v) and stirred for 20 min. Subsequently Cbl-2xPEG-N<sub>3</sub> (**24**) (27 mg, 17.4  $\mu\text{mol}$ ) and 6-FAM alkyne (**11**) (6 mg, 14.5  $\mu\text{mol}$ ) were added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (15 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (15 mL), and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (30 mL) and purified gradually with MeCN/H<sub>2</sub>O from 10 to 20% v/v yielding **14** as an orange solid. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD)  $\delta$  8.20 (bs, 1H), 8.19 – 8.12 (m, 2H), 7.72 (bs, 1H), 7.18 (s, 1H), 7.13 (s, 1H), 6.84 (s, 2H), 6.82 – 6.76 (m, 2H), 6.71 – 6.64 (m, 2H), 6.56 (s, 1H), 6.24 (bs, 1H), 6.02 (s, 1H), 4.93 (bs, 1H), 4.68 – 4.55 (m, 2H), 4.55 – 4.44 (m, 3H), 4.41 (bs, 1H), 4.23 (bs, 2H), 4.13 (d, *J* = 11.1 Hz, 2H), 3.82 (s, 2H), 3.71 – 3.57 (m, 2H), 3.50 (bs, 2H), 3.43 (bs, 2H), 3.34 – 3.29 (m, 4H), 3.27 (d, *J* = 10.7, 1H), 3.10 (bs, 2H), 2.93 – 2.78 (m, 2H), 2.69 – 2.44 (m, 9H), 2.58 (s, 6H), 2.42 – 2.31 (m, 3H), 2.27 (s, 3H), 2.21 (s, 3H), 2.16 – 1.70 (m, 6H), 1.89 (s, 3H), 1.41 (s, 3H), 1.38 (s, 3H), 1.36 (s, 3H), 1.33 – 1.21 (m, 2H), 1.26 (bs, 3H), 1.17 (s, 3H), 1.19 – 1.06 (m, 1H), 0.45 (s, 3H). <sup>13</sup>C NMR (126 MHz, CD<sub>3</sub>OD) 181.6, 180.1, 177.6, 177.4, 176.7, 175.6, 175.5, 175.4, 174.6, 174.3, 169.8, 167.2, 166.9, 143.3, 138.3, 135.1, 131.3, 131.1, 130.6, 117.9, 115.3, 112.4, 112.2, 108.7, 105.2, 103.6, 95.6, 88.3, 86.4, 81.3, 76.3, 75.4, 74.6, 71.3, 71.2, 70.8, 70.5, 70.2, 64.1, 60.3, 57.7, 57.0, 55.2, 52.5, 51.7, 46.6, 44.0, 43.0, 41.8, 40.1, 36.2, 33.5, 33.2, 33.0, 32.3, 29.5, 27.5, 27.4, 20.9, 20.53, 20.45, 20.3, 20.0, 19.9, 17.5, 17.0, 16.4, 16.1. HRMS (ESI) *m/z* [*M* + 2Na]<sup>2+</sup> calcd for C<sub>94</sub>H<sub>115</sub>CoN<sub>19</sub>O<sub>23</sub>PNa<sub>2</sub>, 1006.8634; found, 1006.8627.

## HPLC Method:

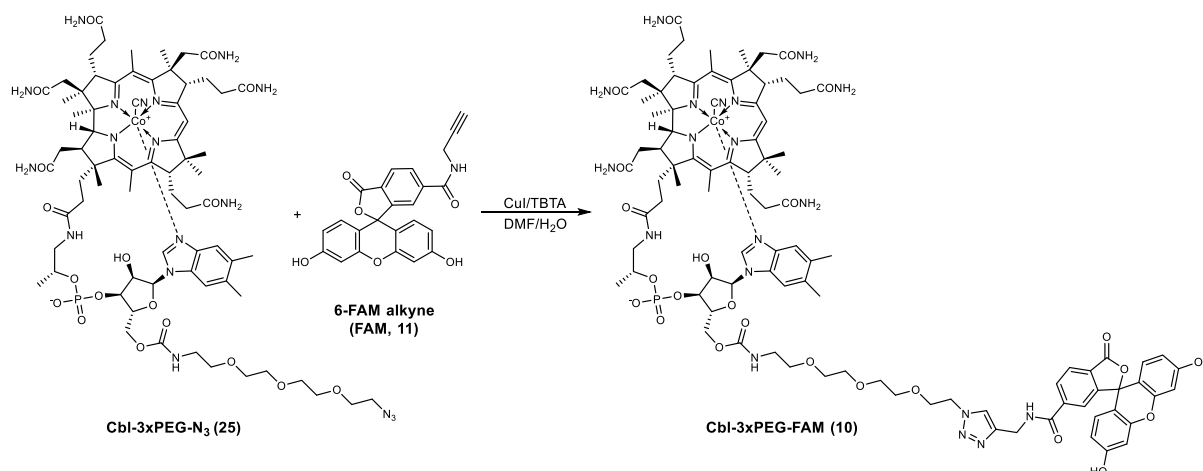
Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN [%]	$\lambda$ [nm]	R <sub>t</sub> [min]
Initial	99	1	361	11.87
15	30	70		



Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-2xPEG-FAM - S 2850: Canel 1)

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
11,867	3306,287	237,538	100,0	100,0	0,22	951
Total	3306,287	237,538	100,0	100,0		

## 3.5 Cbl-3xPEG-FAM (10)

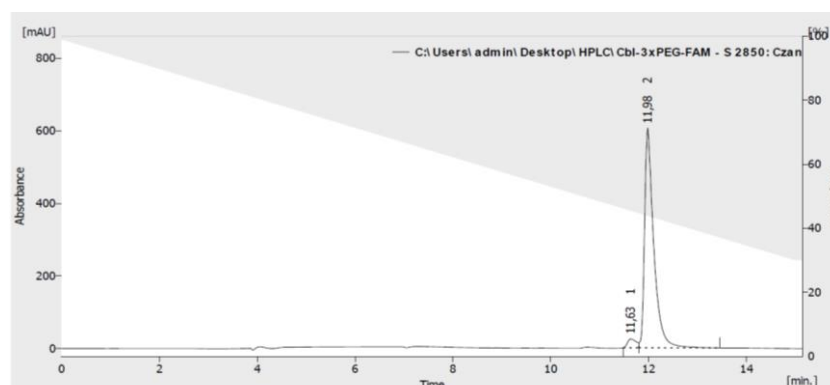


**Cbl-3xPEG-FAM:** CuI (1 mg, 5  $\mu$ mol) and TBTA (5 mg, 10  $\mu$ mol) were dissolved in DMF/H<sub>2</sub>O (1 mL, 3:1 v/v) and stirred for 20 min. Subsequently Cbl-3xPEG-N<sub>3</sub> (**25**) (28 mg, 17.5  $\mu$ mol) and 6-FAM alkyne (**11**) (6 mg, 14.5  $\mu$ mol) were added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (15 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (15 mL), and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (30 mL) and purified gradually with MeCN/H<sub>2</sub>O from 10 to 20% v/v yielding **10** as an orange solid. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD)  $\delta$  8.25 (bs, 1H), 8.11 (s, 1H), 7.75 (bs, 2H), 7.20 (s, 1H), 7.14 (s, 1H), 6.70 (s, 2H), 6.61 (s, 2H), 6.58 – 6.45 (m, 3H), 6.23 (bs, 1H), 6.02 (s, 1H), 4.65 – 4.44 (m, 5H), 4.32 – 4.17 (m, 3H), 4.17 – 4.06 (m, 2H), 3.87 (bs, 2H), 3.70 – 3.65 (m, 2H), 3.65 – 3.58 (m, 2H), 3.52 – 3.36 (m, 12H), 3.28 (m, 1H), 3.25 – 3.14 (m, 2H), 2.95 – 2.82 (m, 2H), 2.71 – 2.41 (m, 8H), 2.58 (s, 6H), 2.42 – 2.32 (m, 2H), 2.28 (s, 3H), 2.22 (s, 3H), 2.17 – 1.79 (m, 6H), 1.88 (s, 3H), 1.79 – 1.70 (m, 1H), 1.42 (s, 3H), 1.38 (s, 3H), 1.36 (s, 3H), 1.33 – 1.20 (m, 5H), 1.17 (s, 3H), 1.14–1.05 (m, 1H), 0.46 (s, 3H). <sup>13</sup>C NMR (126 MHz, CD<sub>3</sub>OD)  $\delta$  181.6, 180.2, 177.6, 177.4, 176.6, 175.2, 175.3, 167.2, 166.9, 154.0, 143.4, 138.3, 135.7,

134.0, 131.4, 130.3, 117.9, 112.5, 110.9, 108.7, 105.2, 103.7, 95.6, 86.4, 76.4, 71.4, 71.37, 71.32, 71.2, 71.1, 70.9, 60.3, 57.7, 56.9, 55.2, 52.5, 51.9, 43.0, 41.8, 40.1, 33.3, 32.3, 29.5, 27.5, 27.4, 20.9, 20.6, 20.6, 20.3, 19.9, 17.5, 17.1, 16.4, 16.1. HRMS (ESI)  $m/z$   $[M + H + Na]^{2+}$  calcd for  $C_{96}H_{120}CoN_{19}O_{24}PNa$ , 1017.8855; found, 1017.8862.

### HPLC Method:

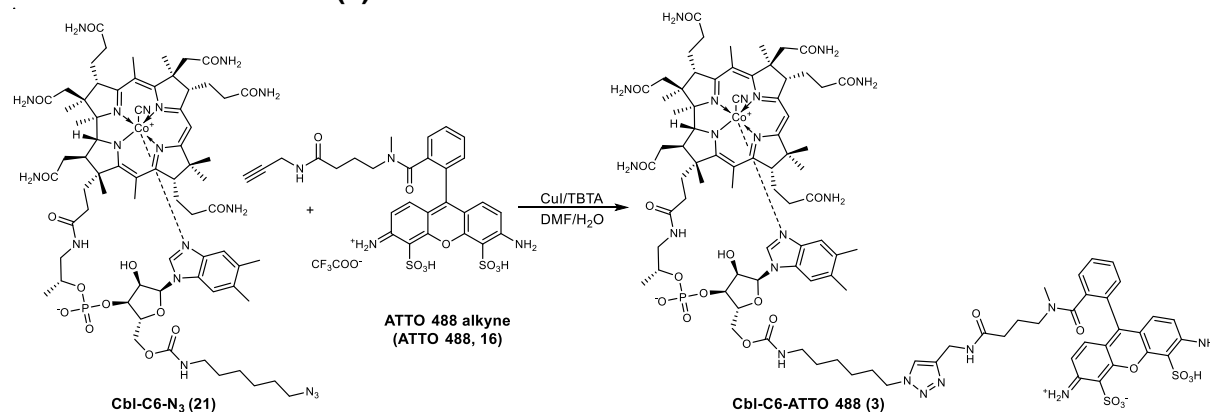
Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN [%]	$\lambda$ [nm]	R <sub>t</sub> [min]
Initial	99	1	361	11.98
15	30	70		



Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-3xPEG-FAM - S 2850 - Czan 1)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	11,633	290,747	24,181	3,6	3,8	0,25	568
2	11,983	7871,066	605,778	96,4	96,2	0,20	568
Total		8161,813	629,959	100,0	100,0		

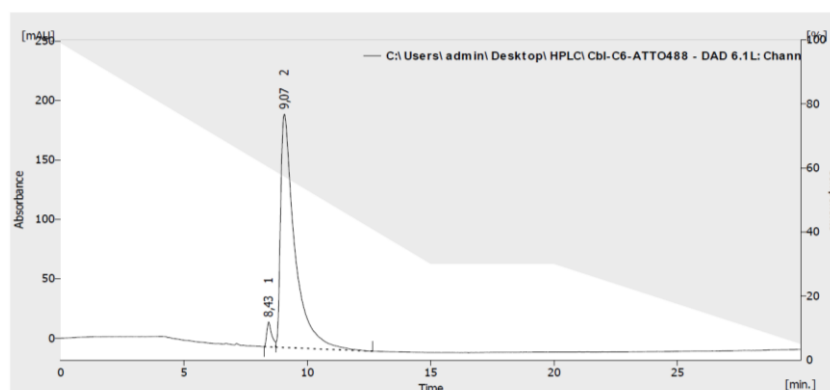
### 3.6 Cbl-C6-ATTO 488 (3)



**Cbl-C6-ATTO 488:** Preparation of a catalyst solution: CuI (1 mg, 5  $\mu$ mol) and TBTA (5 mg, 10  $\mu$ mol) were dissolved in DMF (2 mL) and stirred for 20 min. Cbl-C6-N<sub>3</sub> (**21**) (3 mg, 1.97  $\mu$ mol) and ATTO 488 alkyne (**16**) (0.5 mg, 0.68  $\mu$ mol) were dissolved in DMF/H<sub>2</sub>O (200  $\mu$ L, 1:1, v/v) and subsequently freshly prepared catalyst solution (300  $\mu$ L) was added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (10 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (10 mL), and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (10 mL) and purified gradually with MeCN/H<sub>2</sub>O from 10 to 20% v/v yielding **3** as an orange solid. HRMS (ESI)  $m/z$   $[M + 2Na]^{2+}$  calcd for  $C_{98}H_{120}CoN_{22}O_{24}PS_2Na_2^{2+}$ , 1097.3805; found, 1097.3818.

## HPLC Method:

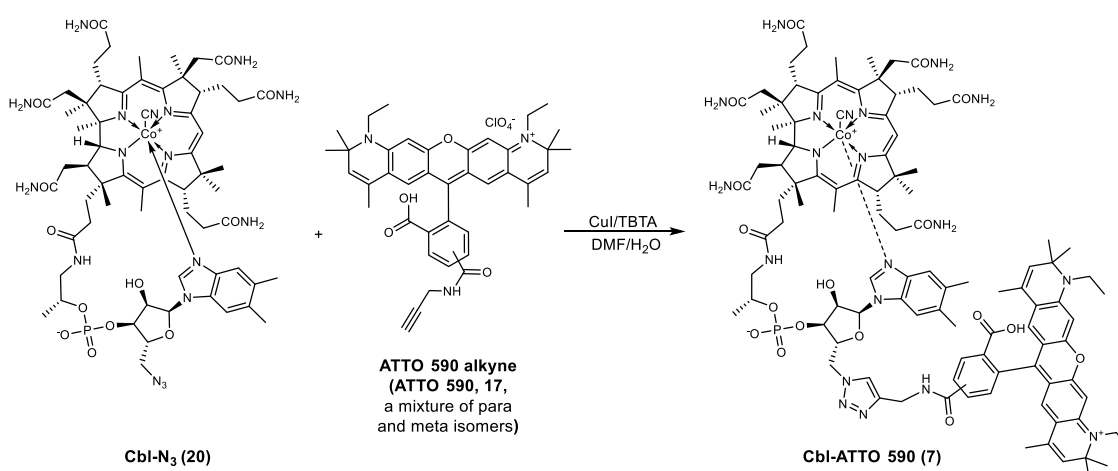
Time [min]	H <sub>2</sub> O+0.2%TFA [%]	MeCN [%]	λ [nm]	R <sub>t</sub> [min]
Initial	99	1	488	9.07
15	30	70		
20	30	70		
30	5	95		



Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-C6-ATTO488 - DAD 6.1L: Channel 3)

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity	
1	8,433	287,697	3,5	9,6	0,22	729	
2	9,067	7857,586	196,321	96,5	90,4	0,55	480
Total		8145,283	217,246	100,0	100,0		

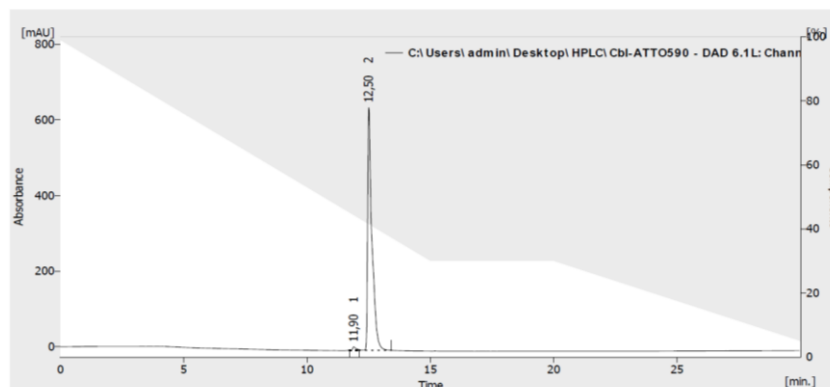
## 3.7 Cbl-ATTO 590 (7)



**Cbl-ATTO 590:** Preparation of a catalyst solution: CuI (1 mg, 5 μmol) and TBTA (5 mg, 10 μmol) were dissolved in DMF (2 mL) and stirred for 20 min. Cbl-N<sub>3</sub> (**20**) (3 mg, 2.20 μmol) and ATTO 590 alkyne (**17**) (0.5 mg, 0.68 μmol) were dissolved in DMF/H<sub>2</sub>O (200 μL, 1:1, v/v) and subsequently freshly prepared catalyst solution (300 μL) was added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (10 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (10 mL), and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (10 mL) and purified gradually with MeCN/H<sub>2</sub>O from 15 to 30% v/v yielding **7** as a violet solid. HRMS (ESI) m/z [M + H]<sup>2+</sup> calcd for C<sub>103</sub>H<sub>130</sub>CoN<sub>20</sub>O<sub>17</sub>P<sup>+</sup>, 1004.4491; found, 1004.4499.

