

DART.2: bidirectional synaptic pharmacology with thousandfold cellular specificity

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SUPPLEMENTARY DISCUSSION

Community Guidelines for Extending DART

DART.2 is compatible with essentially any drug that acts on the cellular surface. To facilitate community design, 'Rx fragments' are made separately from 'HTL modules' for later assembly via *alkyne-azide* click chemistry¹⁸. With regard to the HTL module, we previously established the requirement for a long chemical linker between Rx and HTL. In particular, PEG₁₂ was too short to reach the AMPAR, whereas PEG₂₄ and PEG₃₆ were both acceptable³. Since then, PEG₃₆ has sufficed for all receptors tested. Given its common utility, the long linker is grouped into the 'HTL module' (e.g., *azide*^{DART.2} contains *azide-PEG₃₆-HTL.2*), enabling the majority of investigators to focus on developing small **Rx^{alkyne}** reagents with traditional drug-like properties (**Fig 2c**).

To convert a pharmaceutical into an **Rx^{alkyne}**, one must chemically modify the drug by adding a tether, with the goal of achieving an **AC₅₀** in the μM range. If desired, one may further attenuate the pharmacophore to enhance cellular specificity (as done for **gabazine.7^{alkyne}**). A second parameter is conformational impact of a drug once bound. Here, we found that two full-strength PAMs (diazepam and CMPDA) retained their functional identity upon tether addition. However, we also found that a weak allosteric drug (flumazenil) was altered by the tether (**Extended Data Fig 6b**). While tentative, we speculate that full-strength allosteric drugs may be more resilient to a tether than their partial-allosteric counterparts, as pushing a parameter to its saturation can yield resilience to perturbation. Additional examples are needed to assess applicability to other drug classes.

Finally, we advise that the name of an **Rx** fragment should uniquely identify its structure, with the convention of including the name of its commonly known precursor followed by a version number that defines conjugation and spacer details (e.g., **gabazine.7^{alkyne}**). In the interest of readability, we settled on consistent use of a period to delimit the version number, as many precursor names contain numbers (e.g., **Alexa647.1^{alkyne}**). After the final *alkyne-azide* assembly step, the resulting full-length reagent should retain both version numbers for clarity (e.g., **gabazine.7^{alkyne}** + *azide*^{DART.2} → **gabazine.7^{DART.2}**). In sum, we envision that distributed teams of scientists will be able to grow the platform in a manner that is organized, collaborative, and scalable.

Technical challenges in implementing the postsynaptic strategy

As summarized in **Fig 1c**, combining cell-type specificity, endogenous-receptor specificity, and acute onset has been difficult. Methods that block expression of the GABA_AR or AMPAR (**Fig 1c**, first row) require weeks for protein turnover and are subject to compensatory changes⁴. Attempts to accelerate protein turnover via ubiquitin degradation⁵ have been limited to cytosolic proteins. For instance, the degradation of gephyrin⁶ has been shown to redistribute GABA_ARs to extrasynaptic locations; however, this approach does not block or allosterically potentiate GABA_ARs themselves. Similarly, tools that induce postsynaptic spine shrinkage⁷ lack the receptor specificity and bidirectional precision of pharmacology. Engineered variants of the GABA_AR and AMPAR can tether drugs to themselves⁸⁻¹⁰; nevertheless their cell-specific deployment has been difficult. Cell-type-specific viral delivery^{11,12} allows one to manipulate the overexpressed engineered receptors, but does not provide access to the endogenous receptors (**Fig 1c**, second row). Constitutive knock-in, as implemented for an engineered GABA_AR¹³, did not provide cell-type specificity (**Fig 1c**, third row). DART offers a unique combination of cell-type specificity, endogenous receptor specificity, and acute onset (**Fig 1c**, fourth row)³.

Cell-Specificity Insights

We improved cellular specificity via optimization of HTL and Rx moieties, albeit with two surprises. First, the 40-fold **CC₅₀** improvement seen with a biotin payload (**Fig 2a**), became a 10-fold **CC₅₀** benefit with YM90K (**Fig 2d-e**). We suspect this discrepancy can be attributed to avidity²⁷, wherein transient YM90K interactions can assist the inefficient HTL.1 moiety enabling **YM90K.1^{DART.1}** to outperform HTL.1 on its own. YM90K would not provide the same help to HTL.2, which singlehandedly drives capture in **YM90K.1^{DART.2}** at low doses. Thus avidity

is unequal in the two cases. To compare **HTL.1** vs **HTL.2** fairly, a biotin payload is preferred owing to its consistent lack of interaction with cells.

Next, with regard to **Rx** optimization, we found that **gabazine.7^{DART.2}** requires 15-times as many molecules in the ambient (**AC₅₀**), yet only 2-fold more when tethered (**CC₅₀**) in comparison to **gabazine.1^{DART.2}** (**Extended Data Fig 2**). This was surprising because we had anticipated that both parameters would be impacted similarly. The discrepancy runs counter to what would be predicted by avidity effects, and could not be explained with binding saturation (detailed in the next section). We thus considered pharmaceutical ligand depletion, which is known to arise as volume is miniaturized⁵⁰. In particular, an extended **gabazine^{DART.2}** chemical is ~16 nm in length, which can be used to define a ‘nanodomain’ volume over which a GABA_AR can interact with tethered drug (**Extended Data Fig 2a**). Since this volume is tiny ($\sim 9 \times 10^{-21}$ L), there would be few drug molecules in a nanodomain, each contributing a substantial concentration (~ 180 μ M). In this parameter regime, one must account for each individual drug. In particular, a drug molecule can either modulate the channel (GABA_AR-bound), or contribute to the mobile concentration (GABA_AR-unbound), but it cannot do both simultaneously. Because ligand depletion is a subtractive phenomenon (governed by the number of binding sites on the GABA_AR), replenishing the depletion would require an additive (not multiplicative) right-shift in the dose-response. As such, **CC₅₀** would be impacted most for **gabazine.1^{DART.2}**, narrowing the gap to **gabazine.7^{DART.2}** on the logarithmic dosing axis (**Extended Data Fig 2a**).

Given that ligand depletion is accentuated as volume shrinks, we asked whether our dataset could provide a constraint on the nanodomain volume. Indeed, the 16 nm radius, estimated from molecular structure, recapitulates the 2-fold change in **CC₅₀** data across **gabazine^{DART}** variants (**Extended Data Fig 2b-d**). If the nanodomain volume were increased, the fold-change in **CC₅₀** would broaden, approaching the 15-fold change seen in the ambient. Smaller nanodomains would restrict **CC₅₀** more tightly than in the data. Thus, the volume parameter is well constrained. The model also explains why tethered **gabazine.1^{DART.2}** achieves 97% block, whereas **gabazine.7^{DART.2}** achieves ~72% block at saturation. Both findings are consistent with a stoichiometry of ~4 **HTP** proteins per nanodomain (**Extended Data Fig 2a**). This number sets the maximum concentration in the nanodomain, which we estimate can be as high as ~720 μ M ($= 4 \times 180$ μ M) if all 4 **Rx** molecules remain mobile. For high-affinity gabazine variants, up to 2 **Rx** would be depleted upon binding the GABA_AR, leaving ~360 μ M ($= 2 \times 180$ μ M) mobile. These estimates provide a conceptual framework to better understand, design, and use **DART** reagents.

The high tethered **Rx** concentrations estimated by this analysis heightens the importance of experimentally confirming receptor specificity on ***HTP** cells. Interestingly, the ligand depletion phenomenon (**Extended Data Fig 2**) suggests that the desired receptor could outcompete off-target sites for a limited number of drugs, thus improving receptor specificity over that seen with traditional pharmacology. Nevertheless, given the unknowns of receptor density and drug binding in the tethered regime, we recommend experimental characterization in the cell type of interest, as done for **gabazine.7^{DART.2}** on ***HTP** VTA_{DA} neurons (**Extended Data Fig 3b-c**).

Nanodomain Tethered-Drug Model

The results in **Extended Data Fig 2** (summarized to the right) contain a surprise: large changes in **AC₅₀** correspond to small changes in **CC₅₀**.

- **gabazine.7^{DART.2}**
- **gabazine.5^{DART.2}**
- **gabazine.1^{DART.2}**

AC₅₀	CC₅₀
180 μ M	60 nM $\times$ 15 min
72 μ M	42 nM $\times$ 15 min
12 μ M	30 nM $\times$ 15 min

Equations 1-2 state the paradox in explicit terms. First, for a given gabazine variant, its action on the GABA_AR must obey the same Langmuir binding curve in both *ambient* and *tethered* configurations—the only difference being that the tethered concentration (**C_{EFF}**) would be a boosted version of the ambient concentration (**C_{ambient}**). However, if this boost were linear (**C_{EFF}** = $\beta \times$ **C_{ambient}**) then **AC₅₀** and **CC₅₀** would also be linearly proportional

($AC_{50} = \beta \times CC_{50}$). Moreover, if we consider nonlinearities wherein C_{EFF} saturates with increasing $C_{ambient}$, we find that this could only broaden the spread of CC_{50} , exacerbating the paradox.

#	Equation	50% block	Description
1	$AmbientFit = AC_{50} / (C_{ambient} + AC_{50})$	$C_{ambient} = AC_{50}$	^{dd} HTP ambient dose-response curve (dashed fit in Extended Data Fig 2)
2	$TetheredFit = AC_{50} / (C_{EFF} + AC_{50})$	$C_{EFF} = AC_{50}$ $C_{ambient} = CC_{50} / t$	⁺ HTP tethered dose-response curve (solid fit in Extended Data Fig 2)

We considered pharmaceutical ligand depletion, which is known to arise in miniaturized volumes⁵⁰. In particular, we assumed a physical ~16 nm length scale over which a GABA_AR can interact with a fully extended tethered drug (**Extended Data Fig 2a**, left cartoon). Within these dimensions, there would be few **Rx** molecules in a nanodomain, each contributing a substantial concentration. As such, one must account for each individual molecule. In particular, only the GABA_AR-unbound (N_U) subset of **Rx** molecules contribute to C_{EFF} , as only these can collide to drive new binding events. Conversely, only the GABA_AR-bound (N_B) subset can modulate the channel. However, binding comes with a price, as even one GABA_AR-bound drug ($N_B = 1$) would substantially lower the mobile concentration (C_{EFF}) in a nanodomain.

Equilibrium of this model obeys **Equations 3-5**, which describe how a given total number of nanodomain drugs (N_T) is equilibrated into GABA_AR-bound (N_B) and unbound (N_U) subsets. **Equation 6** describes the capture kinetics, whereby $C_{ambient}$ determines N_T . Thus, the only parameter allowed to vary for each **gabazine^{DART}** variant is AC_{50} (in red), while the physical parameters (in blue) are common to all.

#	Equation	Description
3	$C_{EFF} = N_B \times 0 + N_U \times C_x$	Effective mobile concentration in the nanodomain
4	$N_U = N_T - N_B$	Number of nanodomain Rx unbound to GABA _A R
5	$N_B = N_{B,max} \times C_{EFF} / (C_{EFF} + AC_{50})$	Number of nanodomain Rx bound to GABA _A R.
6	$N_T = N_{T,max} \times (1 - e^{-C_{ambient} \times 15 \text{ min} \times k_T})$	Number of nanodomain Rx total

The data for all three reagents could be jointly fit with the common parameter set shown below (**Extended Data Fig 2**, solid and dashed fits). The values are tightly constrained by data.

Name	Value	Description
C_x	180 μ M	Conc. per Rx. Related to nanodomain radius: $r_{nano} = 92.6 [\text{nm} \times \mu\text{M}^{1/3}] / C_x^{1/3}$
$N_{B,max}$	2	Maximum # of Rx bound to GABA _A R (from GABA _A R structure/function).
$N_{T,max}$	4	Maximum # of Rx total in the nanodomain (= number of HTP in nanodomain).
k_T	$6.6 \times 10^5 \text{ M}^{-1}\text{min}^{-1}$	Tethering rate constant (rate of gabazine^{DART} capture by HTP)

SUPPLEMENTARY TABLES

Table S1. HTL Structure Function.

Each molecule was synthesized, and **CC₅₀** was estimated in neuronal culture (as shown in **Fig 2a**).

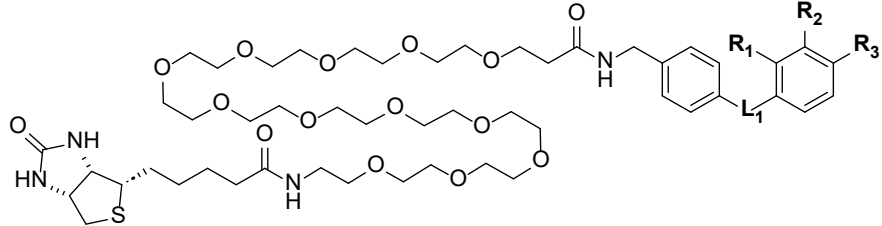
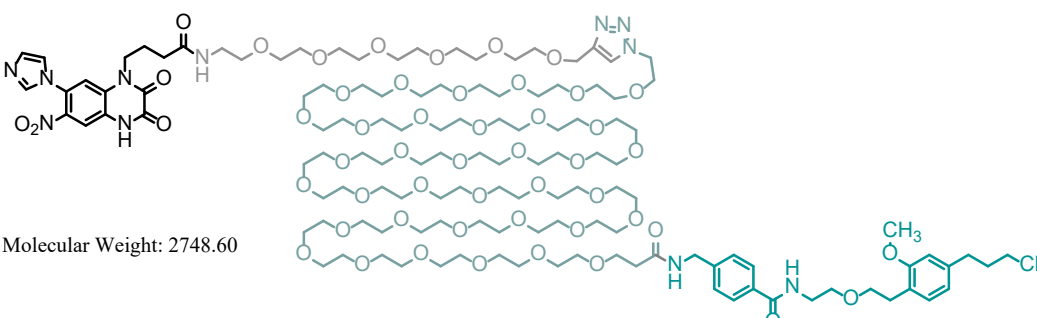
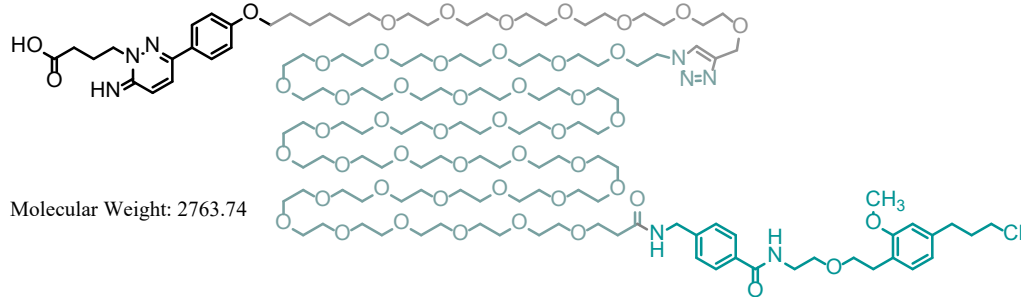
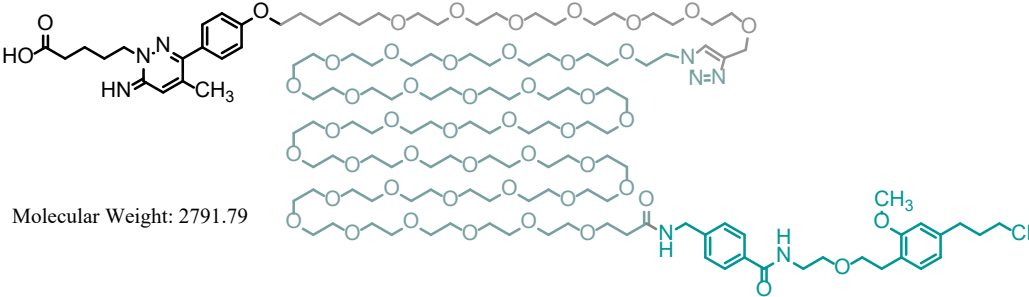
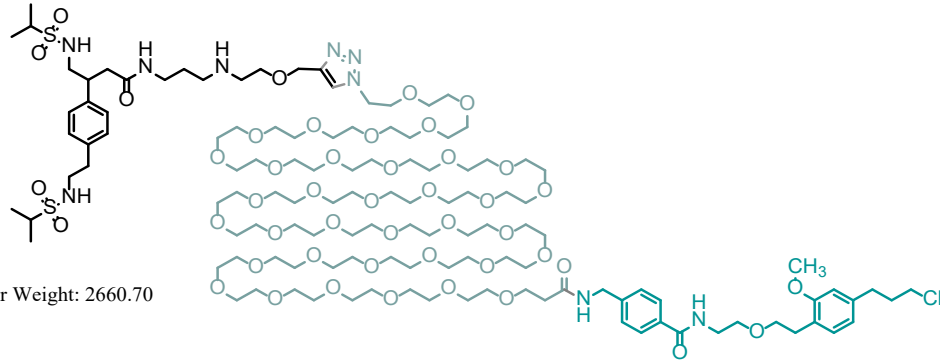
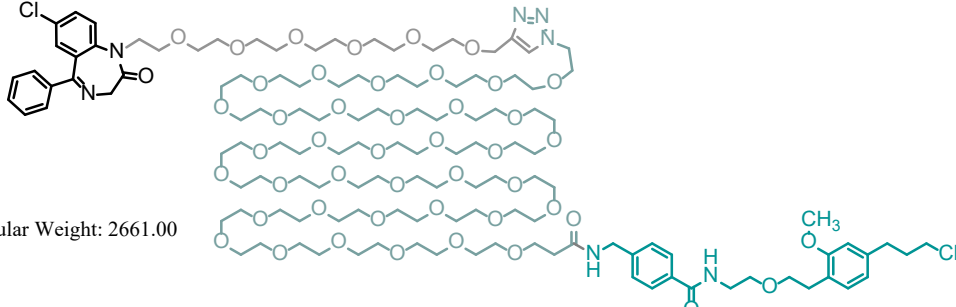
					
	L ₁	R ₁	R ₂	R ₃	CC ₅₀ (nM × 15 min)
HTL.2	-C-C-O-C-C-	-O-Me	-H	-C-C-C-Cl	10
other variants tested:	-C-C-O-C-C-	-O-Me	-H	-C=C-C-C-Cl	20
	-C-C-O-C-C-	-O-Me	-H	-C-C-C-C-Cl	30
	-C-C-C-C-O-	-O-Me	-H	-C-C-C-C-Cl	30
	-C-C-C-C-O-	-O-Me	-H	-C-C-C-Cl	50
	-C-C-C-C-O-	-O-Me	-H	-C=C-C-C-Cl	80
	-C-C-C-C-O-	-OH	-H	-C-C-C-C-Cl	30
	-C-C-C-C-O-	-F	-H	-C-C-C-C-Cl	45
	-C-C-C-C-O-	-O-Et	-H	-C-C-C-C-Cl	70
	-C-C-C-C-O-	-NH ₂	-H	-C-C-C-C-Cl	70
	-C-C-C-C-O-	-Cl	-H	-C-C-C-C-Cl	100
	-C-C-C-C-O-	-H	-H	-C-C-C-C-Cl	110
	-C-C-C-C-O-	-Et	-H	-C-C-C-C-Cl	200
	-C-C-C-C-O-	-NO ₂	-H	-C-C-C-C-Cl	300
	-C-C-C-C-O-	-Me	-H	-C-C-C-C-Cl	1,000
	-C-C-C-C-O-	-O-CF ₃	-H	-C-C-C-C-Cl	1,500
	-C-C-C-C-O-	-N-Me ₂	-H	-C-C-C-C-Cl	4,500
	-C-C-O-C-C-	-O-Me	-C-C-C-C-Cl	-H	130
	-C-C-O-C-C-	-O-Me	-C-C-C-C-C-Cl	-H	880
	-C-C-O-C-	-O-Me	-C-C-C-C-Cl	-H	420
	-C-C-O-C-	-O-Me	-C-C-C-C-C-Cl	-H	620

Table S2. DART.2 catalog

YM90K.1^{DART.2}	 Molecular Weight: 2748.60
gabazine.1^{DART.2}	 Molecular Weight: 2763.74
gabazine.7^{DART.2}	 Molecular Weight: 2791.79
CMPDA.2^{DART.2}	 Molecular Weight: 2660.70
diazepam.1^{DART.2}	 Molecular Weight: 2661.00

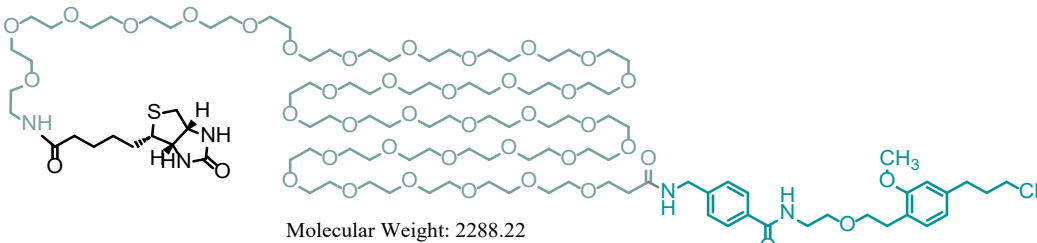
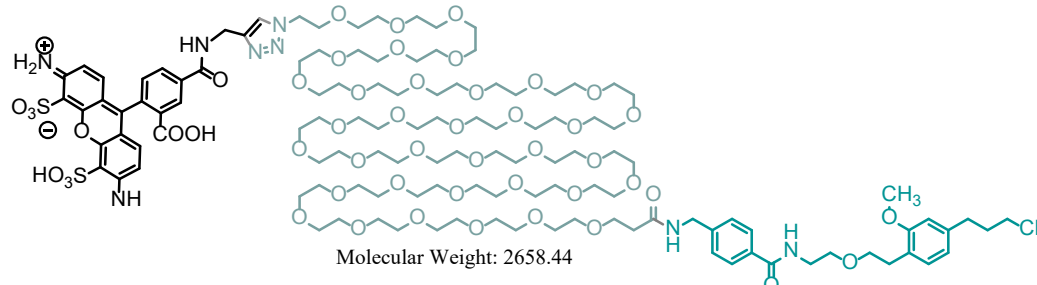
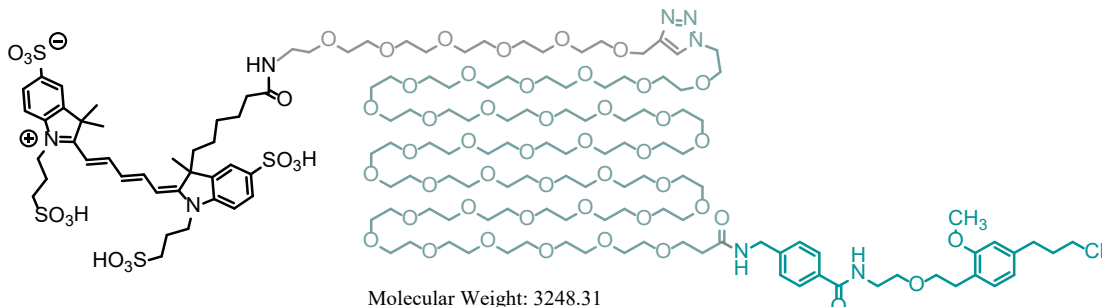
biotin.1 DART.2	 Molecular Weight: 2288.22
Alexa488.1 DART.2	 Molecular Weight: 2658.44
Alexa647.1 DART.2	 Molecular Weight: 3248.31

Table S3. KEY RESOURCES

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
AAV _{rh10} -CAG-DIO- ⁺ HTP _{GPI} -2A-dTomato-WPRE	this paper	AAV10-X0117A, Lot 191001, 200807, 210215
AAV _{rh10} -CAG-DIO- ^{dd} HTP _{GPI} -2A-dTomato-WPRE	this paper	AAV10-M6360D, Lot 200203, 210420
AAV _{7m8} -CAG-DIO- ⁺ HTP _{GPI} -2A-dTomato-WPRE	this paper	AAV7m8-X0117A, Lot 191219, 220321
AAV _{7m8} -CAG-DIO- ^{dd} HTP _{GPI} -2A-dTomato-WPRE	this paper	AAV7m8-M6360D, Lot 200219
AAV _{rh10} -CAG-DIO- ⁺ HTP _{GPI} -IRES-dTomatoF-W3SL	this paper	AAV10-M6771A, Lot 220328
AAV _{rh10} -CAG-DIO- ^{dd} HTP _{GPI} -IRES-dTomatoF-W3SL	this paper	AAV10-M6829B, Lot 220328
AAV ₉ -SYN-jGCaMP8s-WPRE	Addgene	RRID: Addgene_162374
Chemicals, peptides, and recombinant proteins		
biotin-PEG ₁₂ -HTL.1	this paper	Lot 180110
biotin-PEG ₁₂ -HTL.2	this paper	Lot 180220
azide-PEG ₃₆ -HTL.1 (<i>azide</i> ^{DART.1})	this paper	Lot 180222
azide-PEG ₃₆ -HTL.2 (<i>azide</i> ^{DART.2})	this paper	Lot 180810
YM90K.1 ^{DART.1}	this paper	Lot 171031
YM90K.1 ^{DART.2}	this paper	Lot 180712, 200725
gabazine.1 ^{DART.2}	this paper	Lot 180926
gabazine.5 ^{DART.2}	this paper	Lot 201109, 220406
gabazine.7 ^{DART.2}	this paper	Lot 201109, 220512
diazepam.1 ^{DART.2}	this paper	Lots 190331, 210628
flumazenil.1 ^{DART.2}	this paper	Lot 170430, 190331
CMPDA.1 ^{DART.1}	this paper	Lot 180103
CMPDA.2 ^{DART.2}	this paper	Lot 200725
Alexa488.1 ^{DART.2}	this paper	Lot 190730, 210820
Alexa647.1 ^{DART.2}	this paper	Lot 200213, 220316
blank.1 ^{DART.2}	this paper	Lot 210418

AP-V	Sigma	Cat# A5282
CPP-(R)	Tocris	Cat# 0247
DNQX	Sigma	Cat# D0540
NBQX	Abcam	Cat# ab120046
Picrotoxin	Sigma	Cat# P1675
SR 95531 hydrobromide (gabazine)	Tocris	Cat# 1262
Streptavidin-AlexaFluor 488	Molecular Probes/	Cat# S11223
High-molecular weight Poly-D lysine	Sigma	Cat# P7405
Papain enzyme	Worthington	Cat# PAP2
Experimental models: Organisms/strains		
Tg(Drd1a-cre)EY262Gsat/Mmucd	MMRRC	RRID:MMRRC_017264-UCD
B6.SJL-Slc6a3tm1.1(cre)Bkmn/J	Jackson Labs	RRID:IMSR_JAX:006660
B6.129P2- <i>Pvalb</i> ^{tm1(cre)Arbr} /J	Jackson Labs	RRID:IMSR_JAX:017320
STOCK <i>Sst</i> ^{tm2.1(cre)Zjh} /J	Jackson Labs	RRID:IMSR_JAX:013044
CBA/CaJ	Jackson Labs	RRID:IMSR_JAX:000654
B6.Cg-lgs7tm148.1(tetO-GCaMP6f,CAG-tTA2)Hze/J	Jackson Labs	RRID:IMSR_JAX:030328
BACa6Cre-C	Aller et al. ²²	NA
CD® (Sprague Dawley) IGS Rat	Charles River	RRID:RGD_734476
Recombinant DNA		
aavCAG- *HTP _{NLG(432-648)ERXL} -2A-dTomato-WPRE	this paper	M3810A(GenBank PP719192)
aavCAG- ddHTP _{NLG(432-648)ERXL} -2A-dTomato-WPRE	this paper	M6374A(GenBank PP719194)
aavCAG-DIO- *HTP _{GPI} -2A-dTomato-WPRE	this paper	X0117A(GenBank PP719197)
aavCAG-DIO- ddHTP _{GPI} -2A-dTomato-WPRE	this paper	M6360D(GenBank PP719193)
aavCAG-DIO- *HTP _{GPI} -IRES-dTomatoF-W3SL	this paper	M6771A(GenBank PP719195)
aavCAG-DIO- ddHTP _{GPI} -IRES-dTomatoF-W3SL	this paper	M6829B(GenBank PP719196)
aavSYN-ChR2-HA-dsfGFP-WPRE	our prior work ³	M2520A
aavSYN-iCre-WPRE	Addgene	RRID: Addgene_122518
aavCAG-GCaMP6s-WPRE	Addgene	RRID: Addgene_100844

Software and algorithms		
MATLAB	MathWorks, Inc	Version 2018a
Ethovision	Noldus	Version XT 10
Clampfit	Axon Instruments	Version 10.7
Image J	NIH	N/A
Micromanager	NIH	N/A
Scanbox	Neurolabware	N/A
Chemdraw	PerkinElmer	Version 19.1.1.21
Instant JChem	ChemAxon	N/A
ACD Spectrus Processor	ACD Labs	N/A
CalmAn	Github	N/A

Table S4. Detailed Author Contributions

Attribution Process: Each Principle Investigator (PI, right column) worked with their corresponding team members to outline individual contributions across a standard set of CRediT domains. Contributions were entered and edited on a live google-sheet, allowing all individuals to discuss and reach consensus. Decisions regarding the delineation between authorship and acknowledgement were decided by the supervising PI of each individual, and authorship order was decided by the lead PI with input and discussions with all authors.