## HPLC Method:

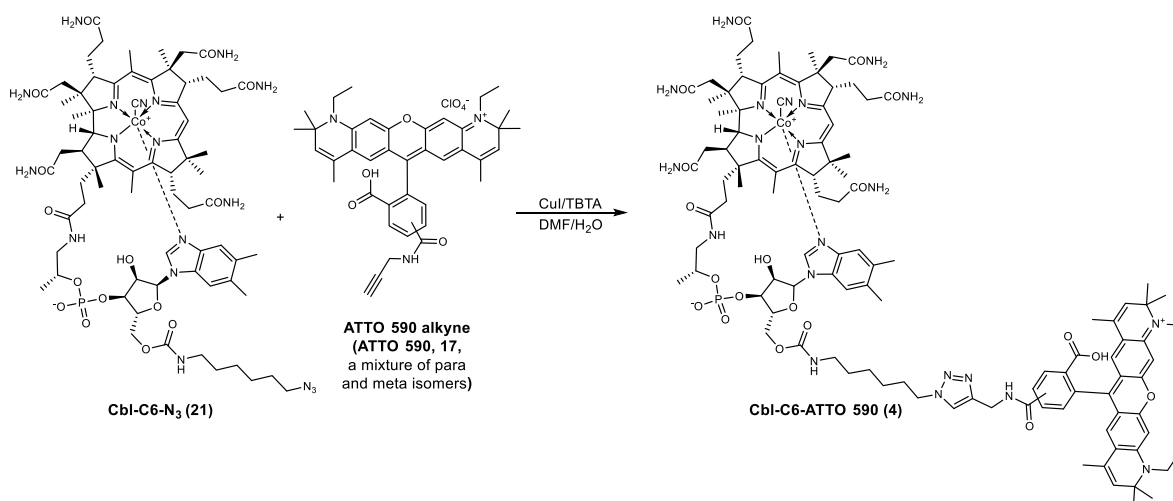
Time [min]	H <sub>2</sub> O+0.2%TFA [%]	MeCN [%]	λ [nm]	R <sub>t</sub> [min]
Initial	99	1	590	12.50
15	30	70		
20	30	70		
30	5	95		



Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-ATTO590 - DAD 6.1L: Channel 3)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	11,900	88,896	9,427	1,1	1,4	0,15	989
2	12,500	7728,290	641,767	98,9	98,6	0,17	413
	Total	7817,186	651,194	100,0	100,0		

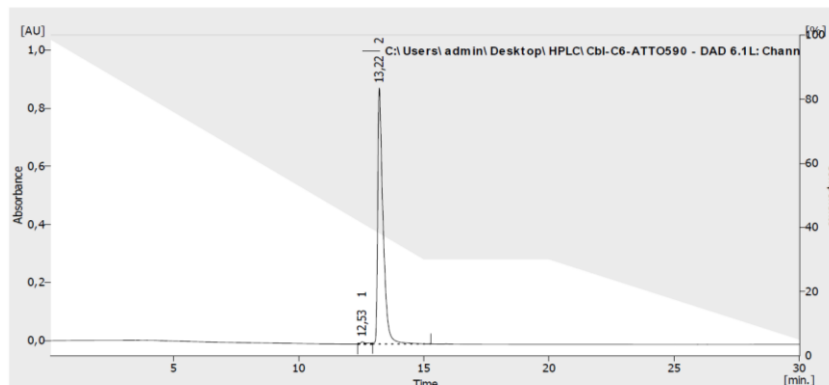
### 3.8 Cbl-C6-ATTO 590 (4)



**Cbl-C6-ATTO 590:** Preparation of a catalyst solution: CuI (1 mg, 5 μmol) and TBTA (5 mg, 10 μmol) were dissolved in DMF (2 mL) and stirred for 20 min. Cbl-C6-N<sub>3</sub> (**21**) (3 mg, 1.97 μmol) and ATTO 590 alkyne (**17**) (0.5 mg, 0.68 μmol) were dissolved in DMF/H<sub>2</sub>O (200 μL, 1:1, v/v) and subsequently freshly prepared catalyst solution (300 μL) was added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (10 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (10 mL), and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (10 mL) and purified gradually with MeCN/H<sub>2</sub>O from 15 to 40% v/v yielding **4** as a violet solid. HRMS (ESI) m/z [M + H]<sup>2+</sup> calcd for C<sub>110</sub>H<sub>143</sub>CoN<sub>21</sub>O<sub>19</sub>P<sup>+</sup>, 1075.9964; found, 1075.9967.

#### HPLC Method:

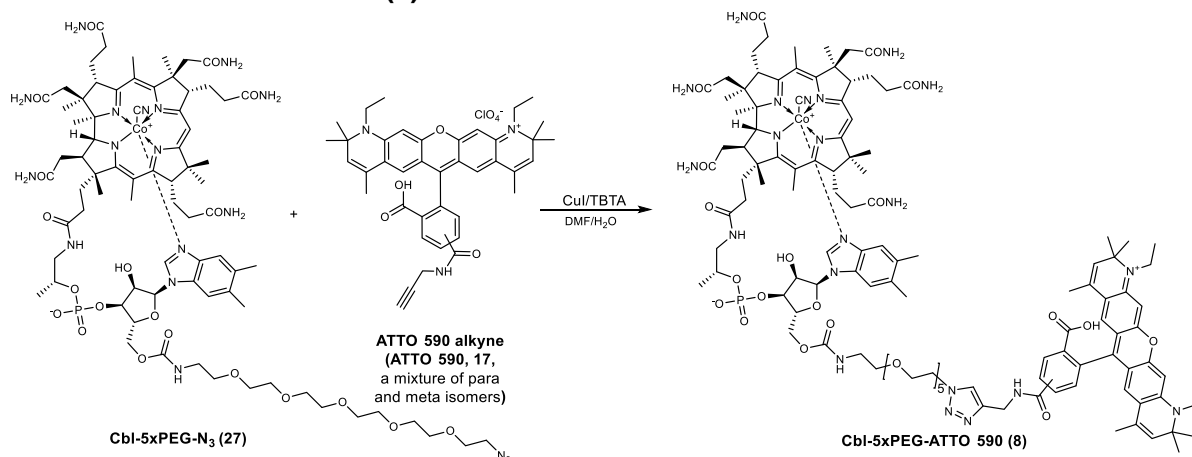
Time [min]	H <sub>2</sub> O+0.2%TFA [%]	MeCN[%]	λ [nm]	R <sub>t</sub> [min]
Initial	99	1	590	13.22
15	30	70		
20	30	70		
30	5	95		



Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-C6-ATTO590 - DAD 6.1L: Channel 3)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	12,533	113,565	7,654	0,9	0,9	0,18	994
2	13,217	13048,298	881,392	99,1	99,1	0,22	152
	Total	13161,864	889,046	100,0	100,0		

### 3.9 Cbl-5xPEG-ATTO 590 (8)

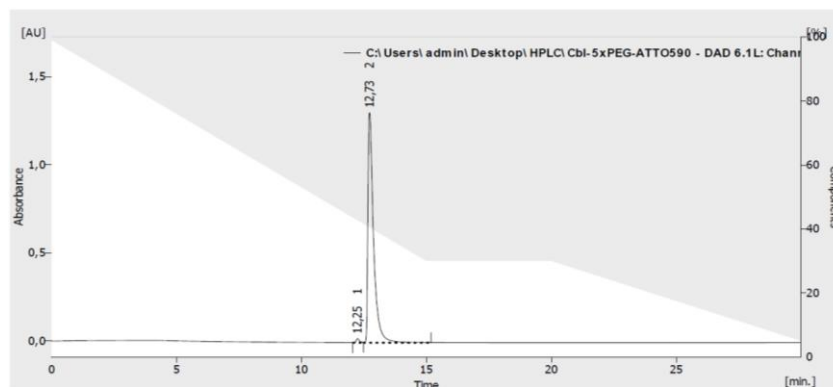


**Cbl-5xPEG-ATTO 590:** Preparation of a catalyst solution: CuI (1 mg, 5 μmol) and TBTA (5 mg, 10 μmol) were dissolved in DMF (2 mL) and stirred for 20 min. Cbl-5xPEG-N<sub>3</sub> (27) (3 mg, 1.78 μmol) and ATTO 590 alkyne (17) (0.5 mg, 0.68 μmol) were dissolved in DMF/H<sub>2</sub>O (200 μL, 1:1, v/v) and subsequently freshly prepared catalyst solution (300 μL) was added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (10 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (10 mL), and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (10 mL) and purified gradually with MeCN/H<sub>2</sub>O from 15 to 30% v/v yielding **8** as a violet solid. HRMS (ESI) m/z [M + Na]<sup>2+</sup> calcd for C<sub>116</sub>H<sub>154</sub>CoN<sub>21</sub>O<sub>24</sub>PNa<sup>+</sup>, 1169.0216; found, 1169.0219

#### HPLC Method:

Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN [%]	λ [nm]	R <sub>t</sub> [min]
Initial	99	1	590	12.73
15	30	70		

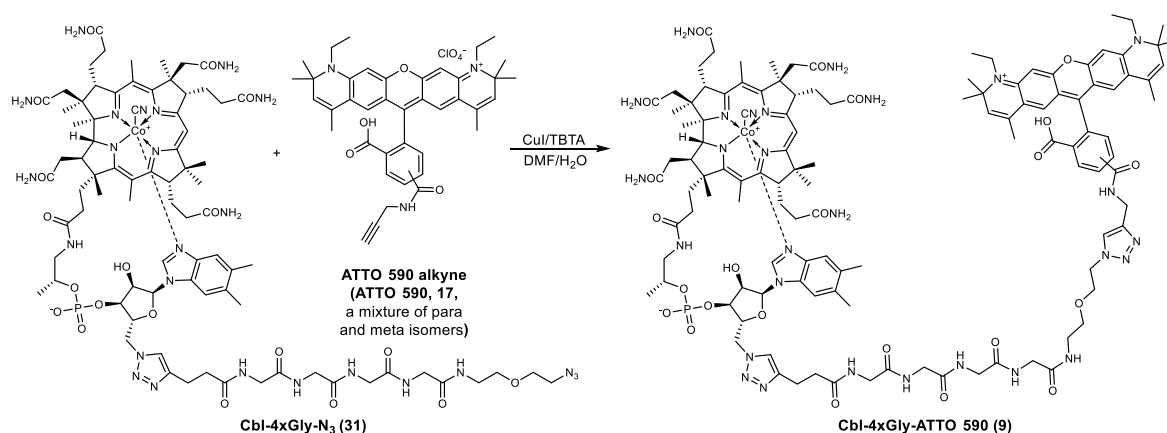
20	30	70	
30	5	95	



Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-5xPEG-ATTO590 - DAD 6.1L: Channel 3)

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	12,250	207,748	23,311	1,0	1,8	0,15
2	12,733	20468,553	1304,040	99,0	98,2	0,23
Total	20676,302	1327,351	100,0	100,0		

### 3.10 Cbl-4xGly-ATTO 590 (9)

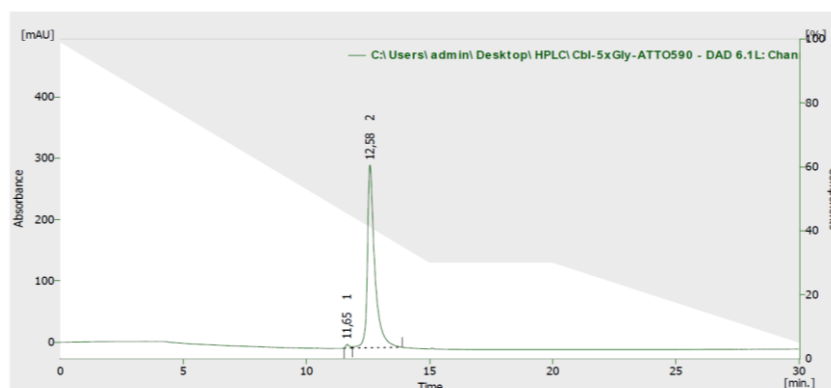


**Cbl-4xGly-ATTO 590:** Preparation of a catalyst solution: CuI (1 mg, 5  $\mu$ mol) and TBTA (5 mg, 10  $\mu$ mol) were dissolved in DMF (2 mL) and stirred for 20 min. Cbl-4xGly-N<sub>3</sub> (**31**) (4 mg, 2.20  $\mu$ mol) and ATTO 590 alkyne (**17**) (0.5 mg, 0.68  $\mu$ mol) were dissolved in DMF/H<sub>2</sub>O (400  $\mu$ L, 3:1, v/v) and subsequently freshly prepared catalyst solution (300  $\mu$ L) was added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (10 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (10 mL), and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (10 mL) and purified gradually with MeCN/H<sub>2</sub>O from 15 to 40% v/v yielding **9** as a violet solid. HRMS (ESI) m/z [M + H]<sup>2+</sup> calcd for C<sub>120</sub>H<sub>156</sub>CoN<sub>28</sub>O<sub>23</sub>P<sup>+</sup>, 2447.0957; found, 2447.0932.

#### HPLC Method:

Time [min]	H <sub>2</sub> O+0.5%TFA [%]	MeCN [%]	$\lambda$ [nm]	R <sub>t</sub> [min]
Initial	99	1	590	12.58
15	30	70		

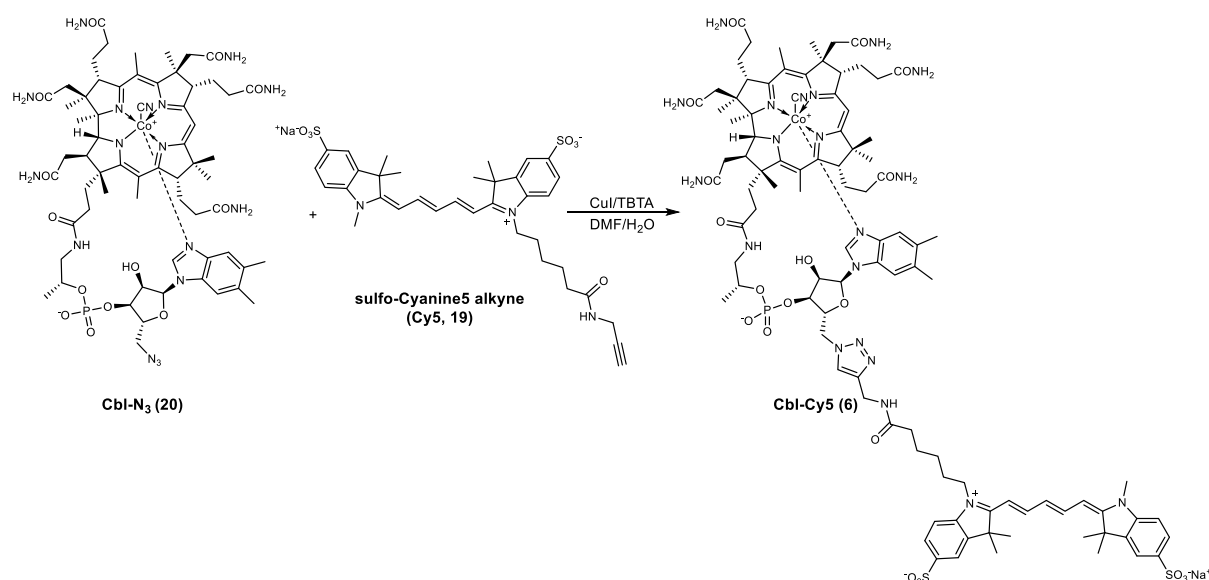
20	30	70	
30	5	95	



Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-5xGly-ATTO590 - DAD 6.1L: Chan 3)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	11,650	61,377	6,124	1,0	2,0	0,17	998
2	12,583	6236,030	297,715	99,0	98,0	0,28	345
Total		6297,407	303,838	100,0	100,0		

### 3.11 Cbl-Cy5 (6)



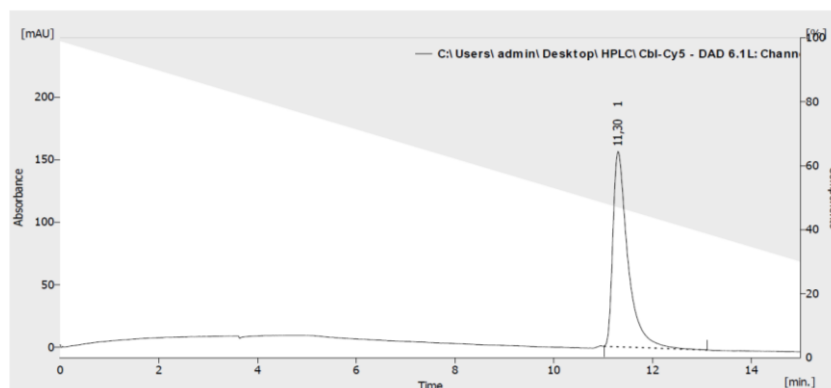
**Cbl-Cy5:** Preparation of a catalyst solution: CuI (1 mg, 5  $\mu\text{mol}$ ) and TBTA (5 mg, 10  $\mu\text{mol}$ ) were dissolved in DMF (2 mL) and stirred for 20 min. Cbl-N<sub>3</sub> (**20**) (3 mg, 2.20  $\mu\text{mol}$ ) and sulfo-Cyanine5 alkyne (**19**) (0.5 mg, 0.72  $\mu\text{mol}$ ) were dissolved in DMF/H<sub>2</sub>O (200  $\mu\text{L}$ , 1:1, v/v) and subsequently freshly prepared catalyst solution (300  $\mu\text{L}$ ) was added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (10 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (10 mL), and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (10 mL) and purified gradually with MeCN/H<sub>2</sub>O from 10 to 20% v/v yielding **6** as a blue solid. HRMS (ESI)  $m/z$  [M + 2Na]<sup>2+</sup> calcd for C<sub>98</sub>H<sub>127</sub>CoN<sub>20</sub>O<sub>20</sub>PS<sub>2</sub>Na<sub>3</sub>, 1063.3864; found, 1063.3871.