Name	Contribution (Authorship & Acknowledgements)	PI
Brenda C. Shields	Fig 2a: Performed and co-developed biotin-HTL dose-response assays, which guided the design and identification HTL.2. Fig 2d-f, Extended Data Fig 2b-d, and Extended Data Fig 6a-d: Co-developed and performed the all-optical AMPAR and GABA_AR pharmacology assays, including data curation and data analysis. Reagent Development: Co-designed, cloned, and optimized DNA constructs, including development of the ^{dd}HTP protein (Fig 2a), its cloning into the GPI-2A and GPI-IRES backbones, their packaging into AAV and in-vitro viral titer characterization and analysis. Wrote and edited original draft of the associated sections of the manuscript and methods. Maintenance and distribution of viral and chemical reagents, histology training, lab management. CRediT: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing Original Draft (Introduction, Methods), Writing Review & Editing, Visualization, Supervision, Project Administration	MRT

Haidun Yan	Conceived, designed, and performed acute brain slice electrophysiology experiments that appear in Fig 3e, Extended Data Fig 2b-c, retina electrophysiology in Fig 5 and Extended Data Fig 6e-f, and dual patch experiments in Fig 6. Analyzed electrophysiology data, performed statistical analyses, prepared figures, and wrote the original draft of the associated sections of the manuscript and methods. CRedit: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing Original Draft (Methods), Writing Review & Editing, Visualization	MRT, GDF
Shaun S.X. Lim	Reagent Development: designed and cloned plasmids encoding the HTP_{GPI} construct and its packaging into AAV. Explored various HTP_{GPI} plasmid designs, which led to key insights that were critical for data in Fig 4e-g. Performed in-vitro viral titer characterization and analysis. Optimized surgical procedures to improve AAV expression (surgeries, histology, imaging), and assisted with histology for ventricular dosing experiments (sample preparation and imaging). Analyzed data for SPR assays (Fig 2b). CRedit: Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Writing Original Draft (Results, Discussion), Writing Review & Editing, Visualization	MRT
Sasha C. Burwell	Optimized AAV serotype, promoter, and surgical procedure for VTA dopamine neurons. Fig 3e: Designed, built, and coded wheel speed assay, and performed gabazine.7^{DART.2} VTA_{DA} experiments (surgeries, drug administration, and treadmill behavior) and histology (sample preparation, imaging, image segmentation, image analysis). Performed statistical analysis of behavioral data and prepared figures. Fig 3f: Performed virus injection surgeries for voltage clamp experiments. Other: Piloted cisterna magna cannulation and infusion. Preliminary piloting of intraventricular cannulation strategies. CRedit: Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Data Curation, Writing Review & Editing, Visualization	MRT
Celine M. Cammarata	Optimized viral parameters in SOM::Cre mice. Designed and troubleshot surgical procedures for ventricular cannula implant compatible with window imaging. Performed widefield pharmacokinetics imaging and analysis. Performed two-photon calcium imaging, alignment, segmentation, analysis, statistics, and prepared figures for Fig 4e-g and Extended Data Fig 5. CRedit: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing Original Draft (Methods), Writing Review & Editing, Visualization, Supervision	LLG
Elizabeth A. Fleming	Fig 3a-d. Designed methodology and performed surgical procedures for local infusion during two-photon imaging of the cerebellar cortex including headpost, window, cannula implants, and viral injections. Performed two-photon imaging and statistical analysis. Prepared figures. Performed viral injections for slice recordings. CRedit: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing Review & Editing, Visualization, Funding Acquisition (NIH: 1F31NS113742-01A1)	CH
S. Aryana Yousefzadeh	Extended Data Fig, 3d-e (HTP turnover) and Extended Data Fig 4a-c (diffusion of different payloads): Designed and performed experiments (surgeries and drug infusions) and histology (sample preparation, streptavidin staining, imaging, and image segmentation). Other: Preliminary piloting and troubleshooting of intraventricular cannulation strategies. CRedit: Conceptualization, Methodology, Validation, Investigation, Data Curation.	MRT

Victoria Z. Goldenshtein	Designed and performed Localized Surface Plasmon Resonance (LSPR) assays, including data curation and visualization that appear in Fig. 2b. Designed and cloned DNA vectors containing GFP-ddHTP/GFP-HTP, expressed and purified proteins for LSPR assays. CRedit: Methodology, Validation, Investigation, Data Curation, Writing Original Draft (Methods), Visualization.	MRT
Elizabeth W. Kahuno	Fig 4a-b: performed YM90K.1^{DART.2} experiments in behaving mice (mouse surgeries and open field behavior), and subsequent brain histology (sample preparation, imaging, and image segmentation). Preliminary tests of AAV serotype and titer to optimize expression and minimize toxicity. Established stereotactic surgeries, and optimized cannula design. Maintained IACUC protocols, mouse care, and reagent ordering. CRedit: Methodology, Validation, Investigation, Data Curation, Project Administration	MRT
Purav P. Vagadia	Synthesized the YM90K, gabazine, Alexa488, Alexa647, blank, diazepam, and Flumazenil DART compounds described in this work. Designed the synthetic routes and analytically characterized the intermediates and final compounds for these compounds. Organized the chemical data and wrote the experimental procedures and supplemental chemistry section describing the above-listed compounds. CRedit: Methodology, Formal Analysis, Investigation, Writing Original Draft (Methods), Writing Review & Editing	GES
Marie H. Loughran	Synthesized first and several subsequent batches of HTL.2; synthesized CMPDA.1^{DART.1} and the first batch of CMPDA.2^{DART.2} . CRedit: Methodology, Validation, Investigation	ABR
Lei Zhiquan	Re-synthesized gabazine. 5^{DART.2} and gabazine. 7^{DART.2} . Analytically characterized the intermediates and final compounds for these reagents, and ran 2D NMR experiments to confirm structures. Helped interpret spectroscopic chemical data for all of the YM90K, gabazine, Alexa488, Alexa647, blank, diazepam, and Flumazenil DART reagents. Contributed to writing some of the experimental procedures and supplemental chemistry data. CRedit: Methodology, Formal Analysis, Investigation, Writing Original Draft (Methods)	GES
Mark E. McDonnell	Supervised associate (MHL) enabling target prep; devised synthetic routes; troubleshooted chemistry; codesigned target structural modifications. Wrote, reviewed, and edited the supplementary chemical synthesis. CRedit: Methodology, Validation, Supervision	ABR
Miranda L. Scalabrino	Performed intraocular injections and optimized viral serotypes, promoters and titers for injections to express HTP in retinal ganglion cells. CRedit: Methodology, Validation	GDF
Mishek Thapa	Performed intraocular injections and optimized viral parameters for retina. CRedit: Methodology, Validation, Investigation	GDF
Tammy M. Hawley	Fig 4e-g and Extended Data Fig 5. Optimized viral parameters in SOM::Cre mice. Designed, troubleshooted, and performed surgical procedures for headpost, window, ventricular cannula implant, and intra-cortical injections. Performed two-photon and widefield imaging of visual cortex, collection and imaging of histology. CRedit: Conceptualization, Methodology, Validation, Investigation	LLG

Greg D. Field	Senior Author. Supervised retina experiments. CRedit : Conceptualization, Methodology, Resources, Writing Review & Editing, Supervision, Project Administration, Funding Acquisition (NIH: R34 NS111645; NIH: R01 EY031396; Duke University: Startup Funds)
Court Hull	Senior Author. Supervised cerebellum experiments. Performed granule cell slice recordings (Fig 3b, Extended Data Fig 3a). CRedit : Conceptualization, Methodology, Resources, Writing Review & Editing, Supervision, Project Administration, Funding Acquisition (NIH: R01 NS096289, R01 NS112917, Duke University Startup Funds).
Gary E. Schiltz	Senior Author. Supervised the synthetic chemistry to prepare the YM90K, gabazine, Alexa488, Alexa647, blank, diazepam, and Flumazenil DART reagents. Provided preparative HPLC, lyophilizer, and normal phase purification resources beyond grant 1RF1MH117055 to support the purification of DART compounds. Contributed to the design of synthetic routes to prepare some DART compounds and their analytical characterization. Reviewed and edited the final manuscript, particularly with regards to the supplementary chemical synthesis section. CRedit : Methodology, Formal Analysis, Resources, Writing Review & Editing, Supervision, Project Administration
Lindsey L. Glickfeld	Senior Author. Supervised visual cortex experiments. Iterated with MRT on the manuscript. CRedit : Conceptualization, Methodology, Resources, Writing Review & Editing, Supervision, Project Administration, Funding Acquisition (NIH: R01 EY031716; Duke University: Holland Trice Scholars Award and Startup Funds)
Allen B. Reitz	Senior Author. Supervised MHL and MEM, co-designed chemical targets and synthetic routes; solved synthetic problems. Reviewed and edited the supplementary chemical synthesis. CRedit : Methodology, Resources, Supervision, Project Administration
Michael R. Tadross	Senior Author & Lead Contact. Proposed HTL library and HTL.2 structure (Table S1). Proposed structures of diazepam ^{DART.2} , gabazine ^{DART.2} series, and performed structural modeling to design CMPDA.2 ^{DART.2} . With MHL, MEM, ABR, PPV, LZ and GES, developed synthetic routes. With BCS and SSXL, designed and optimized DNA constructs. With BCS, conceived biotin-HTL, AMPAR, and GABA _A R assays. With BCS, SAY, and EWK, designed tracer ^{DART} experiments. Wrote software for analysis of cultured-neuron data, open-field behavior and histology, and generated Fig 1, Fig 2, Fig 4a-d, and Extended Data Fig 6 . Designed experiments and edited figures with SCB, HY, EAF, CH (Fig 3, Extended Data Fig 3), with CMC, and LLG (Fig 4e-g, Extended Data Fig 5) and with HY and GDF (Fig 5). Derived nanodomain model and prepared Extended Data Fig 2 with input from SSXL. Wrote and revised the paper, with input from all authors. CRedit : Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing Original Draft, Writing Review & Editing, Visualization, Supervision, Project Administration, Funding Acquisition (Duke Startup and DIBS Awards. NIH: 1RF1MH117055, 1DP2MH1194025, 1R01NS107472, 1R61DA051530)

SUPPLEMENTARY PROTOCOLS

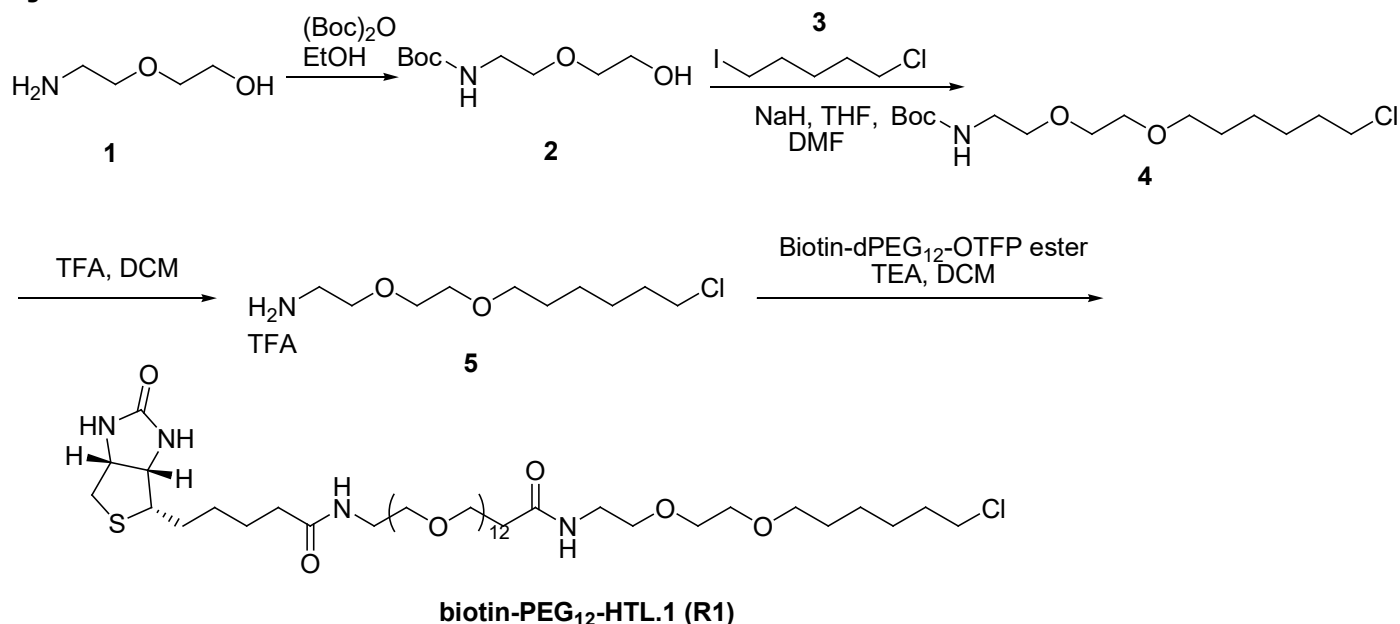
General Chemical Synthesis Information

All chemical reagents were obtained from commercial suppliers and used without further purification unless otherwise stated. Anhydrous solvents were purchased from Sigma-Aldrich, and dried over 3 Å molecular sieves when necessary. Normal-phase flash column chromatography was performed using Biotage KP-Sil 50 µm silica gel columns and ACS grade solvents on a Biotage Isolera flash purification system or Teledyne Combiflash using Silicycle silica gel columns. Reverse phase preparative HPLC was performed with the following conditions: Phenomenex Gemini-NX C18, 110 Å, 150 x 21.2 mm; 5 µm. Eluting with a gradient of acetonitrile:water specified for each DART with 0.1 % formic acid over 25 min, then 1 min at 100 % acetonitrile. For Alexa647.1^{DART.2} (R11), 0.1 % TFA rather than 0.1 % formic acid was used and the flow rate was 20 mL/min. For Alexa488.1^{DART.2} (R10), the following prep HPLC conditions were used: Phenomenex Kinetex C18 100 Å, 50 x 30 mm; 5 µm, and a gradient of 15 to 80 % acetonitrile in water (0.1 % FA) at 50 mL/min for 12 min. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60 F254 plates and visualized by UV light. Proton (1 H), and carbon (13C) NMR spectra were recorded on a 500 MHz Bruker Avance III with direct cryoprobe spectrometer. Chemical shifts were reported in ppm (δ) and were referenced using residual nondeuterated solvent as an internal standard (CDCl₃ at 7.24 ppm for 1 H-NMR and 77.0 for 13C-NMR. CD₃OD at 3.33 ppm for 1 H-NMR and 47.6 for 13C-NMR. DMSO-d₆ at 2.52 ppm for 1 H-NMR and 39.9 ppm for 13C-NMR). Proton coupling constants are expressed in hertz (Hz). The following abbreviations are used to denote spin multiplicity for proton NMR: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br.s = broad singlet, dd = doublet of doublets, dt = doublet of triplets, quin = quintet, tt = triplet of triplets. Low-resolution liquid chromatography/mass spectrometry (LCMS) was performed on a Waters Acquity-H UPLC/MS system with a 2.1 mm × 50 mm, 1.7 µm, reversed phase BEH C18 column and LCMS grade solvents. A gradient elution from 95% water +0.1% formic acid/5% acetonitrile +0.1% formic acid to 95% acetonitrile +0.1% formic acid/5% water +0.1% formic acid over 2 min plus a further minute continuing this mixture at a flow rate of 0.85 mL/min was used as the eluent (for Alexa647.1^{DART.2} (R11), 0.1 % TFA rather than 0.1 % FA was used). Total ion current traces were obtained for electrospray positive and negative ionization (ESI+/ESI-). High-resolution mass spectra were obtained using an Agilent 6210 LC-TOF spectrometer in the positive ion mode using electrospray ionization with an Agilent G1312A HPLC pump and an Agilent G1367B autosampler at the Integrated Molecular Structure Education and Research Center (IMSERC), Northwestern University. All microwave-assisted reactions were carried out in a Biotage® initiator. All IUPAC compound names were generated using Chemdraw Version 19.1.1.21 or Instant J Chem.

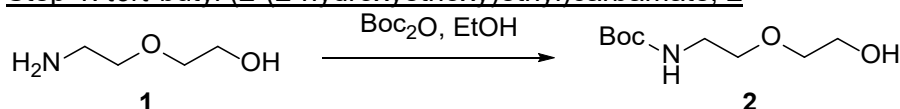
Synthesis details are provided for the following

- R1 — biotin-PEG₁₂-HTL.1
- R2 — biotin-PEG₁₂-HTL.2
- R3 — azide-PEG₃₆-HTL.1 (*azide*^{DART.1})
- R4 — azide-PEG₃₆-HTL.2 (*azide*^{DART.2})
- R5 — YM90K.1^{DART.1}
- R6 — YM90K.1^{DART.2}
- R7 — gabazine.1^{DART.2}
- R8 — gabazine.5^{DART.2}
- R9 — gabazine.7^{DART.2}
- R10 — Alexa488.1^{DART.2}
- R11 — Alexa647.1^{DART.2}
- R12 — blank.1^{DART.2}
- R13 — diazepam.1^{DART.2}
- R14 — flumazenil.1^{DART.2}
- R15 — CMPDA.1^{DART.1}
- R16 — CMPDA.2^{DART.2}

Synthesis of R1—biotin-PEG₁₂-HTL.1

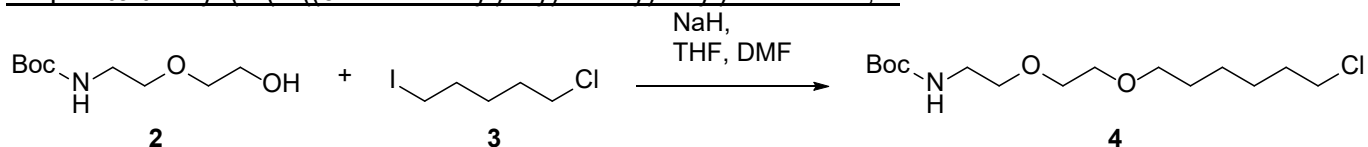


Step 1: *tert*-butyl (2-(2-hydroxyethoxy)ethyl)carbamate, **2**



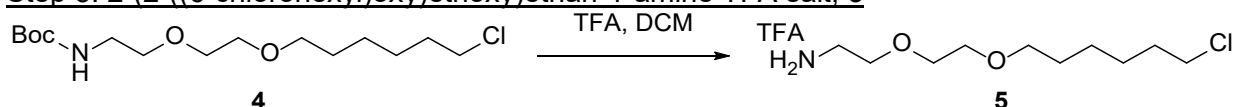
To a solution of 2-(2-aminoethoxy)ethan-1-ol (3.2 g, 30.4 mmol) in EtOH (10 mL) was added di-*tert*-butyl dicarbonate (6.64 g, 30.4 mmol) and reaction mixture was maintained at room temperature for 24 h. After the complete consumption of starting material (LC-MS), the volatiles were removed *in vacuo*, and the residue was concentrated. Purification by flash chromatography (24 g silica cartridge, 0-10% MeOH/ CH_2Cl_2) afforded the title compound in 5.8 g, 92% yield. (ES-LCMS) m/z 228 ($\text{M}+\text{Na}$)⁺.

Step 2: *tert*-butyl (2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamate, **4**



In a flame-dried 250 mL round-bottom flask under nitrogen was placed sodium hydride (60% dispersion in mineral oil, 0.76 g, 19.0 mmol) followed by dry THF (30 mL). The mixture was cooled in an ice bath to 0 °C and a solution of *tert*-butyl (2-(2-hydroxyethoxy)ethyl)carbamate (3 g, 14.6 mmol) in dry THF (5 mL) was slowly added. After the reaction was stirred for one hour at 0 °C, a solution of 1-chloro-5-iodopentane (5.41 g, 21.9 mmol) in dry THF (5 mL) was slowly added. The reaction was kept at 0 °C overnight then poured into water and the product extracted with ethyl acetate (3x). The combined organic extracts were filtered to remove dark brown precipitates then washed with brine, dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude material was purified by flash chromatography (40 g silica column, 0-100% EtOAc/hexane gradient) to obtain the product as a light yellow oil (3.1 g, 65% yield). (ES-LCMS) m/z 343 ($\text{M}+\text{Na}$)⁺.

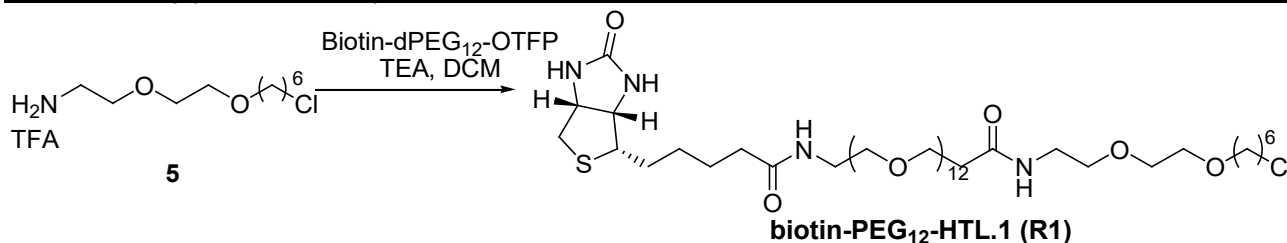
Step 3: 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-amine TFA salt, **5**



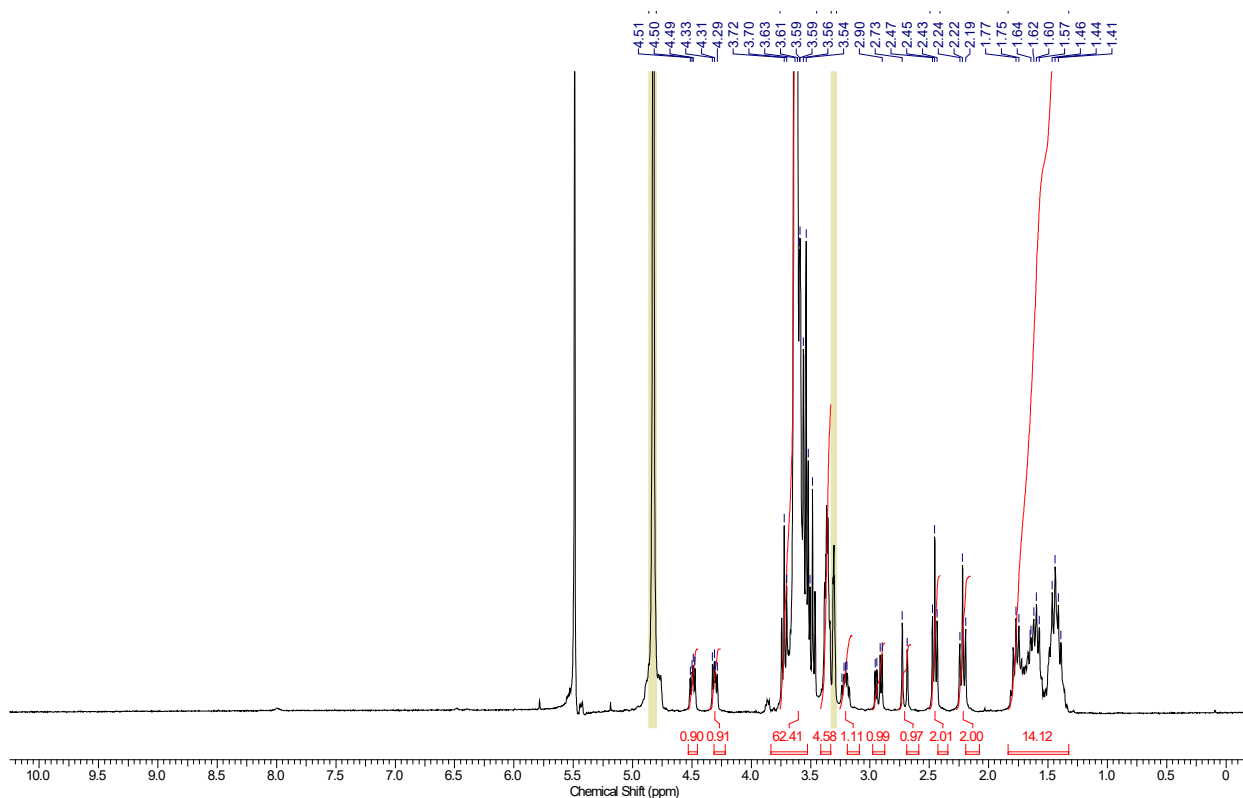
To a solution of *tert*-butyl (2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamate (3 g, 9.28 mmol) in dichloromethane (20.0 mL) added trifluoroacetic acid (5 mL) and maintained at room temperature for 3.0 h. The reaction was concentrated under vacuum and triturated with two 5 mL portions of ether and ether decanted

away and residual ether of was concentrated to yield a dark yellow product 3.11 g, 104% yield. ^1H NMR (CHLOROFORM- d , 300MHz): δ = 11.01 - 11.63 (m, 1 H), 3.71 - 3.80 (m, J =10.0 Hz, 2 H), 3.65 - 3.71 (m, 2 H), 3.63 (br. s., 2 H), 3.43 - 3.57 (m, 4 H), 3.16 - 3.31 (m, 2 H), 1.76 (s, 2 H), 1.59 (s, 2 H), 1.25 - 1.50 ppm (m, 4 H); (ES-LCMS) m/z 224 ($M+\text{Na}$) $^+$.

Step 4: *N*-(2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)-1-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)-3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxanonatriacontan-39-amide, **R1**

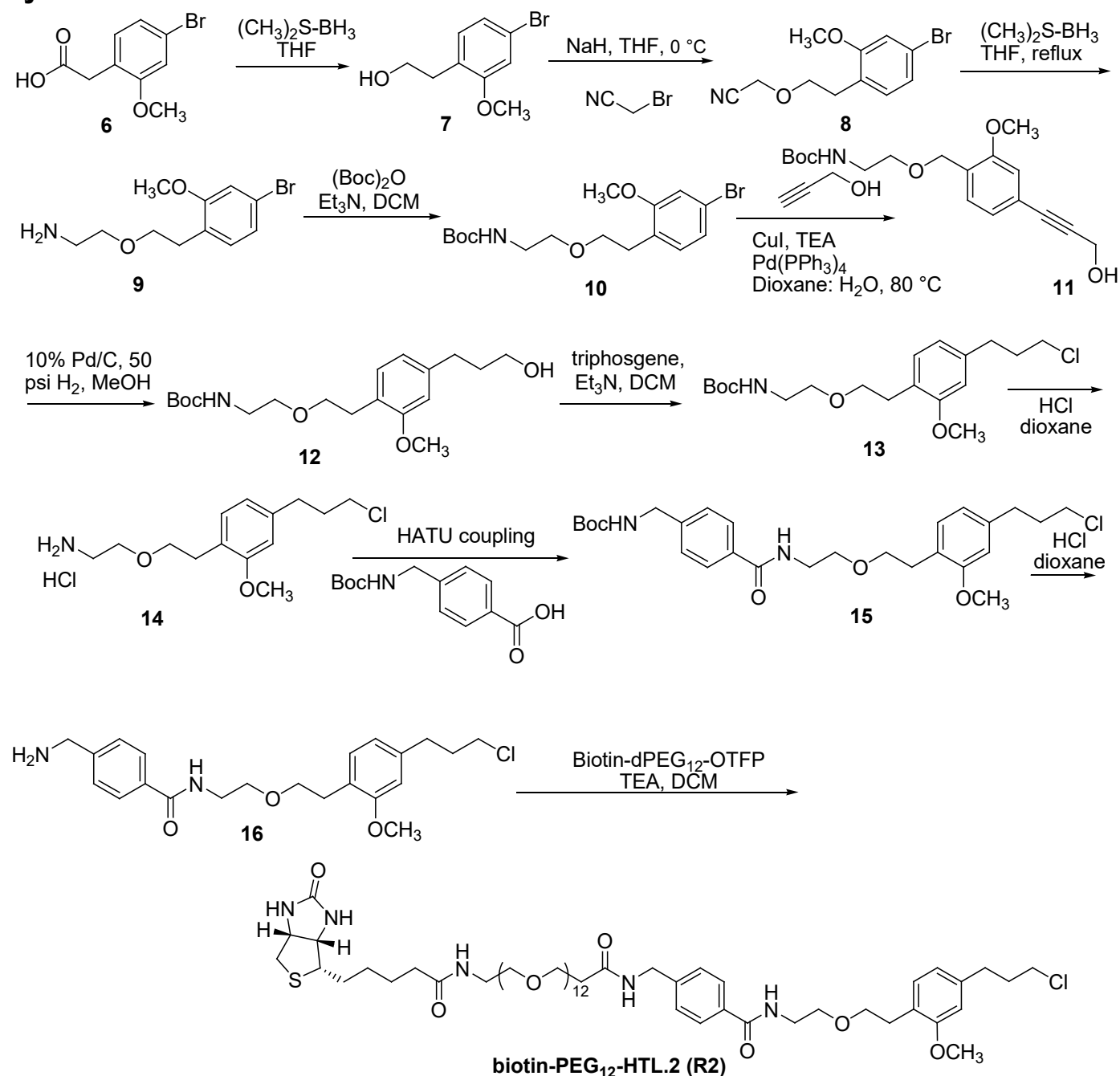


DIPEA (125 μL , 0.717 mmol) was added to a solution of 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-amine TFA salt (**5**) (20 mg, 0.089 mmol) and biotin-dPEG12-OTFP ester (71 mg, 0.071 mmol, Quanta Biodesign) in DCM (1 mL). After two hours the solvent was removed *in vacuo* and the residue was purified by Gilson prep HPLC (Sunfire Prep C18 OBD, 10 μm , 30 x 150 mm column, 15-75% ACN/ H_2O (both containing 0.1% TFA) gradient). The product fractions were combined and concentrated to remove the acetonitrile before freeze drying overnight to obtain a colorless semi-solid product (20 mg, 21 % yield). ES-LCMS: m/z = 1049.5($M+\text{H}$) $^+$; ^1H NMR (METHANOL- d_4 , 300MHz): δ = 4.45 - 4.53 (m, 1 H), 4.26 - 4.35 (m, 1 H), 3.45 - 3.76 (m, 58 H), 3.33 - 3.41 (m, 5 H), 3.21 (dd, J =8.8, 4.7 Hz, 1 H), 2.87 - 2.98 (m, 1 H), 2.66 - 2.76 (m, 1 H), 2.45 (s, 2 H), 2.22 (s, 2 H), 1.32 - 1.84 ppm (m, 14 H); HRMS (ESI $^+$): m/z calcd for $\text{C}_{47}\text{H}_{90}\text{ClN}_4\text{O}_{17}\text{S}$: 1049.5711 [$M+\text{H}$] $^+$; found: 1049.5704, [$M+\text{H}$] $^+$

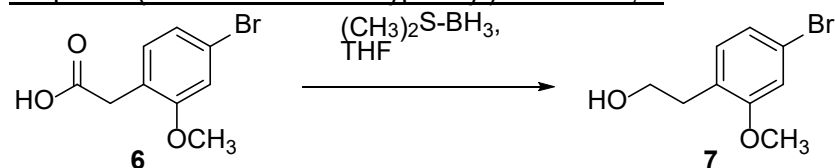


^1H NMR of biotin-PEG₁₂-HTL.1 (R1)

Synthesis of R2-biotin-PEG₁₂-HTL.2

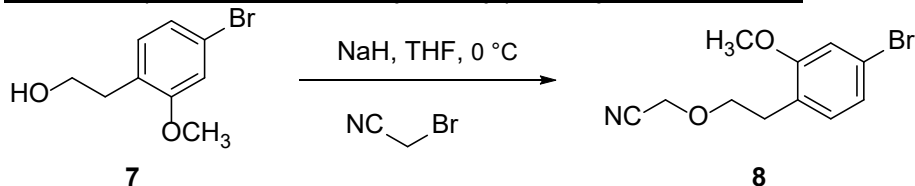


Step 1: 2-(4-bromo-2-methoxyphenyl)ethan-1-ol, **7**



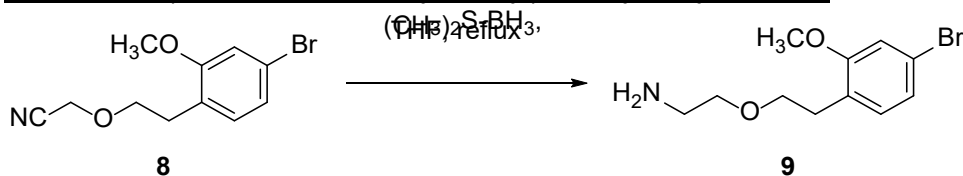
To a solution of 2-(4-bromo-2-methoxyphenyl)acetic acid (10.0 g, 41.0 mmol, CombiBlocks) in THF (100 mL) at 0 °C was added dropwise borane dimethylsulfide (21.1 mL, 246.0 mmol), keeping the temperature below 5 °C during the addition. The reaction was allowed to stir overnight as the ice bath warmed to ambient temperature. The reaction was poured into dry methanol (100 mL), stirred for 5 minutes and then the solvent was removed *in vacuo*. The crude product was obtained as a yellowish brown oil (13.45 g, 98% yield). ¹H NMR (METHANOL-d₄, 300MHz): δ = 6.94 - 7.09 (m, *J*=8.8 Hz, 3 H), 3.78 (s, 3 H), 3.67 (t, *J*=7.0 Hz, 2 H), 2.77 ppm (t, *J*=7.0 Hz, 2 H); ES-LCMS: *m/z* 232 (M+H)⁺

Step 2: [2-(4-Bromo-2-methoxy-phenyl)-ethoxy]-acetonitrile, **8**



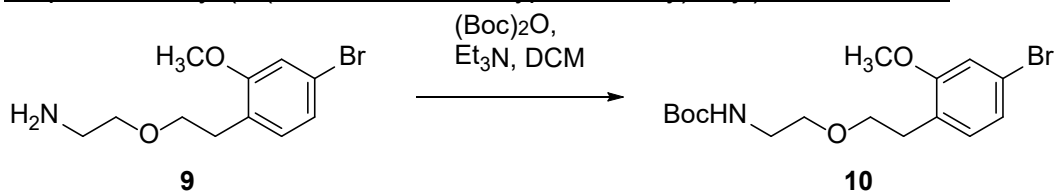
In a flame-dried 100 mL round-bottom flask under nitrogen was placed sodium hydride (60% dispersion in mineral oil, 9.35 g, 70.1 mmol) followed by dry THF (90 mL). The mixture was cooled in an ice bath to 0 °C and a solution of 2-(4-bromo-2-methoxy-phenyl)-ethanol (13.45 g, 58.4 mmol) in dry THF (50 mL) was slowly added. After the reaction was stirred for one hour at 0 °C, a solution of bromoacetonitrile (4.89 mL, 70.1 mmol) in dry THF (50 mL) was slowly added. The reaction was kept at 0 °C overnight then poured into water and the product extracted with ethyl acetate (3x). The combined organic extracts were filtered to remove dark brown precipitates then washed with brine, dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude material was purified by flash chromatography (40 g silica column, 0-2% MeOH/DCM gradient) to obtain the product as a colorless oil (8.57 g, 54% yield). ¹H NMR (CHLOROFORM-d, 300MHz): δ = 7.03 (br. s., 3 H), 4.23 (br. s., 2 H), 3.67 - 3.91 (m, 5 H), 2.89 ppm (d, *J*=5.9 Hz, 2 H); ES-LCMS: *m/z* 271.5 (M+H)⁺

Step 3: 2-[2-(4-Bromo-2-methoxy-phenyl)-ethoxy]-ethylamine, **9**



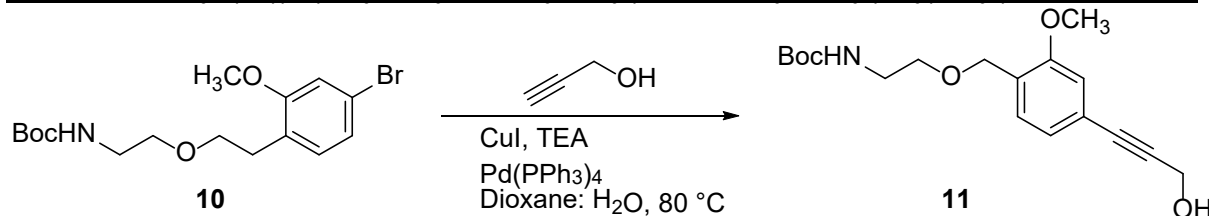
A 2M solution of borane dimethyl sulfide complex in THF (47.8 mL, 95.5 mmol) was added to a solution of [2-(4-bromo-2-methoxy-phenyl)-ethoxy]-acetonitrile (8.57 g, 31.8 mmol) in THF (50 mL) and the mixture was heated at 70 °C for 3 hours. The reaction was cooled and then quenched with methanol (50 mL). The reaction was concentrated *in vacuo* and then concentrated twice more from methanol. The crude oil was placed under high vacuum overnight to afford compound **9** (5.2 g, 60% yield). ES-LCMS: *m/z* 274.5, 276.4 (M, M+2H)⁺

Step 4: *tert*-butyl (2-(4-bromo-2-methoxyphenethoxy)ethyl)carbamate, **10**



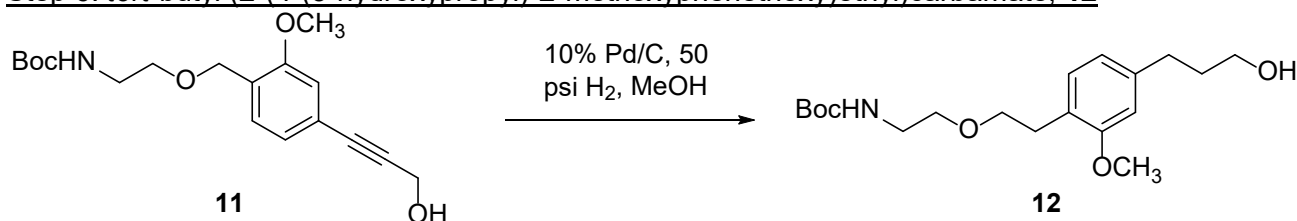
To a solution of 2-(4-bromo-2-methoxyphenethoxy)ethan-1-amine (8.6 g, 31.3 mmol) in DCM (50 mL) was added di-*tert*-butyl dicarbonate (8.9 g, 40.8 mmol) and the reaction mixture was maintained at 45 °C for 24 h. After the complete consumption of starting material (LC-MS), the volatiles were removed *in vacuo*, and the residue was dissolved in dichloromethane (30 mL). The solution was washed successively with 1% HCl (60 mL), brine (2 x 30 mL), and deionized water (30 mL), then dried over MgSO₄, filtered, and concentrated. Purification by flash chromatography (40 g silica cartridge, 0-100% EtOAc/Hexanes) afforded the title compound in 9.0 g, 77% yield. ¹H NMR (CHLOROFORM-d, 300MHz): δ = 6.92 - 7.12 (m, 3 H), 4.70 - 4.93 (m, 1 H), 3.73 - 3.93 (m, 3 H), 3.54 - 3.66 (m, 2 H), 3.43 - 3.53 (m, 2 H), 3.21 - 3.33 (m, 2 H), 2.76 - 2.90 (m, 2 H), 1.44 ppm (br. s., 9 H); (ESI) *m/z* 397, 399 (M+Na, M+Na+2)⁺.

Step 5: *tert*-butyl (2-((4-(3-hydroxyprop-1-yn-1-yl)-2-methoxybenzyl)oxy)ethyl)carbamate, **11**



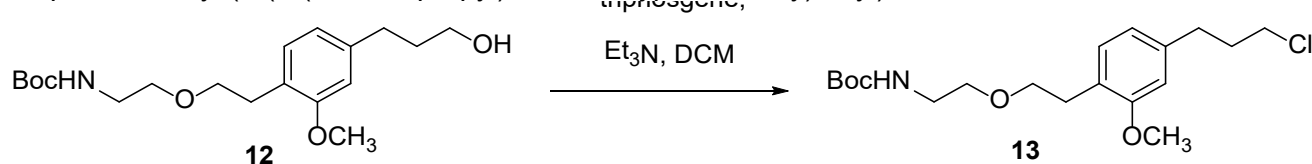
To mixture of compounds *tert*-butyl (2-(4-bromo-2-methoxyphenethoxy)ethyl)carbamate (7.0 g, 19.0 mmol), K₂CO₃ (5.25 g, 38.0 mmol), copper(I) iodide (0.36 g, 1.90 mmol), Pd(PPh)₄ (1.06 g, 0.95 mmol), and dioxane/H₂O (100 mL/10 mL) were added to a round bottom flask. The reaction mixture was placed under vacuum and then vented with nitrogen (3 times). The prop-2-yn-1-ol (13.3 g, 190.0 mmol) was added and the reaction heated to 75°C overnight. Reaction monitored by LC-MS until complete. The solvent was removed under reduced pressure and crude residue purified by flash chromatography (40 g silica cartridge, 0-70% EtOAc/Hexanes) to afford the title compound in 2.16 g 33% yield. ¹H NMR (CHLOROFORM-d, 300MHz): δ = 7.03 - 7.11 (m, 1 H), 6.93 - 7.01 (m, 1 H), 6.86 - 6.92 (m, 1 H), 4.77 - 5.01 (m, 1 H), 4.46 (br. s., 2 H), 4.24 - 4.33 (m, 1 H), 3.78 (s, 3 H), 3.59 (s, 2 H), 3.46 (br. s., 2 H), 3.20 - 3.31 (m, 2 H), 2.85 (s, 2 H), 1.43 ppm (s, 9 H); (ESI) *m/z* 372 (M+Na)⁺.

Step 6: *tert*-butyl (2-(4-(3-hydroxypropyl)-2-methoxyphenethoxy)ethyl)carbamate, **12**



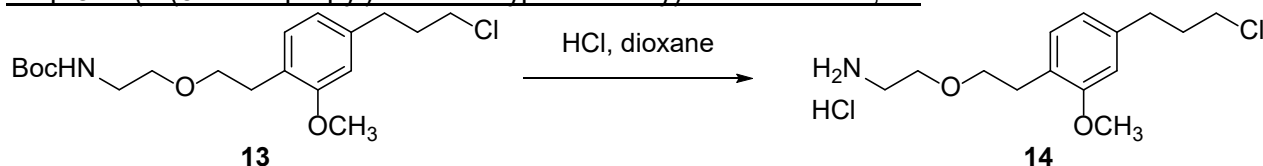
tert-Butyl (2-((4-(3-hydroxyprop-1-yn-1-yl)-2-methoxybenzyl)oxy)ethyl)carbamate (1.8 g, 5.15 mmol) in EtOH (20 mL) was hydrogenated using 5% Pt/C (~100 mg) in a small Parr vessel (250 mL) with hydrogen (55 psi) overnight. The reaction was filtered through celite while under nitrogen and the filtrate was then concentrated *in vacuo*. The crude product was purified by flash chromatography (24 g silica, 0-100% EtOAc/hexane gradient) to obtain product **12** (1.11 g, 61% yield). ¹H NMR (CHLOROFORM-d, 300MHz): δ = 6.99 - 7.15 (m, 1 H), 6.69 (m, 2 H), 4.91 (br. s., 1 H), 3.80 (s, 3 H), 3.56 - 3.72 (m, *J*=6.2, 6.2 Hz, 4 H), 3.49 (t, *J*=5.0 Hz, 2 H), 3.27 (d, *J*=5.3 Hz, 2 H), 2.85 (t, *J*=7.3 Hz, 2 H), 2.61 - 2.74 (m, *J*=7.6, 7.6 Hz, 1 H), 1.81 - 1.95 (m, 2 H), 1.44 ppm (s, 9 H); ES-LCMS: *m/z* 377 (M+Na)⁺

Step 7: *tert*-butyl (2-(4-(3-chloropropyl)-2-methoxyphenethoxy)ethyl)carbamate, **13**



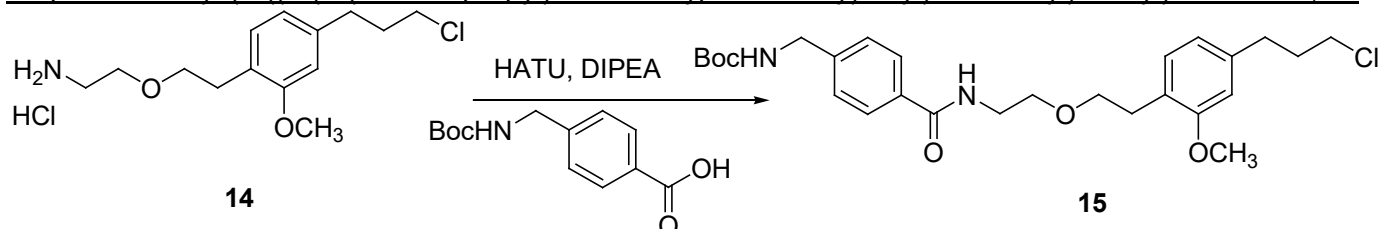
To *tert*-butyl (2-(4-(3-hydroxypropyl)-2-methoxyphenethoxy)ethyl)carbamate (1.11 g, 3.14 mol) and triethylamine (1.09 mL, 7.85 mmol) in DCM (20 mL) was added triphosgene (0.46 g, 1.57 mmol) and the reaction stirred at room temperature for 1h. Aqueous sodium bicarbonate was added and the water layer extracted twice with dichloromethane. The organic extracts were combined, dried (Na₂SO₄), and concentrated under reduced pressure to provide crude product. Purification by flash chromatography (24 g silica cartridge, 0-10% MeOH/CH₂Cl₂) afforded the title compound (1.05 g, 89% yield). ¹H NMR (CHLOROFORM-d, 300MHz): δ = 7.07 (d, *J*=7.6 Hz, 1 H), 6.65 - 6.77 (m, 2 H), 4.85 (br. s., 1 H), 3.82 (s, 3 H), 3.44 - 3.66 (m, 6 H), 3.28 (q, *J*=4.9 Hz, 2 H), 2.86 (t, *J*=7.0 Hz, 2 H), 2.75 (t, *J*=7.3 Hz, 2 H), 2.01 - 2.14 (m, 2 H), 1.45 ppm (s, 9 H); (ES-LCMS) *m/z* 395 (M+Na)⁺.

Step 8: 2-(4-(3-chloropropyl)-2-methoxyphenethoxy)ethan-1-amine, **14**



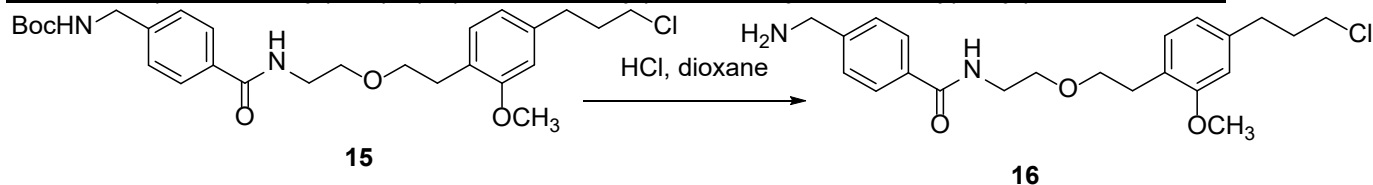
To a solution of *tert*-butyl (2-(4-(3-chloropropyl)-2-methoxyphenethoxy)ethyl)carbamate (203 mg, 0.55 mmol) in dichloromethane (2.0 mL) was added 4N HCl in dioxane (0.5 mL) and maintained at room temperature for 3.0h. The reaction was concentrated under vacuum and triturated with two portions of ether (5 ml) and the ether decanted off to yield product (160.2 mg, 99% yield). ¹H NMR (CHLOROFORM-*d*, 300MHz): δ = 8.29 (br. s., 2 H), 7.07 (d, *J*=7.6 Hz, 1 H), 6.62 - 6.78 (m, 2 H), 3.80 (s, 3 H), 3.62 - 3.76 (m, *J*=5.0, 5.0 Hz, 4 H), 3.52 (t, *J*=6.4 Hz, 2 H), 3.20 (br. s., 2 H), 2.88 (t, *J*=7.0 Hz, 2 H), 2.73 (t, *J*=7.3 Hz, 2 H), 1.92 - 2.20 ppm (m, 2 H); (ES-LCMS) *m/z* 272 (M+H)⁺.

Step 9: *tert*-butyl (4-((2-(4-(3-chloropropyl)-2-methoxyphenethoxy)ethyl)carbamoyl)benzyl)carbamate, **15**



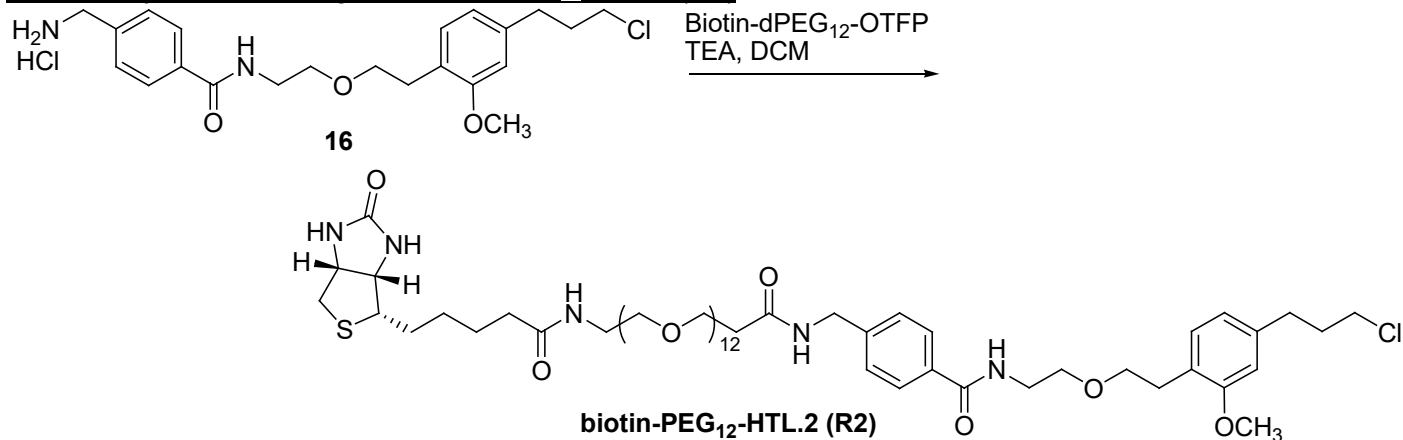
2-(4-(3-Chloropropyl)-2-methoxyphenethoxy)ethan-1-amine (276 mg, 1.01 mmol), 4-Boc-aminomethyl)benzoic acid (305.9 mg, 1.21 mmol), HATU (964.5 mg, 2.53 mmol), and DIPEA (708 μ L, 4.05 mmol) were combined in DMF (5 mL). The mixture was stirred for 4 hours then poured into water and the product extracted with ethyl acetate (3x). The combined organic extracts were washed with brine then dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude material was purified by flash chromatography (24 g silica, 15-70% EtOAc/hexane gradient) to obtain the product (339.8 mg, 66% yield). ¹H NMR (CHLOROFORM-*d*, 300MHz): δ = 7.68 (d, *J*=7.6 Hz, 2 H), 7.34 (d, *J*=8.2 Hz, 2 H), 7.08 (d, *J*=7.6 Hz, 1 H), 6.67 (br. s., 2 H), 6.44 (br. s., 1 H), 4.86 - 5.00 (br. s., 1 H), 4.22 - 4.47 (m, 2 H), 3.80 (s, 3 H), 3.47 - 3.71 (m, 8 H), 2.61 - 2.98 (m, *J*=6.7, 6.7 Hz, 4 H), 2.05 (t, *J*=6.7 Hz, 2 H), 1.47 ppm (s, 9 H); (ES-LCMS) *m/z* 506 (M+H)⁺.

Step 10: 4-(aminomethyl)-N-(2-(4-(3-chloropropyl)-2-methoxyphenethoxy)ethyl)benzamide, **16**

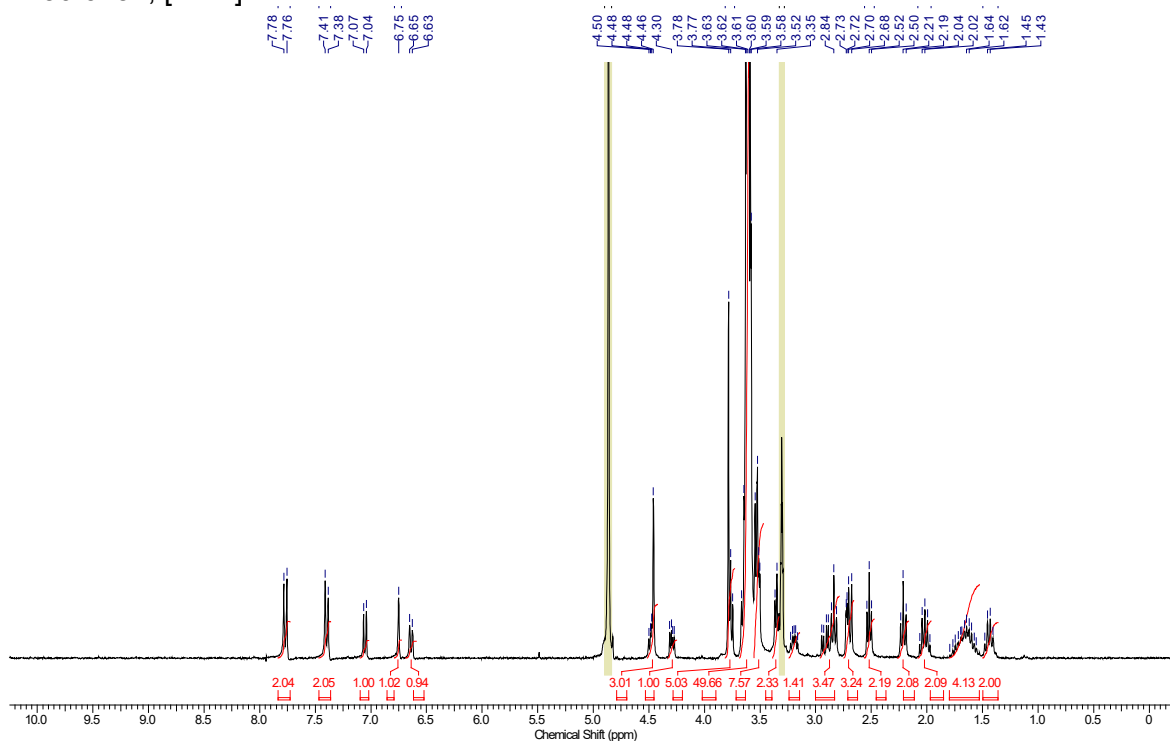


To a solution of *tert*-butyl (2-(4-(3-chloropropyl)-2-methoxyphenethoxy)ethyl)carbamate (203 mg, 0.55 mmol) in dichloromethane (2.0 mL) was added 4N HCl (0.5 mL) in dioxane and the reaction maintained at room temperature for 3.0 h. The reaction was then concentrated under vacuum and triturated with two portions of ether (5 ml) that were subsequently decanted off to yield product (160.2 mg, 99% yield). ¹H NMR (CHLOROFORM-*d*, 300MHz): δ = 8.41 (br. s., 2 H), 7.52 (br. s., 2 H), 7.31 (br. s., 2 H), 6.96 - 7.04 (br. s., 1 H), 6.59 - 6.70 (m, 2 H), 3.99 (s., 2 H), 3.70 - 3.78 (s, 3 H), 3.39 - 3.67 (m, 8 H), 2.83 (m, 2 H), 2.57 - 2.74 (m, 2 H), 1.90 - 2.09 ppm (m, *J*=5.3 Hz, 2 H); (ES-LCMS) *m/z* 406 (M+H)⁺.

Step 11: Synthesis of reagent Biotin-PEG₁₂-HTL.2 (R2)



To a solution of solution of 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-amine HCl salt (**16**) (20 mg, 0.089 mmol) and biotin-dPEG₁₂-OTFP (71 mg, 0.071 mmol, Quanta Biodesign) in DCM (1 mL) was added DIPEA (69 μ L, 0.395 mmol) and the reaction stirred at room temperature overnight. The solvent was then removed *in vacuo* and the residue was purified by Gilson prep HPLC (Sunfire Prep C18 OBD, 10 μ m, 30 x 150 mm column, 15-75% ACN/H₂O gradient (both solvents containing 0.1% TFA). The product fractions were combined and concentrated to remove acetonitrile before freeze drying overnight to obtain a colorless semi-solid product (20 mg, 21 % yield). ¹H NMR (METHANOL-d₄, 300MHz): δ = 7.77 (d, *J*=8.2 Hz, 2 H), 7.40 (d, *J*=8.2 Hz, 2 H), 7.05 (d, *J*=7.6 Hz, 1 H), 6.75 (s, 1 H), 6.64 (d, *J*=7.6 Hz, 1 H), 4.42 - 4.51 (m, 3 H), 4.29 (dd, *J*=7.9, 4.4 Hz, 1 H), 3.73 - 3.81 (m, 4 H), 3.56 - 3.68 (m, 48 H), 3.47 - 3.56 (m, 6 H), 3.36 (d, *J*=5.3 Hz, 2 H), 3.15 - 3.24 (m, *J*=8.5, 5.0 Hz, 1 H), 2.79 - 2.96 (m, *J*=12.9, 4.7 Hz, 3 H), 2.66 - 2.75 (m, *J*=2.3 Hz, 3 H), 2.52 (t, *J*=6.2 Hz, 2 H), 2.21 (t, *J*=7.3 Hz, 2 H), 1.96 - 2.08 (m, 2 H), 1.53 - 1.80 (m, *J*=2.3 Hz, 4 H), 1.44 ppm (q, *J*=7.0 Hz, 2 H); (ES-LCMS) *m/z* 1231.63 (M+H)⁺; HRMS (ESI): *m/z* calcd for C₅₉H₉₇ClN₅O₁₈S: 1230.6236 [M+H]⁺; found: 1230.6232, [M+H]⁺

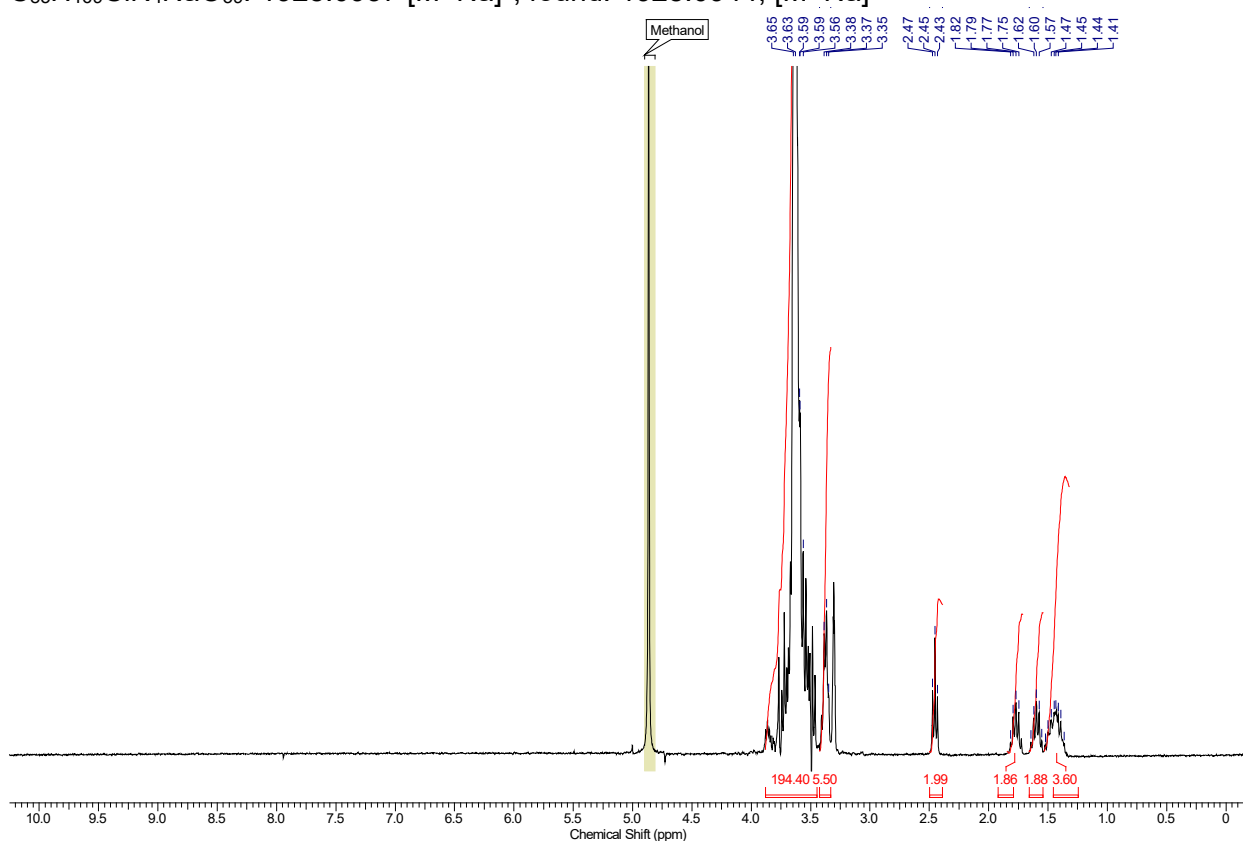


¹H NMR of Biotin-PEG₁₂-HTL.2 (R2)

Synthesis of R3—azide-PEG₃₆-HTL.1 (*azide*^{DART.1})

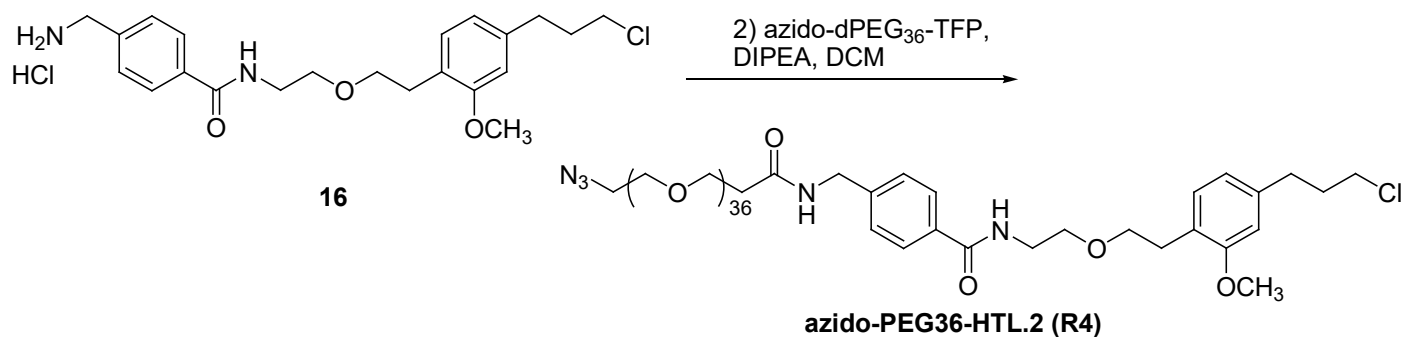


DIPEA (125 μ L, 0.717 mmol) was added to a solution of 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-amine TFA salt (**5**) (20.5 mg, 0.109 mmol) and biotin-dPEG₃₆-OTFP (202 mg, 0.109 mmol, Quanta Biodesign) in DCM (2 mL). After two hours the solvent was removed *in vacuo* and the residue was purified by Gilson prep HPLC (Sunfire Prep C18 OBD, 10 μ m, 30 x 150 mm column, 15-80% MeCN/H₂O gradient (both solvents 0.1% TFA). The product fractions were combined and concentrated to remove the acetonitrile before freeze drying overnight to obtain a colorless semi-solid for the product (54 mg, 23 % yield). ES-LCMS: m/z = 1929.3(M+Na)⁺; ¹H NMR (METHANOL-d₄, 300MHz): δ = 3.45 - 3.88 (m, 158 H), 3.33 - 3.42 (m, 6 H), 2.39 - 2.50 (m, 2 H), 1.71 - 1.84 (m, 2 H), 1.54 - 1.66 (m, 2 H), 1.32 - 1.53 ppm (m, 4 H); HRMS (ESI): m/z calcd for C₈₅H₁₆₉ClN₄NaO₃₉: 1928.0937 [M+Na]⁺; found: 1928.0944, [M+Na]⁺

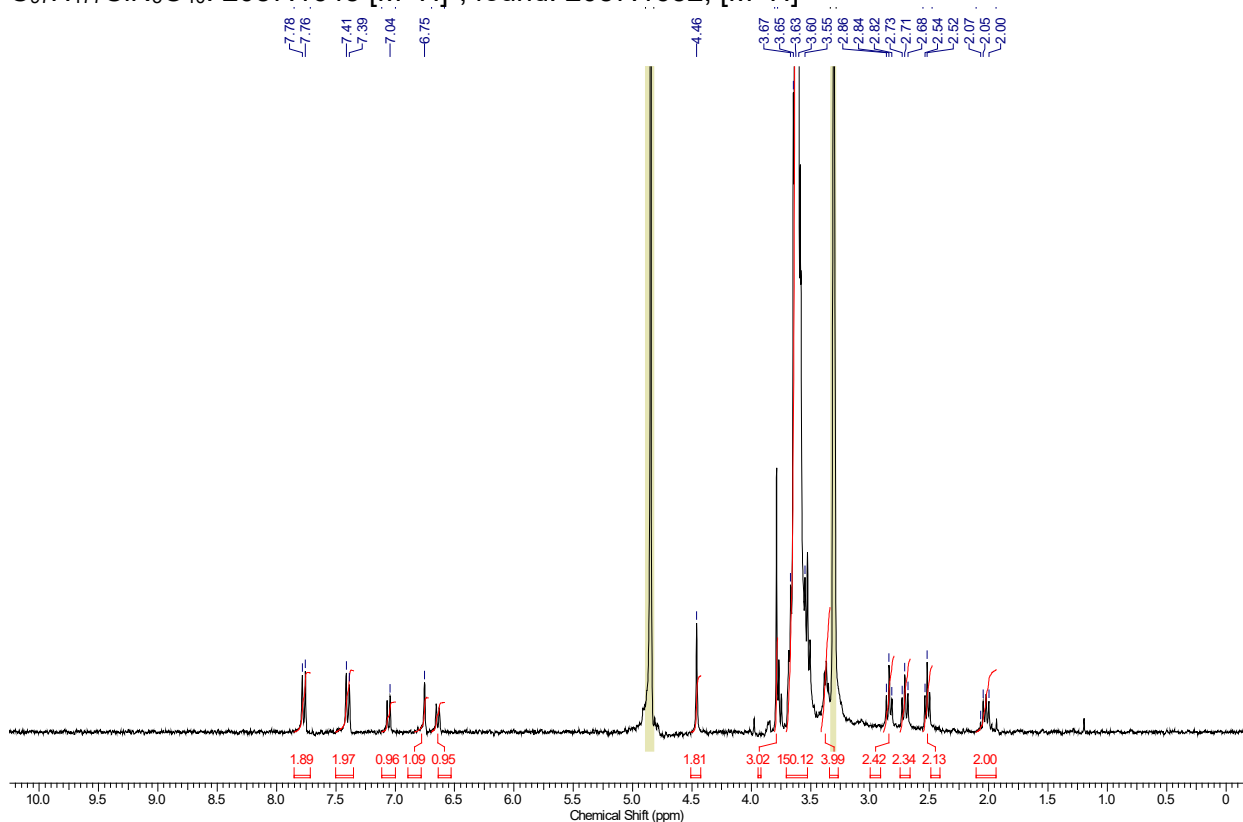


¹H NMR of azide-PEG₃₆-HTL.1 (*azide*^{DART.1}, R3)

Synthesis of R4—azide-PEG₃₆-HTL.2 (*azide*^{DART.2})

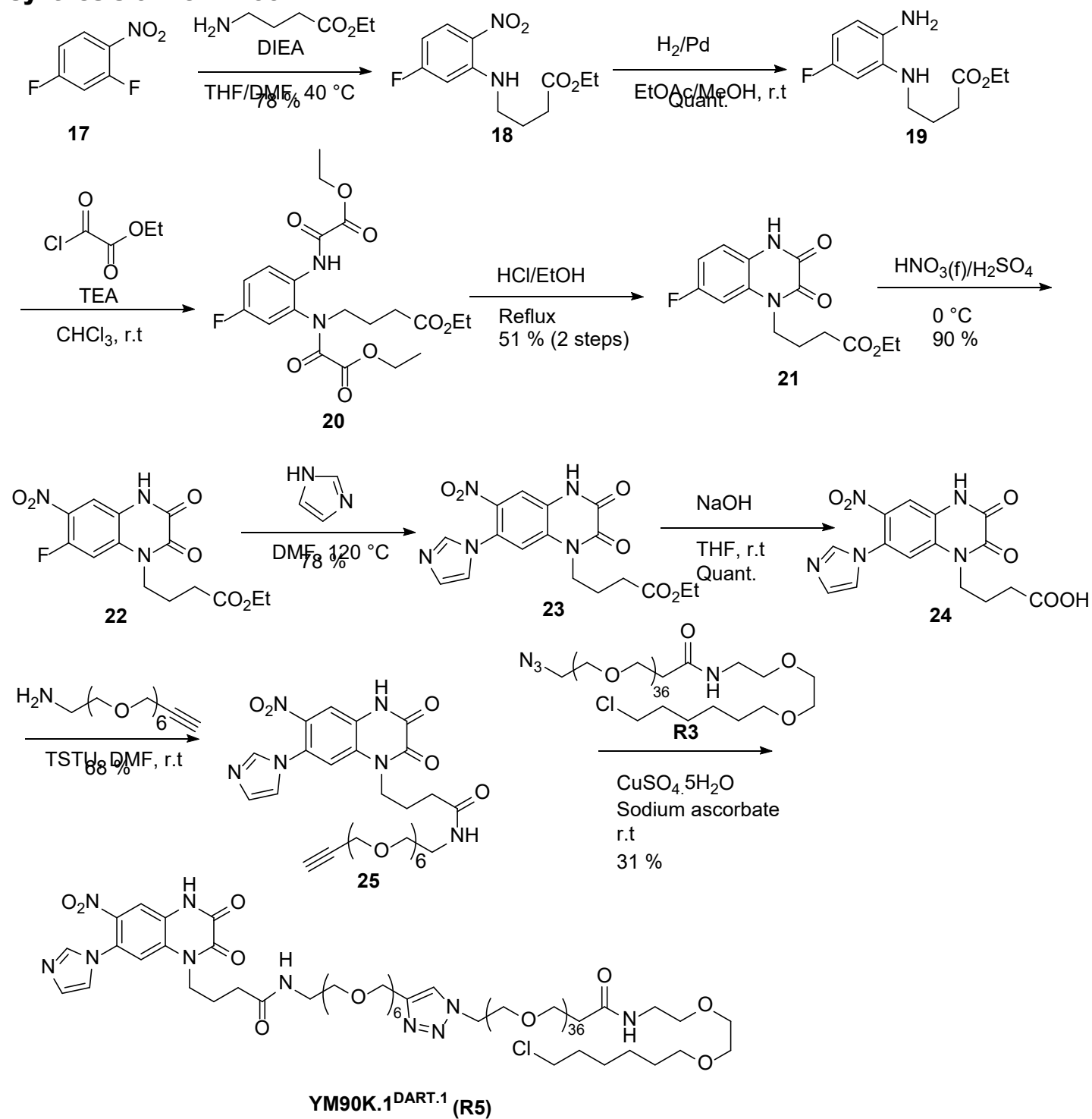


DIPEA (125 μ L, 0.717 mmol) was added to a solution of 2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-amine TFA salt (**5**) (20.5 mg, 0.109 mmol) and biotin-dPEG₃₆-OTFP ester (202 mg, 0.109 mmol, Quanta Biodesign) in DCM (2 mL). After two hours the solvent was removed *in vacuo* and the residue was purified by Gilson prep HPLC (Sunfire Prep C18 OBD, 10 μ m, 30 x 150 mm column, 15-80% ACN/H₂O gradient (both 0.1% TFA). The product fractions were combined and concentrated to remove the acetonitrile before freeze drying overnight to obtain a colorless semi-solid for the product (54 mg, 24%). ¹H NMR (METHANOL-d₄, 300MHz): δ = 7.77 (d, *J*=8.2 Hz, 2 H), 7.40 (d, *J*=8.2 Hz, 2 H), 7.04 (s, 1 H), 6.75 (s, 1 H), 6.59 - 6.69 (m, 1 H), 4.43 - 4.51 (m, 2 H), 3.78 - 3.80 (m, 3 H), 3.53 - 3.71 (m, 150 H), 3.34 - 3.41 (m, 4 H), 2.80 - 2.88 (m, 2 H), 2.66 - 2.75 (m, 2 H), 2.47 - 2.55 (m, 2 H), 2.02 ppm (s, 2 H); (ES-LCMS) *m/z* 1053.74 ((*M*+Na)/2)⁺; HRMS (ESI): *m/z* calcd for C₉₇H₁₇₇ClN₅O₄₀: 2087.1645 [*M*+H]⁺; found: 2087.1652, [*M*+H]⁺

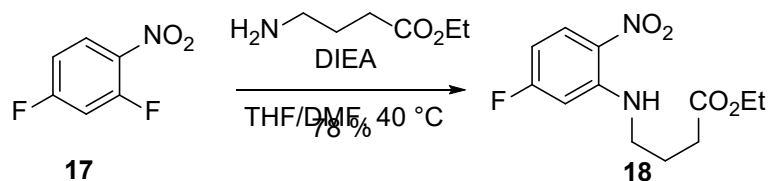


¹H NMR of azido-PEG₃₆-HTL.2 (*azide*^{DART.2}, R4)

Synthesis of R5—YM90K.1^{DART.1}

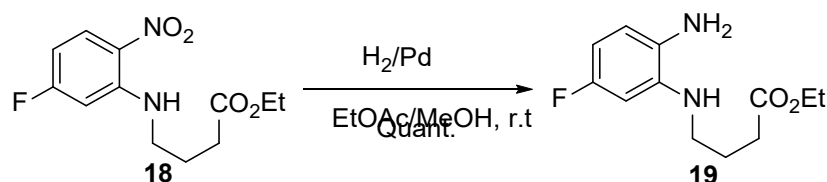


Step 1: Synthesis of ethyl 4-((5-fluoro-2-nitrophenyl)amino)butanoate, **18** (reported in reference 1)



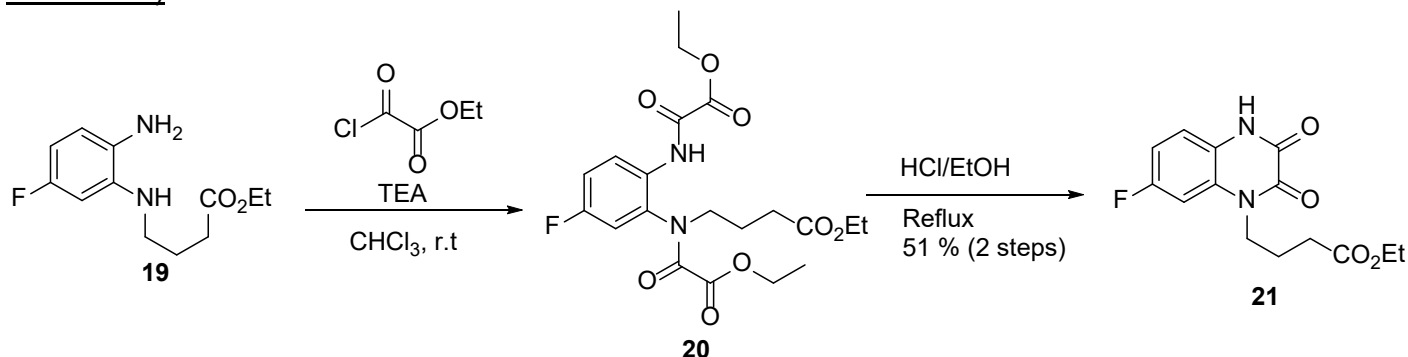
To a vial was added 2,4-difluoro-1-nitrobenzene, **17** (1.993 g, 1.374 mL, 12.53 mmol) and THF (10 mL)/DMF (5 mL) after which ethyl-4-aminobutanoate.HCl (2.000 g, 11.93 mmol) and triethylamine (1.449 g, 2.0 mL, 14.32 mmol) were added. The reaction was stirred at 40°C overnight after which the mixture was concentrated, taken up in DCM (~200 mL), washed with water (2 x 20 mL), brine (20 mL), filtered through an isolate phase separator, and concentrated to a yellow oil which was purified by silica gel chromatography eluting with 0 to 50 % ethyl acetate in hexanes to give ethyl-4-((5-fluoro-2-nitrophenyl)amino)butanoate, **18** (2.5 g, 78 %) as a yellow/orange oil. ¹H NMR (500 MHz, Chloroform-*d*) δ 8.19 (dd, *J* = 9.5, 6.1 Hz, 2H), 6.52 (dd, *J* = 11.5, 2.6 Hz, 1H), 6.35 (ddd, *J* = 9.7, 7.2, 2.5 Hz, 1H), 4.14 (q, *J* = 7.2 Hz, 2H), 3.32 (q, *J* = 6.6 Hz, 2H), 2.44 (t, *J* = 7.0 Hz, 2H), 2.02 (p, *J* = 7.1 Hz, 2H), 1.30 – 1.20 (m, 3H). (ES-LCMS) *m/z* 271.2 (M+H)⁺.