#### HPLC Method:

Time [min]	MeCN[%]	$\lambda$ [nm]	R <sub>t</sub> [min]
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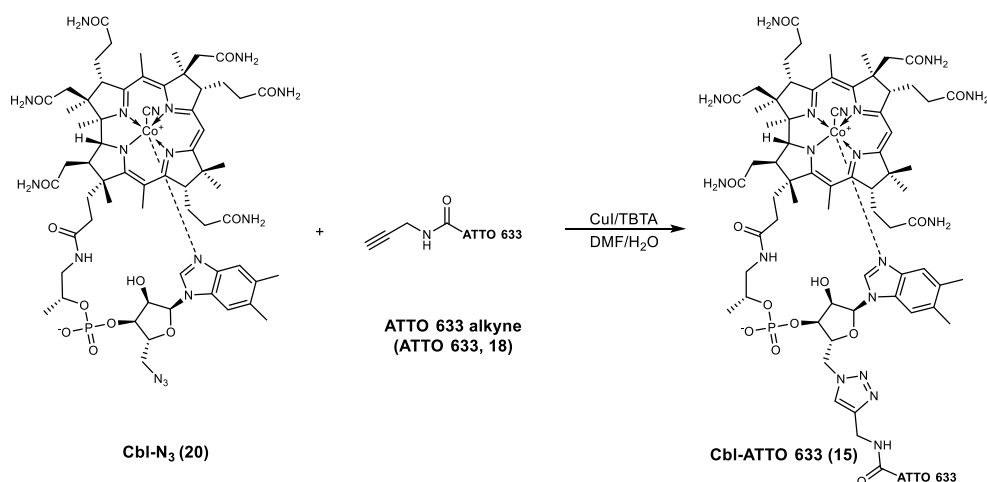
<b>Initial</b>	99	1	646	11.30
<b>15</b>	30	70		



Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-Cy5 - DAD 6.1L: Channel 3)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	11,300	3283,150	155,931	100,0	100,0	0,30	613
	Total	3283,150	155,931	100,0	100,0		

### 3.12 Cbl-ATTO 633 (15)

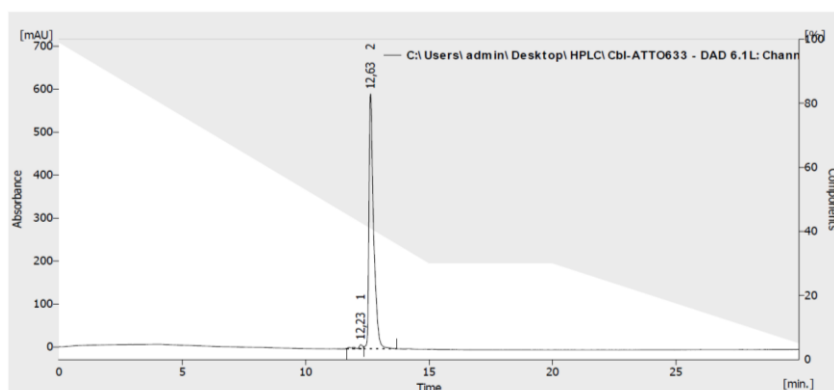


**Cbl-ATTO 633:** Preparation of a catalyst solution: CuI (1 mg, 5  $\mu$ mol) and TBTA (5 mg, 10  $\mu$ mol) were dissolved in DMF (2 mL) and stirred for 20 min. Cbl-N<sub>3</sub> (**20**) (3 mg, 2.20  $\mu$ mol) and ATTO 633 alkyne (**18**) (0.5 mg, 0.72  $\mu$ mol) were dissolved in DMF/H<sub>2</sub>O (200  $\mu$ L, 1:1, v/v) and subsequently freshly prepared catalyst solution (300  $\mu$ L) was added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (10 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (10 mL), and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (10 mL) and purified by RP column chromatography gradually with MeCN/H<sub>2</sub>O from 15 to 40% v/v yielding **15** as a blue solid. LRMS (ESI) m/z [M + Na + H]<sup>2+</sup> found, 995.96.

#### HPLC Method:

Time [min]	H <sub>2</sub> O+0.2%TFA [%]	MeCN [%]	$\lambda$ [nm]	R <sub>t</sub> [min]
<b>Initial</b>	99	1	633	12.63
<b>15</b>	30	70		

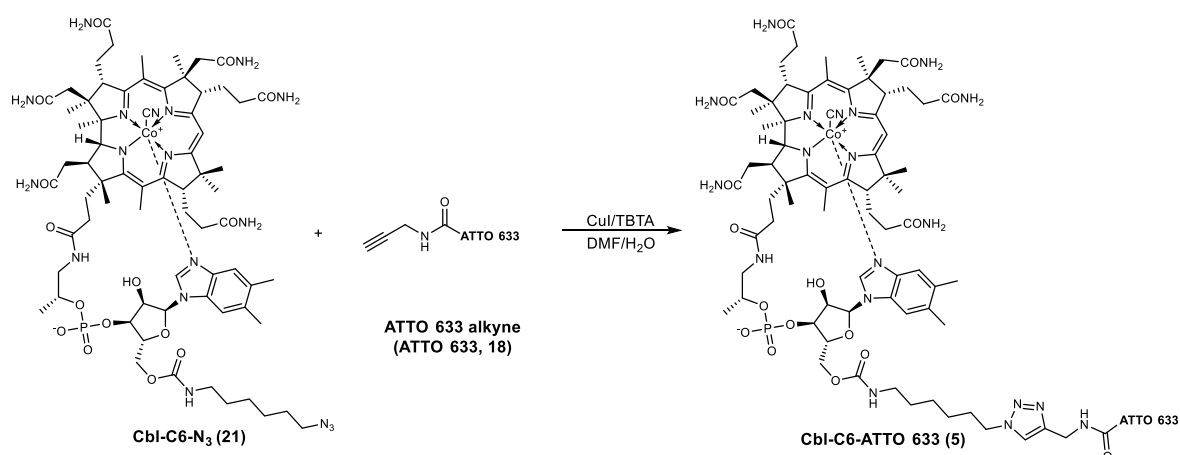
20	30	70		
30	5	95		



Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-ATTO633 - DAD 6.1L: Channel 3)

Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
12,233	130,633	9,482	1,8	1,6	0,18	855
12,633	7783,171	592,556	98,2	98,4	0,20	508
Total	7922,804	602,037	100,0	100,0		

### 3.13 Cbl-C6-ATTO 633 (5)

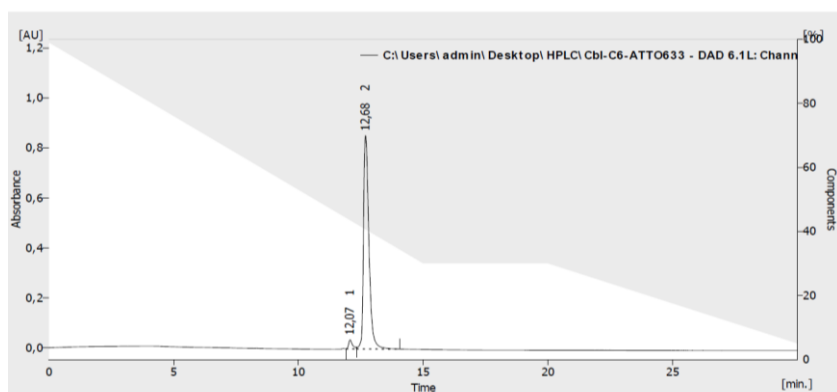


**Cbl-C6-ATTO 633:** Preparation of a catalyst solution: CuI (1 mg, 5  $\mu$ mol) and TBTA (5 mg, 10  $\mu$ mol) were dissolved in DMF (2 mL) and stirred for 20 min. Cbl-C6-N<sub>3</sub> (**21**) (3 mg, 1.97  $\mu$ mol) and ATTO 633 alkyne (**18**) (0.5 mg, 0.72  $\mu$ mol) were dissolved in DMF/H<sub>2</sub>O (200  $\mu$ L, 1:1, v/v) and subsequently freshly prepared catalyst solution (300  $\mu$ L) was added and the reaction mixture was stirred overnight. Then it was diluted with DMF (1 mL), poured into AcOEt (10 mL) and the precipitate was centrifuged and dried. The crude solid was dissolved in MeOH (1 mL), precipitated with Et<sub>2</sub>O (10 mL), and then centrifuged. The dried solid was then dissolved in H<sub>2</sub>O, loaded onto RP column (10 mL) and purified crude product was purified by RP column chromatography gradually with MeCN/H<sub>2</sub>O from 20 to 50% v/v yielding **5** as a blue solid. LRMS (ESI) m/z [M + Na + H]<sup>2+</sup> found, 1067.51.

#### HPLC Method:

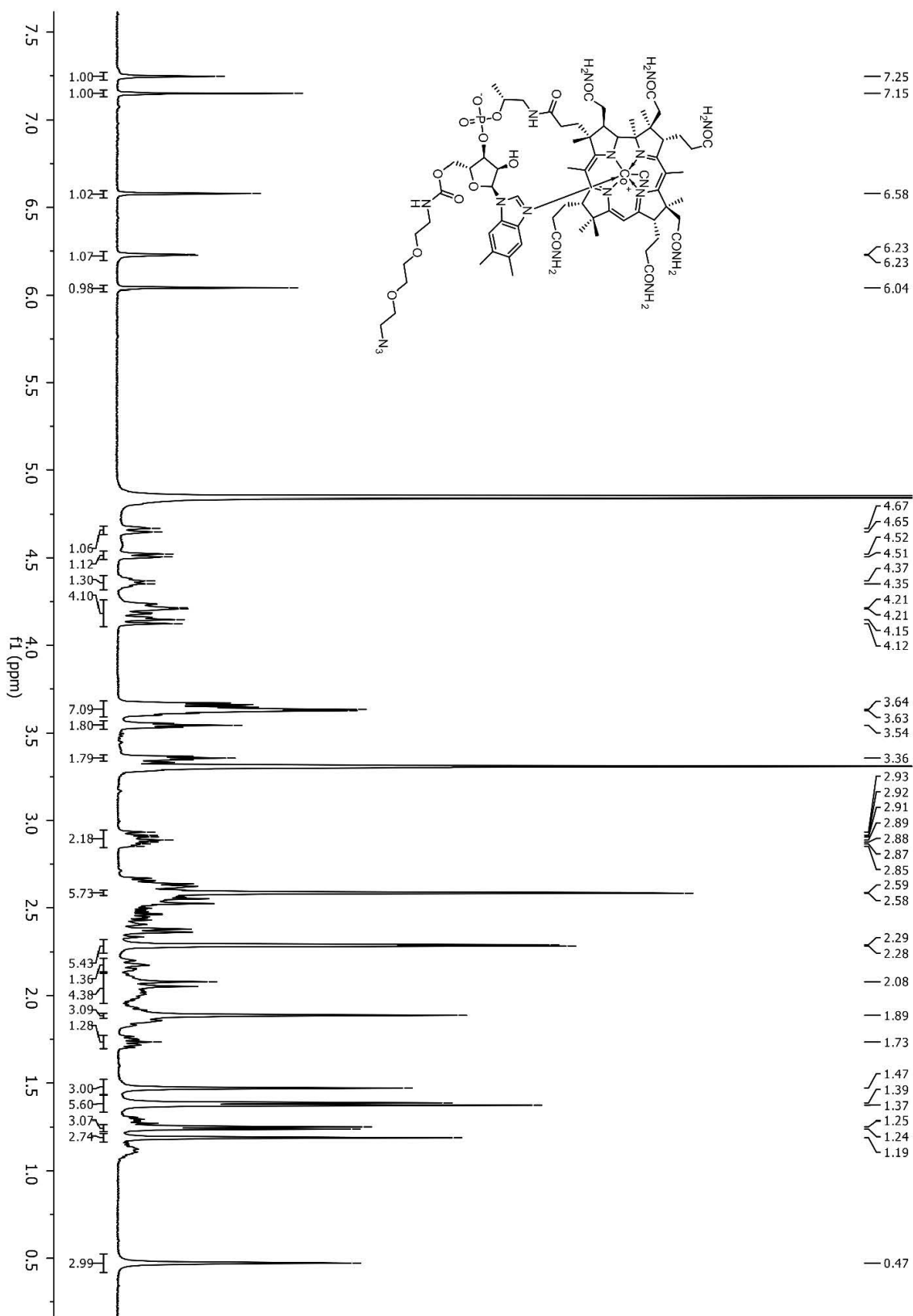
Time [min]	MeCN[%]	$\lambda$ [nm]	R <sub>t</sub> [min]
Initial	99	633	12.68
15	30		