Step 2: Synthesis of ethyl 4-((2-amino-5-fluorophenyl)amino)butanoate, **19** (reported in reference 1)



To a flask containing palladium on carbon (0.30 g, 0.28 mmol) was added (under a stream of nitrogen) a solution of ethyl 4-((5-fluoro-2-nitrophenyl)amino)butanoate, **18** (2.5 g, 9.3 mmol) in methanol (30 mL)/ethyl acetate (30 mL). The reaction was stirred under an atmosphere of hydrogen overnight after which it was filtered through celite and the filtrate concentrated to give ethyl 4-((2-amino-5-fluorophenyl)amino)butanoate, **19** (2.3 g, quant.) as a purple oil. ¹H NMR (500 MHz, Chloroform-*d*) δ 6.59 (dd, *J* = 8.3, 5.6 Hz, 1H), 6.36 – 6.23 (m, 2H), 4.12 (q, *J* = 7.1 Hz, 2H), 3.12 (t, *J* = 6.9 Hz, 2H), 3.07 (s, 2H), 2.44 (t, *J* = 7.1 Hz, 2H), 1.98 (p, *J* = 7.0 Hz, 2H), 1.24 (t, *J* = 7.1 Hz, 4H). (ES-LCMS) *m/z* 241.3 (M+H)⁺.

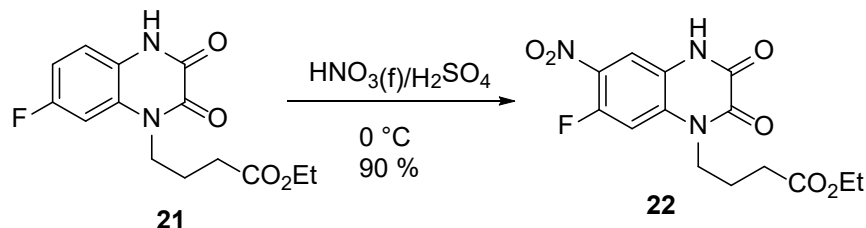
Steps 3 / 4: Synthesis of ethyl 4-(7-fluoro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoate, **21** (reported in reference 1)



A solution of ethyl 4-((2-amino-5-fluorophenyl)amino)butanoate, **19** (2.3 g, 9.6 mmol) and triethylamine (1.9 g, 2.7 mL, 19 mmol) in chloroform (40 mL) was cooled in an ice/water bath and placed under nitrogen. Ethyl 2-chloro-2-oxoacetate (2.6 g, 2.1 mL, 19 mmol) in chloroform (5 mL) was added dropwise. The ice bath was removed upon dropwise addition and the reaction was allowed to stir at room temperature for 1.5 h after LC/MS indicated the desired intermediate, **20**. The reaction was diluted with chloroform (15 mL) and transferred into a separatory funnel. The organic layer was washed with water (15 mL), saturated aqueous NaHCO₃ solution (2 x 15 mL), saturated NH₄Cl solution (2 x 15 mL), brine (15 mL), and filtered through an

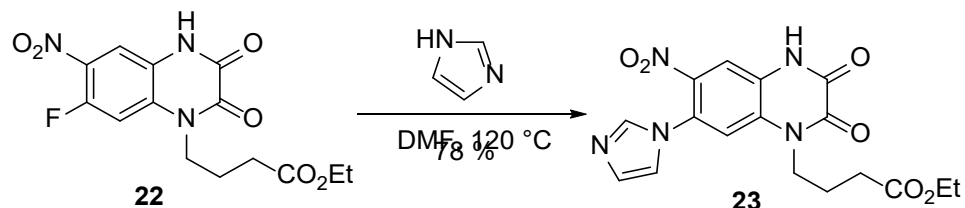
isolute phase separator. The filtrate was concentrated to a black/purple oil which was taken up in ethanol (30 mL). Concentrated HCl (0.5 mL) was added and the reaction was heated to 100°C for 1 h after which LC/MS indicated starting material. Additional concentrated HCl (0.5 mL) was added and heating was continued for another 1.5 after the reaction was allowed to cool to room temperature and solids precipitated out of solution. The mixture was filtered and the collected solids washed with ethanol to give ethyl 4-(7-fluoro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoate, **21** (1.45 g, 51 %) as a tan solid. ¹H NMR (500 MHz, DMSO-d₆) δ 12.04 (s, 1H), 7.43 (dd, *J* = 11.1, 2.6 Hz, 1H), 7.19 (dd, *J* = 8.8, 5.5 Hz, 1H), 7.06 (td, *J* = 8.5, 2.5 Hz, 1H), 4.17 – 3.98 (m, 4H), 2.47 (t, *J* = 7.2 Hz, 2H), 1.87 (p, *J* = 7.3 Hz, 2H), 1.20 (t, *J* = 7.1 Hz, 3H). (ES-LCMS) *m/z* 295.2 (M+H)⁺.

Step 5: Synthesis of ethyl 4-(7-fluoro-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoate, **22 (reported in reference 1)**



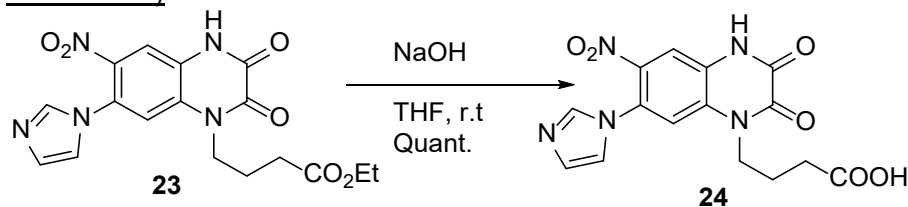
A round bottom flask containing ethyl 4-(7-fluoro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoate, **21** (1.45 g, 4.93 mmol) was immersed in an ice/water bath and concentrated sulfuric acid (15 mL) was added. The mixture was stirred and nitric acid (0.34 g, 0.24 mL, 4.93 mmol) (red, fuming) was added dropwise. The resulting reaction mixture was stirred for 30 min after which it was poured into a mixture of ice and water, and yellow solids precipitated out. The suspension was thoroughly stirred then filtered to give ethyl 4-(7-fluoro-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoate, **22** (1.51 g, 90.3 %) as a light yellow solid. ¹H NMR (500 MHz, DMSO-d₆) δ 12.25 (s, 1H), 7.92 (d, *J* = 7.1 Hz, 1H), 7.73 (d, *J* = 13.4 Hz, 1H), 4.19 – 3.99 (m, 4H), 2.49 (t, *J* = 7.2 Hz, 2H), 1.87 (p, *J* = 7.3 Hz, 2H), 1.20 (t, *J* = 7.1 Hz, 3H). (ES-LCMS) *m/z* 340.2 (M+H)⁺.

Step 6: Synthesis of ethyl 4-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoate, **23 (reported in reference 1)**



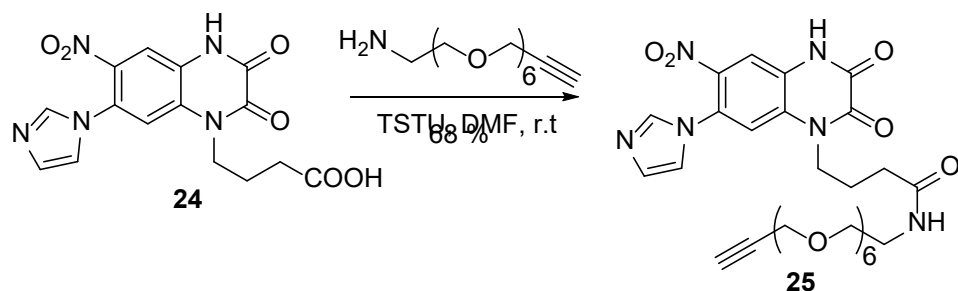
To a vial containing ethyl 4-(7-fluoro-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoate, **22** (1.5 g, 4.4 mmol) was added DMF (10 mL) after which imidazole (0.63 g, 9.3 mmol) was added and the reaction was heated overnight at 80°C after which LC/MS still indicated starting material. Additional imidazole (100 mg, 0.7 mmol) was added and the temperature was increased to 100°C for 2 h after which LC/MS still starting material. Additional imidazole (100 mg, 0.7 mmol) was added and heating was continued at 100°C for 2 h then 120°C for 2 h after which the reaction was finally complete. The reaction mixture was poured into a mixture of ice/water and stirred vigorously then filtered. The collected solid was isolated to give ethyl 4-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoate, **23** (1.34 g, 78 %) as an orange solid. ¹H NMR (500 MHz, DMSO-d₆) δ 12.57 (s, 1H), 9.45 (s, 1H), 8.17 (s, 1H), 8.05 (d, *J* = 1.7 Hz, 1H), 8.00 (s, 1H), 7.89 (d, *J* = 1.7 Hz, 1H), 4.13 (t, *J* = 7.5 Hz, 2H), 2.40 (t, *J* = 7.3 Hz, 2H), 1.87 (p, *J* = 7.4 Hz, 2H). (ES-LCMS) *m/z* 388.3 (M+H)⁺.

Step 7: 4-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoic acid, **24** (reported in reference 1)



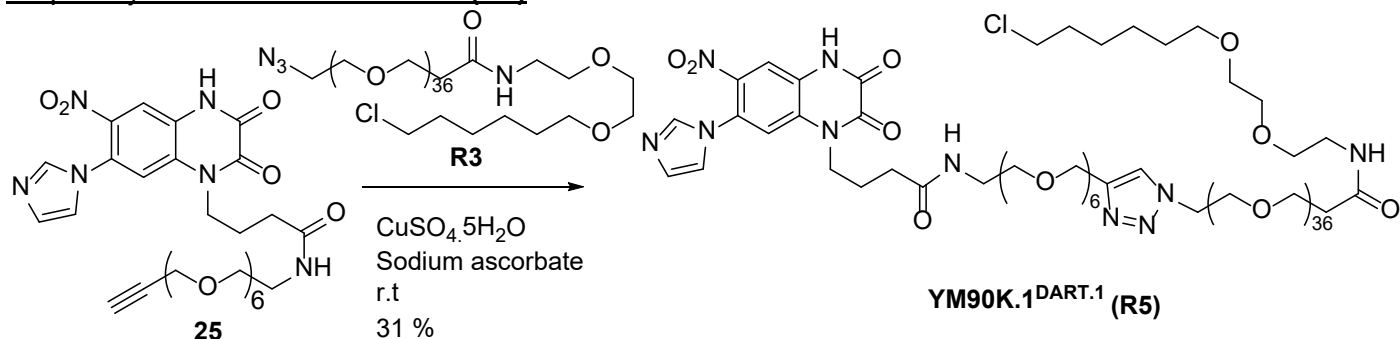
To a vial containing ethyl 4-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoate, **23** (1 g, 3 mmol) was added NaOH (1 M in water, 8 mL, 4 mmol) and THF (2 mL) and the reaction was stirred at room temperature for 4 h. THF was removed under a stream of nitrogen and to the resulting solution was added 2 M HCl (~ 6 mL) [pH ~ 2] and solids precipitated. The mixture was vigorously stirred then filtered to give 4-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoic acid, **24** (1.12 g, quant.) as a yellow solid. ¹H NMR (500 MHz, DMSO-d₆) δ 12.57 (s, 1H), 9.45 (s, 1H), 8.17 (s, 1H), 8.05 (d, *J* = 1.7 Hz, 1H), 8.00 (s, 1H), 7.89 (d, *J* = 1.7 Hz, 1H), 4.13 (t, *J* = 7.5 Hz, 2H), 2.40 (t, *J* = 7.3 Hz, 2H), 1.87 (p, *J* = 7.4 Hz, 2H). (ES-LCMS) *m/z* 360.3 (M+H)⁺.

Step 8: Synthesis of 4-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)-N-(3,6,9,12,15,18-hexaoxahenicos-20-yn-1-yl)butanamide, **25**



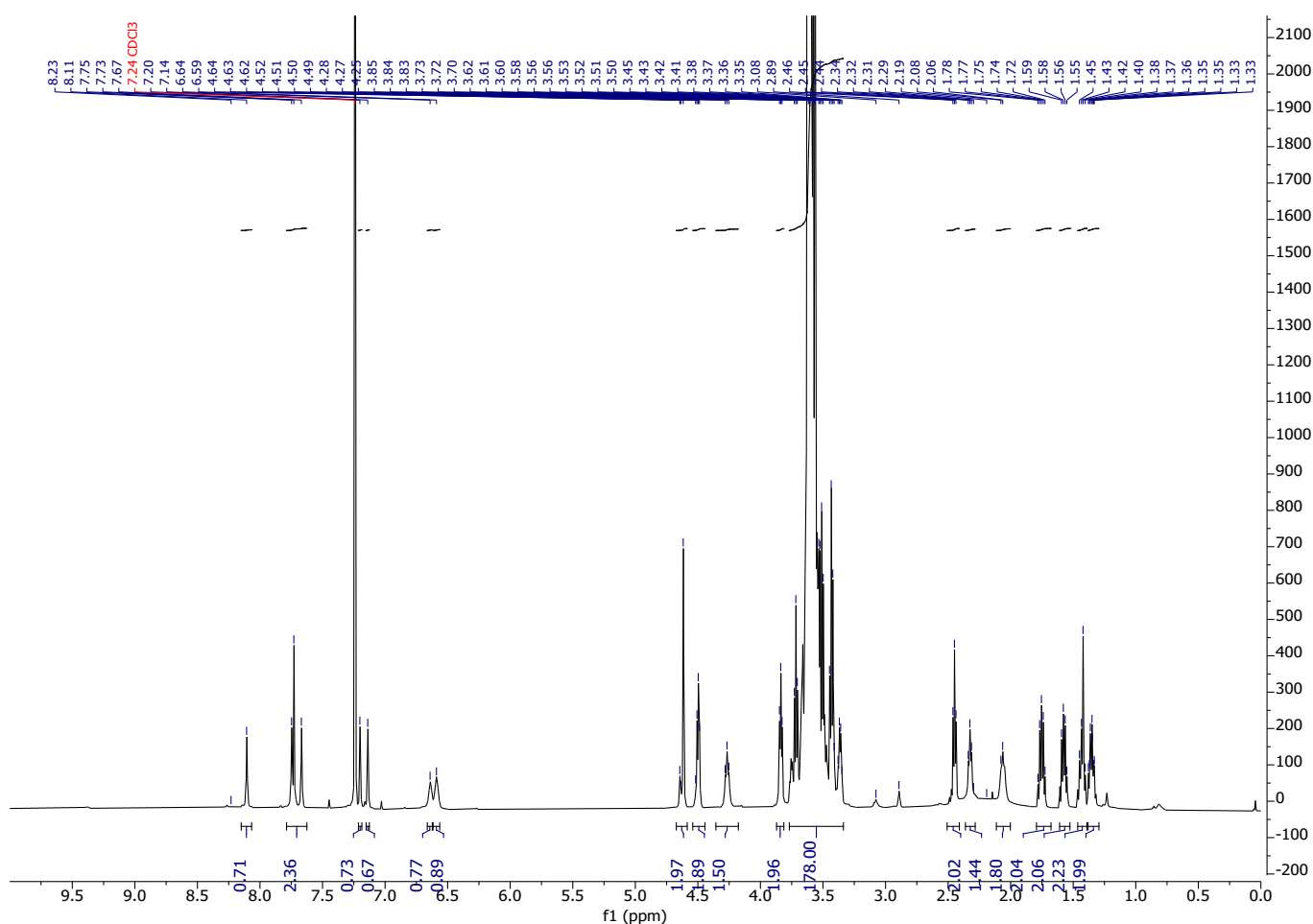
To 4-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)butanoic acid, **24** (100 mg, 278 μmol) was added DMF (1.5 mL), DIPEA (71.9 mg, 97 μL, 557 μmol) and 1-(bis(dimethylamino)(tetrafluoro-15-boraneyl)methoxy)pyrrolidine-2,5-dione (TSTU) (83.8 mg, 278 μmol). The reaction was stirred for 15 min after 3,6,9,12,15,18-hexaoxahenicos-20-yn-1-amine (88.9 mg, 278 μmol) was added and the reaction was stirred at room temperature for 1 h after which it was directly purified by reverse phase prep HPLC eluting from 5 to 50 % acetonitrile in water (0.1 % FA and conditions as described in the general chemical synthesis information) to give 4-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)-N-(3,6,9,12,15,18-hexaoxahenicos-20-yn-1-yl)butanamide, **25** (125 mg, 68 %) as a yellow oil. ¹H NMR (500 MHz, CDCl₃) δ 8.08 (s, 1H), 7.78 (s, 1H), 7.71 (s, 1H), 7.19 (s, 1H), 7.15 (s, 1H), 6.66 (t, *J* = 5.5 Hz, 1H), 4.27 (t, *J* = 7.5 Hz, 2H), 4.13 (d, *J* = 2.4 Hz, 2H), 3.69 – 3.55 (m, 20H), 3.51 (t, *J* = 5.1 Hz, 2H), 3.38 (q, *J* = 5.2 Hz, 2H), 2.49 – 2.26 (m, 3H), 2.11 (q, *J* = 7.0 Hz, 2H). (ES-LCMS) *m/z* 661.4 (M+H)⁺.

Step 9: Synthesis of YM90K.1^{DART.1} (R5**)**



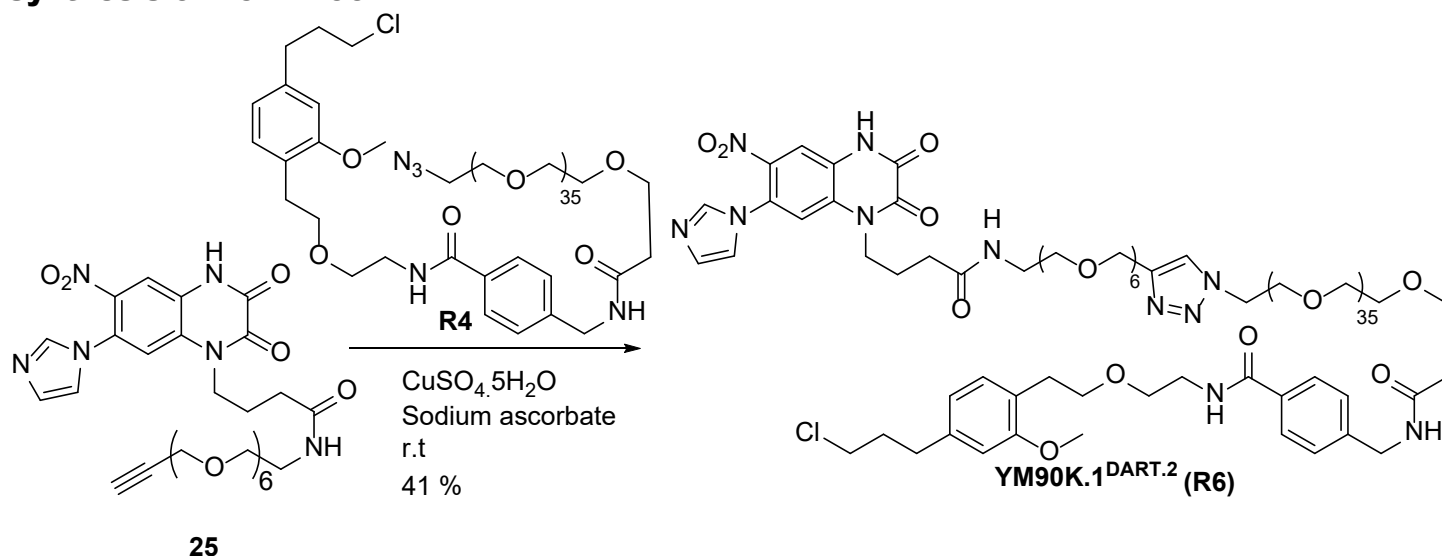
To 4-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)-N-(3,6,9,12,15,18-hexaoxahenicos-20-yn-1-yl)butanamide, **25** (5 mg, 7.6 μ mol) was added EtOH (0.4 mL)/t-BuOH (0.2 mL)/Water (0.2 mL) after which azido-PEG36-HTL1.0, **R3** (14 mg, 7.6 μ mol) was added. Finally, Cu(II) sulfate pentahydrate (0.1 mg, 0.38 μ mol) and sodium ascorbate (0.14 mg, 0.76 μ mol) were added and the reaction mixture was stirred overnight at room temperature. The mixture was directly purified by reverse phase prep HPLC eluting from 5 to 70 % acetonitrile in water (0.1 % FA, 220 nm collection wavelength and other parameters as described in the general chemical synthesis section) and lyophilized to give 1-(4-(24-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)-21-oxo-2,5,8,11,14,17-hexaoxa-20-azatetracosyl)-1H-1,2,3-triazol-1-yl)-N-(2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108-hexatriacontaoxaundecahectan-111-amide, **YM90K.1^{DART.1}**, **R5** (6 mg, 31 %).

^1H NMR (500 MHz, CDCl_3) δ 8.11 (s, 1H), 7.79 – 7.62 (m, 2H), 7.20 (s, 1H), 7.14 (s, 1H), 6.64 (s, 1H), 6.59 (s, 1H), 4.62 (s, 2H), 4.50 (q, J = 5.2 Hz, 2H), 4.27 (t, J = 7.4 Hz, 1H), 3.84 (t, J = 5.1 Hz, 2H), 3.77 – 3.34 (m, 178H), 2.45 (t, J = 6.1 Hz, 2H), 2.32 (t, J = 6.5 Hz, 1H), 2.11 – 2.00 (m, 2H), 1.75 (p, J = 6.9 Hz, 2H), 1.58 (p, J = 6.8 Hz, 2H), 1.43 (q, J = 8.3 Hz, 2H), 1.38 – 1.29 (m, 2H). HRMS (ESI+): m/z calcd for $\text{C}_{115}\text{H}_{211}\text{ClN}_{10}\text{O}_{50}$: 1284.1994 $[\text{M}+2\text{H}]^{2+}$; found: 1284.1989, $[\text{M}+2\text{H}]^{2+}$



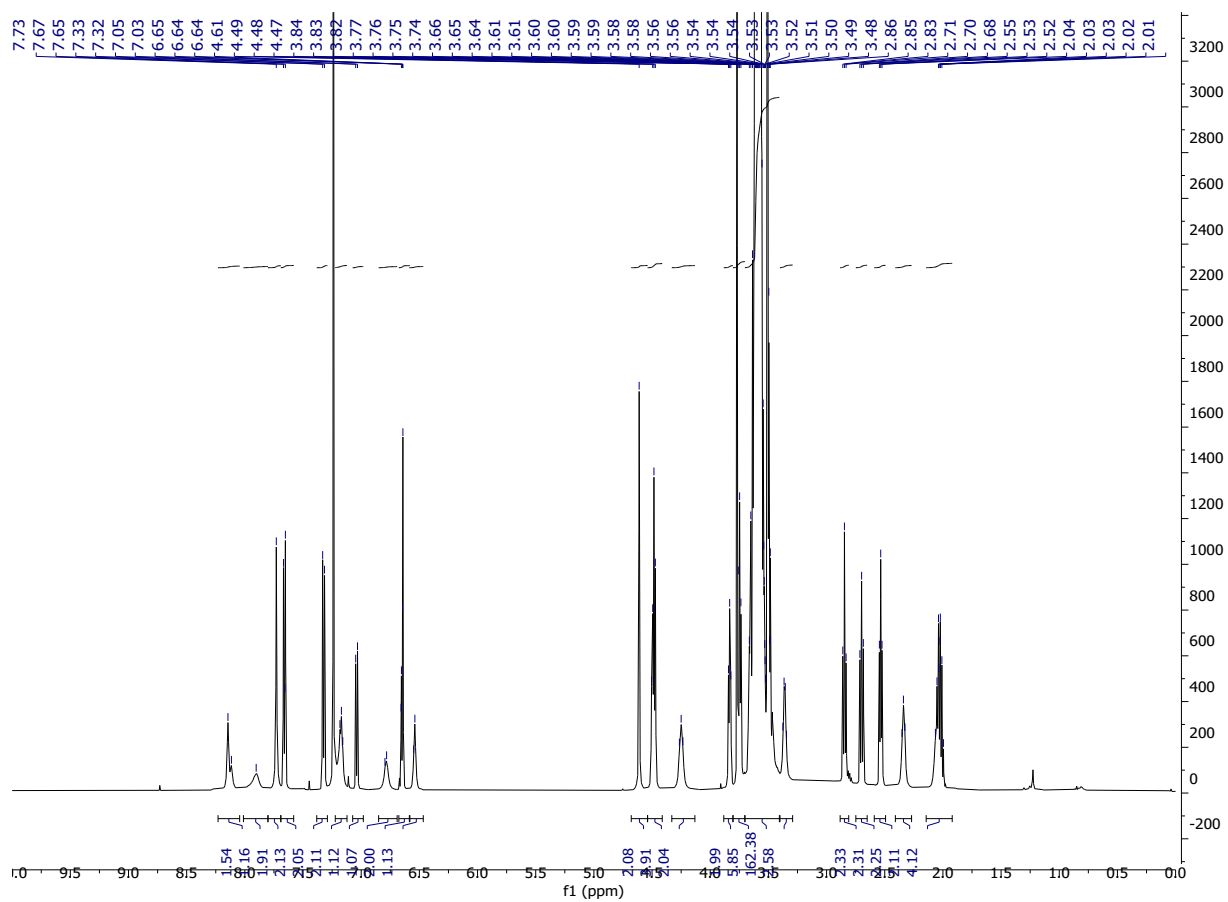
^1H NMR of **YM90K.1^{DART.1}** (**R5**) in CDCl_3

Synthesis of R6—YM90K.1^{DART.2}



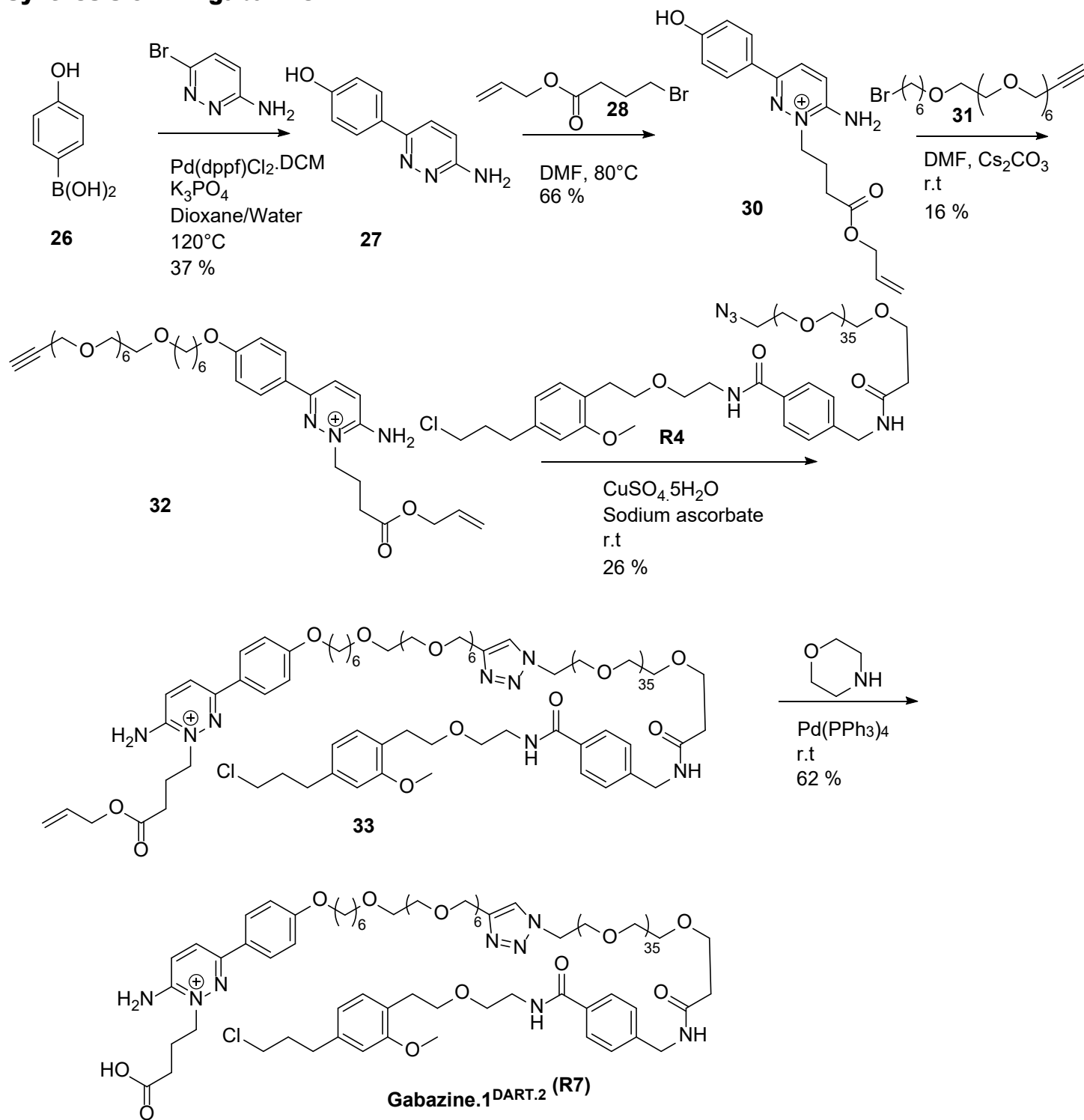
To 4-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)-N-(3,6,9,12,15,18-hexaoxahenicos-20-yn-1-yl)butanamide, **25** (10 mg, 15 μmol) was added EtOH (0.4 mL)/t-BuOH (0.2 mL)/Water (0.2 mL) after which azido-PEG36-HTL2.0, **R4** (32 mg, 15 μmol) was added. Finally, Cu(II) sulfate pentahydrate (0.19 mg, 0.76 μmol) and sodium ascorbate (0.27 mg, 1.5 μmol) were added and the reaction mixture was stirred overnight at room temperature. The mixture was directly purified by reverse phase prep HPLC eluting from 5 to 70 % acetonitrile in water (0.1 % FA, 220 nm collection wavelength and other parameters as described in the general chemical synthesis section) and lyophilized to give 4-(113-(4-(24-(7-(1H-imidazol-1-yl)-6-nitro-2,3-dioxo-3,4-dihydroquinoxalin-1(2H)-yl)-21-oxo-2,5,8,11,14,17-hexaoxa-20-azatetracosyl)-1H-1,2,3-triazol-1-yl)-3-oxo-6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108,111-hexatriacontaoxa-2-azatridecahectyl)-N-(2-(4-(3-chloropropyl)-2-methoxyphenoxy)ethyl)benzamide, **YM90K.1^{DART.2}, R6** (17 mg, 41 %). ¹H NMR (500 MHz, CDCl_3) δ 8.15–8.12 (m, 2H), 7.90 (br.s, 1H), 7.73 (s, 2H), 7.66 (d, J = 8.1 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 7.25 – 7.12 (m, 2H), 7.04 (d, J = 7.8 Hz, 1H), 6.79 (d, J = 7.0 Hz, 1H), 6.65 (d, J = 6.3 Hz, 2H), 6.54 (d, J = 5.4 Hz, 1H), 4.61 (s, 2H), 4.51 – 4.42 (m, 4H), 4.24 (t, J = 7.5 Hz, 2H), 3.83 (t, J = 5.0 Hz, 2H), 3.77 (s, 3H), 3.75 (t, J = 5.7 Hz, 3H), 3.68 – 3.47 (m, 163H), 3.36 (q, J = 5.2 Hz, 2H), 2.85 (t, J = 7.0 Hz, 2H), 2.70 (t, J = 7.4 Hz, 2H), 2.53 (t, J = 5.6 Hz, 2H), 2.33 (d, J = 6.4 Hz, 2H), 2.06 – 2.00 (m, 4H).