20	30	70	
30	5	95	

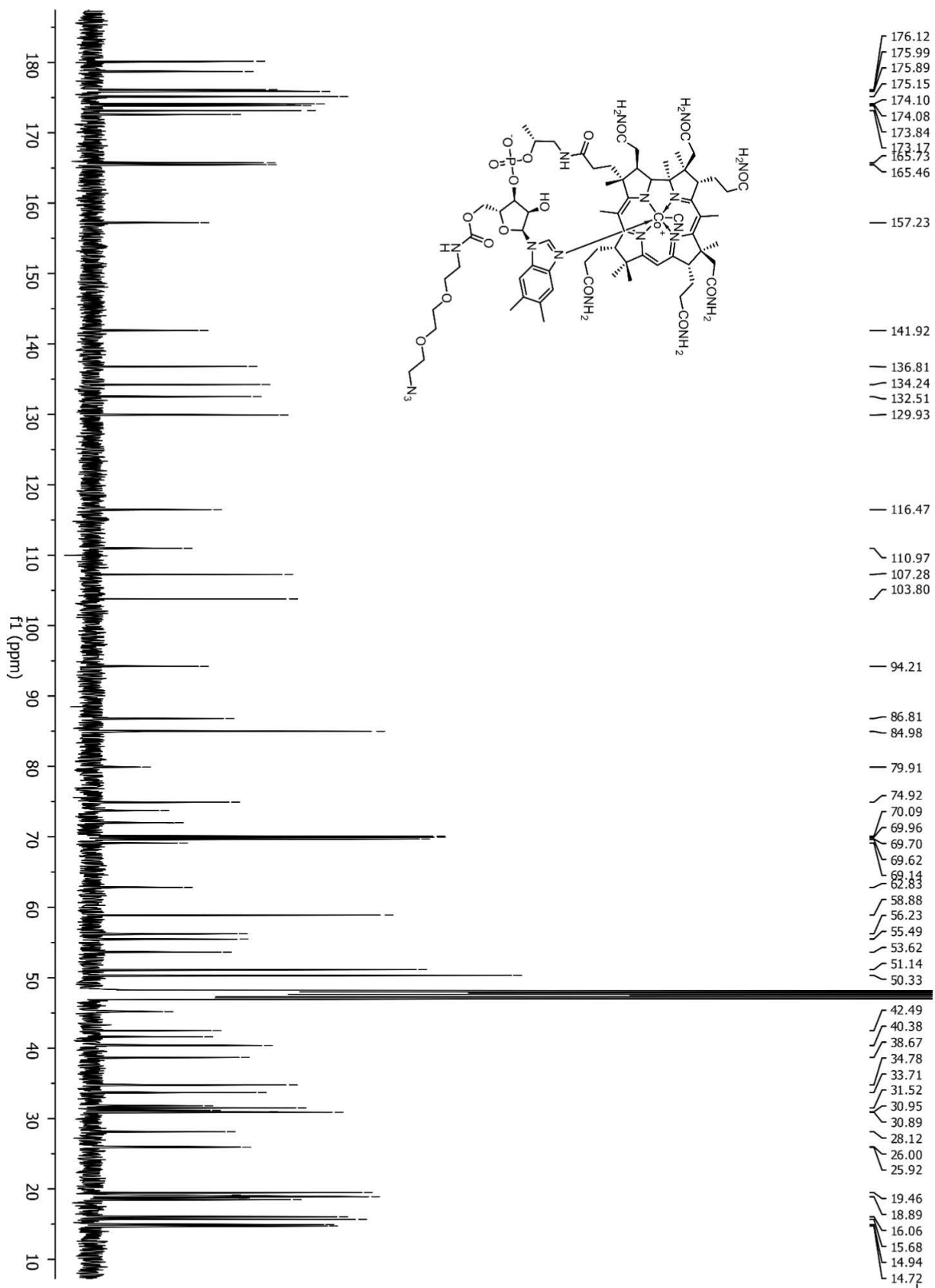


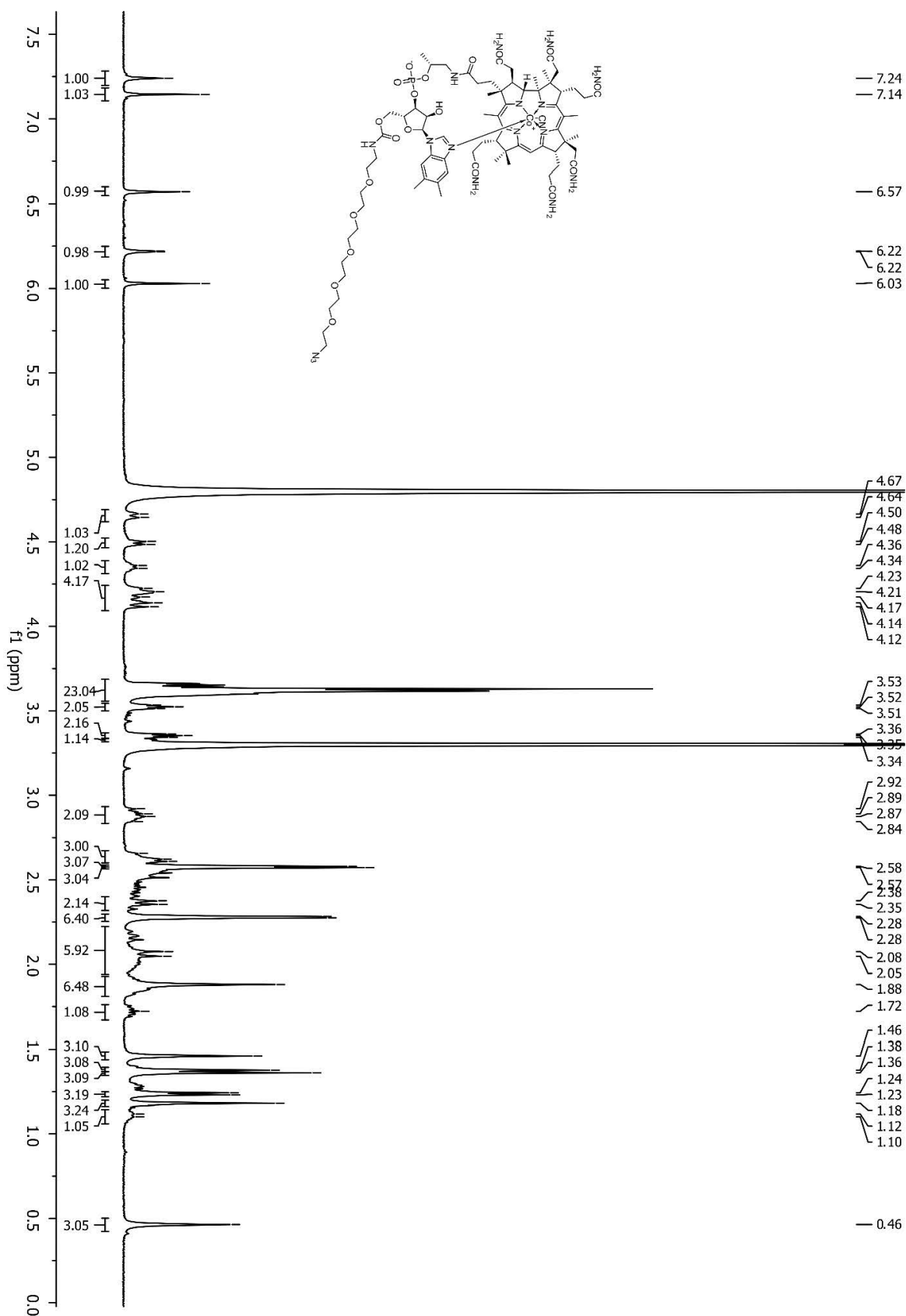
Result Table (Uncal - C:\Users\admin\Desktop\HPLC\Cbl-C6-ATTO633 - DAD 6.1L- Channel 3)

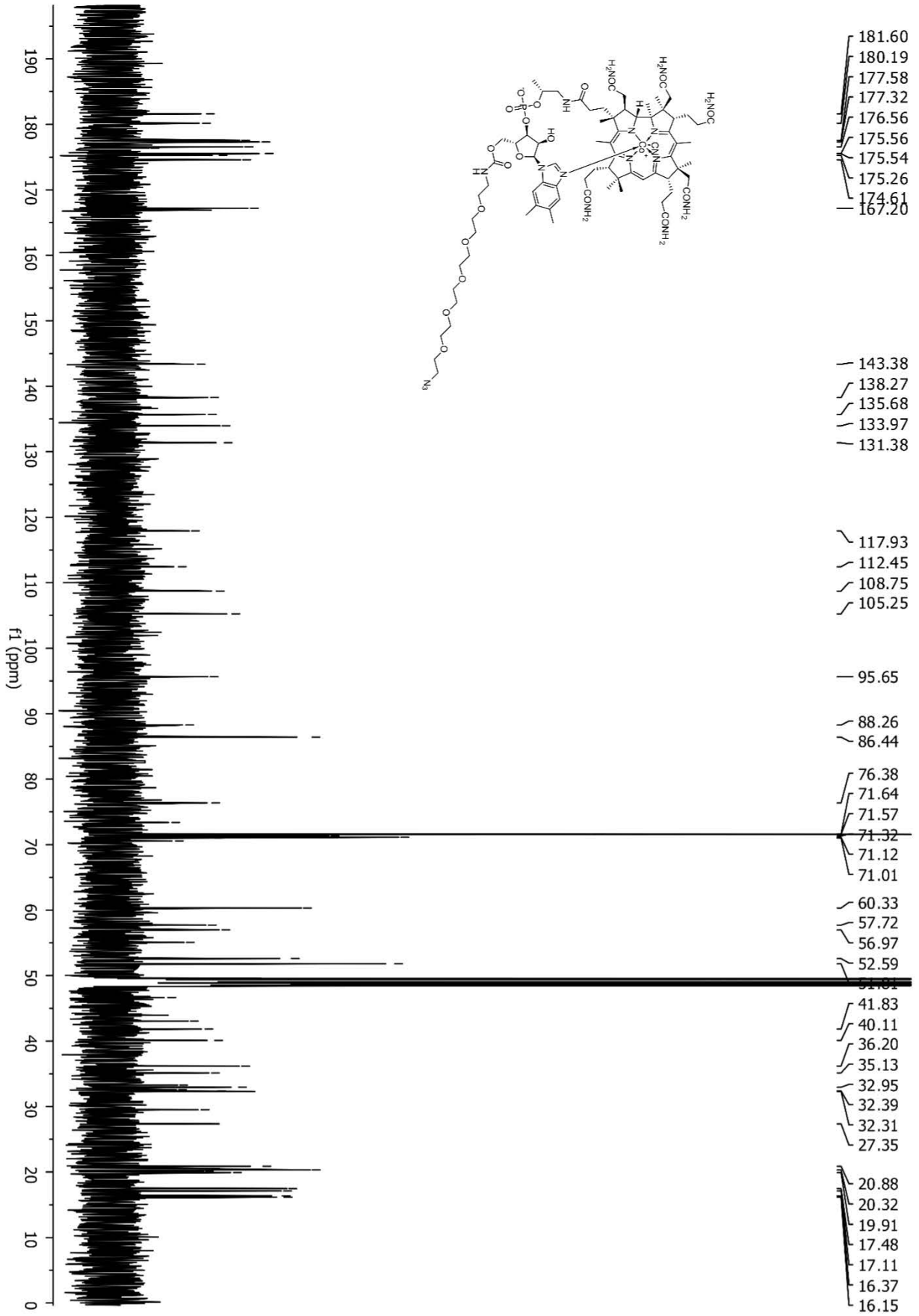
	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	PDA Peak Purity
1	12,067	387,217	36,352	2,9	4,1	0,17	873
2	12,683	12923,830	854,169	97,1	95,9	0,25	518
	Total	13311,047	890,520	100,0	100,0		



4. NMR spectra  
4.1 NMR spectra of Cbl-2xPEG-N<sub>3</sub>(24) recorded for CD<sub>3</sub>OD





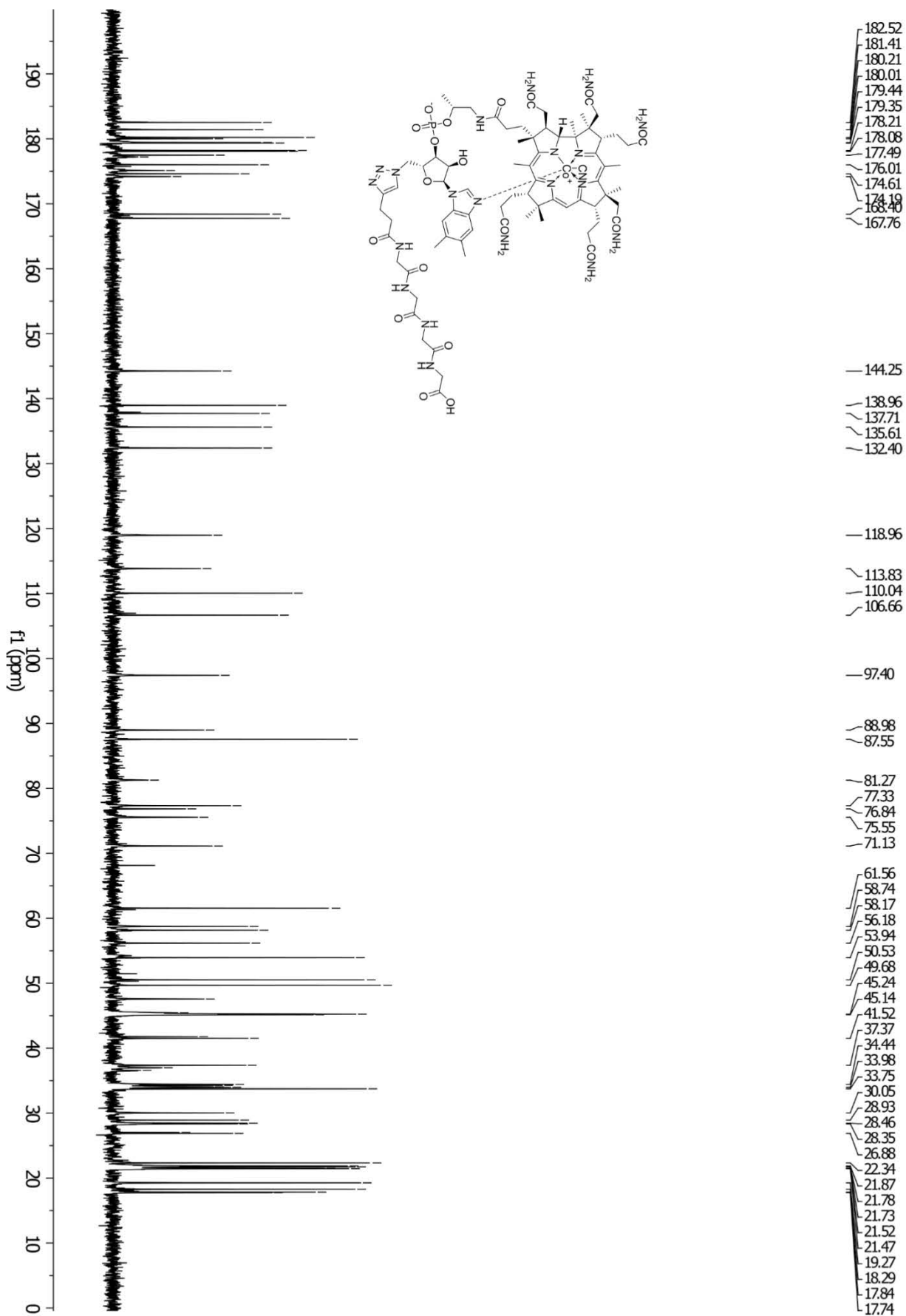


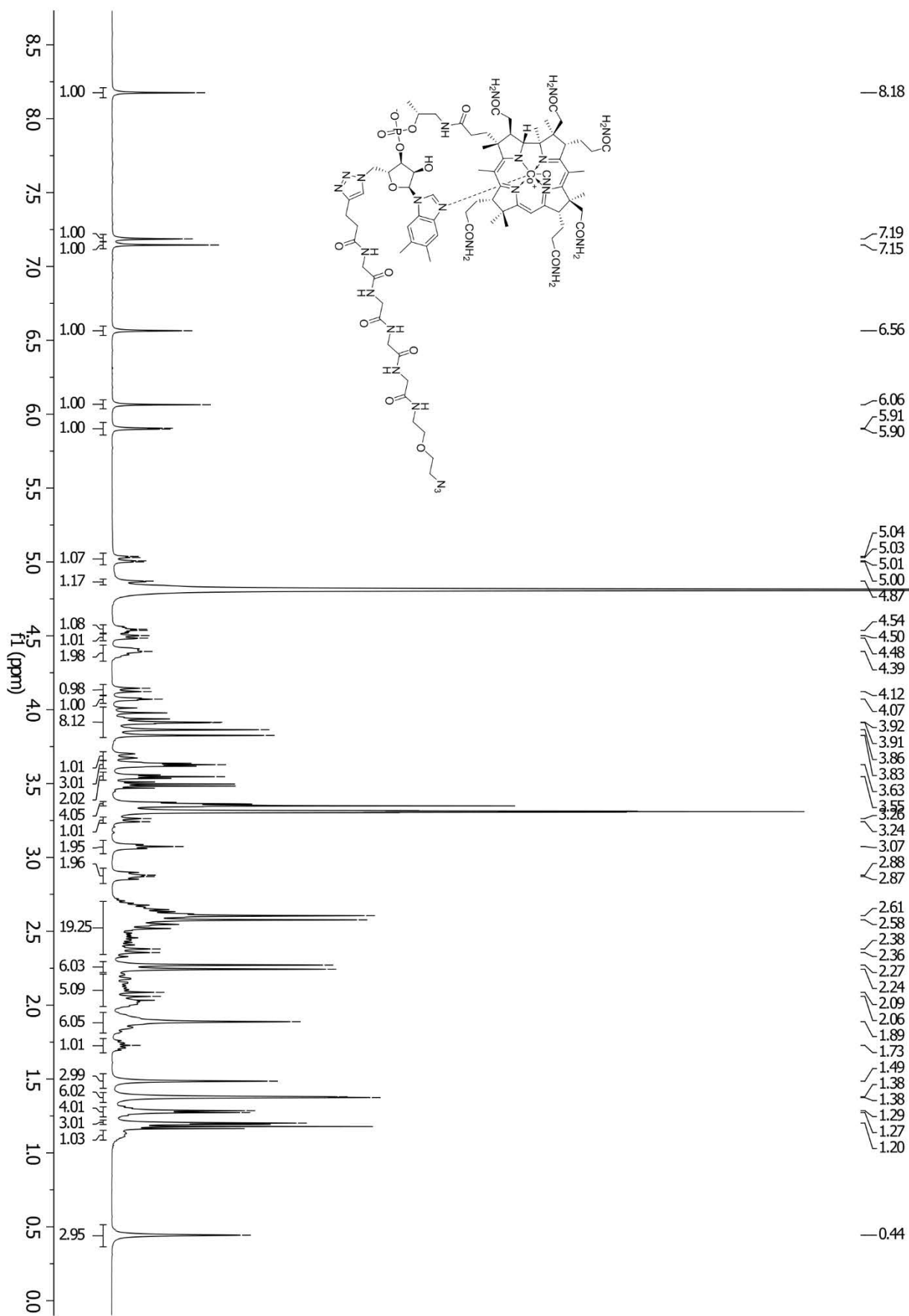
### 4.3 NMR spectra of Cbl-4xGly-OH (29) recorded for D<sub>2</sub>O

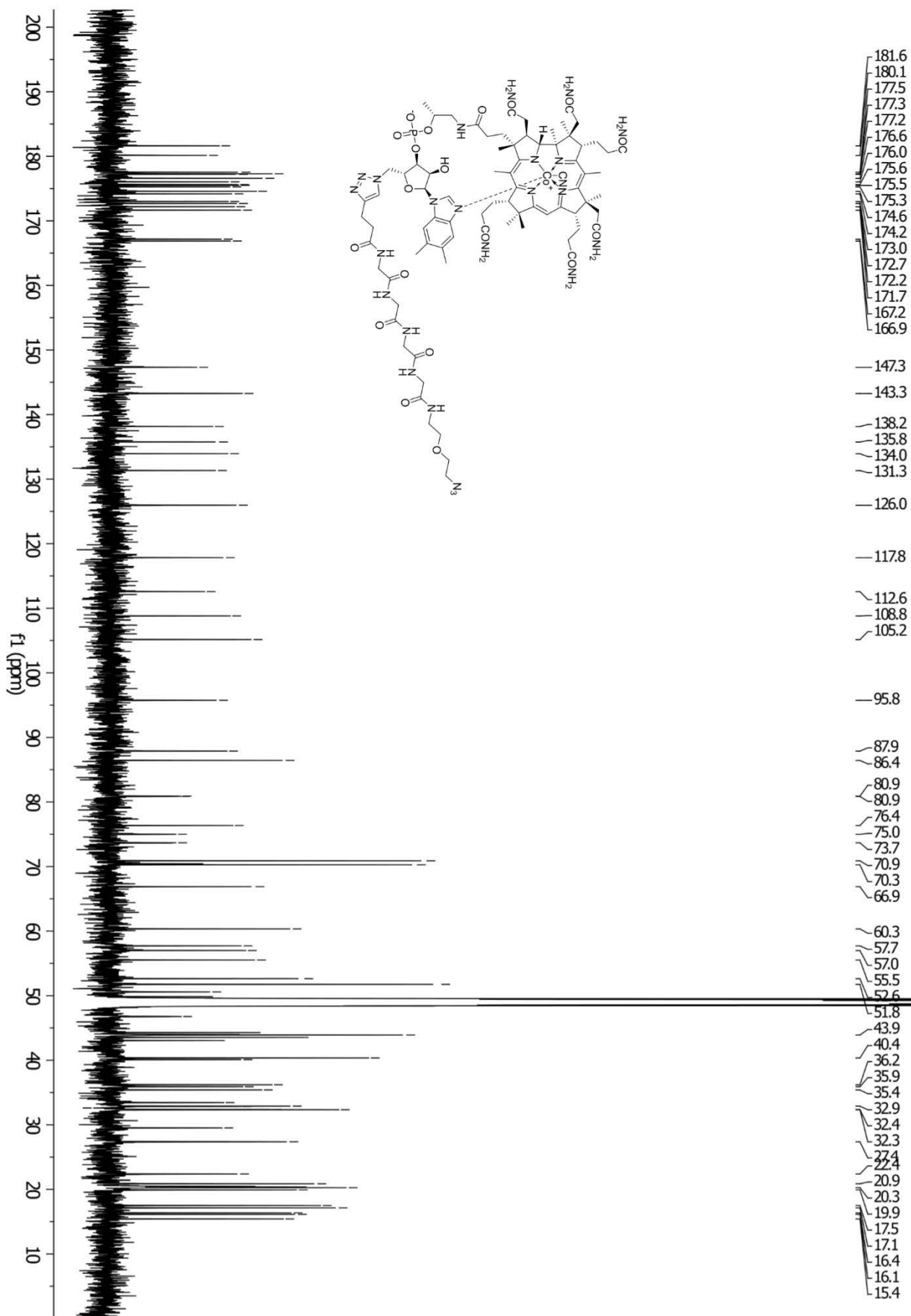
Note: Water signal was suppressed using presaturation (presat)

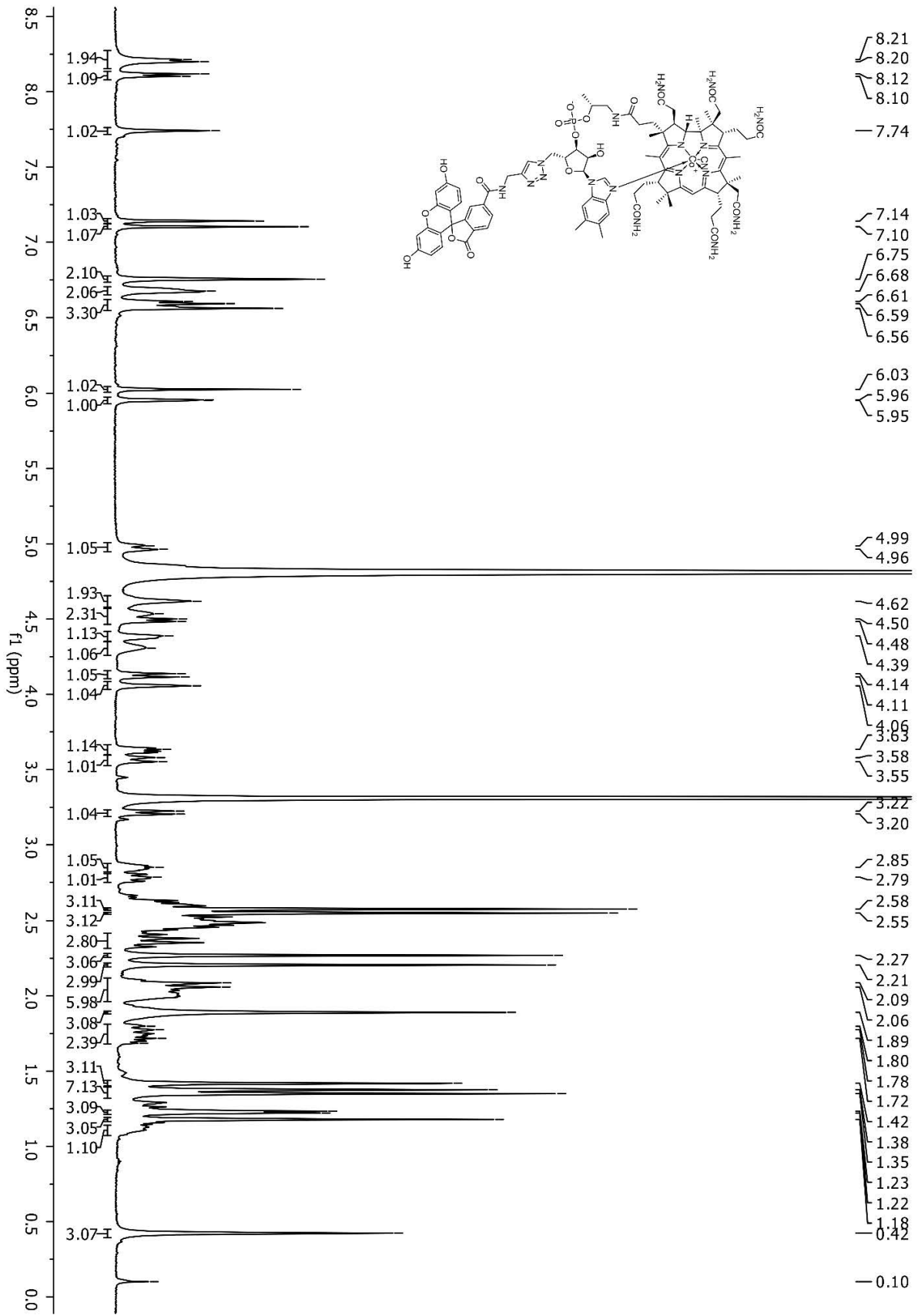


Note: Water signal was suppressed using presaturation, the spectrum was recorded at 80°C