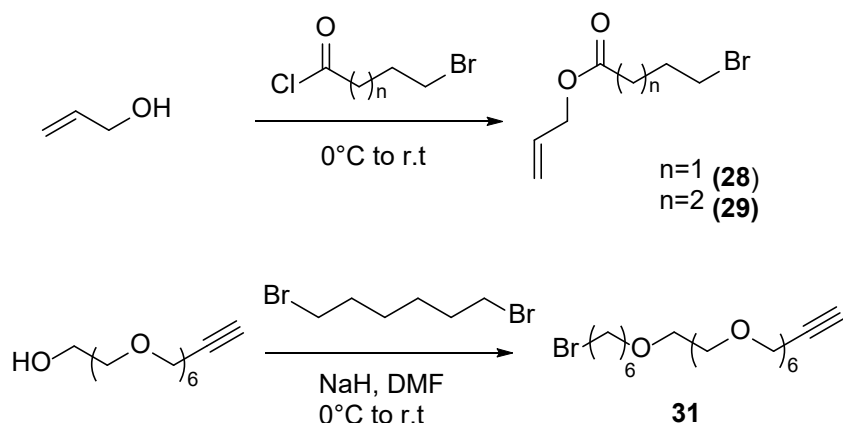
HRMS (ESI+): m/z calcd for $\text{C}_{127}\text{H}_{218}\text{ClN}_{11}\text{O}_{51}$: 1374.7257 $[\text{M}+2\text{H}]^{2+}$; found: 1374.7217, $[\text{M}+2\text{H}]^{2+}$



¹H NMR of YM90K.1^{DART.2} (R6) in CDCl₃

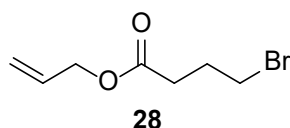
Synthesis of R7–gabazine.1^{DART.2}





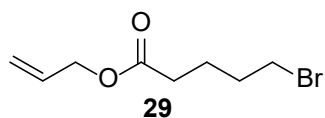
Synthesis of Intermediates towards Gabazine-DARTs

Synthesis of allyl 4-bromobutanoate, **28** (Reported in reference 2)



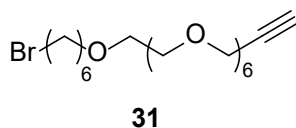
To cooled (ice/water bath) mixture of allyl alcohol (1.9 g, 2.2 mL, 6 equiv., 32 mmol) under nitrogen was added 4-bromobutanoyl chloride (1.0 g, 0.62 mL, 1 equiv., 5.0 mmol) dropwise. The reaction was allowed to stir at 0 °C for 2 h followed by room temperature for 3 h after TLC (1:1 EtOAc:Hex, iodine/silica stain) indicated conversion. The reaction mixture was directly concentrated to give allyl 4-bromobutanoate, **28** (1.035 g, 4.998 mmol, 93 %) as a brown oil. ^1H NMR (500 MHz, CDCl_3) δ 5.90 (ddt, J = 16.7, 11.1, 5.6 Hz, 1H), 5.27 (dd, J = 38.1, 13.8 Hz, 2H), 4.59 (d, J = 5.8 Hz, 2H), 3.46 (t, J = 6.3 Hz, 2H), 2.54 (t, J = 7.1 Hz, 2H), 2.18 (p, J = 6.7 Hz, 2H). (ES-LCMS) m/z 209.2 ($M+2\text{H}$)⁺.

Synthesis of allyl 5-bromopentanoate, **29** (Reported in reference 2)



To a cooled (ice/water bath) mixture of allyl alcohol (1.7 g, 2.0 mL, 6 equiv., 30 mmol) under nitrogen was added 5-bromopentanoyl chloride (1.0 g, 0.62 mL, 1 equiv., 5.0 mmol) dropwise. The reaction was allowed to stir at 0 °C for 2 h followed by room temperature for 3 h after TLC (1:1 EtOAc:Hex, iodine/silica stain) indicated conversion. The reaction mixture was directly concentrated to give allyl 5-bromopentanoate, **29** (0.77 g, 3.48 mmol, 69 %) as a brown oil. ^1H NMR (500 MHz, CDCl_3) δ 5.94-5.86 (m, 1H), 5.30 (d, J = 17.1 Hz, 1H), 5.22 (d, J = 10.4 Hz, 1H), 4.57 (d, J = 5.6 Hz, 2H), 3.40 (t, J = 6.5 Hz, 2H), 2.37 (t, J = 7.2 Hz, 2H), 1.89 (q, J = 7.0 Hz, 2H), 1.79 (q, J = 7.3 Hz, 2H). (ES-LCMS) m/z 223.1 ($M+2\text{H}$)⁺.

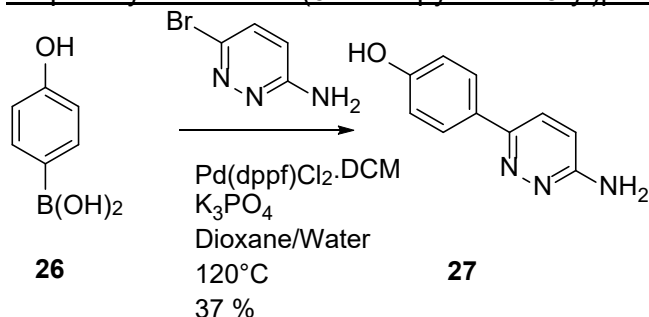
Synthesis of 28-bromo-4,7,10,13,16,19,22-heptaooxaoctacos-1-yne, **31**



To 3,6,9,12,15,18-hexaoxahenicos-20-yn-1-ol (1.00 g, 1 equiv., 3.12 mmol) in DMF (12 mL) in an ice/water bath and under nitrogen was added NaH (137 mg, 60% Wt, 1.1 equiv., 3.43 mmol) and the mixture was stirred

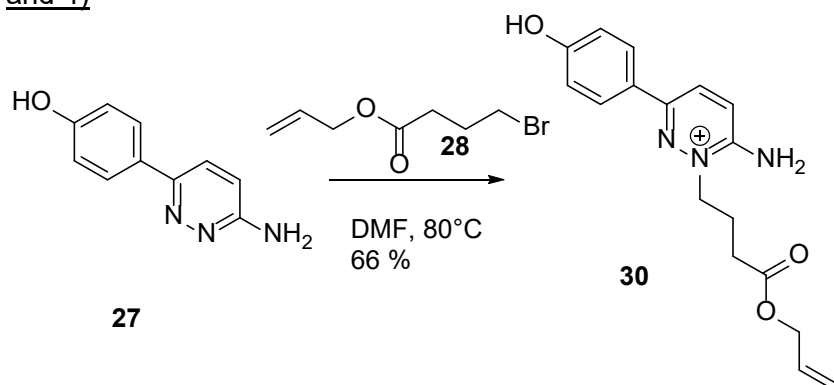
for 20 min after which 1,6-dibromohexane (1.22 g, 768 μ L, 1.6 equiv., 4.99 mmol) was added quickly. After 2 h of stirring as the ice bath expired, the reaction was quenched with saturated aqueous ammonium chloride solution and diluted with ethyl acetate and water. The aqueous layer was extracted once with ethyl acetate. Combined organics were washed with water, 10 % LiCl (aqueous) solution, brine, filtered through an isolute phase separator, and concentrated to a residue which was purified by silica gel chromatography eluting with 0 to 15 % MeOH in DCM to give 28-bromo-4,7,10,13,16,19,22-heptaooctacos-1-yne, **31** (865 mg, 1.79 mmol, 57.3 %) as a light yellow oil. ^1H NMR (500 MHz, CDCl_3) δ 4.18 (d, J = 2.4 Hz, 2H), 3.69 – 3.59 (m, 22H), 3.55 (dd, J = 5.9, 3.8 Hz, 2H), 3.43 (t, J = 6.6 Hz, 2H), 3.38 (t, J = 6.8 Hz, 2H), 2.41 (t, J = 2.4 Hz, 1H), 1.86-1.80 (m, 2H), 1.60-1.54 (m, 2H), 1.46 – 1.39 (m, 2H), 1.37 – 1.30 (m, 2H). (ES-LCMS) m/z 485.3 ($\text{M}+2\text{H}$) $^+$.

Step 1: Synthesis of 4-(6-aminopyridazin-3-yl)phenol, **27**



To (4-hydroxyphenyl)boronic acid, **26** (2 g, 14.5 mmol) was added K_3PO_4 (6.16 g, 29 mmol), $\text{Pd(dppf)Cl}_2 \cdot \text{DCM}$ (0.71 g, 0.87 mmol), 6-bromopyridazin-3-amine (2.47 g, 14.2 mmol) and dioxane (44 mL)/water (28 mL), and the reaction was heated in the microwave at 120°C for 10 min (note: separated into 4 portions which were each heated separately at 120°C for 10 min). The combined reaction mixture was diluted with ethyl acetate (50 mL), filtered through a pad of silica, and the contents transferred to a separatory funnel. The organic layer was washed with water (20 mL), brine (20 mL), and concentrated to a black solid which was triturated from dichloromethane. This solid was further purified by silica gel chromatography eluting with 0 to 35 % methanol in dichloromethane (product eluted in a wide band) to give 4-(6-aminopyridazin-3-yl)phenol, **27** (1 g, 5.34 mmol, 36.8 % yield) as a dark, brown solid. ^1H NMR (500 MHz, CD_3OD) δ 7.73 (dd, J = 9.0, 2.5 Hz, 3H), 7.00 (d, J = 9.3 Hz, 1H), 6.92 – 6.84 (m, 2H). (ES-LCMS) m/z 188.1 ($\text{M}+\text{H}$) $^+$.

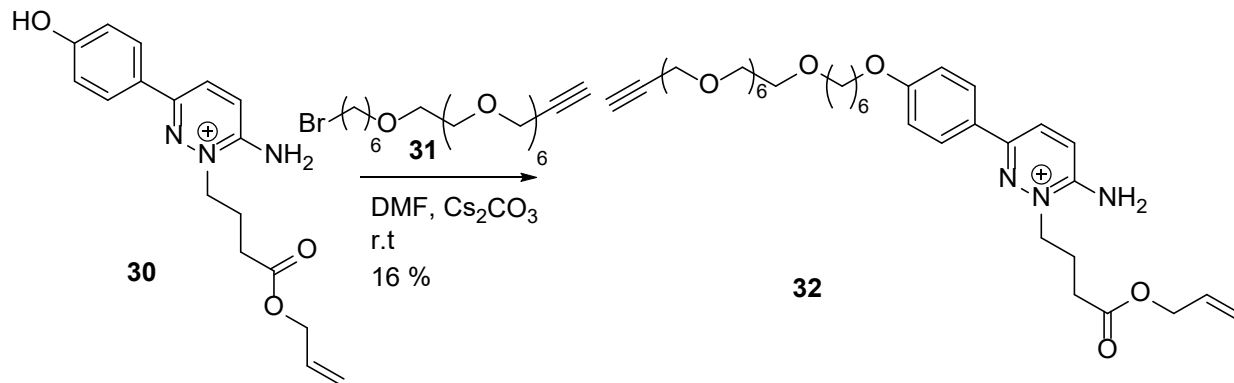
Step 2: Synthesis of allyl 4-(3-(4-hydroxyphenyl)-6-iminopyridazin-1(6H)-yl)butanoate, **30** (see references 3 and 4)



To 4-(6-aminopyridazin-3-yl)phenol, **27** (1 g, 5.34 mmol) was added allyl 4-bromobutanoate, **28** (1.14 g, 5.51 mmol) and DMF (26.7 mL) and the reaction was heated at 80°C for 7 h after which it was concentrated to remove DMF. The resulting thick, black oil was then triturated from acetonitrile give allyl 4-(3-(4-hydroxyphenyl)-6-iminopyridazin-1(6H)-yl)butanoate, **30** (1.1 g, 3.51 mmol, 65.7 % yield) as a brown solid. Since there are three nitrogens and a phenolic oxygen which may all react with the alkyl bromide, the assignment and confirmation of this product was described in the synthesis of **Gabazine.7**^{DART.2} (**R9**). ^1H NMR (500 MHz, Methanol- d_4) δ 8.23 (d, J = 9.5 Hz, 1H), 7.84 (d, J = 8.6 Hz, 2H), 7.55 (d, J = 9.5 Hz, 1H), 6.91 (dd, J = 9.1, 2.7 Hz, 2H), 5.86 (ddt, J = 16.5, 11.0, 5.7 Hz, 1H), 5.33 – 5.13 (m, 2H), 4.50 (d, J = 5.7 Hz, 2H), 4.43

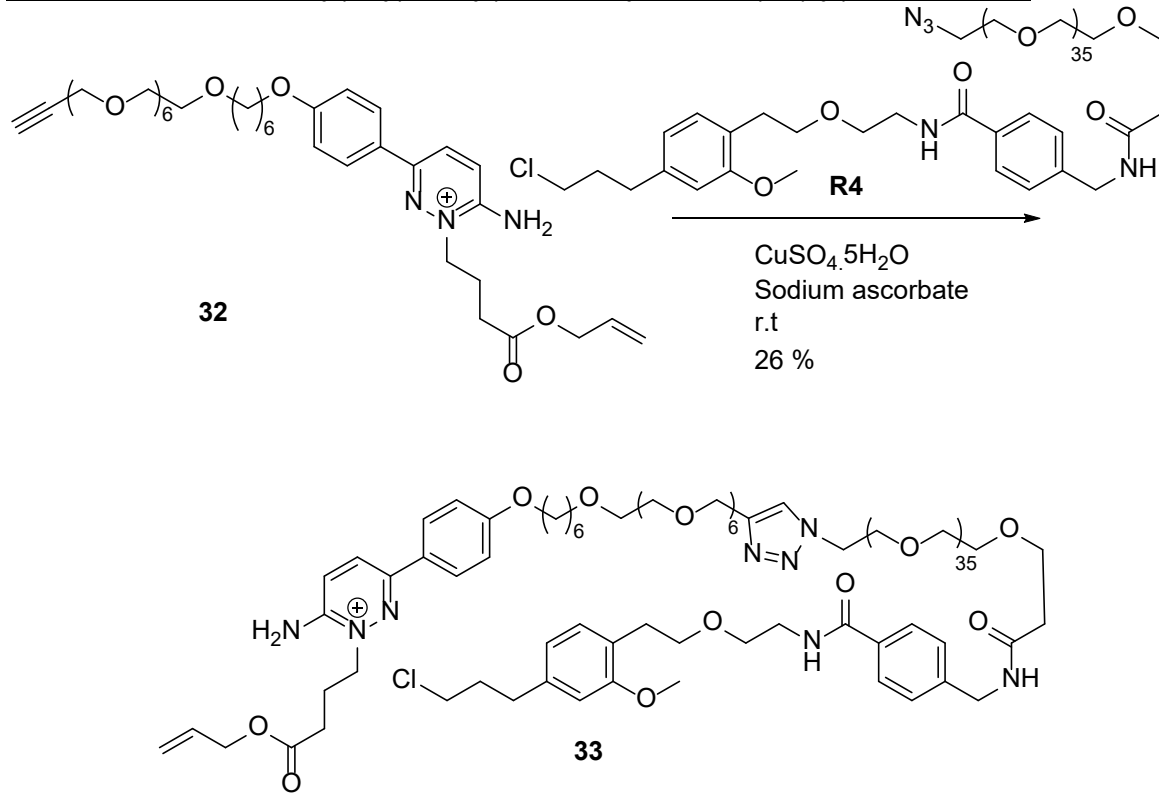
(t, $J = 6.9$ Hz, 2H), 2.63 (t, $J = 6.8$ Hz, 2H), 2.27 (p, $J = 6.8$ Hz, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 172.65, 161.38, 152.44, 150.54, 132.00, 130.83, 127.91, 125.25, 123.22, 117.22, 115.99, 65.08, 55.19, 29.94, 21.12. (ES-LCMS) m/z 314.3 ($\text{M}+\text{H}$) $^+$.

Step 3: Synthesis of allyl 4-(3-(4-(4,7,10,13,16,19,22-heptaooxaoctacos-1-yn-28-yloxy)phenyl)-6-aminopyridazin-1(6H)-yl)butanoate, **32**



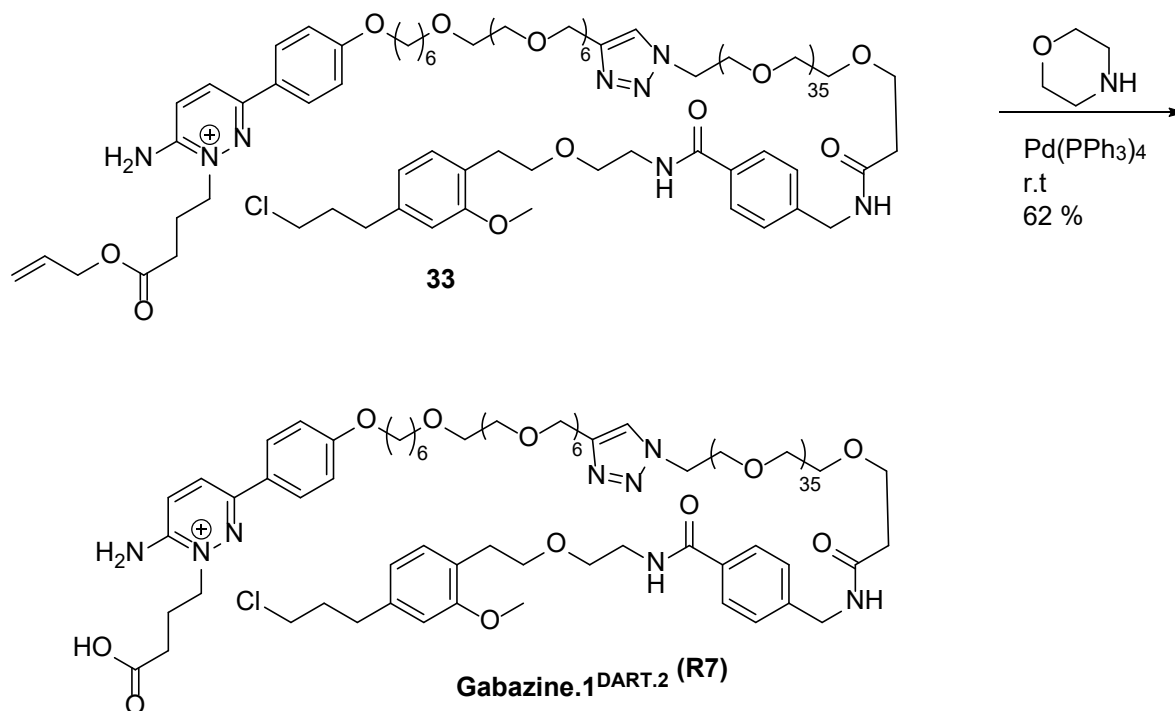
To allyl 4-(3-(4-hydroxyphenyl)-6-aminopyridazin-1(6H)-yl)butanoate, **30** (1.1 g, 3.51 mmol) was added anhydrous DMF (35.1 mL) after which 28-bromo-4,7,10,13,16,19,22-heptaooxaoctacos-1-yne, **31** (2 g, 4.13 mmol) was added. The solution was cooled to 0°C and anhydrous cesium carbonate (3.43 g, 10.53 mmol) was added and the reaction was stirred for 3 h as the ice bath expired after which water (20 mL) and ethyl acetate (60 mL) were added. The organic layer was washed with water (10 mL), brine (2 x 10 mL), dried (sodium sulfate), filtered and concentrated to give crude material as a yellow oil which was purified by reverse phase prep HPLC in several injections eluting with 5 to 50 % acetonitrile in water (0.1 % formic acid conditions and other conditions as described in the general chemical synthesis section) to give allyl 4-(3-(4-(4,7,10,13,16,19,22-heptaooxaoctacos-1-yn-28-yloxy)phenyl)-6-aminopyridazin-1(6H)-yl)butanoate, **32** (412 mg, 0.576 mmol, 16.39 % yield) as a red/yellow oil. ^1H NMR (500 MHz, CD_3OD) δ 8.39 – 8.24 (m, 3H), 8.03 – 7.85 (m, 2H), 7.61 (d, $J = 9.6$ Hz, 1H), 7.18 – 6.99 (m, 2H), 5.86 (ddt, $J = 17.3, 10.5, 5.7$ Hz, 1H), 5.34 – 5.11 (m, 2H), 4.57 – 4.38 (m, 4H), 4.25 – 4.02 (m, 4H), 3.75 – 3.44 (m, 24H), 2.87 (t, $J = 2.4$ Hz, 1H), 2.64 (t, $J = 6.7$ Hz, 2H), 2.29 (p, $J = 6.8$ Hz, 2H), 1.92 – 1.78 (m, 2H), 1.71 – 1.42 (m, 6H). (ES-LCMS) m/z 716.5 ($\text{M}+\text{H}$) $^+$.

Step 4: Synthesis of allyl 4-(3-(4-((1-(1-(1-(4-((2-(4-(3-chloropropyl)-2-methoxyphenoxy)ethyl)carbamoyl)phenyl)-3-oxo-6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108,111-hexatriacontaoxa-2-azatridecahectan-113-yl)-1H-1,2,3-triazol-4-yl)-2,5,8,11,14,17,20-heptaohexacosan-26-yl)oxy)phenyl)-6-iminopyridazin-1(6H)-yl)butanoate, **33**

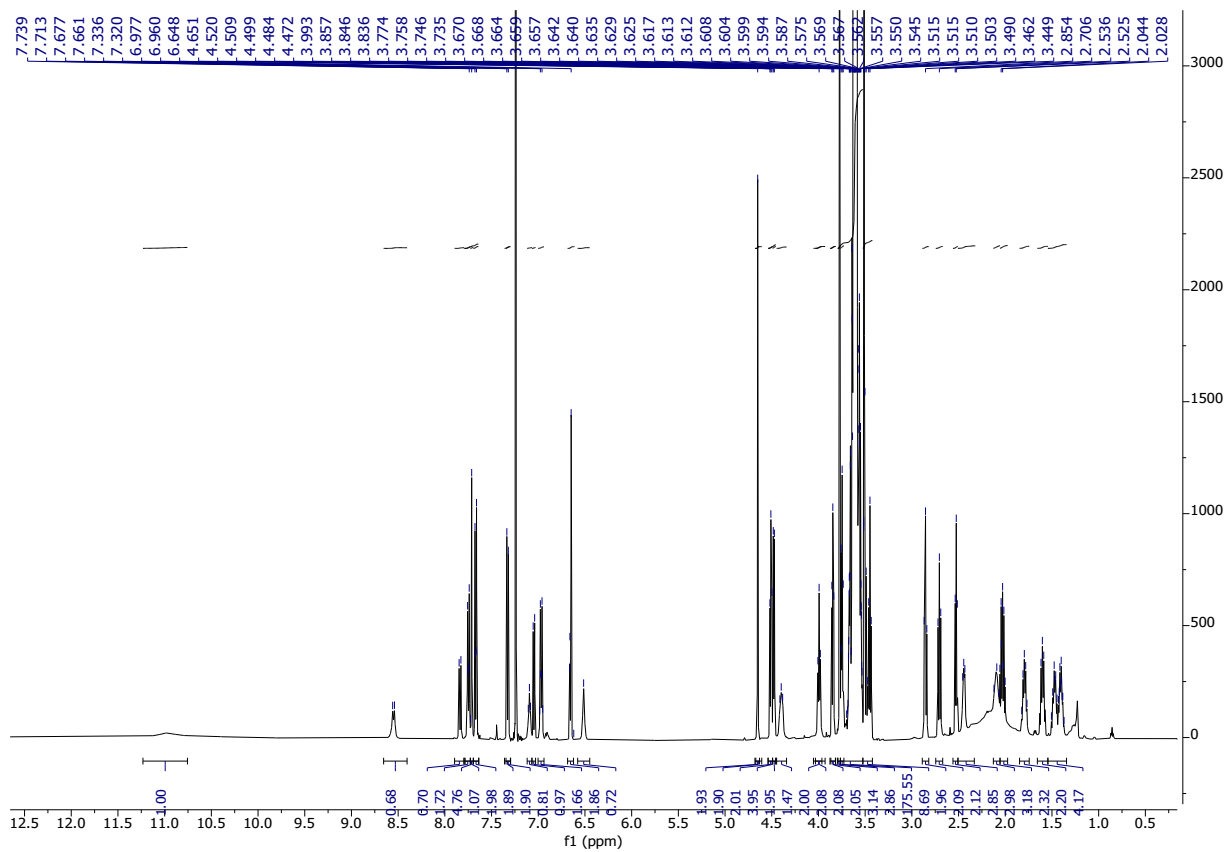


To a vial was added allyl 4-(3-(4-((4,7,10,13,16,19,22-heptaooxaoctacos-1-yn-28-yl)oxy)phenyl)-6-iminopyridazin-1(6H)-yl)butanoate, **32** (40 mg, 56 μ mol) and EtOH/t-BuOH/water (2:1:1, 1.6 mL) after which azido-PEG36-HTL2.0, **R4** (0.12 g, 56 μ mol) was added. Finally, Cu(II) sulfate pentahydrate (0.7 mg, 2.8 μ mol) and sodium ascorbate (0.98 mg, 5.6 μ mol) were added and the reaction mixture was stirred at room temperature for 3 h after which it was purified by reverse phase prep HPLC eluting with 50 to 80 % acetonitrile in water (0.1 % formic acid, 220 nm wavelength for collection, and other conditions reported in the general chemical synthesis section) to give allyl 4-(3-(4-((1-(1-(1-(4-((2-(4-(3-chloropropyl)-2-methoxyphenoxy)ethyl)carbamoyl)phenyl)-3-oxo-6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108,111-hexatriacontaoxa-2-azatridecahectan-113-yl)-1H-1,2,3-triazol-4-yl)-2,5,8,11,14,17,20-heptaohexacosan-26-yl)oxy)phenyl)-6-iminopyridazin-1(6H)-yl)butanoate, **33** (45 mg, 26 %) as a yellow oil. ^1H NMR (500 MHz, CDCl_3) δ 8.44 (s, 2H), 8.17 (d, J = 9.5 Hz, 1H), 7.83 (d, J = 9.5 Hz, 1H), 7.76 – 7.65 (m, 5H), 7.33 (d, J = 7.9 Hz, 2H), 7.05 (d, J = 7.8 Hz, 1H), 7.02 – 6.94 (m, 2H), 6.66 (d, J = 6.5 Hz, 2H), 6.53 (s, 1H), 5.91 – 5.77 (m, 1H), 5.42 – 5.11 (m, 2H), 4.65 (s, 2H), 4.58 (d, J = 5.9 Hz, 2H), 4.51 (t, J = 5.2 Hz, 2H), 4.48 (d, J = 6.0 Hz, 2H), 4.42 (t, J = 7.8 Hz, 2H), 3.99 (d, J = 6.4 Hz, 1H), 3.85 (t, J = 5.1 Hz, 2H), 3.78 (s, 3H), 3.75 (t, J = 5.6 Hz, 2H), 3.69 – 3.42 (m, 172H), 2.86 (t, J = 7.0 Hz, 2H), 2.71 (t, J = 7.4 Hz, 2H), 2.54 (dt, J = 15.9, 6.0 Hz, 4H), 2.23 (p, J = 6.8 Hz, 2H), 2.03 (dq, J = 8.3, 6.5 Hz, 3H), 1.79 (q, J = 6.8 Hz, 2H), 1.60 (p, J = 6.9 Hz, 2H), 1.53 – 1.30 (m, 3H). (ES-LCMS) m/z 571.2 $[(M+5H)]^{5+}$.

Step 5: Synthesis of Gabazine.1^{DART.2} (R7)



To a solution of allyl 4-(3-(4-((1-(1-(1-(4-((2-(4-(3-chloropropyl)-2-methoxyphenethoxy)ethyl)carbamoyl)phenyl)-3-oxo-6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108,111-hexatriacontaoxa-2-azatriecahectan-113-yl)-1H-1,2,3-triazol-4-yl)-2,5,8,11,14,17,20-heptaohexacosan-26-yl)oxy)phenyl)-6-aminopyridazin-1(6H)-yl)butanoate, **33** (52 mg, 19 μ mol) in THF (0.8 mL) was added morpholine (8.1 mg, 8.1 μ L, 93 μ mol) and Pd(PPh₃)₄ (3 mg, 11.1 μ mol). The reaction was stirred under nitrogen for 1 h after which it was purified by reverse phase prep HPLC eluting with 5 to 80 % acetonitrile in water (0.1 % formic acid, 220 nm wavelength for collection, and conditions reported in the general chemical synthesis section) to give 4-(3-(4-((1-(1-(1-(4-((2-(4-(3-chloropropyl)-2-methoxyphenethoxy)ethyl)carbamoyl)phenyl)-3-oxo-6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108,111-hexatriacontaoxa-2-azatriecahectan-113-yl)-1H-1,2,3-triazol-4-yl)-2,5,8,11,14,17,20-heptaohexacosan-26-yl)oxy)phenyl)-6-aminopyridazin-1(6H)-yl)butanoic acid **Gabazine.1^{DART.2}, R7** (32 mg, 62 %). ¹H NMR (500 MHz, CDCl₃) δ 10.97 (br.s, 1H), 8.55 (d, *J* = 9.7 Hz, 1H), 7.84 (d, *J* = 9.6 Hz, 1H), 7.77 – 7.72 (m, 2H), 7.71 (s, 1H), 7.69 – 7.65 (m, 2H), 7.33 (d, *J* = 8.0 Hz, 2H), 7.09 (t, *J* = 5.9 Hz, 1H), 7.05 (d, *J* = 7.9 Hz, 1H), 6.99 – 6.95 (m, 2H), 6.66-6.62 (m, 2H), 6.51 (s, 1H), 4.65 (s, 2H), 4.51 (t, *J* = 5.5 Hz, 2H), 4.48 (d, *J* = 6.0 Hz, 2H), 4.40 (t, *J* = 7.5 Hz, 2H), 3.99 (t, *J* = 6.5 Hz, 2H), 3.85 (t, *J* = 5.2 Hz, 2H), 3.77 (s, 3H), 3.75 (t, *J* = 5.6 Hz, 2H), 3.70 – 3.37 (m, 175H), 3.52-3.44 (m, 9H), 2.85 (t, *J* = 7.0 Hz, 2H), 2.71 (t, *J* = 7.4 Hz, 2H), 2.52 (t, *J* = 5.6 Hz, 2H), 2.50 – 2.41 (m, 2H), 2.12-2.07 (m, 2H), 2.06-2.00 (m, 2H), 1.80 (p, *J* = 6.8 Hz, 2H), 1.65 – 1.54 (m, 2H), 1.51-1.37 (m, 4H), HRMS (ESI⁺): *m/z* calcd for C₁₃₂H₂₂₉ClN₈Na₂O₅₀: 1404.7582 [M+2Na]²⁺; found: 1404.7709 [M+2Na]²⁺



¹H NMR of Gabazine.1^{DART.2} (R7) in CDCl₃

Oc1ccc(B(O)O)cc1 (**26**) $\xrightarrow[\text{K}_3\text{PO}_4, 120^\circ\text{C}]{\text{Pd(dppf)Cl}_2, \text{DCM}}$ Nc1cc(N)nc(Cc2ccc(O)cc2)c1 (**27**) (37 %)

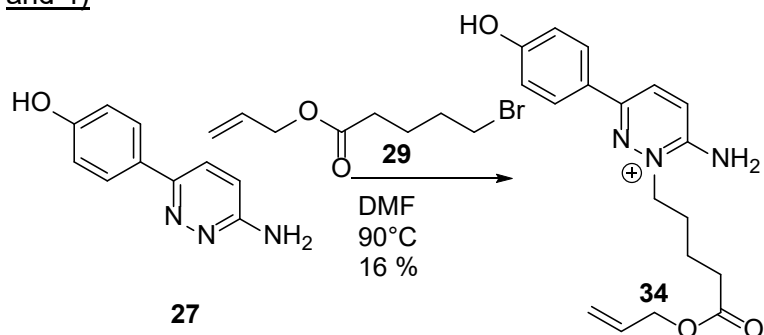
BrCCCCCOC(=O)C=C (**29**) $\xrightarrow[\text{DMF, } 90^\circ\text{C}]{}$ Nc1cc(N)nc(Cc2ccc(O)cc2)[n+]1CCCCCOC(=O)C=C (**34**) (16 %)

BrCCCCCOC(=O)C=C (**34**) $\xrightarrow[\text{DMF, Cs}_2\text{CO}_3, \text{r.t.}]{}$ Nc1cc(N)nc(Cc2ccc(O)cc2)[n+]1CCCCCOC(=O)C=C (**35**) (40 %)

Nc1cc(N)nc(Cc2ccc(O)cc2)[n+]1CCCCCOC(=O)C=C (**35**) $\xrightarrow[\text{MeOH/water, r.t.}]{\text{LiOH}}$ Nc1cc(N)nc(Cc2ccc(O)cc2)[n+]1CCCCCOC(=O)O (**36**)