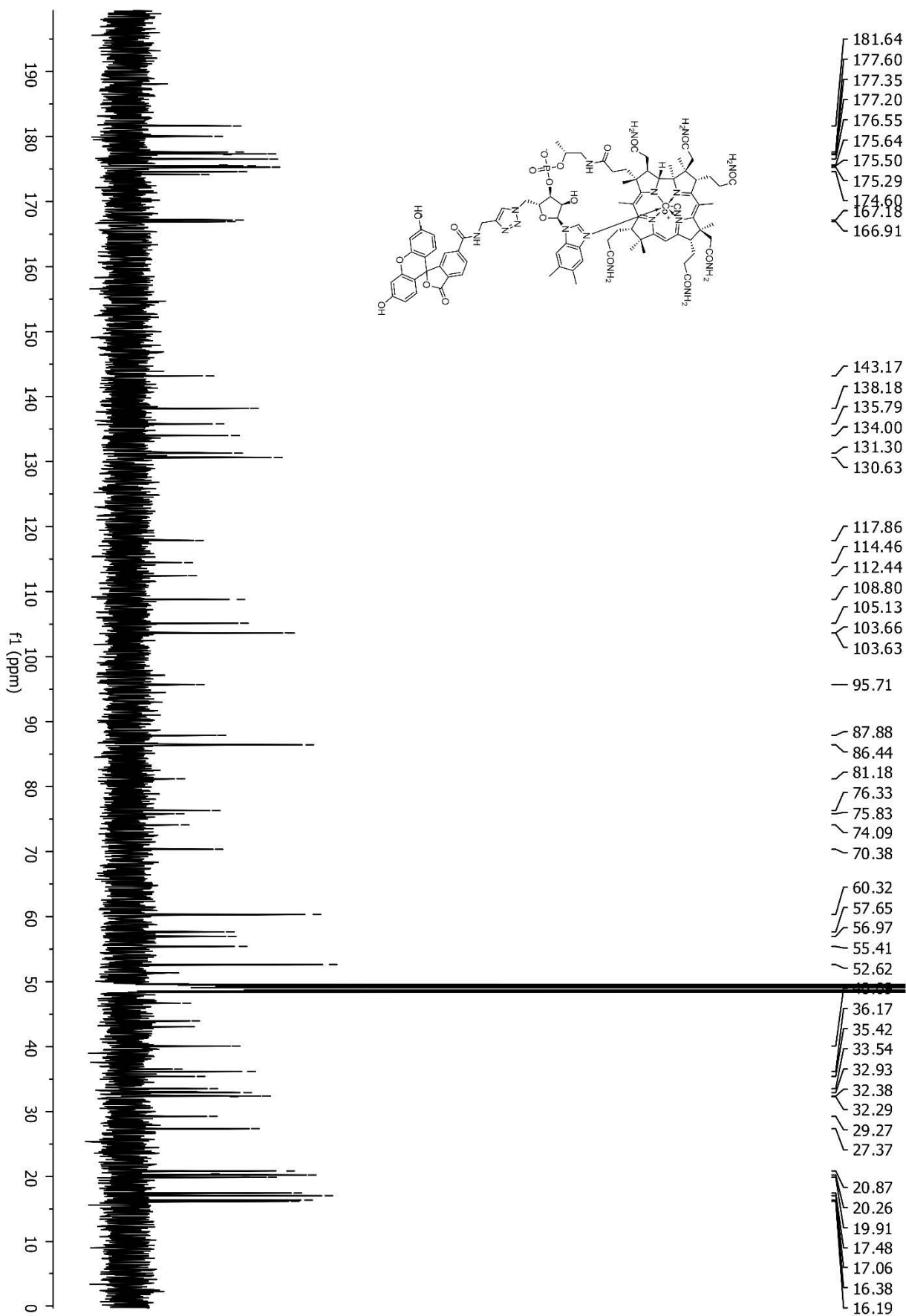




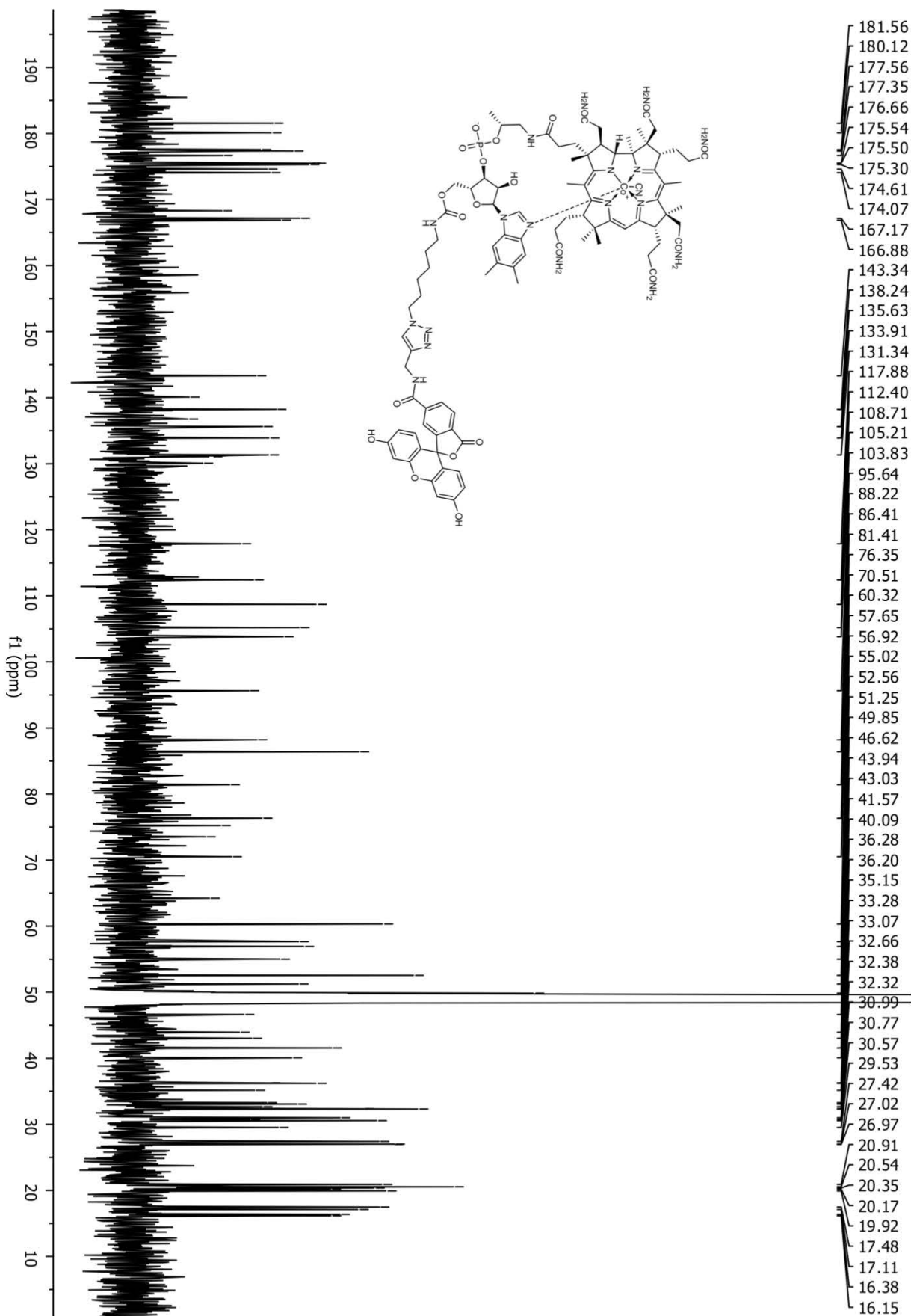




4.5 NMR spectra of Cbl-FAM (12) recorded for CD<sub>3</sub>OD

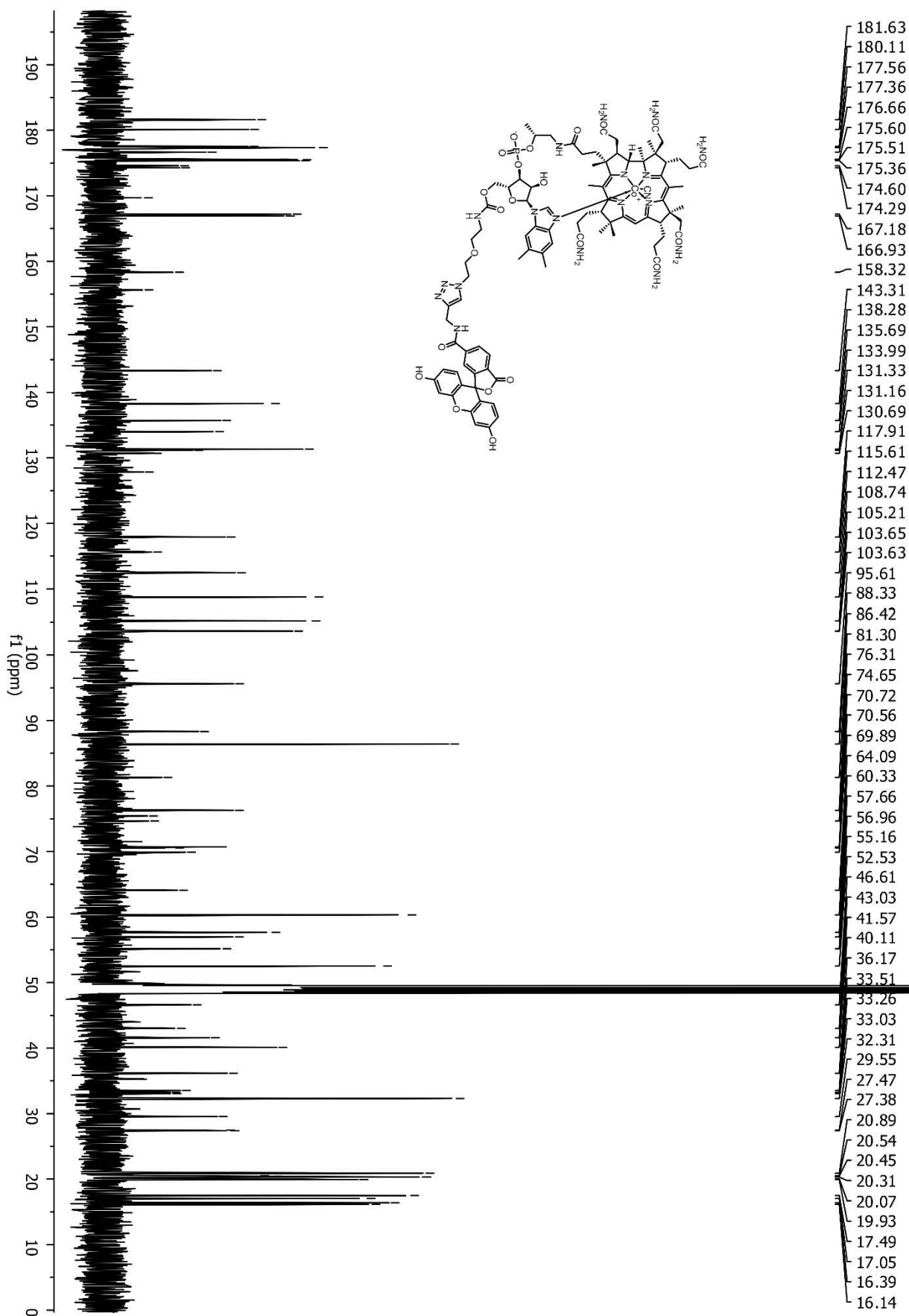


#### 4.6 NMR spectra of Cbl-C6-FAM (2) recorded for CD<sub>3</sub>OD

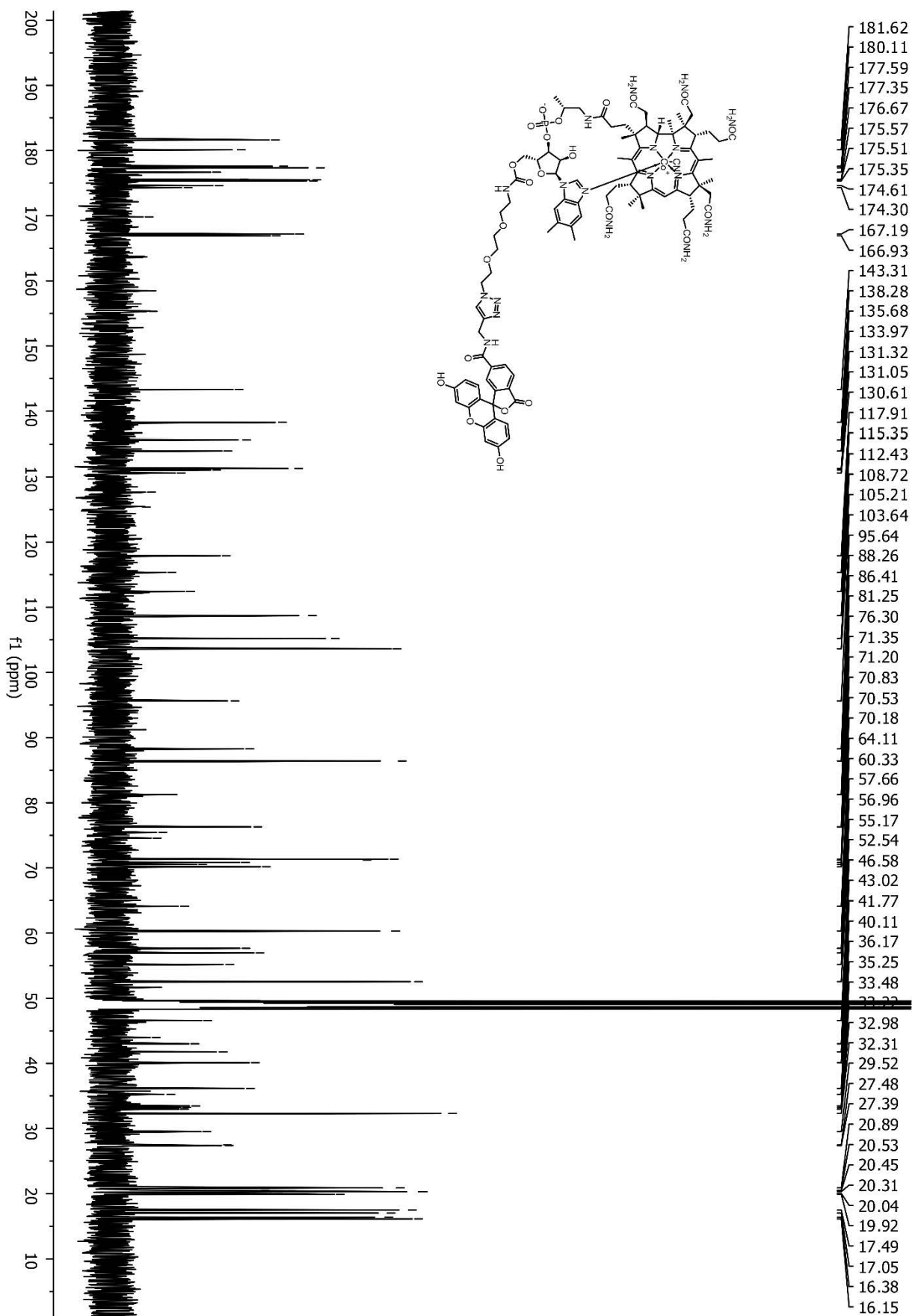




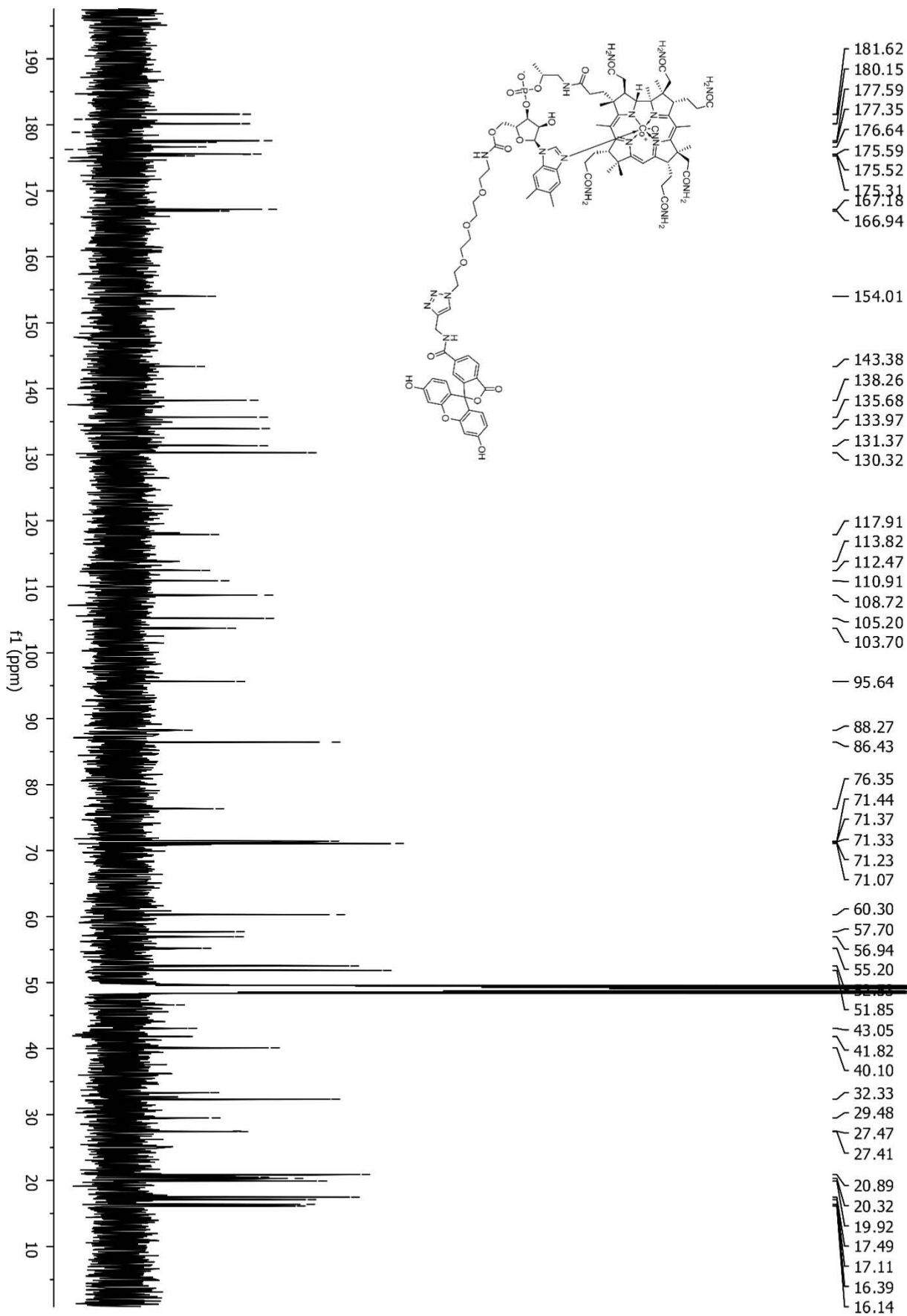
#### 4.7 NMR spectra of Cbl-1xPEG-FAM (13) recorded for CD<sub>3</sub>OD



#### 4.8 NMR spectra of Cbl-2xPEG-FAM (14) recorded for CD<sub>3</sub>OD



#### 4.9 NMR spectra of Cbl-3xPEG-FAM (10) recorded for CD<sub>3</sub>OD

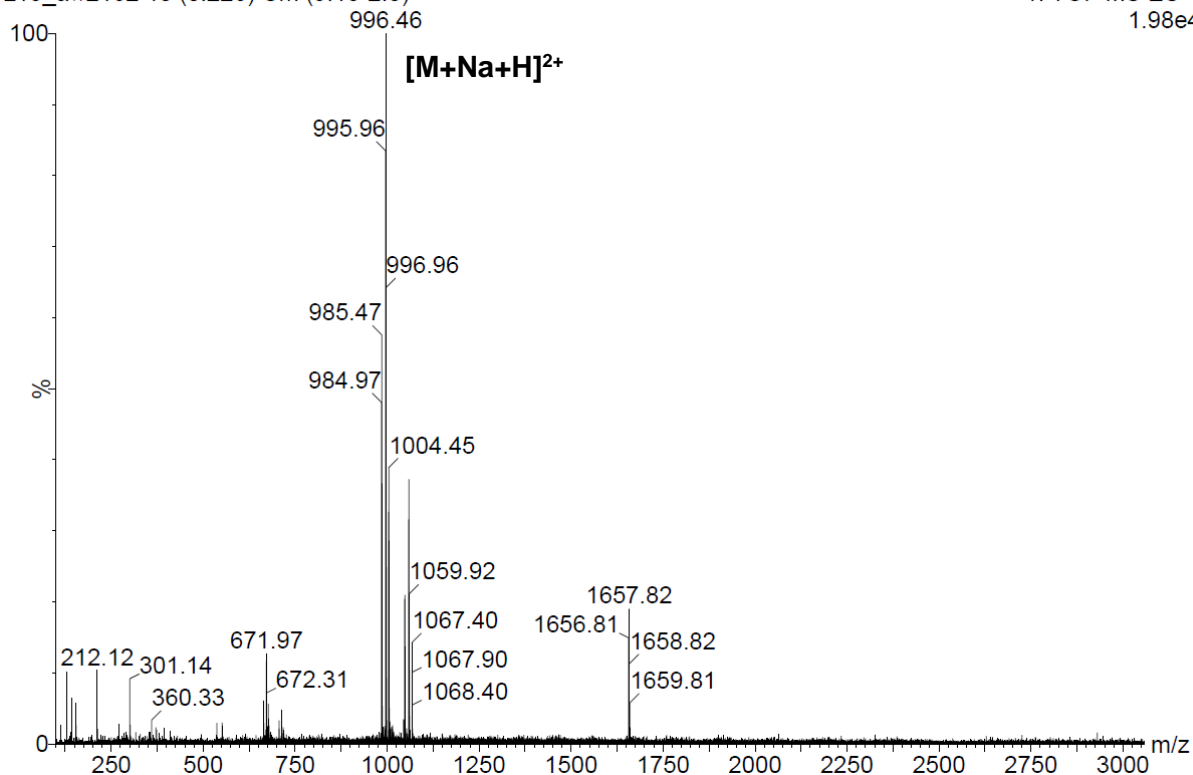


## 5. MS spectra

### 5.1 MS spectrum of Cbl-ATTO 633 (15)

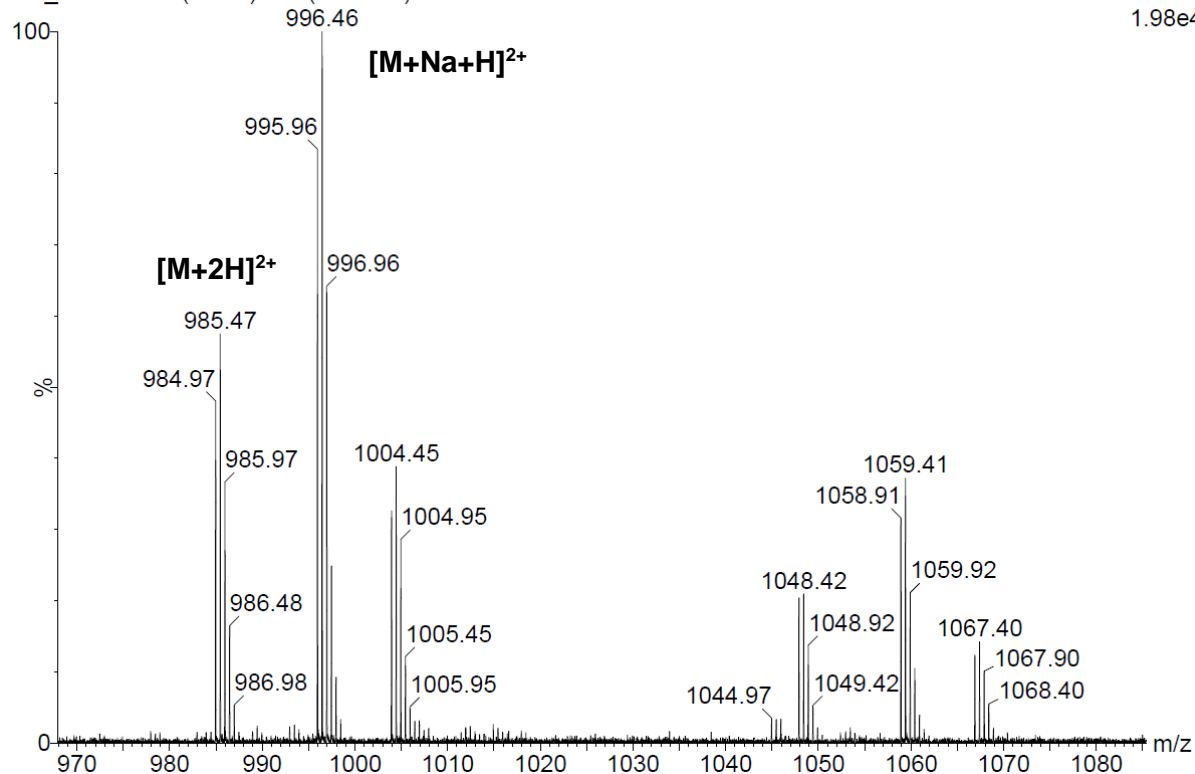
z15\_aw2162 10 (0.226) Cm (9:15-2:8)