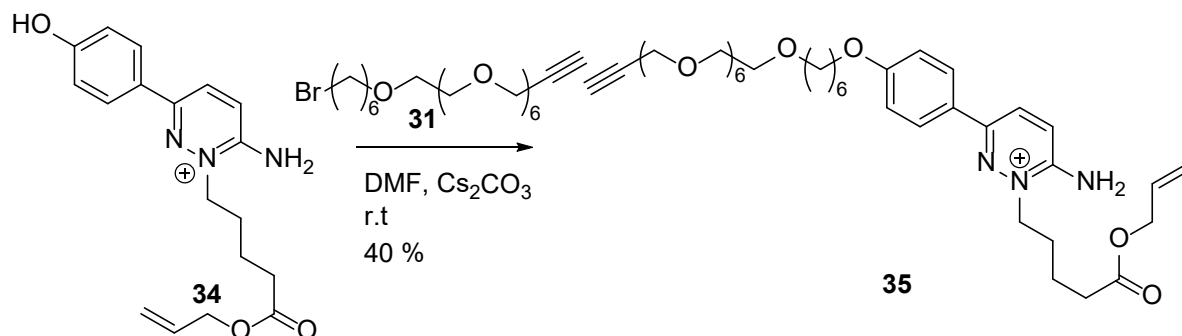
Nc1cc(N)nc(Cc2ccc(O)cc2)[n+]1CCCCCOC(=O)O (**36**) $\xrightarrow[\text{Sodium ascorbate, r.t.}]{\text{CuSO}_4 \cdot 5\text{H}_2\text{O}}$ Nc1cc(N)nc(Cc2ccc(O)cc2)[n+]1CCCCCOC(=O)O (**Gabazine.5^{DART.2} (R8)**) (58 % (2 steps))

Step 2: Synthesis of allyl 5-(3-(4-hydroxyphenyl)-6-aminopyridazin-1(6H)-yl)pentanoate, **34** (see references 3 and 4)



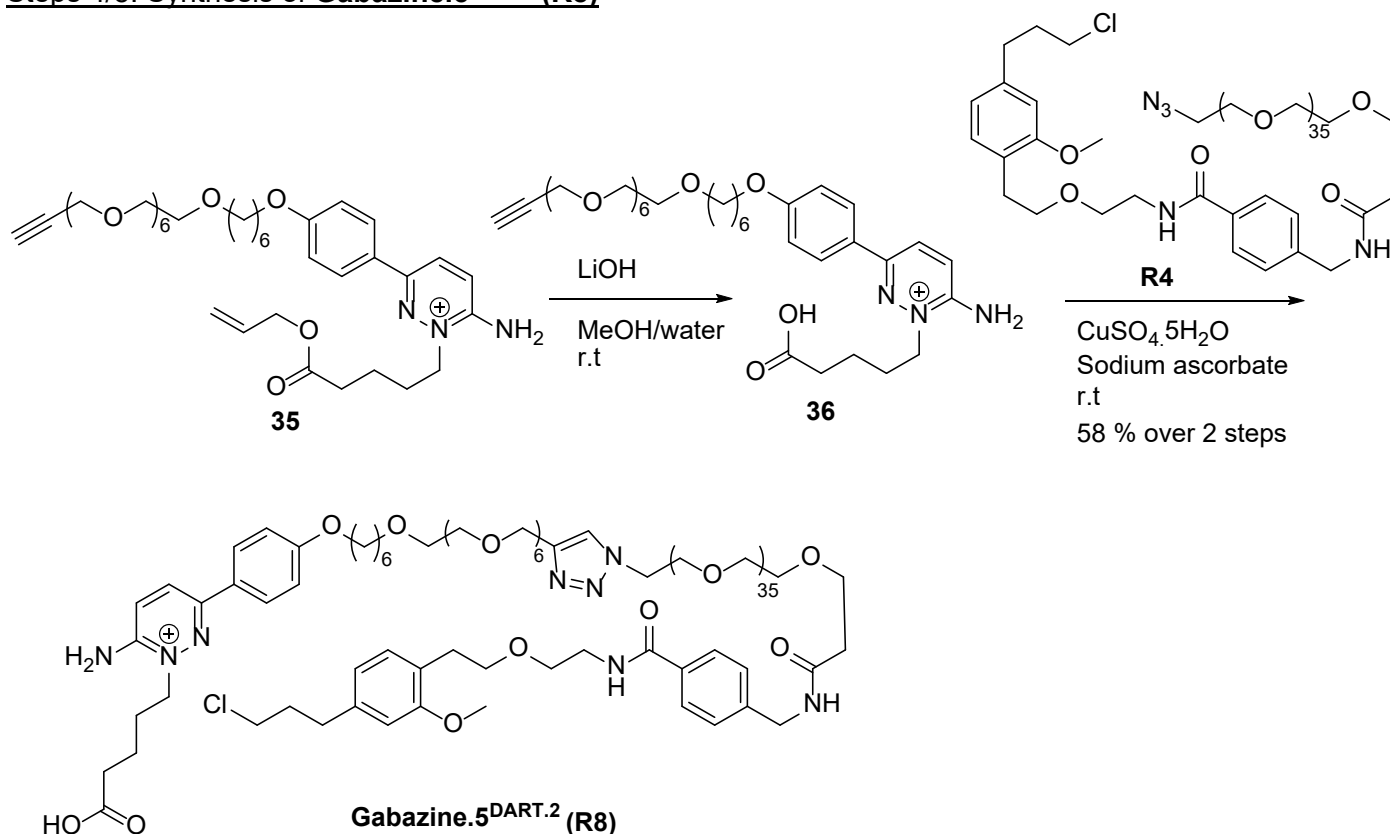
To 4-(6-aminopyridazin-3-yl)phenol, **27** (250 mg, 1.34 mmol) was added allyl 5-bromopentanoate, **29** (295 mg, 1.34 mmol) and DMF (2 mL) and the reaction was heated at 90°C for 2 h. The mixture was directly purified by prep HPLC eluting with 10 to 90 % ACN in water (0.1 % FA, 220 nm wavelength for collection) in three 750 uL injections. The main peak(s) were isolated, and fractions were concentrated to give the desired product, **34** (71 mg, 16 %). Before this main peak, one byproduct with the same mass was isolated. Since three nitrogens in the product as well as the phenolic oxygen all may react with the alkyl bromide, the assignment and confirmation of product was described in the **Gabazine.7^{DART.2} (R9)** section. ¹H NMR (500 MHz, CD₃OD) δ 8.47 (br.s, 1H), 8.27 (d, *J* = 9.3 Hz, 1H), 7.87 (d, *J* = 8.4 Hz, 2H), 7.58 (d, *J* = 9.4 Hz, 1H), 6.94 (d, *J* = 8.4 Hz, 2H), 5.97-5.85 (m, 1H), 5.31 (dd, *J* = 17.3, 1.9 Hz, 1H), 5.21 (d, *J* = 10.5 Hz, 1H), 4.59 (d, *J* = 5.6 Hz, 2H), 4.40 (t, *J* = 7.2 Hz, 2H), 2.51 (t, *J* = 7.3 Hz, 2H), 2.05-1.99 (m, 2H), 1.83-1.77 (m, 2H). ¹³C NMR (126 MHz, CD₃OD) δ 173.05, 160.45, 152.26, 150.88, 132.27, 131.05, 128.00, 125.02, 123.82, 116.95, 115.69, 64.77, 55.54, 32.75, 25.86, 21.21. (ES-LCMS) *m/z* 328.3 (M+H)⁺.

Step 3: Synthesis of allyl 5-(3-(4-((4,7,10,13,16,19,22-heptaooxaoctacos-1-yn-28-yl)oxy)phenyl)-6-aminopyridazin-1(6H)-yl)pentanoate, **35**

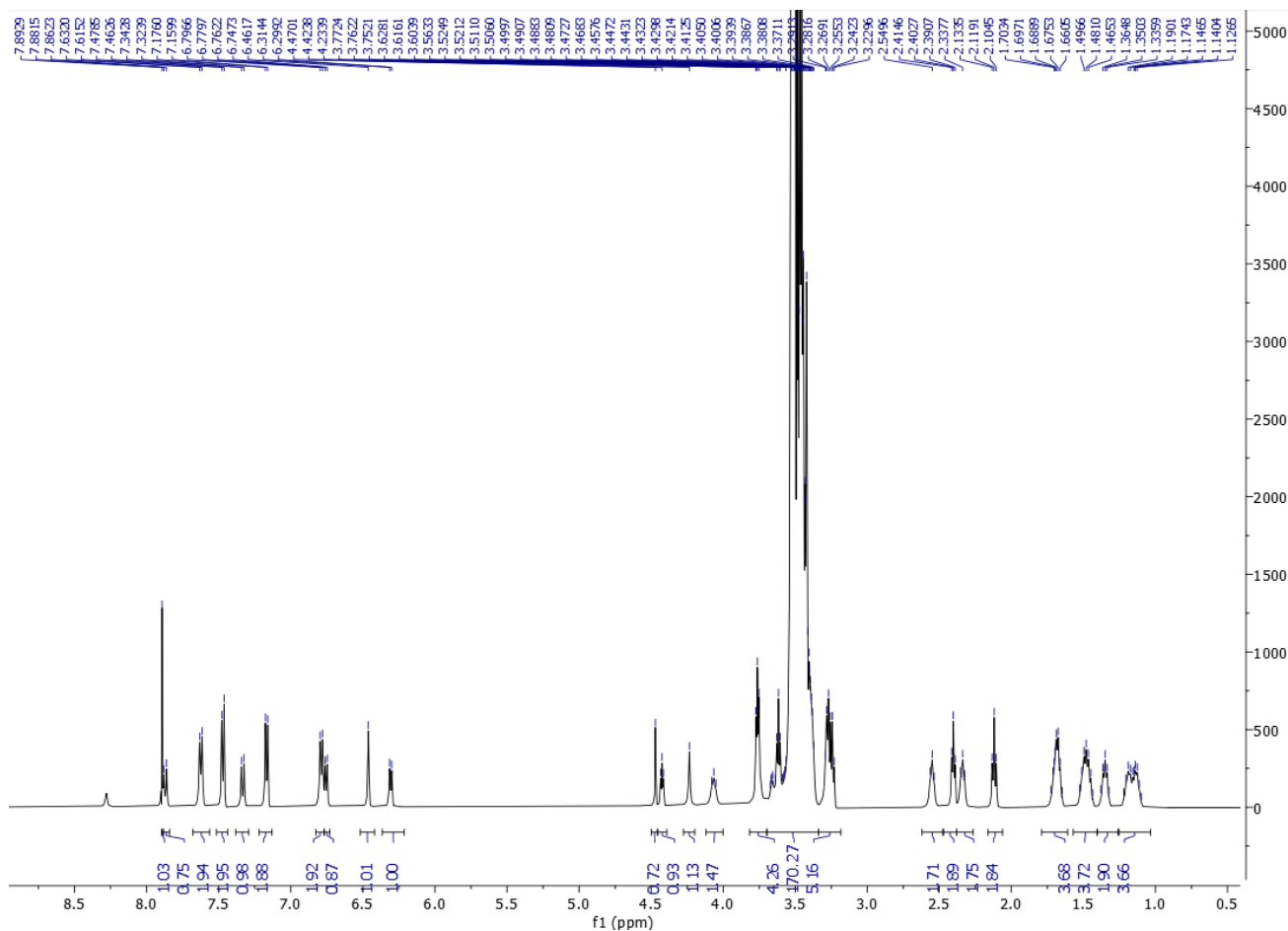


To allyl 5-(3-(4-hydroxyphenyl)-6-aminopyridazin-1(6H)-yl)pentanoate, **34** (40 mg, 0.12 mmol) was added anhydrous DMF (1 mL) after which 28-bromo-4,7,10,13,16,19,22-heptaooxaoctacos-1-yne, **31** (89 mg, 0.18 mmol) and anhydrous cesium carbonate (80 mg, 0.24 mmol) were added and the reaction was stirred for 3h. DMF was removed under a stream of nitrogen, and the crude was purified by reverse phase prep HPLC eluting with 5 to 50 % acetonitrile in water (0.1 % formic acid conditions and other conditions as described in the general chemical synthesis section) to give allyl 5-(3-(4-((4,7,10,13,16,19,22-heptaooxaoctacos-1-yn-28-yl)oxy)phenyl)-6-aminopyridazin-1(6H)-yl)pentanoate, **35** (36 mg, 49 μmol, 40 % yield) as a red/yellow oil. ¹H NMR (500 MHz, CD₃OD) δ 8.53 (s, 1H), 8.30 (d, *J* = 9.6 Hz, 1H), 8.02 – 7.90 (m, 2H), 7.60 (d, *J* = 9.5 Hz, 1H), 7.16 – 7.03 (m, 2H), 5.98 – 5.90 (m, 1H), 5.31 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.21 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.59 (dt, *J* = 5.7, 1.5 Hz, 2H), 4.41 (t, *J* = 7.2 Hz, 2H), 4.20 (d, *J* = 2.4 Hz, 2H), 4.09 (t, *J* = 6.4 Hz, 2H), 3.69 – 3.63 (m, 21H), 3.61 – 3.59 (m, 2H), 3.52 (t, *J* = 6.5 Hz, 2H), 2.86 (t, *J* = 2.4 Hz, 1H), 2.51 (t, *J* = 7.3 Hz, 2H), 2.06 – 2.00 (m, 2H), 1.87-1.77 (m, 4H), 1.67-1.61 (m, 2H), 1.58-1.52 (m, 2H), 1.51 – 1.45 (m, 2H). (ES-LCMS) *m/z* 730.6 (M+H)⁺.

Steps 4/5: Synthesis of **Gabazine.5^{DART.2} (R8)**

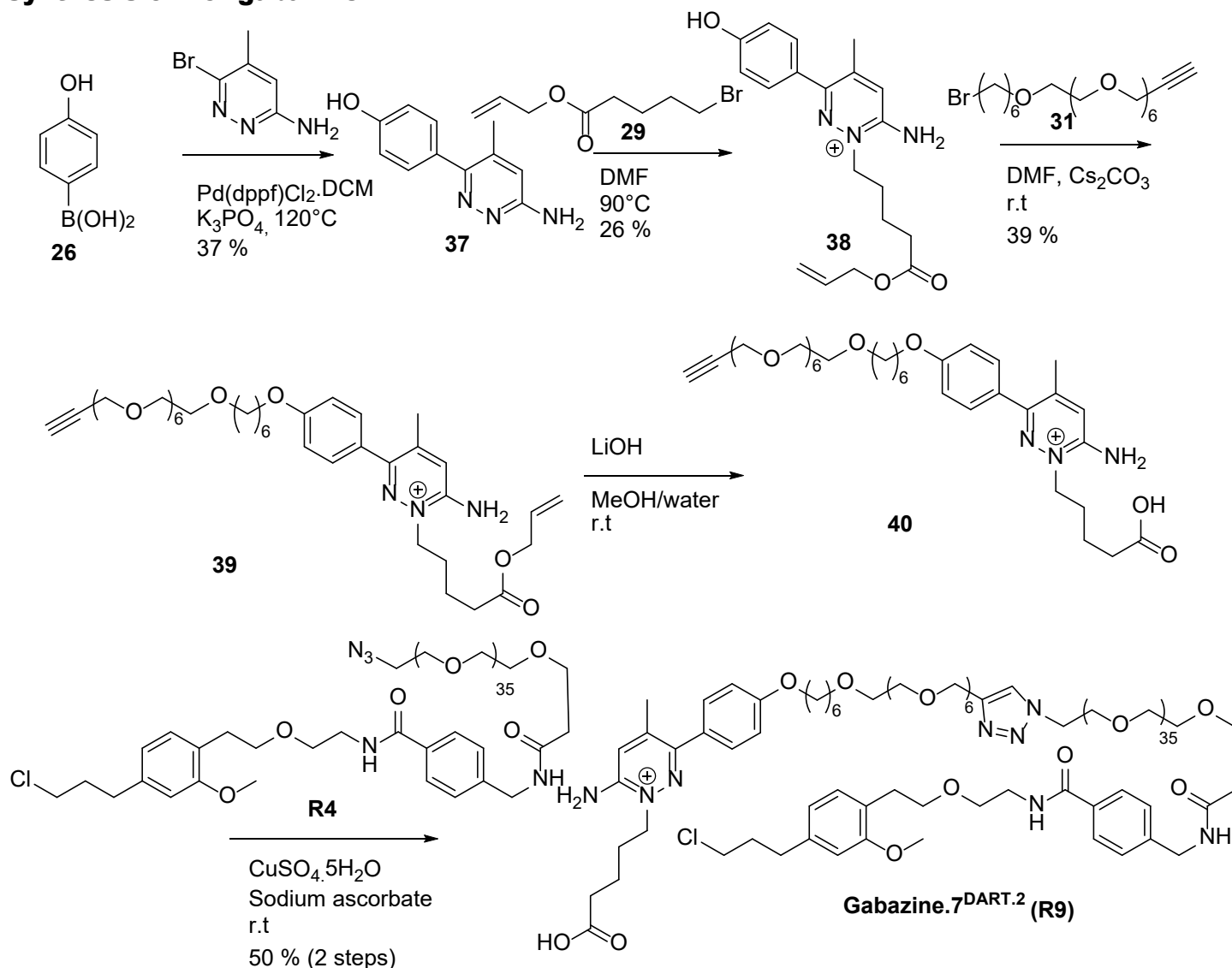


To allyl 5-(3-(4-((4,7,10,13,16,19,22-heptaooxaoctacos-1-yn-28-yl)oxy)phenyl)-6-iminopyridazin-1(6H)-yl)pentanoate, **35** (15 mg, 20 μmol) in $\text{MeOH}/\text{H}_2\text{O}$ (1/1, 2 mL) was added lithium hydroxide hydrate (5.1 mg, 120 μmol) at r.t., and the reaction was stirred for 2h. LCMS indicated formation of **36** and the pH was adjusted to 8 by addition of 1N HCl (110 μL). The mixture was concentrated under a stream of nitrogen. To the crude mixture was added $\text{EtOH}/t\text{-BuOH}/\text{water}$ (2:1:1, 1 mL) after which azido-PEG36-HTL2.0, **R4** (42 mg, 20 μmol) was added. Finally, Cu(II) sulfate pentahydrate (0.45 mg, 1.8 μmol) and sodium ascorbate (0.32 mg, 1.8 μmol) were added and the reaction mixture was stirred at room temperature overnight after which it was purified by reverse phase prep HPLC eluting with 20 to 90 % acetonitrile in water (0.1 % formic acid, 200 nm wavelength for collection, and other conditions reported in the general chemical synthesis section) to give 5-(3-(4-((1-(1-(1-(4-((2-(4-(3-chloropropyl)-2-methoxyphenoxy)ethyl)carbamoyl)phenyl)-3-oxo-6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108,111-hexatriacontaoxa-2-azatriecahectan-113-yl)-1H-1,2,3-triazol-4-yl)-2,5,8,11,14,17,20-heptaooxahexacosan-26-yl)oxy)phenyl)-6-iminopyridazin-1(6H)-yl)pentanoic acid, **Gabazine.5^{DART.2}, R8** (32.0 mg, 2 steps 58%) ^1H NMR (500 MHz, D_2O , water suppression used- the peaks close to the water peak between 4.4 and 4.6 ppm were partially suppressed) δ 7.89 (s, 1H), 7.87 (d, J = 9.6 Hz, 1H), 7.62 (d, J = 8.4 Hz, 2H), 7.47 (d, J = 8.0 Hz, 2H), 7.33 (d, J = 9.4 Hz, 1H), 7.17 (d, J = 8.1 Hz, 2H), 6.79 (d, J = 8.5 Hz, 2H), 6.75 (d, J = 7.4 Hz, 1H), 6.46 (s, 1H), 6.31 (d, J = 7.6 Hz, 1H), 4.47 (s, 2H), 4.42 (t, J = 5.0 Hz, 2H), 4.23 (s, 2H), 4.07 (t, J = 7.4 Hz, 2H), 3.76 (t, J = 5.1 Hz, 4H), 3.70 – 3.34 (m, 170H), 3.29-3.23 (m, 5H), 2.55 (t, J = 6.6 Hz, 2H), 2.40 (t, J = 6.0 Hz, 2H), 2.34 (t, J = 7.5 Hz, 2H), 2.12 (t, J = 7.3 Hz, 2H), 1.72-1.65 (m, 4H), 1.53-1.43 (m, 4H), 1.38-1.32 (m, 2H), 1.22-1.10 (m, 4H). HRMS (ESI⁺): m/z calcd for $\text{C}_{133}\text{H}_{232}\text{ClN}_8\text{NaO}_{50}$: 1400.2733 $[\text{M}+\text{H}+\text{Na}]^{2+}$; found: 1400.2734 $[\text{M}+\text{H}+\text{Na}]^{2+}$

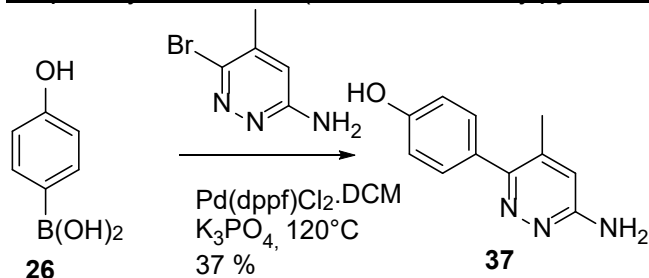


¹H NMR of Gabazine.5^{DART.2} (R8) in D₂O (water suppression)

Synthesis of R9—gabazine.7^{DART.2}

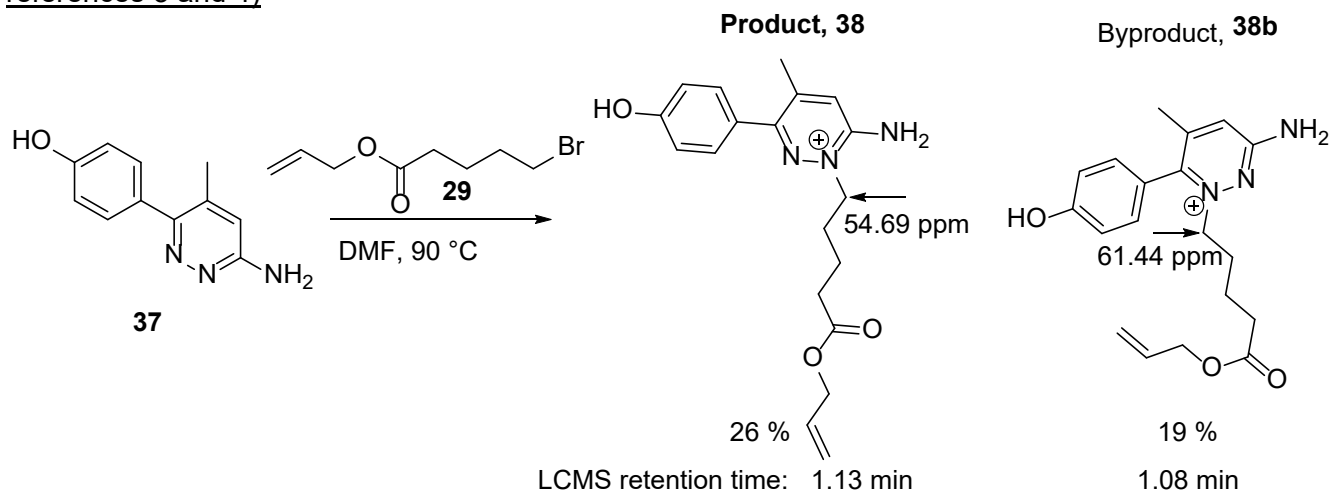


Step 1: Synthesis of 4-(6-amino-4-methylpyridazin-3-yl)phenol, **37**



To (4-hydroxyphenyl)boronic acid, **26** (1 g, 7.25 mmol) was added K_3PO_4 (3.08 g, 14.5 mmol), $\text{Pd(dppf)Cl}_2 \cdot \text{DCM}$ (0.355 g, 0.435 mmol), 6-bromo-5-methylpyridazin-3-amine (0.937 g, 6.53 mmol) and dioxane (7 mL)/water (4 mL). The suspension was stirred and degassed for 5 min after which it was heated at 120°C for 17 min, then concentrated. The material was then taken up in water and a slight amount of DCM, stirred thoroughly, then filtered. The mixture was filtered to give 4-(6-amino-4-methylpyridazin-3-yl)phenol, **37** (1.132 g, 5.62 mmol, 77% yield) as a dark, brown solid, containing some aryl halide. Used directly without further purification. ^1H NMR (500 MHz, CD_3OD) δ 7.30 (d, $J = 8.6$ Hz, 2H), 6.89 (d, $J = 8.6$ Hz, 2H), 6.86 (d, $J = 1.1$ Hz, 1H), 2.23 (d, $J = 1.0$ Hz, 3H). (ES-LCMS) m/z 202.2 ($\text{M}+\text{H}^+$)

Step 2: Synthesis of allyl 5-(3-(4-hydroxyphenyl)-6-imino-4-methylpyridazin-1(6H)-yl)pentanoate, **38** (see references 3 and 4)

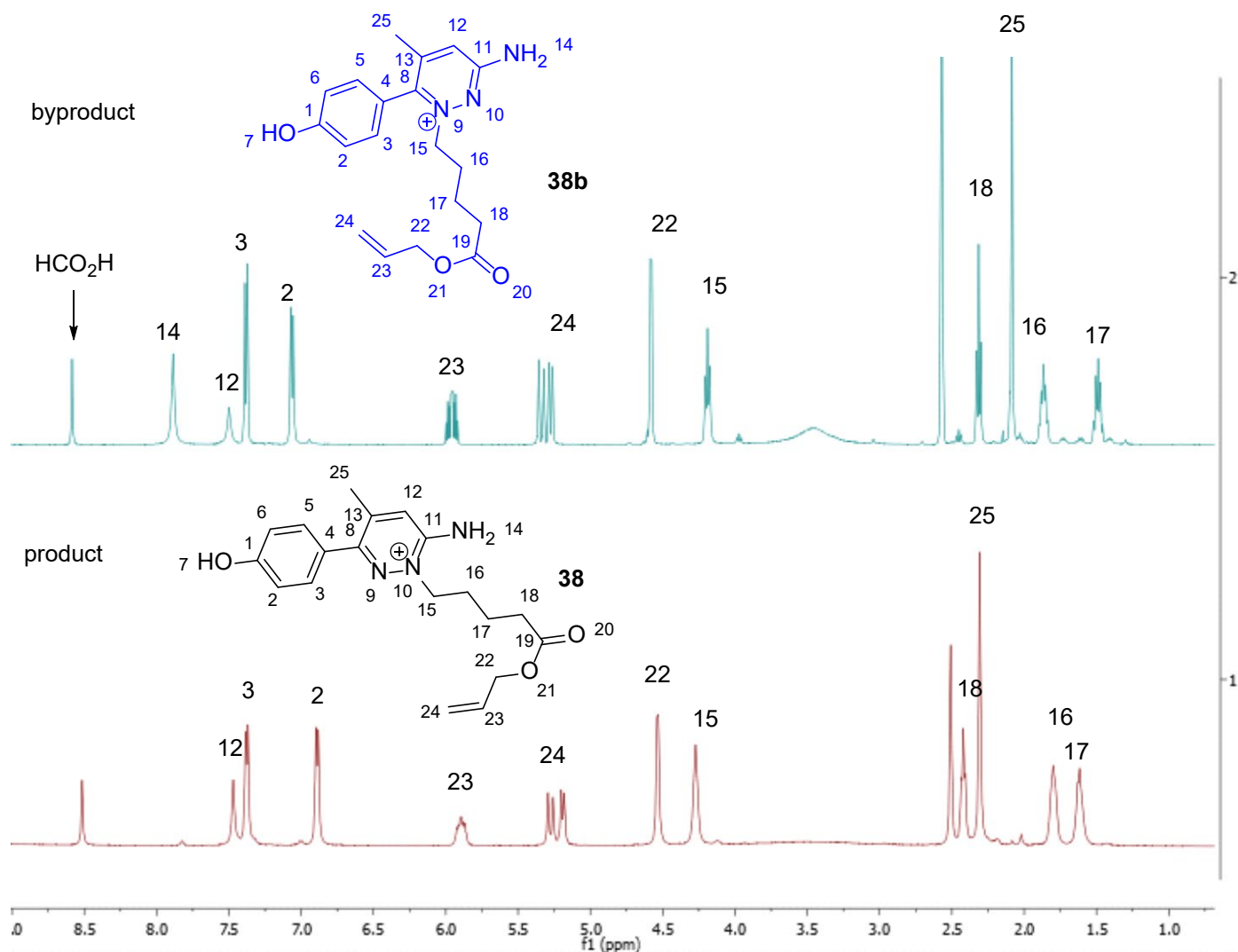


To 4-(6-amino-4-methylpyridazin-3-yl)phenol, **37** (250 mg, 1.24 mmol) was added allyl 5-bromopentanoate, **29** (275 mg, 1.24 mmol) and DMF (2 mL) and the reaction was heated at 90°C for 2 h. The mixture was directly purified by prep HPLC eluting with 10 to 90 % ACN in water (0.1 % FA, 220 nm wavelength for collection, and the rest of the conditions as described in the general chemistry synthesis section) in three 750 uL injections. Relevant fractions were combined and concentrated. The undesired byproduct eluted first and the desired product eluted later. Since three nitrogens and the phenolic oxygen may all react with the alkyl bromide, the structures were fully assigned and confirmed by H/C NMR, HSQC, and HMBC in DMSO-d₆. The most distinguishing peak for the product and byproduct is the CH₂ that is directly connected to the nitrogen which reacted. The ¹³C NMR of the CH₂ immediately adjacent to the nucleophilic nitrogen has a shift of 54.69 ppm in the desired product vs 61.44 ppm in the undesired byproduct. The ¹³C NMR shift of this CH₂ in the desired compound is the same as the shift of a CH₂ adjacent to the same nucleophilic nitrogen in a similar compound reported in reference 4 in which the structure was confirmed by X-ray crystallography. The regioselectivity of the alkylation of pyridazine has been documented in reference 3. The reactivities of the structurally related analogs, Gabazine 1, Gabazine 5, and Gabazine 7, are very similar: in all cases, the undesired byproduct eluted first on RP HPLC. Additionally, the shifts of the CH₂ directly connected to the same nitrogen are very similar for all 3 analogs (see figure below). It worthwhile to mention that the products and byproducts exist as tautomers under acidic and basic conditions. Furthermore, the undesired byproduct does not react in the following alkylation step (the reaction with the alkyl bromide in DMF with cesium carbonate) while the desired product does react, further confirming our structural assignments. Desired product **38** yield: 108 mg (26 %). Byproduct **38b** yield: 80 mg (19 %). The NMR shifts of each compound in both CD₃OD and DMSO-d₆ are reported below.