1: TOF MS ES+  
1.98e4



z15\_aw2162 10 (0.226) Cm (9:15-2:8)

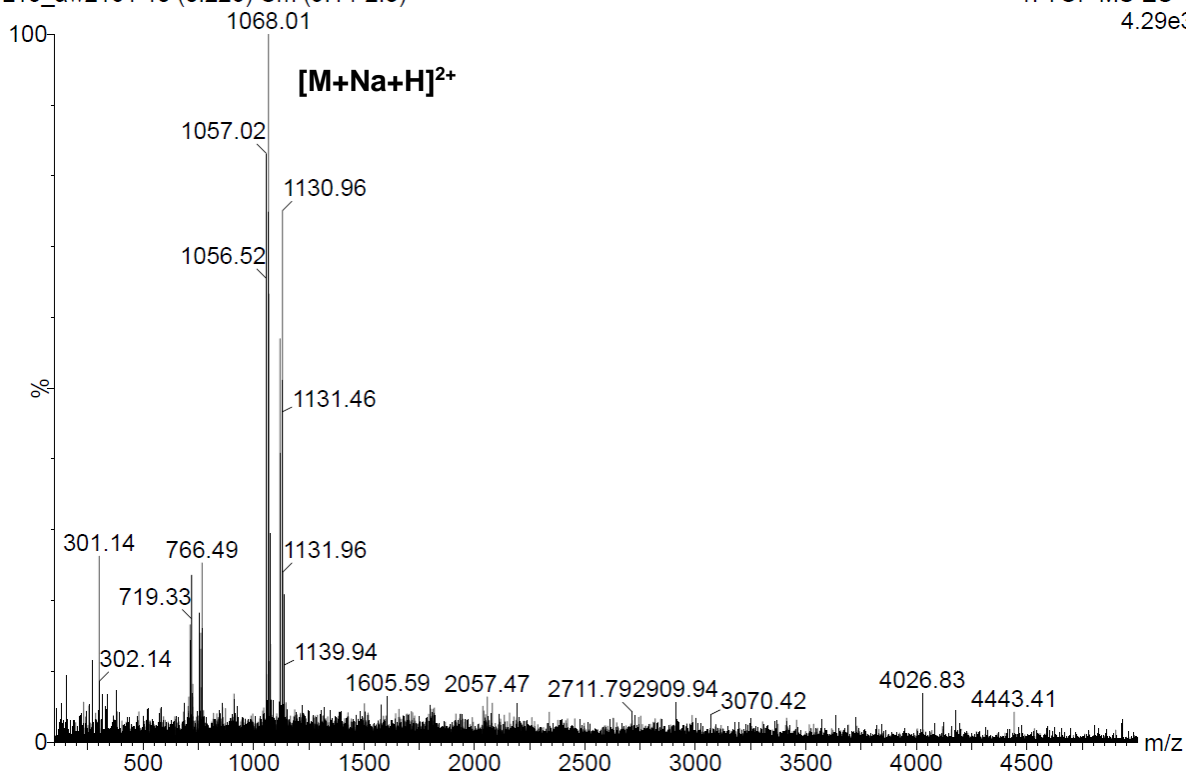
1: TOF MS ES+  
1.98e4



## 5.2 MS spectrum of Cbl-C6-ATTO 633 (5)

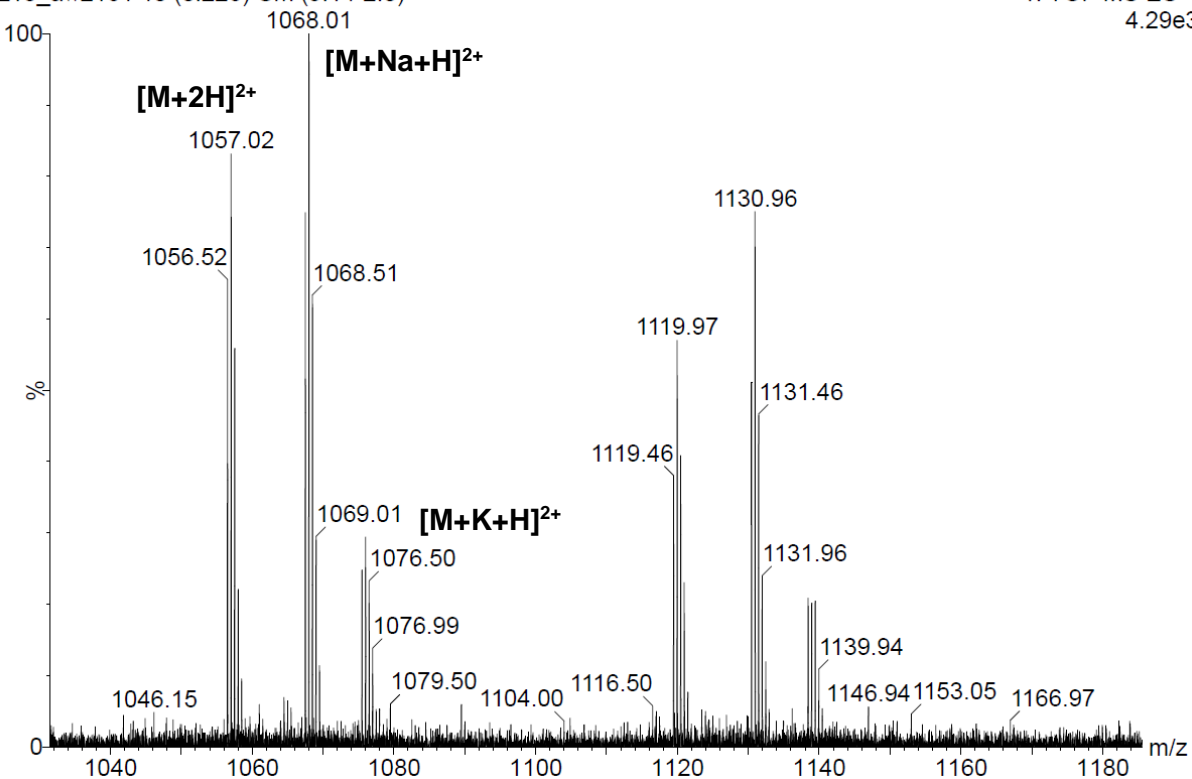
z15\_aw2161 10 (0.226) Cm (9:14-2:8)

1: TOF MS ES+  
4.29e3

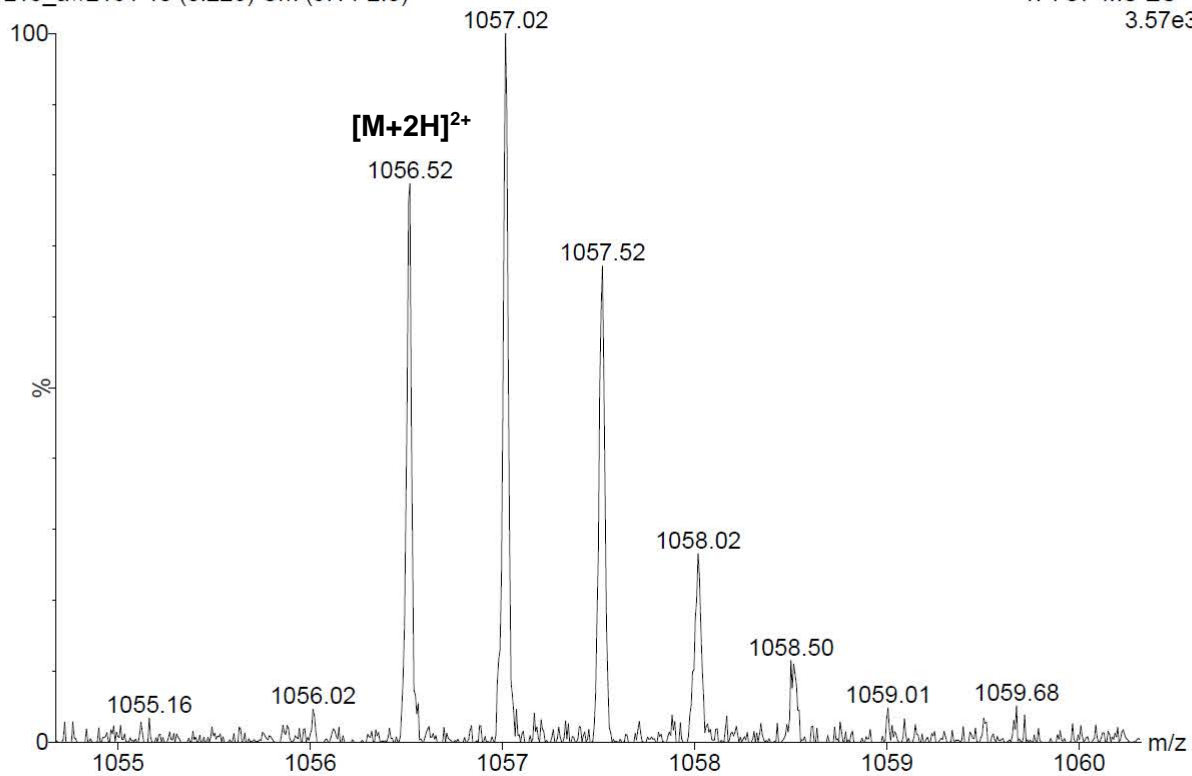


z15\_aw2161 10 (0.226) Cm (9:14-2:8)

1: TOF MS ES+  
4.29e3







## **9. Oświadczenia autorów publikacji**





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## Aleksandra Wierzba, mgr inż.

Oświadczam, że mój wkład w powstanie poniższych prac polegał na:

1. A. J. Wierzba, S. Hassan, D. Gryko, *Synthetic Approaches Toward Vitamin B<sub>12</sub> Conjugates*, *Asian J. Org. Chem.* **2018**, 10.1002/ajoc.201800579, praca zaakceptowana

Opracowałam koncepcję przeglądu oraz zgromadziłam literaturę naukową. Przygotowałam części zatytułowane *Conjugation at the ribonucleotide fragment* oraz *Double-conjugated cobalamins*. Uczestniczyłam w pisaniu części *Conjugation at the central Co ion* oraz *Conjugation at the macrocyclic core* oraz w poprawianiu przeglądu.

2. A. J. Wierzba, K. Maximova, A. Wincenciuk, M. Równicki, M. Wojciechowska, E. Nexø, J. Trylska, D. Gryko, *Does a Conjugation Site Affect Transport of Vitamin B<sub>12</sub>-PNA Conjugates into Bacterial Cells*, *Chem. Eur. J.* **2018**, 10.1002/chem.201804304

Współpracowałam koncepcję badań poprzez dobór pozycji przyłączenia PNA do kobalaminy. Opracowałam metodologię syntezy związków **9**, **12-23**. Otrzymałam oraz oczyściłam związki **3-11**, **16**, **21-23**. Scharakteryzowałam wszystkie nowe pochodne witaminy B<sub>12</sub> z terminalną grupą azydkową. Interpretowałam wyniki badań biologicznych. Przygotowałam manuskrypt wraz z opisem części eksperymentalnej.

3. E. Braselmann, A. J. Wierzba, J. T. Polaski, M. Chromiński, Z. E. Holmes, S. Hung, D. Batan, J. R. Wheeler, R. Parker, R. Jimenez, D. Gryko, R. T. Batey, A. E. Palmer, *A Multicolor Riboswitch-Based Platform for Imaging of RNA in Live Mammalian Cells*, *Nat. Chem. Bio.* **2018**, 14, 964–971

Uczestniczyłam w projektowaniu koniugatów witaminy B<sub>12</sub> z barwnikami fluorescencyjnymi poprzez dobór łączników, ich syntezę i opracowanie metodologii przyłączania barwników do kobalaminy. Przeprowadziłam syntezy pochodnych witaminy B<sub>12</sub> opisanych w manuskrypcie, a także scharakteryzowałam otrzymane związki. Przygotowałam część manuskryptu dotyczącą części eksperymentalnej (Supplementary Note).

4. A. J. Wierzba, A. Wincenciuk, M. Karczewski, V. I. Vullev, D. Gryko, *meso-Modified Cobalamins: Synthesis, Structure, and Properties*, *Chem. Eur. J.* , 24, 10344 –10356

Opracowałam koncepcję badań. Opracowałam syntezę wszystkich pochodnych witaminy B<sub>12</sub> opisanych w manuskrypcie. Wykonałam część eksperymentów mających na celu optymalizację reakcji nitrowania kobalaminy. Otrzymałam oraz oczyściłam związki **2**, **3**, **5**, **8-9**. Analizowałam wyniki uzyskane z rentgenowskiej analizy strukturalnej, zinterpretowałam

5. M. Równicki, M. Wojciechowska, A. J. Wierzba, J. Czarnecki, D. Bartosik, D. Gryko, J. Trylska, *Vitamin B<sub>12</sub> as a Carrier of Peptide Nucleic Acid (PNA) into Bacterial Cells*, *Sci. Rep.* **2017**, 7, 7644

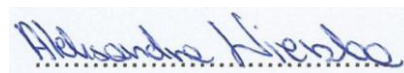
Współpracowałam koncepcję badań. Zsyntezowałam, oczyściłam i scharakteryzowałam pochodne witaminy B<sub>12</sub>-N<sub>3</sub>, B<sub>12</sub>-(CH<sub>2</sub>)<sub>6</sub>-N<sub>3</sub>, B<sub>12</sub>-(CH<sub>2</sub>)<sub>12</sub>-N<sub>3</sub>, B<sub>12</sub>-PEG2-N<sub>3</sub> oraz B<sub>12</sub>-SS-Py. Przygotowałam część eksperymentalną. Uczestniczyłam w pisaniu i poprawianiu manuskryptu.

6. T. Pieńko, A. J. Wierzba, M. Wojciechowska, D. Gryko, J. Trylska, *Conformational Dynamics of Cyanocobalamin and Its Conjugates with Peptide Nucleic Acids*, *J. Phys. Chem. B* **2017**, 121, 2968–2979

Opracowałam i przeprowadziłam syntezę związków **5-7**. Scharakteryzowałam otrzymane związki. Zinterpretowałam serię widm dwuwymiarowych NMR dla pochodnej **7**. Przygotowałam część manuskryptu dotyczącą porównania wyników uzyskanych z widm NMR z wynikami otrzymanymi z dynamiki molekularnej.

7. A. J. Wierzba, M. Wojciechowska, J. Trylska, D. Gryko, *Vitamin B<sub>12</sub> Suitably Tailored for Disulfide-Based Conjugation*, *Bioconjugate Chem.* **2016**, 27, 189–197

Opracowałam koncepcję badań. Przeprowadziłam wszystkie eksperymenty optymalizacyjne dotyczące syntezy związku **6** oraz syntezy disiarczków **5**. Otrzymałam i oczyściłam związki **5a-d**. Scharakteryzowałam wszystkie otrzymane pochodne. Przeprowadziłam eksperymenty mające na celu redukcję wiązań disiarczkowych w pochodnych **5c** i **5e**. Przygotowałam manuskrypt wraz z opisem części eksperymentalnej.





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Warszawa 29.10.2018

**Dorota Gryko, prof.**

Oświadczam, że mój wkład w powstanie poniższych prac polegał na współuczestniczeniu w planowaniu koncepcji badań, analizie danych i korekcie manuskryptów.