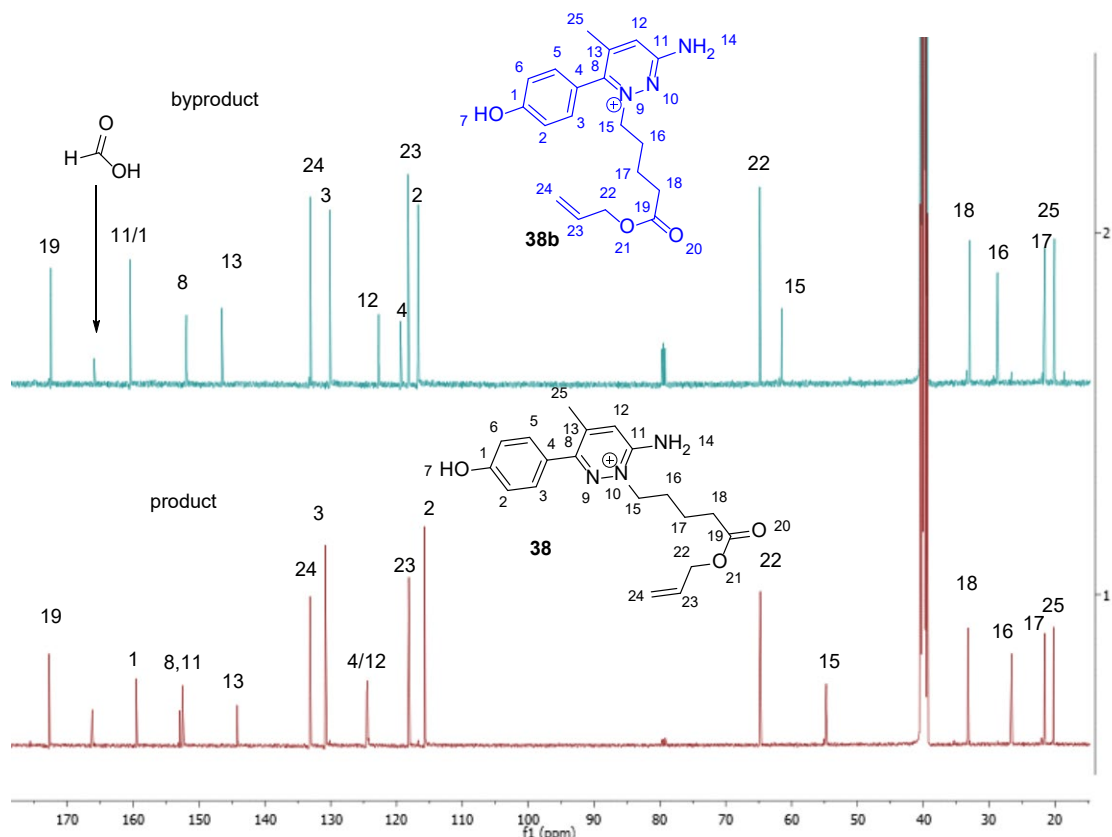
Desired Product: ¹H NMR (500 MHz, **CD₃OD**) δ 8.35 (br.s, 1H), 7.43 – 7.39 (m, 3H), 6.93 (d, *J* = 8.6 Hz, 2H), 5.97-5.89 (m, 1H), 5.30 (dq, *J* = 17.2, 1.7 Hz, 1H), 5.21 (dt, *J* = 10.5, 1.5 Hz, 1H), 4.58 (dt, *J* = 5.6, 1.5 Hz, 2H), 4.36 (t, *J* = 7.2 Hz, 2H), 2.48 (t, *J* = 7.3 Hz, 2H), 2.42 (s, 3H), 2.01 – 1.95 (m, 2H), 1.79-1.73 (m, 2H). ¹³C NMR (126 MHz, **CD₃OD**) δ 173.06, 159.15, 154.36, 152.42, 145.60, 132.27, 130.21, 124.56, 123.28, 116.95, 115.04, 64.77, 55.03, 32.73, 25.92, 21.18, 18.99. ¹H NMR (500 MHz, **DMSO-d₆**) δ 8.48 (s, 1H), 7.50 (s, 1H), 7.39 (d, *J* = 8.3 Hz, 2H), 6.91 (d, *J* = 8.4 Hz, 2H), 5.95-5.87 (m, 1H), 5.29 (dt, *J* = 17.2, 1.7 Hz, 1H), 5.21 (dd, *J* = 10.5, 1.6 Hz, 1H), 4.55 (dd, *J* = 4.4, 2.8 Hz, 2H), 4.29 (t, *J* = 7.2 Hz, 2H), 2.43 (t, *J* = 7.4 Hz, 2H), 2.32 (s, 3H), 1.85-1.79 (m, 2H), 1.67-1.60 (m, 2H). ¹³C NMR (126 MHz, **DMSO-d₆**) δ 172.67, 159.57, 152.94, 152.48, 144.20, 133.10, 130.75, 124.41, 124.34, 118.06, 115.70, 64.67, 54.69, 33.18, 26.49, 21.52, 20.13. (ES-LCMS) *m/z* 342.3 (M+H)⁺

Byproduct: ¹H NMR (500 MHz, **CD₃OD**) δ 8.32 (br.s, 1H), 7.41 (s, 1H), 7.36 – 7.25 (m, 2H), 7.12 – 6.98 (m, 2H), 5.98-5.90 (m, 1H), 5.31 (dt, *J* = 17.3, 1.7 Hz, 1H), 5.23 (dd, *J* = 10.4, 1.6 Hz, 1H), 4.57 (dd, *J* = 5.7, 1.6 Hz, 2H), 4.27 (t, *J* = 7.4 Hz, 2H), 2.31 (t, *J* = 7.2 Hz, 2H), 2.13 (s, 3H), 1.99-1.92 (m, 2H), 1.59-1.53 (m, 2H). ¹³C NMR (126 MHz, **CD₃OD**) δ 172.80, 160.60, 160.04, 151.83, 146.60, 132.27, 129.52, 122.20, 119.60,

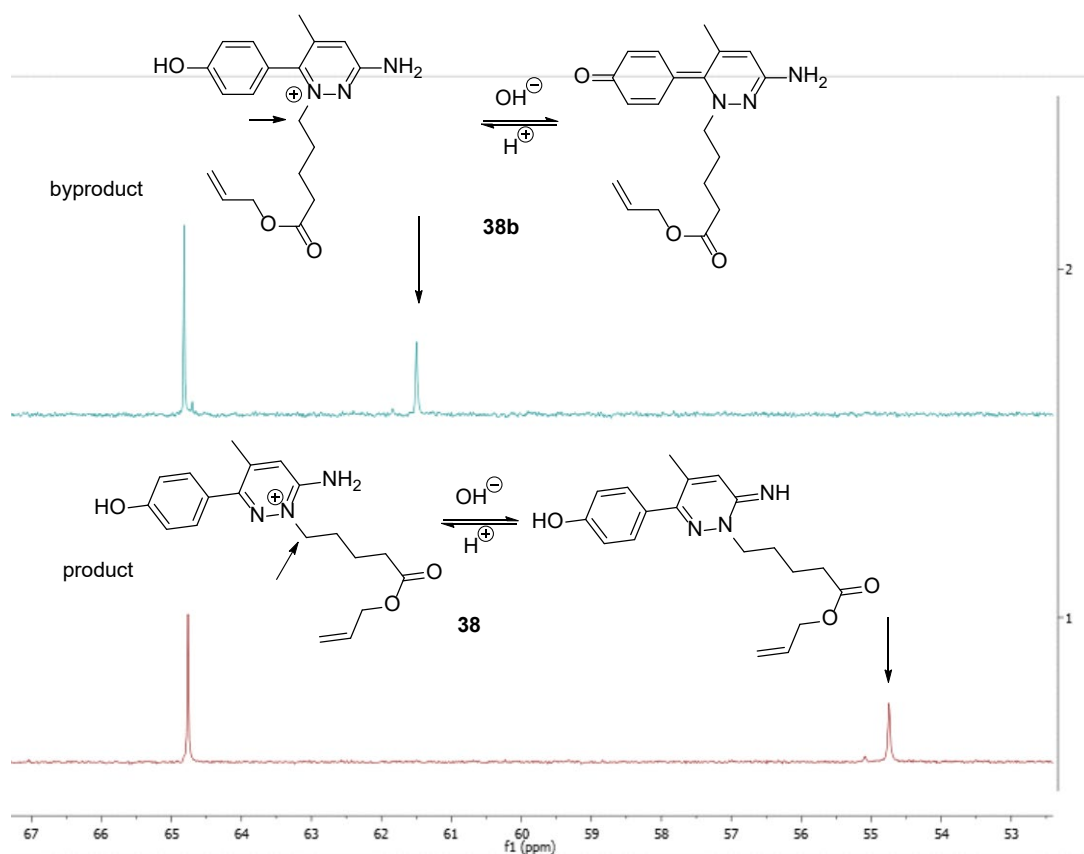
116.97, 116.25, 64.77, 61.52, 32.48, 28.54, 21.11, 18.70. ^1H NMR (500 MHz, **DMSO- d_6**) δ 8.53 (br.s, 1H), 7.85 (s, 2H), 7.49 (s, 1H), 7.38 (d, $J = 8.4$ Hz, 2H), 7.06 (d, $J = 8.3$ Hz, 2H), 5.99-5.92 (m, 1H), 5.34 (dq, $J = 17.2$, 1.7 Hz, 1H), 5.27 (dt, $J = 10.4$, 1.5 Hz, 1H), 4.58 (dt, $J = 5.5$, 1.5 Hz, 2H), 4.19 (t, $J = 7.4$ Hz, 2H), 2.31 (t, $J = 7.3$ Hz, 2H), 2.08 (s, 3H), 1.90 – 1.84 (m, 2H), 1.52-1.46 (m, 2H). ^{13}C NMR (126 MHz, **DMSO- d_6**) δ 172.41, 160.41, 160.35, 151.86, 146.48, 133.06, 130.10, 122.68, 119.39, 118.17, 116.68, 64.73, 61.44, 32.91, 28.64, 21.46, 19.97. (ES-LCMS) m/z 342.3 (M+H) $^+$



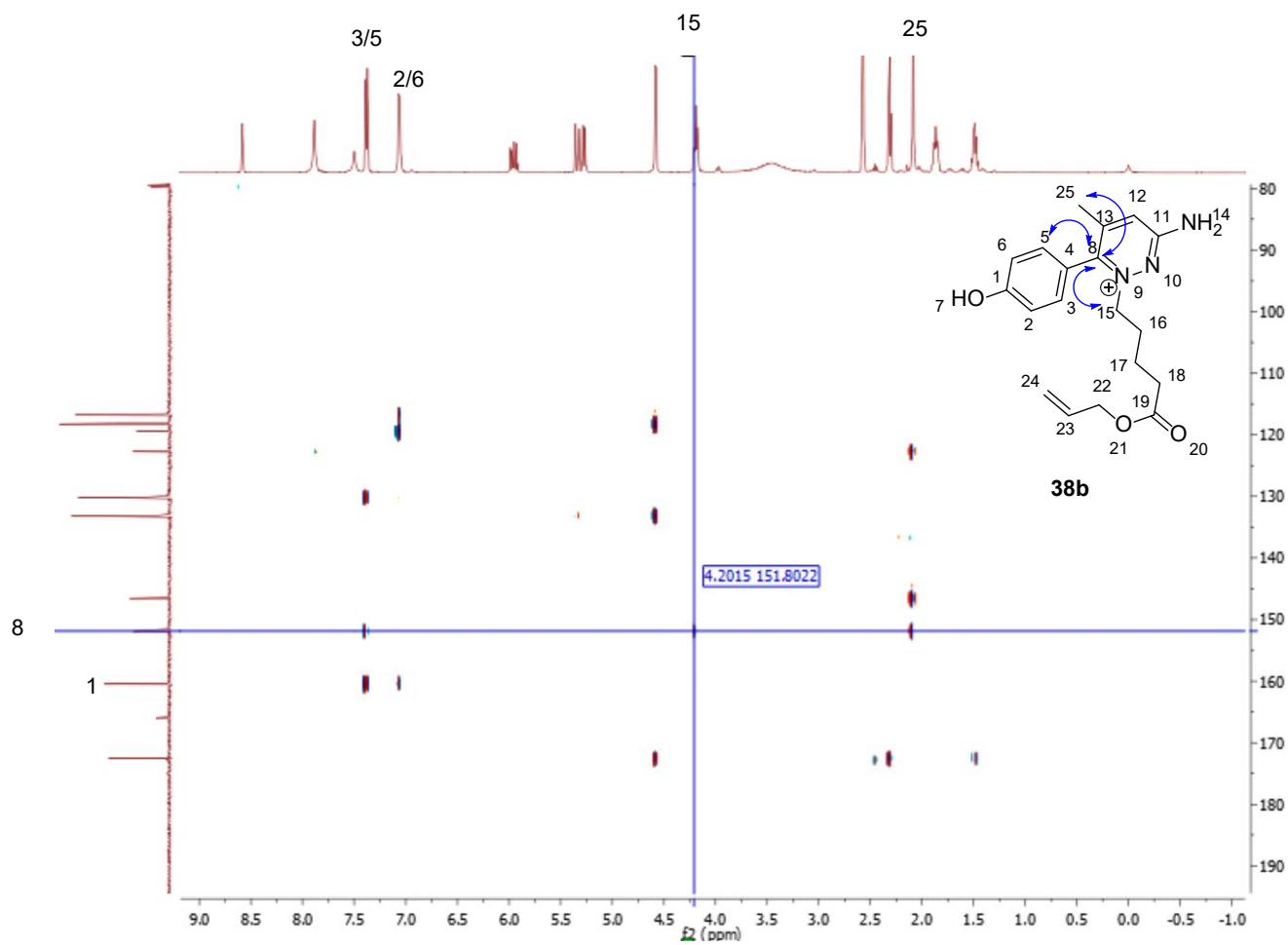
Comparison of ^1H NMR for product (**38**) and byproduct (**38b**) in **DMSO- d_6**



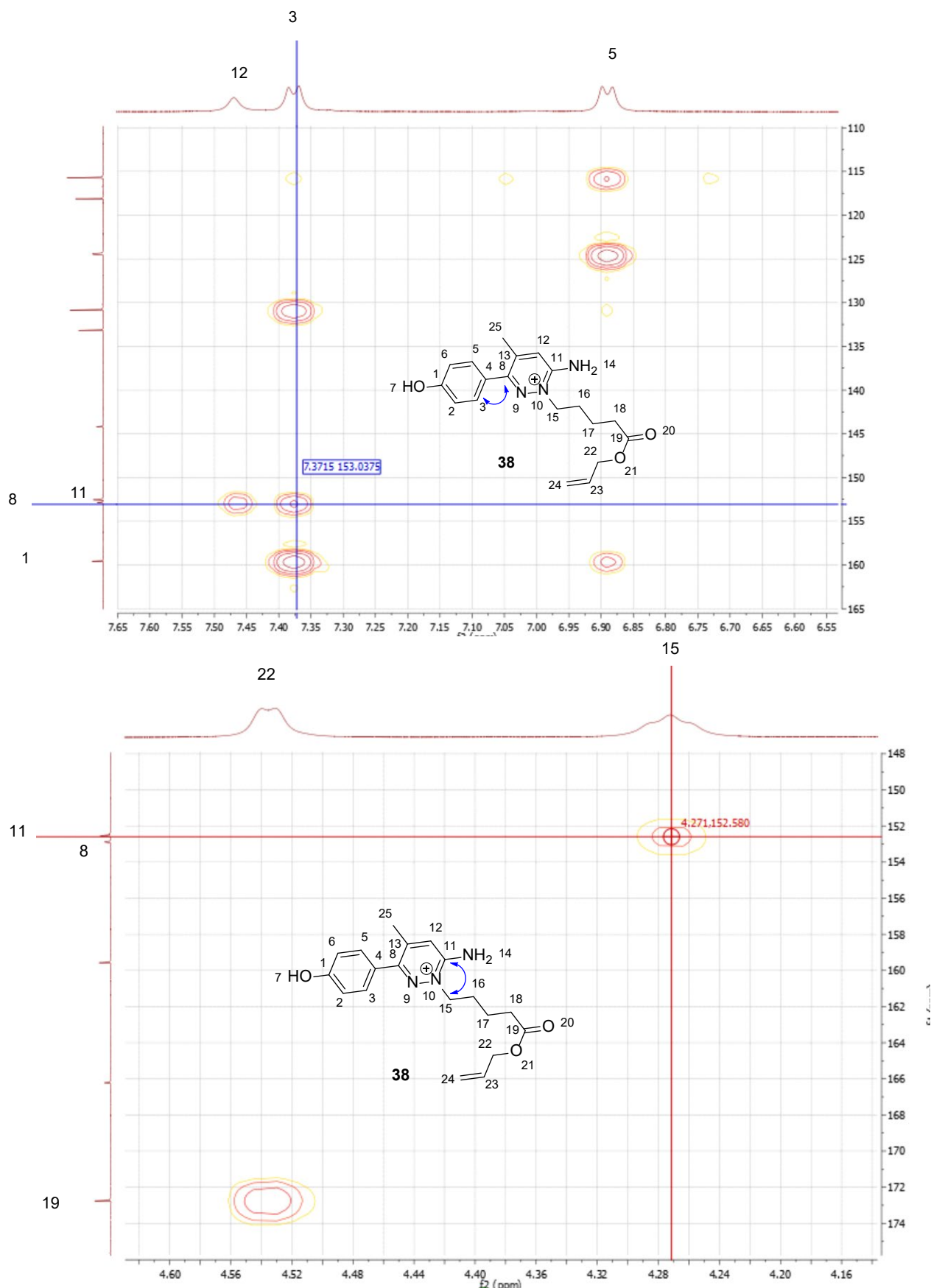
Comparison of ^{13}C NMR for product (38) and byproduct (38b) in DMSO-d_6



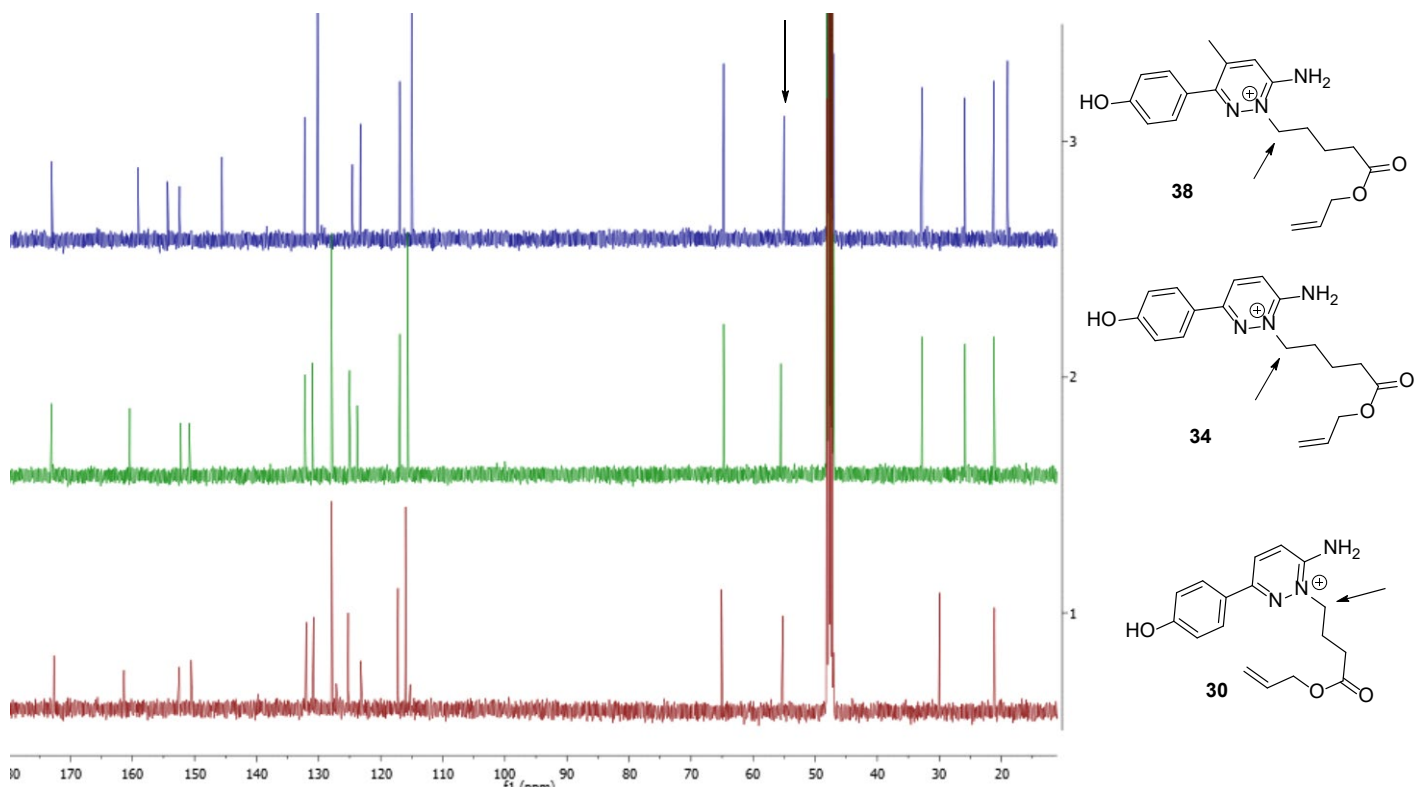
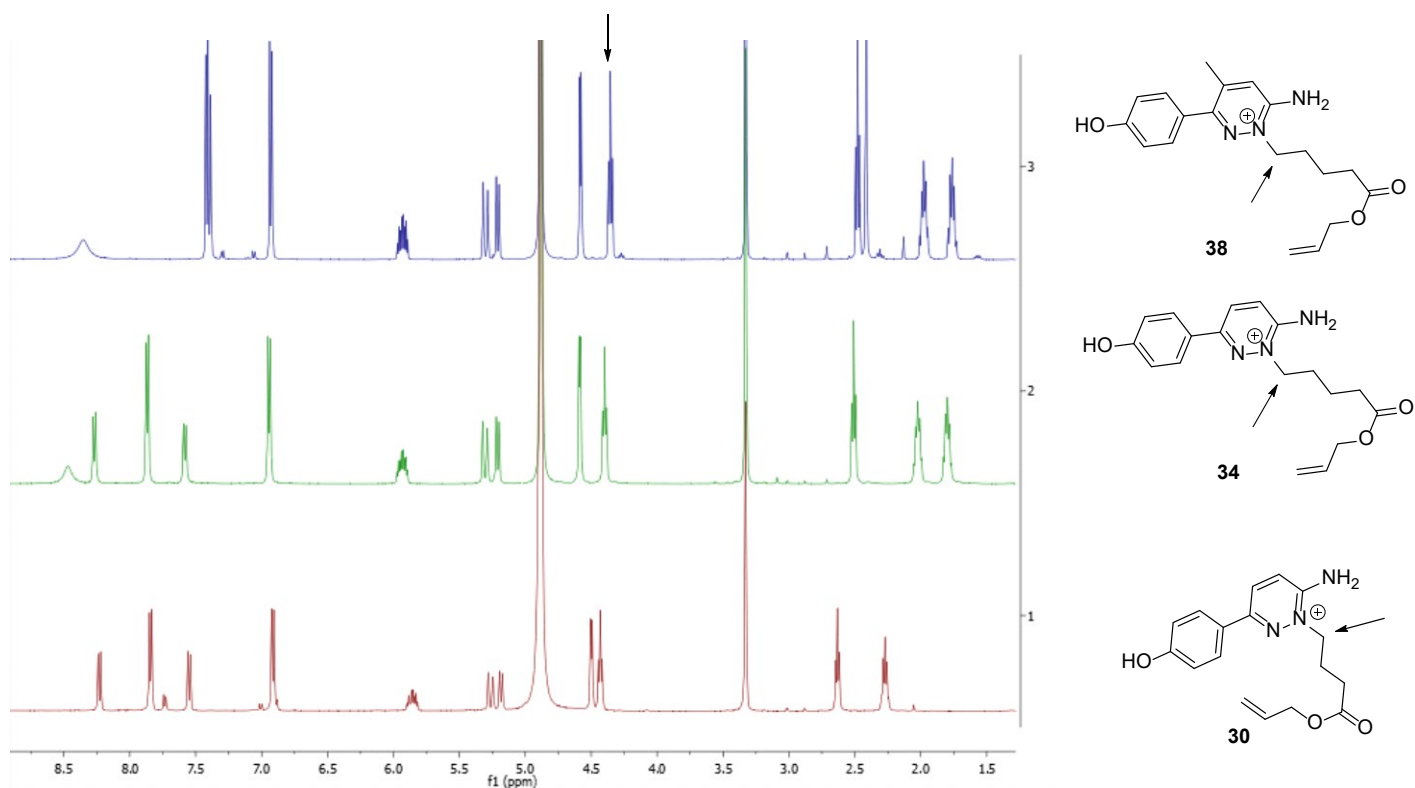
The tautomers and zoomed in versions of ^{13}C NMR spectra for product (38) and byproduct (38b) in DMSO-d_6



The correlation of H5 and C8 & H15 and C8 in HMBC(DMSO- d_6) confirms the structure of the byproduct (38b)

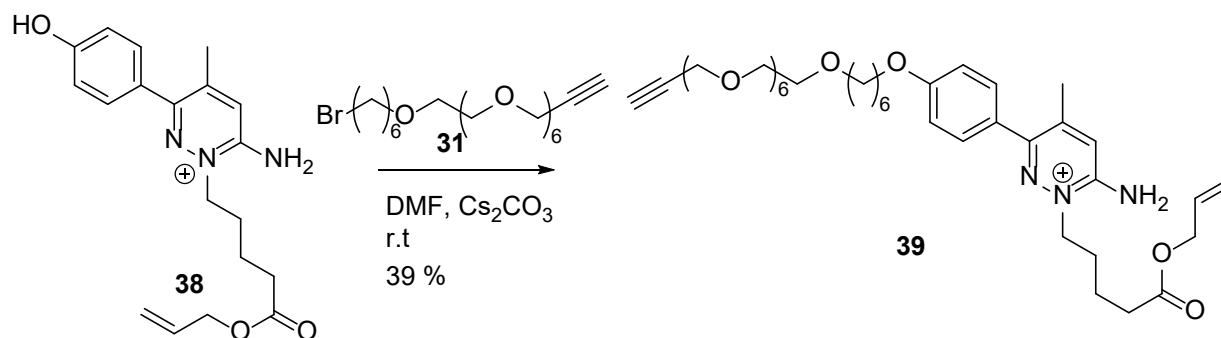


The correlation of H3 and C8 (top) & H15 and C11 (bottom) in HMBC(DMSO-d₆) confirmed the structure of product, **38**



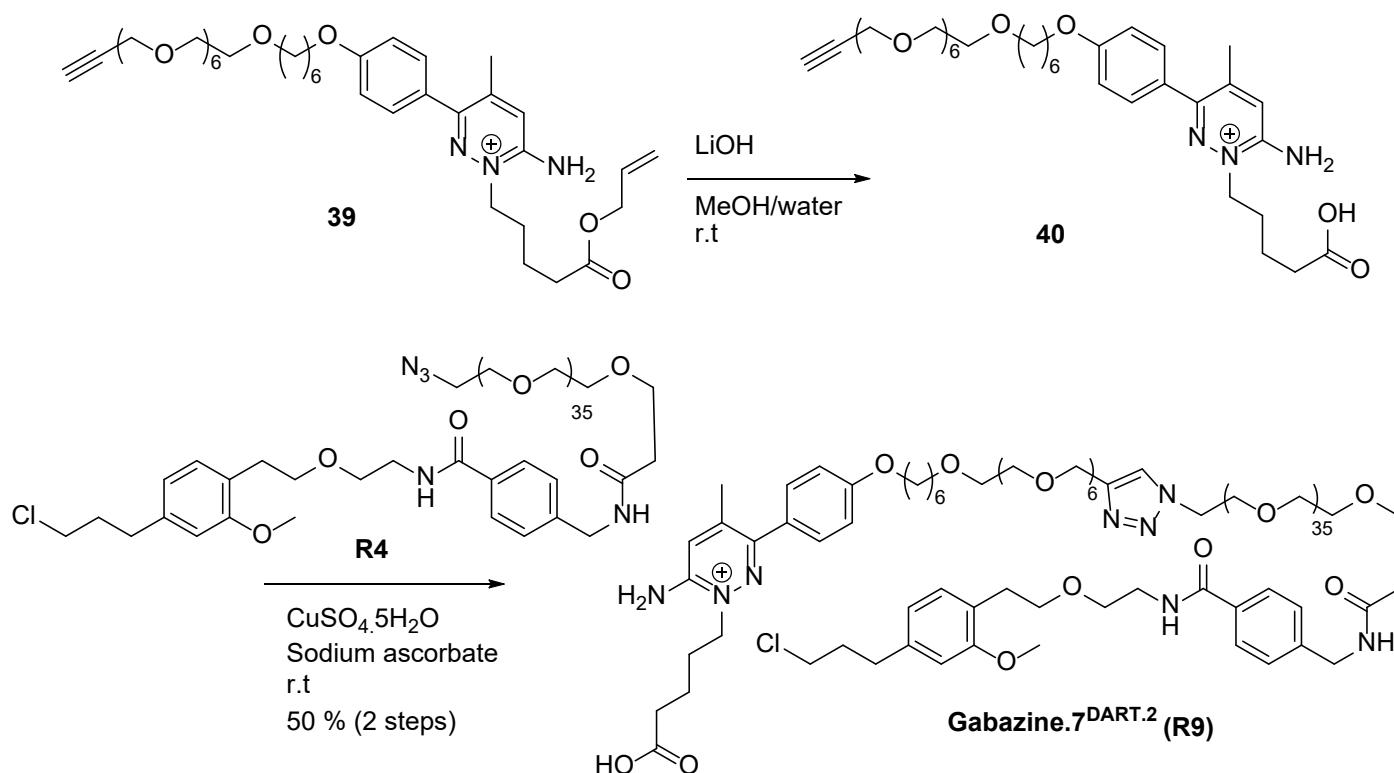
Comparison of ^1H NMR (top), ^{13}C NMR (bottom) in CD_3OD of desired intermediates for Gabazine.1 (30), Gabazine.5 (34), and Gabazine.7 (38)

Step 3: allyl 5-(3-(4-((4,7,10,13,16,19,22-heptaooxaoctacos-1-yn-28-yl)oxy)phenyl)-6-imino-4-methylpyridazin-1(6H)-yl)pentanoate, **39**

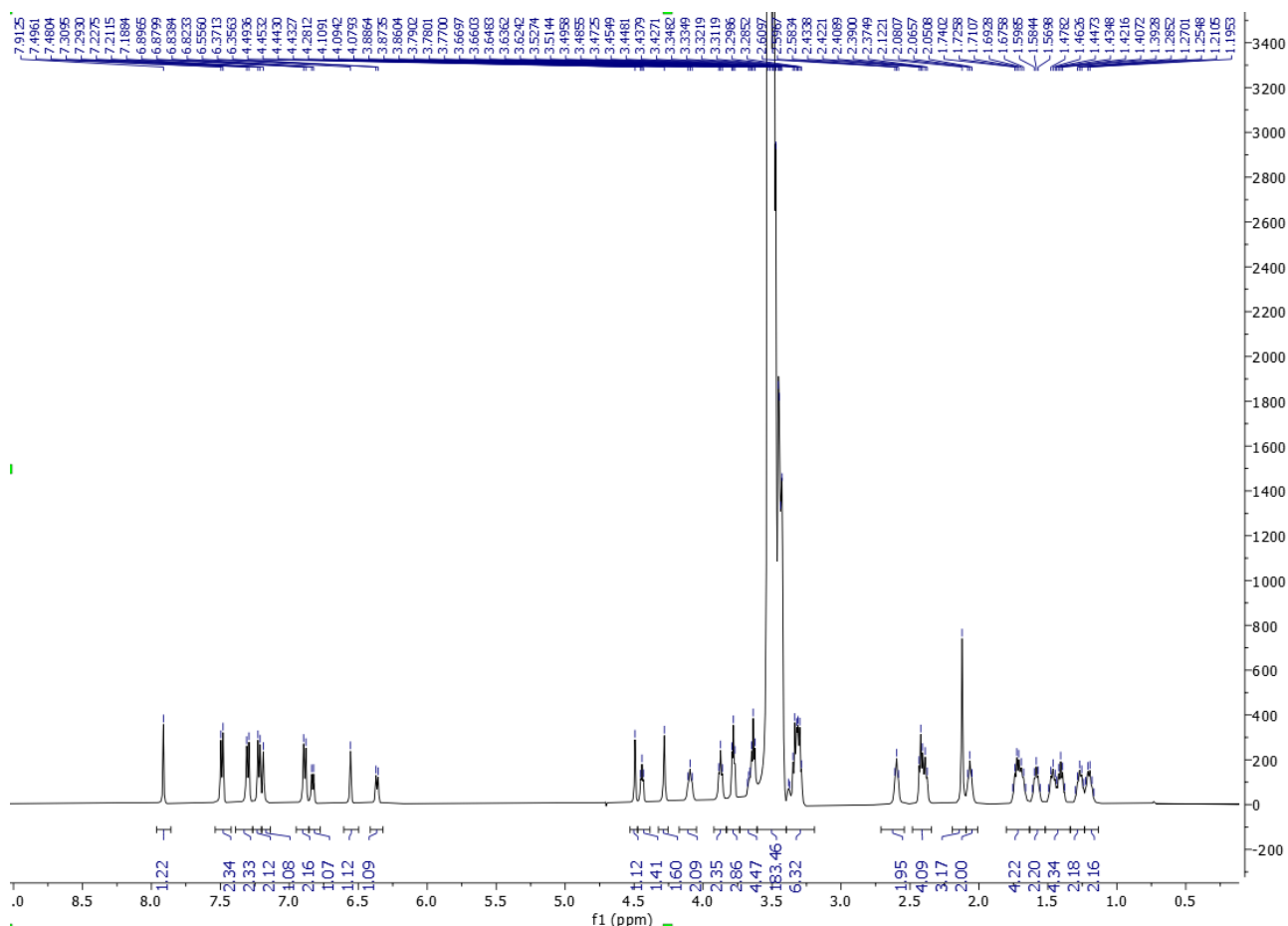


To allyl 5-(3-(4-hydroxyphenyl)-6-imino-4-methylpyridazin-1(6H)-yl)pentanoate, **38** (40 mg, 0.12 mmol) was added anhydrous DMF (1 mL) after which 28-bromo-4,7,10,13,16,19,22-heptaooxaoctacos-1-yne, **31** (85 mg, 0.18 mmol) and anhydrous cesium carbonate (76 mg, 0.23 mmol) were added and the reaction was stirred for 3 h. DMF was removed under a stream of nitrogen, and the crude was purified by reverse phase prep HPLC eluting with 5 to 50 % acetonitrile in water (0.1 % formic acid conditions and other conditions as described in the general chemical synthesis section) to give allyl 5-(3-(4-((4,7,10,13,16,19,22-heptaooxaoctacos-1-yn-28-yl)oxy)phenyl)-6-imino-4-methylpyridazin-1(6H)-yl)pentanoate, **39** (34 mg, 46 μ mol, 39 % yield) as a red/yellow oil. ¹H NMR (500 MHz, CD₃OD) δ 8.49 (s, 1H), 7.57 – 7.45 (m, 2H), 7.39 (d, *J* = 1.3 Hz, 1H), 7.09 – 7.06 (m, 2H), 5.97-5.89 (m, 1H), 5.30 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.21 (dq, *J* = 10.4, 1.4 Hz, 1H), 4.59 (dt, *J* = 5.7, 1.5 Hz, 2H), 4.36 (t, *J* = 7.2 Hz, 2H), 4.20 (d, *J* = 2.4 Hz, 2H), 4.08 (t, *J* = 6.4 Hz, 2H), 3.70 – 3.64 (m, 21H), 3.61 – 3.59 (m, 2H), 3.52 (t, *J* = 6.5 Hz, 2H), 2.86 (t, *J* = 2.4 Hz, 1H), 2.48 (t, *J* = 7.3 Hz, 2H), 2.42 (s, 3H), 2.01 – 1.95 (m, 2H), 1.88 – 1.82 (m, 2H), 1.79-1.73 (m, 2H), 1.67-1.62 (m, 2H), 1.59-1.53 (m, 2H), 1.51-1.45 (m, 2H). ES-LCMS) *m/z* 744.6 (M+H)⁺

Steps 4/5: Synthesis of Gabazine.7^{DART.2} (**R9**)