1. A. J. Wierzba, S. Hassan, D. Gryko, *Synthetic Approaches Toward Vitamin B<sub>12</sub> Conjugates*, *Asian J. Org. Chem.* **2018**, 10.1002/ajoc.201800579, praca zaakceptowana
2. A. J. Wierzba, K. Maximova, A. Wincenciuk, M. Równicki, M. Wojciechowska, E. Nexø, J. Trylska, D. Gryko, *Does a Conjugation Site Affect Transport of Vitamin B<sub>12</sub>-PNA Conjugates into Bacterial Cells*, *Chem. Eur. J.* **2018**, 10.1002/chem.201804304
3. E. Braselmann, A. J. Wierzba, J. T. Polaski, M. Chromiński, Z. E. Holmes, S. Hung, D. Batan, J. R. Wheeler, R. Parker, R. Jimenez, D. Gryko, R. T. Batey, A. E. Palmer, *A Multicolor Riboswitch-Based Platform for Imaging of RNA in Live Mammalian Cells*, *Nat. Chem. Bio.* **2018**, 14, 964–971
4. A. J. Wierzba, A. Wincenciuk, M. Karczewski, V. I. Vullev, D. Gryko, *meso-Modified Cobalamins: Synthesis, Structure, and Properties*, *Chem. Eur. J.* **2018**, 24, 10344–10356
5. M. Równicki, M. Wojciechowska, A. J. Wierzba, J. Czarnecki, D. Bartosik, D. Gryko, J. Trylska, *Vitamin B<sub>12</sub> as a Carrier of Peptide Nucleic Acid (PNA) into Bacterial Cells*, *Sci. Rep.* **2017**, 7, 7644
6. T. Pieńko, A. J. Wierzba, M. Wojciechowska, D. Gryko, J. Trylska, *Conformational Dynamics of Cyanocobalamin and Its Conjugates with Peptide Nucleic Acids*, *J. Phys. Chem. B* **2017**, 121, 2968–2979
7. A. J. Wierzba, M. Wojciechowska, J. Trylska, D. Gryko, *Vitamin B<sub>12</sub> Suitably Tailored for Disulfide-Based Conjugation*, *Bioconjugate Chem.* **2016**, 27, 189–197



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Sidra Hassan, PhD

I hereby declare that my contribution to below mentioned publication is as follows:

A. J. Wierzba, S. Hassan, D. Gryko, *Synthetic Approaches Toward Vitamin B<sub>12</sub> Conjugates*,  
*Asian J. Org. Chem.* **2018**, 10.1002/ajoc.201800579

I co-wrote part entitled *Conjugation at the central Co ion and Conjugation at the macrocyclic core.*

  
.....  
31-10-2018

Monika Wojciechowska, dr

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### Monika Wojciechowska, dr

Oświadczam, że mój wkład w powstanie poniższych prac polegał na:

1. A. J. Wierzba, K. Maximova, A. Wincenciuk, M. Równicki, M. Wojciechowska, E. Nexø, J. Trylska, D. Gryko, *Does a Conjugation Site Affect Transport of Vitamin B<sub>12</sub>-PNA Conjugates into Bacterial Cells*, *Chem. Eur. J.* **2018**, 10.1002/chem.201804304

- wykonałam eksperymenty mające na celu syntezę koniugatów nr **25**, **26** i **28** oraz oligomeru PNA **24**.

2. M. Równicki, M. Wojciechowska, A. J. Wierzba, J. Czarnecki, D. Bartosik, D. Gryko, J. Trylska, *Vitamin B<sub>12</sub> as a Carrier of Peptide Nucleic Acid (PNA) into Bacterial Cells*, *Sci. Rep.* **2017**, 7, 7644

- współpracowałam koncepcję syntezy koniugatów B<sub>12</sub>-PNA,  
- wykonałam eksperymenty mające na celu syntezę oligomerów PNA, koniugatów B<sub>12</sub>-PNA,  
- przygotowałam część manuskryptu dotyczącą syntez PNA i ich koniugatów,  
- uczestniczyłam w pisaniu i poprawianiu manuskryptu.

3. T. Pieńko, A. J. Wierzba, M. Wojciechowska, D. Gryko, J. Trylska, *Conformational Dynamics of Cyanocobalamin and Its Conjugates with Peptide Nucleic Acids*, *J. Phys. Chem. B* **2017**, 121, 2968–2979

- współpracowałam koncepcję syntezy monomeru nr **5** oraz koniugatu B<sub>12</sub>-monomer PNA nr **7**.

4. A. J. Wierzba, M. Wojciechowska, J. Trylska, D. Gryko, *Vitamin B<sub>12</sub> Suitably Tailored for Disulfide-Based Conjugation*, *Bioconjugate Chem.* **2016**, 27, 189–197

- współpracowałam koncepcję syntezy koniugatów B<sub>12</sub>-PNA,  
- wykonałam eksperymenty mające na celu syntezę oligomeru PNA (Cys-PNA-Lys-NH<sub>2</sub>), peptydu Cys-Phe-Phe-Lys-Lys-NH<sub>2</sub> oraz koniugatów B<sub>12</sub>-PNA **5e** oraz **5f**,  
- przygotowałam część manuskryptu dotyczącą syntezy PNA, peptydu i koniugatów **5e** oraz **5f**.







Prof. dr hab. Joanna Trylska  
Centrum Nowych Technologii  
Uniwersytet Warszawski  
joanna@cent.uw.edu.pl

**Joanna Trylska, prof.**

Oświadczam, że mój wkład w powstanie poniższych prac polegał na:

1. A. J. Wierzba, K. Maximova, A. Wincenciuk, M. Równicki, M. Wojciechowska, E. Nexø, J. Trylska, D. Gryko, *Does a Conjugation Site Affect Transport of Vitamin B<sub>12</sub>-PNA Conjugates into Bacterial Cells*, *Chem. Eur. J.* **2018**, 10.1002/chem.201804304

uczestniczyłam w opracowaniu planu badań, uczestniczyłam w dyskusjach dotyczących interpretacji wyników badań biologicznych, uczestniczyłam w pisaniu manuskryptu

2. M. Równicki, M. Wojciechowska, A. J. Wierzba, J. Czarnecki, D. Bartosik, D. Gryko, J. Trylska, *Vitamin B<sub>12</sub> as a Carrier of Peptide Nucleic Acid (PNA) into Bacterial Cells*, *Sci. Rep.* **2017**, 7, 7644

uczestniczyłam w opracowaniu planu badań, uczestniczyłam w dyskusjach dotyczących interpretacji wyników badań, uczestniczyłam w pisaniu manuskryptu.

3. T. Pieńko, A. J. Wierzba, M. Wojciechowska, D. Gryko, J. Trylska, *Conformational Dynamics of Cyanocobalamin and Its Conjugates with Peptide Nucleic Acids*, *J. Phys. Chem. B* **2017**, 121, 2968–2979

uczestniczyłam w opracowaniu planu badań, uczestniczyłam w interpretacji wyników symulacji dynamiki molekularnej, uczestniczyłam w pisaniu manuskryptu

4. A. J. Wierzba, M. Wojciechowska, J. Trylska, D. Gryko, *Vitamin B<sub>12</sub> Suitably Tailored for Disulfide-Based Conjugation*, *Bioconjugate Chem.* **2016**, 27, 189–197

uczestniczyłam w edycji ostatecznej wersji manuskryptu oraz w interpretacji wyników dotyczących oligomeru peptydowego kwasu nukleinowego



San Diego, 16.10.2018

**Marcin Równicki, mgr**

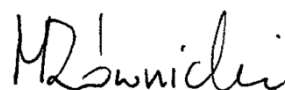
Oświadczam, że mój wkład w powstanie poniższych prac polegał na:

1. A. J. Wierzba, K. Maximova, A. Wincenciuk, M. Równicki, M. Wojciechowska, E. Nexø, J. Trylska, D. Gryko, *Does a Conjugation Site Affect Transport of Vitamin B<sub>12</sub>-PNA Conjugates into Bacterial Cells*, *Chem. Eur. J.* **2018**, 10.1002/chem.201804304

Wykonałem eksperymenty mające na celu ustalenie wydajności pobierania koniugatów witamina B<sub>12</sub>-PNA oraz hamowania biosyntezy białka u bakterii *Escherichia coli* oraz zinterpretowałem wyniki, przygotowałem część manuskryptu dotyczącą ww. eksperymentów.

2. M. Równicki, M. Wojciechowska, A. J. Wierzba, J. Czarnecki, D. Bartosik, D. Gryko, J. Trylska, *Vitamin B<sub>12</sub> as a Carrier of Peptide Nucleic Acid (PNA) into Bacterial Cells*, *Sci. Rep.* **2017**, 7, 7644

Współpracowałem koncepcję badań biologicznych, wykonałem eksperymenty mające na celu ustalenie wydajności pobierania koniugatów witamina B<sub>12</sub>-PNA oraz hamowania biosyntezy białka u bakterii Gram-ujemnych, interpretowałem wyniki eksperymentów biologicznych oraz przygotowałem wstęp do ww. manuskryptu, część dotycząca badań biologicznych (opis wyników oraz metod) a także dyskusję.

  
.....

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e-mail: jczarnecki@biol.uw.edu.pl

**Jakub Czarnecki, dr**

Oświadczam, że mój wkład w powstanie poniższej pracy polegał na: zaplanowaniu i konstrukcji (wraz z prof. dr hab. Dariuszem Bartosikiem i mgr Marcinem Równickim) plazmidu testowego, pozwalającego na monitorowanie translacji białka fluorescencyjnego RFP.

M. Równicki, M. Wojciechowska, A. J. Wierzba, J. Czarnecki, D. Bartosik, D. Gryko, J. Trylska, *Vitamin B<sub>12</sub> as a Carrier of Peptide Nucleic Acid (PNA) into Bacterial Cells*, *Sci. Rep.* **2017**, 7, 7644

Warszawa, 17.10.2018

Jakub Czarnecki

.....

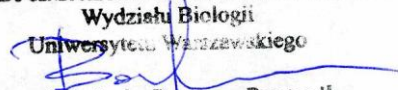
prof. dr hab. Dariusz Bartosik  
Zakład Genetyki Bakterii  
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**Dariusz Bartosik, prof.**

Oświadczam, że mój wkład w powstanie poniższej pracy polegał na: zaplanowaniu i konstrukcji (wraz z dr Jakubem Czarneckim i mgr Marcinem Równickim) plazmidu testowego, pozwalającego na monitorowanie translacji białka fluorescencyjnego RFP.

*Rep. 2017, 7, 7644*

Warszawa, 17.10.2018

**KIEROWNIK**  
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02-097 Warsaw

**Ksenia Maximova, PhD**

I hereby declare that my contribution to below mentioned publication is as follows:

A. J. Wierzba, K. Maximova, A. Wincenciuk, M. Równicki, M. Wojciechowska, E. Nexø, J. Trylska, D. Gryko, *Does a Conjugation Site Affect Transport of Vitamin B<sub>12</sub>-PNA Conjugates into Bacterial Cells*, *Chem. Eur. J.* **2018**, 10.1002/chem.201804304

I carried out the synthesis of PNA scrambled **24a** and conjugates **27**, **29-32**, purified and analyzed the compounds. I participated in writing/correcting the manuscript.



Tomasz Pieńko  
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**Tomasz Pieńko, mgr**

Oświadczam, że mój wkład w powstanie poniższej pracy polegał na:

T. Pieńko, A. J. Wierzba, M. Wojciechowska, D. Gryko, J. Trylska, *Conformational Dynamics of Cyanocobalamin and Its Conjugates with Peptide Nucleic Acids*, *J. Phys. Chem. B* **2017**, *121*, 2968–2979

- 1) wykonaniu symulacji dynamiki molekularnej cyjanokobalaminy oraz jej koniugatów z peptydowym kwasem nukleinowym w celu zbadania ich struktury w roztworze wodnym
- 2) zanalizowaniu i interpretacji wyników symulacji
- 3) przygotowaniu części manuskryptu dotyczącej symulacji dynamik molekularnej, a także wstępu i wniosków
- 4) udziale w ostatecznym poprawianiu całego manuskryptu

  
.....



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---

**Aleksandra Wincenciuk, mgr**

Oświadczam, że mój wkład w powstanie poniższych prac polegał na:

1. A. J. Wierzba, K. Maximova, A. Wincenciuk, M. Równicki, M. Wojciechowska, E. Nexø, J. Trylska, D. Gryko, *Does a Conjugation Site Affect Transport of Vitamin B<sub>12</sub>-PNA Conjugates into Bacterial Cells*, *Chem. Eur. J.* **2018**, 10.1002/chem.201804304

Otrzymałam oraz oczyściłam związki **12-14** oraz **17-19**.

2. A. J. Wierzba, A. Wincenciuk, M. Karczewski, V. I. Vullev, D. Gryko, *meso-Modified Cobalamins: Synthesis, Structure, and Properties*, *Chem. Eur. J.* **2018**, 24, 10344–10356

Wykonałam część eksperymentów mających na celu optymalizację reakcji nitrowania kobalaminy oraz optymalizację reakcji otrzymywania iminy **4**. Zsyntezowałam oraz oczyściłam związki **6** oraz **7**.

Aleksandra... Wincenciuk





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---

**Maksymilian Karczewski, dr**

Oświadczam, że mój wkład w powstanie poniższej pracy polegał na:

A. J. Wierzba, A. Wincenciuk, M. Karczewski, V. I. Vullev, D. Gryko, *meso-Modified Cobalamins: Synthesis, Structure, and Properties*, *Chem. Eur. J.* **2018**, *24*, 10344 –10356

Przeprowadziłem obliczenia kwantowo-mechaniczne oraz dokonałem analizy i interpretacji otrzymanych danych. Przygotowałem również część manuskryptu dotyczącą badań obliczeniowych.



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October 16, 2018

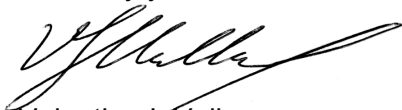
To: Whom it may concern  
From: Valentine Vullev, Ph.D.

I hereby declare that my contribution to below mentioned publication is as follows:

A. J. Wierzba, A. Wincenciuk, M. Karczewski, V. I. Vullev, D. Gryko, *meso-Modified Cobalamins: Synthesis, Structure, and Properties*, *Chem. Eur. J.* **2018**, 24, 10344 –10356

I interpreted the results of cyclic voltammetry and spectroelectrochemical measurements. I co-wrote the part *Photophysical properties of meso-substituted cobalamins* and wrote the part entitled *Electrochemical properties of meso-substituted cobalamins*. I was also involved in correcting the manuscript.

Sincerely yours,



Valentine I. Vullev  
Professor of Bioengineering, Chemistry,  
Biochemistry, and Materials Science and Engineering  
Fulbright U.S. Scholar Fellow

Department of Biochemistry/Biofrontiers Institute  
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3415 Colorado Ave  
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E-mail: esther.breselmann@colorado.edu

**Esther Braselmann, PhD**

I hereby declare that my contribution to below mentioned publication is as follows:

E. Braselmann, A. J. Wierzba, J. T. Polaski, M. Chromiński, Z. E. Holmes, S. Hung, D. Batan, J. R. Wheeler, R. Parker, R. Jimenez, D. Gryko, R. T. Batey, A. E. Palmer, *A Multicolor Riboswitch-Based Platform for Imaging of RNA in Live Mammalian Cells*, *Nat. Chem. Bio.* **2018**, *14*, 964–971

I conceptualized and designed the study, designed organic probes, performed in vitro work, designed and performed cellular work, analyzed data with input from all authors and wrote the manuscript with edits from all authors.

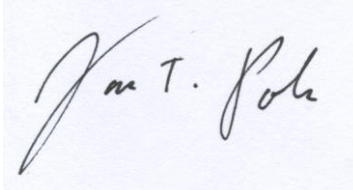
A handwritten signature in black ink, appearing to read 'Esther Braselmann', written over a horizontal dotted line.

**Jacob T. Polaski, PhD**

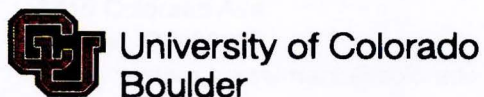
I hereby declare that my contribution to below mentioned publication is as follows:

E. Braselmann, A. J. Wierzba\*, J. T. Polaski\*, M. Chromiński, Z. E. Holmes, S. Hung, D. Batan, J. R. Wheeler, R. Parker, R. Jimenez, D. Gryko, R. T. Batey, A. E. Palmer, *A Multicolor Riboswitch-Based Platform for Imaging of RNA in Live Mammalian Cells*, *Nat. Chem. Bio.* **2018**, *14*, 964–971, \* equal contributions.

I conceptualized and designed the study, rationally designed riboswitch variants, designed organic probes and purified riboswitch variants for in vitro work.



.....



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**Amy E. Palmer, Prof.**

**I hereby declare that my contribution to below mentioned publication is as follows:**

E. Braselmann, A. J. Wierzba, J. T. Polaski, M. Chromiński, Z. E. Holmes, S. Hung, D. Batan, J. R. Wheeler, R. Parker, R. Jimenez, D. Gryko, R. T. Batey, A. E. Palmer, *A Multicolor Riboswitch-Based Platform for Imaging of RNA in Live Mammalian Cells*, *Nat. Chem. Bio.* **2018**, *14*, 964–971

I conceptualized and designed the study, designed organic probes and wrote the manuscript with edits from all authors.

Sincerely,

Amy E. Palmer  
Professor of Biochemistry and BioFrontiers Institute  
University of Colorado Boulder



B. Org. 404/18

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**O-B.404/18**



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