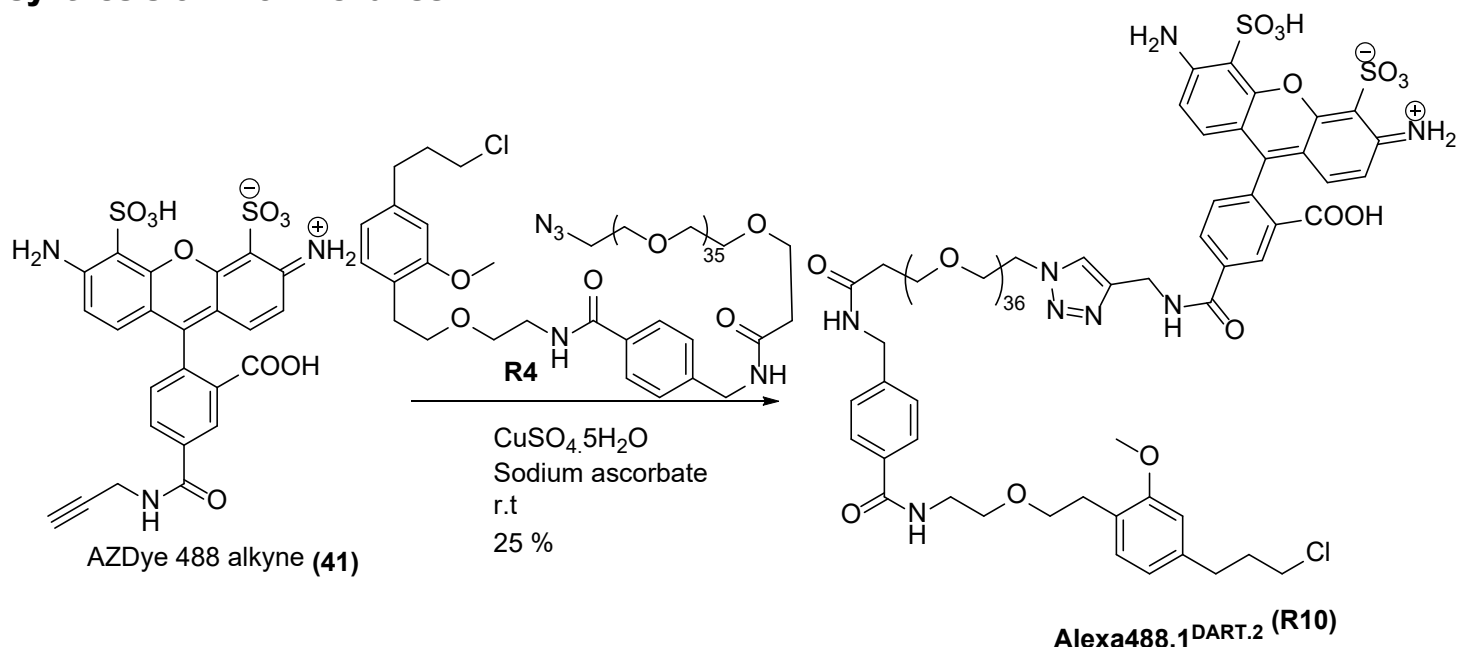


To allyl 5-(3-(4-((4,7,10,13,16,19,22-heptaooxaoctacos-1-yn-28-yl)oxy)phenyl)-6-imino-4-methylpyridazin-1(6H)-yl)pentanoate, **39** (15 mg, 20.0 μmol) in MeOH/water(1:1, 2 ml) was added lithium hydroxide hydrate (5.1 mg, 120 μmol) at room temperature and the reaction was stirred for 2h after which the pH was adjusted to 8 by addition of 1N HCl (110 μL) and solvent was removed under a stream of nitrogen. The crude material **40** was taken up in EtOH/t-BuOH/water (2:1:1, 1 mL) after which azido-PEG36-HTL2.0, **R4** (38 mg, 18 μmol) was added. Finally, Cu(II) sulfate pentahydrate (0.45 mg, 1.8 μmol) and sodium ascorbate (0.32 mg, 1.8 μmol) were added and the reaction mixture was stirred at room temperature overnight after which it was purified by reverse phase prep HPLC eluting with 20 to 90 % acetonitrile in water (0.1 % formic acid, 200 nm wavelength for collection, and other conditions reported in the general chemical synthesis section) to give 5-(3-(4-((1-(1-(1-(4-((2-(4-(3-chloropropyl)-2-methoxyphenethoxy)ethyl)carbamoyl)phenyl)-3-oxo-6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108,111-hexatriacontaoxa-2-azatriecahectan-113-yl)-1H-1,2,3-triazol-4-yl)-2,5,8,11,14,17,20-heptaooxahexacosan-26-yl)oxy)phenyl)-6-imino-4-methylpyridazin-1(6H)-yl)pentanoic acid, **Gabazine.7^{DART.2}**, **R9** (25.0 mg, 50% over 2 steps) ^1H NMR (500 MHz, D_2O , water suppression used- the peaks close to the water peak between 4.4 and 4.6 ppm were partially suppressed) δ 7.91 (s, 1H), 7.49 (d, J = 7.9 Hz, 2H), 7.30 (d, J = 8.2 Hz, 2H), 7.22 (d, J = 8.0 Hz, 2H), 7.19 (s, 1H), 6.89 (d, J = 8.3 Hz, 2H), 6.83 (d, J = 7.6 Hz, 1H), 6.56 (s, 1H), 6.36 (d, J = 7.5 Hz, 1H), 4.49 (s, 2H), 4.44 (t, J = 5.2 Hz, 2H), 4.28 (s, 2H), 4.09 (t, J = 7.4 Hz, 2H), 3.87 (t, J = 6.5 Hz, 2H), 3.78 (t, J = 5.1 Hz, 3H), 3.68-3.62 (m, 5H), 3.53 – 3.43 (m, 183H), 3.38-3.28 (m, 6H), 2.60 (t, J = 6.6 Hz, 2H), 2.43 – 2.37 (m, 4H), 2.12 (s, 3H), 2.07 (t, J = 7.5 Hz, 2H), 1.75-1.66 (m, 4H), 1.61-1.56 (m 2H), 1.49-1.38 (dp, J = 28.1, 7.4 Hz, 4H), 1.30-1.24 (m, 2H), 1.23-1.17 (m, 2H). HRMS (ESI⁺): m/z calcd for $\text{C}_{134}\text{H}_{234}\text{ClN}_8\text{NaO}_{50}$: 1407.2812 $[\text{M}+\text{H}+\text{Na}]^{2+}$; found: 1407.2808 $[\text{M}+\text{H}+\text{Na}]^{2+}$

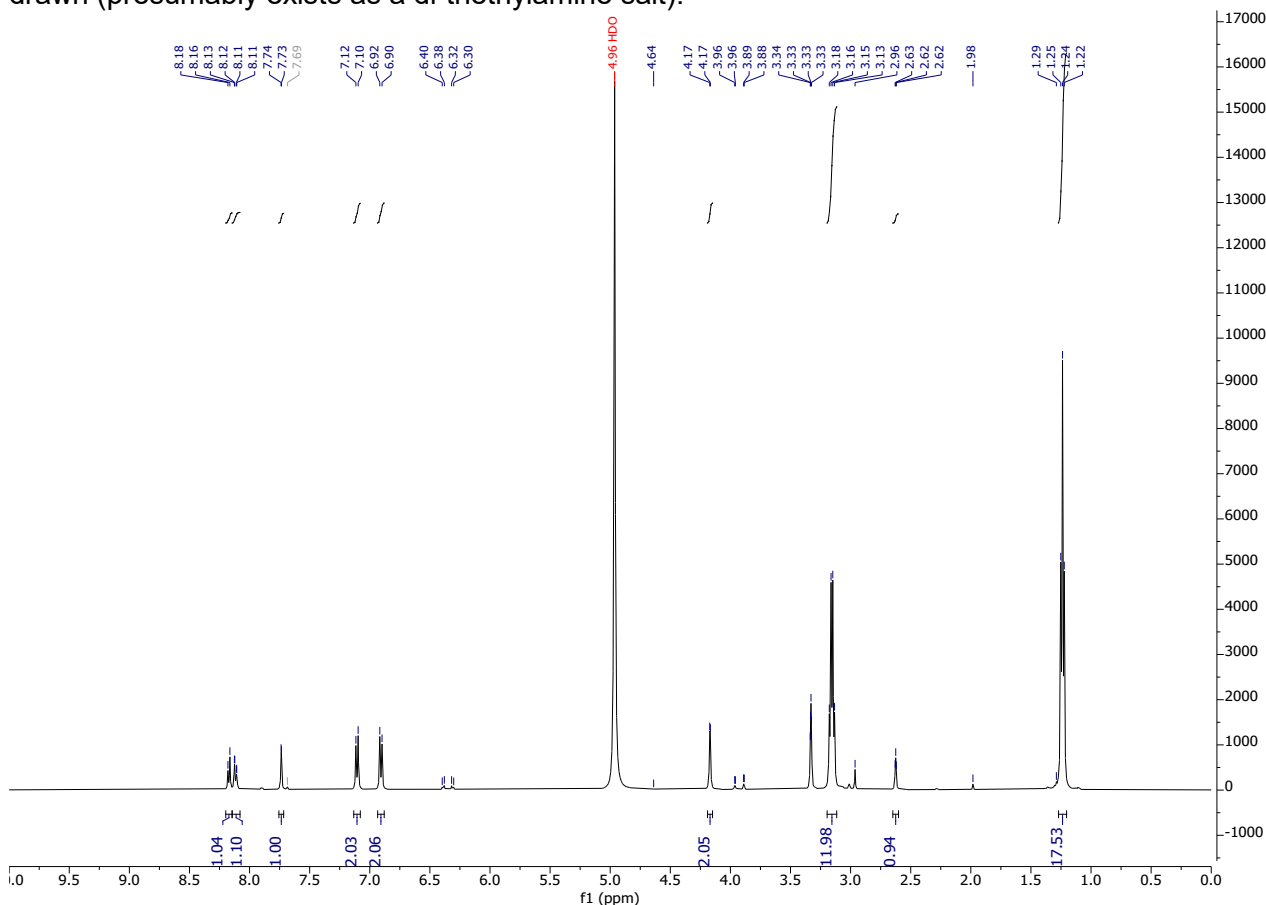


^1H NMR of Gabazine.7^{DART.2} (**R9**) in D_2O (water suppression)

Synthesis of R10—Alexa488.1^{DART.2}



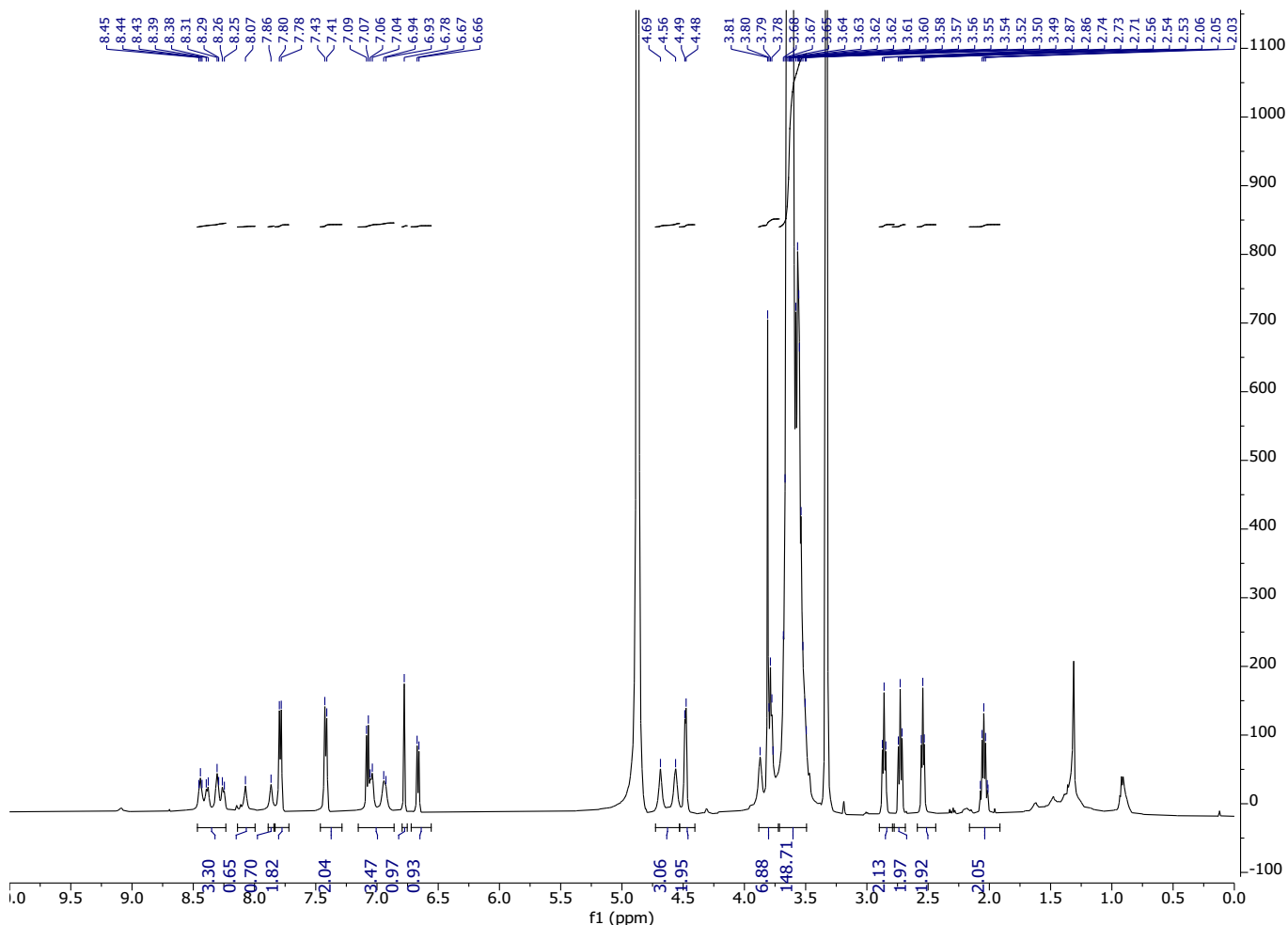
Note: the attachment point of the alkyne in AF488 alkyne, **41** (Click Chemistry Tools; Catalog # 1277-25, MW 773.91 g/mol reported, 571.53 g/mol protonated) or the linker length is not directly disclosed by the vendor, however, based on LCMS as well as ^1H NMR shown below, we are reasonably confident of the structure as drawn (presumably exists as a di-triethylamine salt).



^1H NMR of AZDye 488 Alkyne (**41**) in CD_3OD

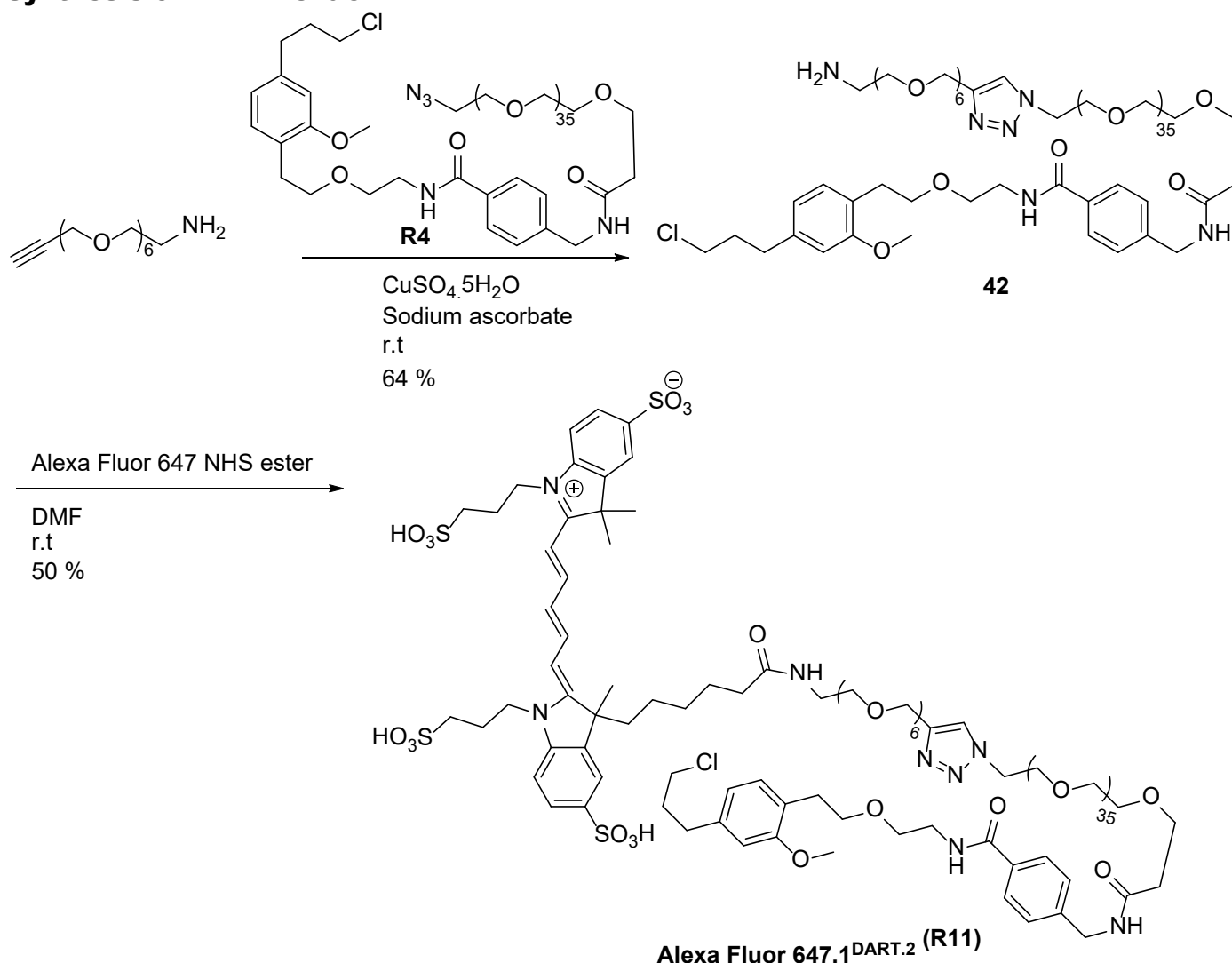
To azido-PEG36-HTL2.0, **R4** (13 mg, 6.5 μmol) was added AF488 alkyne, **41** (5 mg, 6.5 μmol) followed by EtOH/t-BuOH/water (2:1:1, 0.8 mL). Then, copper (II) sulfate pentahydrate (0.1 mg, 0.325 μmol) and sodium

ascorbate (0.1 mg, 0.65 μmol) were added and the reaction was stirred overnight after which it was concentrated under a stream of nitrogen, taken up in acetonitrile/DMSO and purified by 15 to 80 % acetonitrile in water (0.1 % FA) with a Phenomenex 50 x 30 mm column, a 12 min gradient, 50 mL/min flow rate, and collection wavelength of 230 nm. Relevant fractions were concentrated to give 6-amino-9-(2-carboxy-4-(((1-(1-(4-((2-(4-(3-chloropropyl)-2-methoxyphenethoxy)ethyl)carbamoyl)phenyl)-3-oxo-6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108,111-hexatriacontaoxa-2-azatridecahectan-113-yl)-1H-1,2,3-triazol-4-yl)methyl)carbamoyl)phenyl)-3-iminio-5-sulfo-3H-xanthene-4-sulfonate, **Alexa488.1^{DART.2} (R10)** (5 mg, 29 %). ^1H NMR (500 MHz, CD_3OD) δ 8.47 – 8.23 (m, 4H), 8.07 (s, 1H), 7.86 (s, 1H), 7.79 (d, J = 7.8 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.15 – 6.86 (m, 4H), 6.78 (s, 1H), 6.67 (d, J = 7.5 Hz, 1H), 4.62 (d, J = 61.7 Hz, 3H), 4.48 (d, J = 5.4 Hz, 2H), 3.80 (d, J = 11.4 Hz, 7H), 3.71 – 3.49 (m, 149H), 2.86 (t, J = 7.0 Hz, 2H), 2.73 (t, J = 7.4 Hz, 2H), 2.54 (t, J = 6.0 Hz, 2H), 2.05 (p, J = 6.9 Hz, 2H). (ES-LCMS) m/z 665.9 ($\text{M}+4\text{H}$) $^{4+}$; Compound decomposed on HRMS.

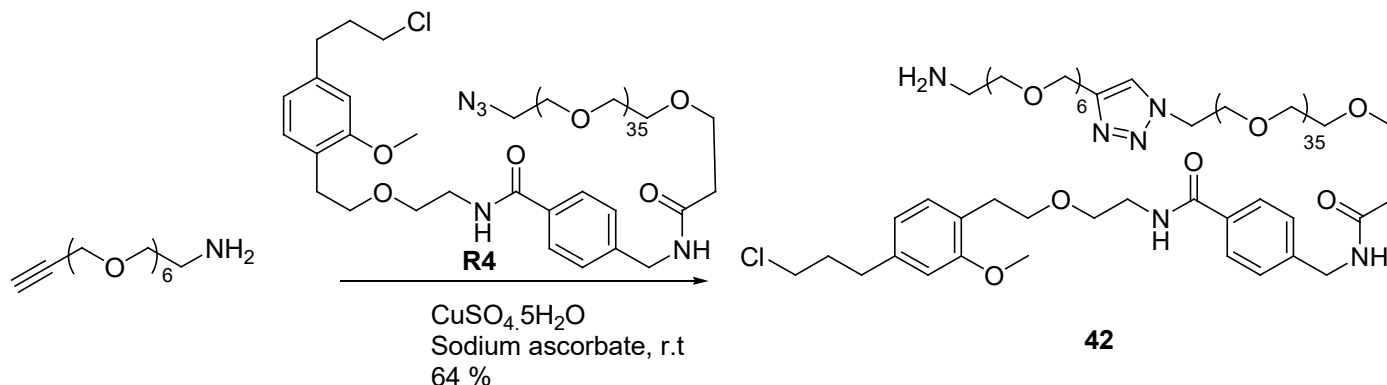


^1H NMR of Alexa488.1^{DART} (R10) in CD_3OD

Synthesis of R11–Alexa647.1^{DART.2}



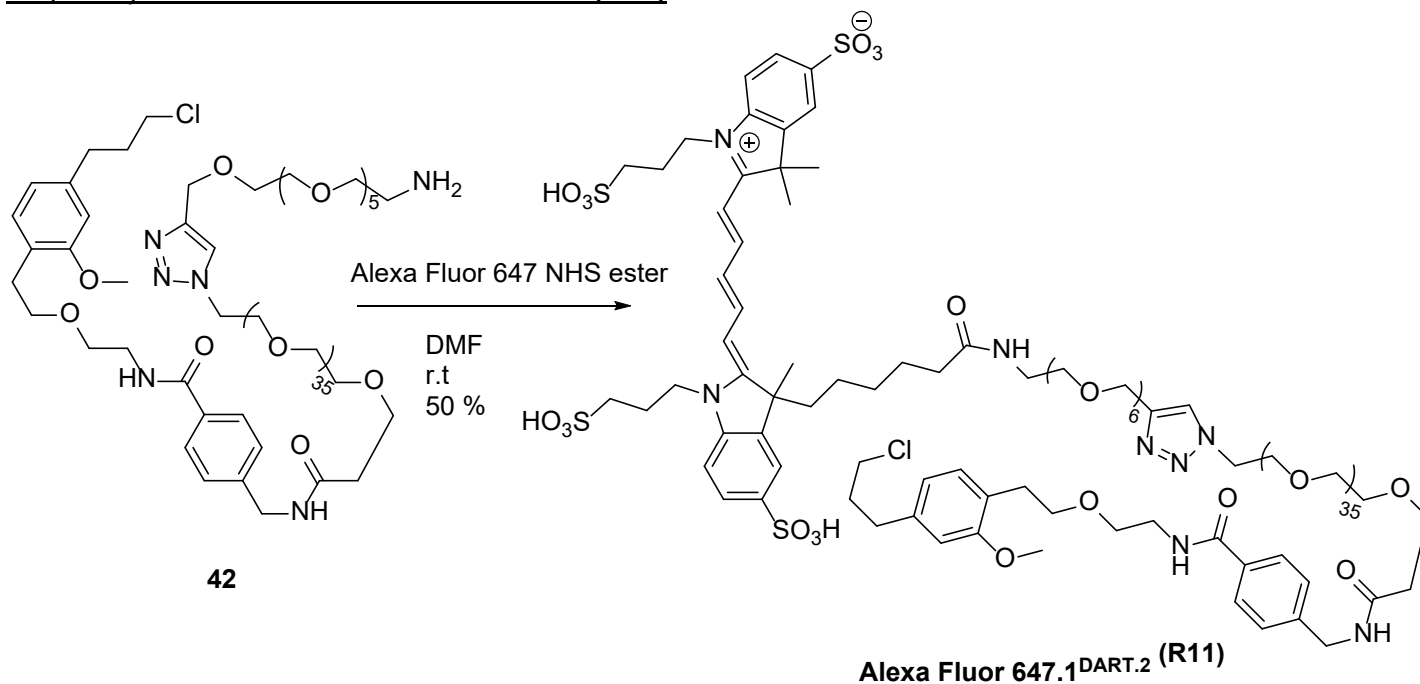
Step 1: Synthesis of 1-(4-(19-amino-2,5,8,11,14,17-hexaoxanonadecyl)-1H-1,2,3-triazol-1-yl)-N-(4-((2-(4-(3-chloropropyl)-2-methoxyphenoxy)ethyl)carbamoyl)benzyl)-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108-hexatriacontaoxaundecahectan-111-amide, **42**



To 3,6,9,12,15,18-hexaoxahenicos-20-yn-1-amine (7.6 mg, 24 μmol) was added azido-PEG36-HTL2.0, **R4** (50 mg, 24 μmol) after which EtOH (0.4 mL)/t-BuOH(0.2 mL)/H₂O(0.2 mL) was added. Then, copper (II) sulfate pentahydrate (0.3 mg, 1.2 μmol) and sodium ascorbate (0.42 mg, 2.4 μmol) were added and the reaction

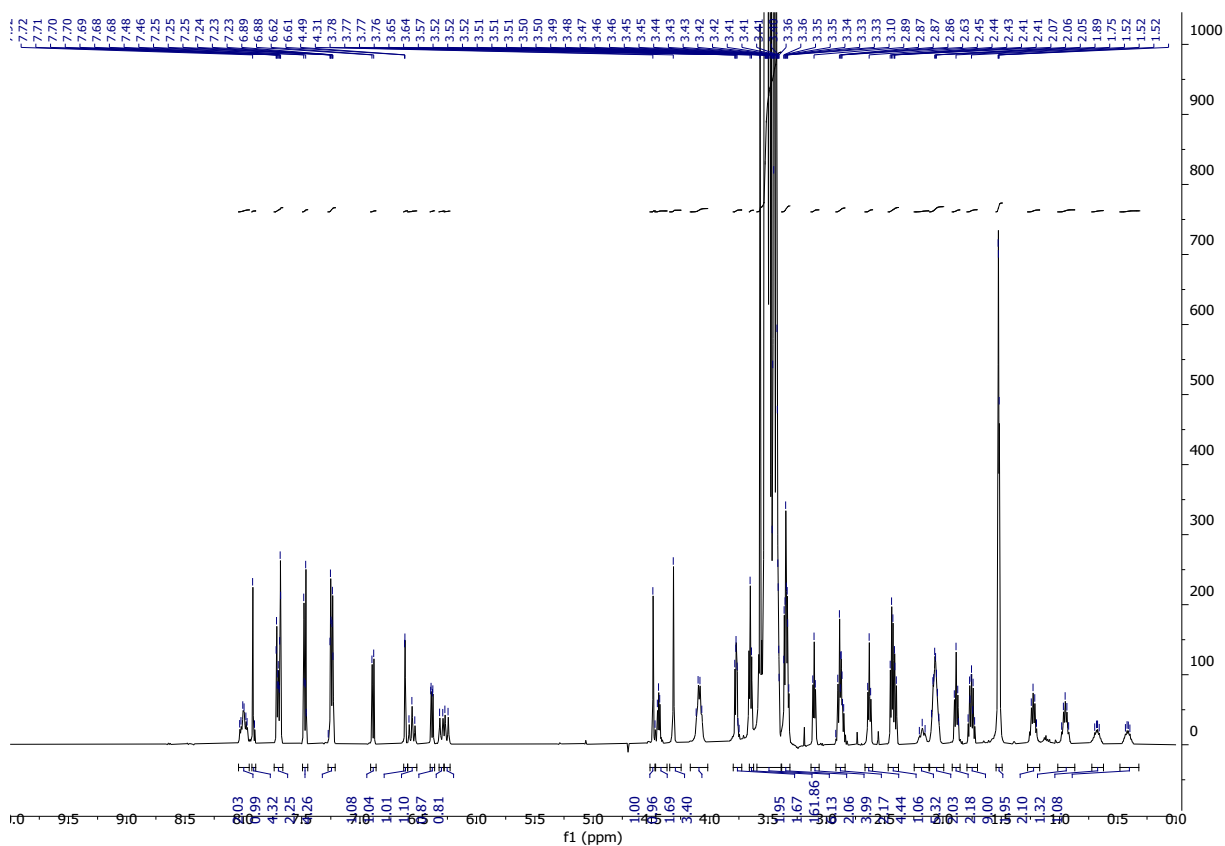
mixture was stirred at room temperature for 2h after which it was directly purified by reverse phase HPLC eluting with 20 to 90 % acetonitrile in water (0.1 % formic acid, 200 nm wavelength for collection and conditions as described in the general chemical synthesis section) to give 1-(4-(19-amino-2,5,8,11,14,17-hexaoxonadecyl)-1H-1,2,3-triazol-1-yl)-N-(4-((2-(4-(3-chloropropyl)-2-methoxyphenoxy)ethyl)carbamoyl)benzyl)-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108-hexatriacontaoxaundecahectan-111-amide, **42** (37 mg, 64 %) as a light brown oil. ¹H NMR (500 MHz, D₂O, water suppression used- the peaks close to the water peak between 4.4 and 4.6 ppm were partially suppressed) δ 7.94 (s, 1H), 7.57 – 7.39 (m, 2H), 7.37 – 7.18 (m, 2H), 6.94 (d, *J* = 7.6 Hz, 1H), 6.65 (d, *J* = 1.6 Hz, 1H), 6.43 (dd, *J* = 7.6, 1.6 Hz, 1H), 4.53 (s, 2H), 4.51 – 4.42 (m, 2H), 4.35 (d, *J* = 4.7 Hz, 2H), 3.82 (dd, *J* = 5.6, 4.5 Hz, 2H), 3.72 – 3.28 (m, 174H), 3.05 (t, *J* = 5.0 Hz, 2H), 2.68 (t, *J* = 6.2 Hz, 2H), 2.45 (td, *J* = 7.6, 6.7, 3.3 Hz, 4H), 1.79 (dq, *J* = 8.5, 6.6 Hz, 2H). (ES-LCMS) *m/z* 602.9 (M+4H)⁴⁺.

Step 2: Synthesis of Alexa Fluor-647.1^{DART.2} (R11)



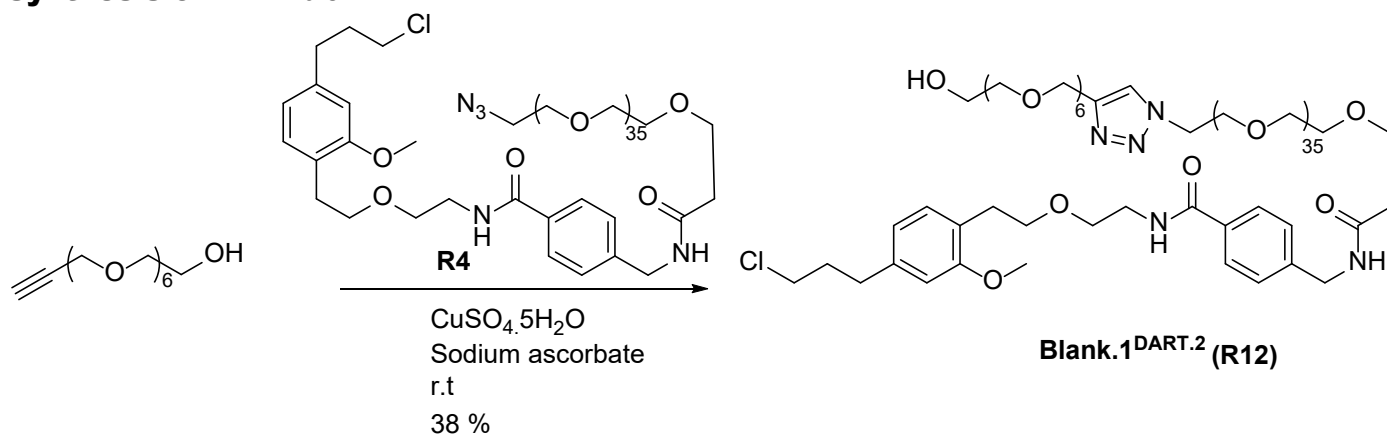
To 1-(4-(19-amino-2,5,8,11,14,17-hexaoxonadecyl)-1H-1,2,3-triazol-1-yl)-N-(4-((2-(4-(3-chloropropyl)-2-methoxyphenoxy)ethyl)carbamoyl)benzyl)-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108-hexatriacontaoxaundecahectan-111-amide, **42** (25 mg, 10.4 μmol) was added DMF (1 mL) followed by AlexaFluor 647 NHS ester (20 mg, 20.8 μmol, ThermoFisher Scientific Catalog No. A37566). The mixture was purged with nitrogen, covered in aluminum foil and stirred overnight after which the reaction mixture was purified by reverse phase prep HPLC eluting with 30 to 60 % acetonitrile in water (0.1 % TFA, 647 nm wavelength for collection and other conditions as described in the general chemical synthesis method) in several injections and concentrated under a stream of nitrogen in the dark to give 2-((1E,3E)-5-(€-3-(1-(1-(4-((2-(4-(3-chloropropyl)-2-methoxyphenoxy)ethyl)carbamoyl)phenyl)-3-oxo-6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108,111-hexatriacontaoxa-2-azatriecahectan-113-yl)-1H-1,2,3-triazol-4-yl)-21-oxo-2,5,8,11,14,17-hexaoxa-20-azahexacosan-26-yl)-3-methyl-5-sulfo-1-(3-sulfopropyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(3-sulfopropyl)-3H-114-indole-5-sulfonic acid, **Alexa Fluor-647.1^{DART.2} (R11)** (17 mg, 50 %) as a deep blue oil which was stored in a vial covered with aluminum foil. ¹H NMR (500 MHz, D₂O, water suppression used- the peaks close to the water peak between 4.25 and 4.5 ppm were partially suppressed) δ 8.00 (td, *J* = 13.2, 5.4 Hz, 2H), 7.92 (s, 1H), 7.73 – 7.66 (m, 4H), 7.49 – 7.45 (m, 2H), 7.24 (dt, *J* = 8.5, 2.1 Hz, 4H), 6.89 (d, *J* = 7.6 Hz, 1H), 6.61 (d, *J* = 1.6 Hz, 1H), 6.55 (t, *J* = 12.5 Hz, 1H), 6.38 (dd, *J* = 7.6, 1.6 Hz, 1H), 6.30 (d, *J* = 13.5Hz, 1H), 6.36 (d, *J* = 13.5Hz, 1H), 4.53 (s, 2H), 4.44 (t, *J* = 5.0 Hz, 2H), 4.31 (s, 2H), 4.09 (q, *J* = 8.1 Hz, 4H), 3.77

(dd, $J = 5.5, 4.4$ Hz, 2H), 3.65 (d, $J = 5.9$ Hz, 2H), 3.59 – 3.39 (m, 162H), 3.34 (ddt, $J = 10.4, 6.9, 3.2$ Hz, 6H), 3.10 (t, $J = 5.3$ Hz, 2H), 2.87 (dt, $J = 10.9, 7.1$ Hz, 4H), 2.63 (t, $J = 6.3$ Hz, 2H), 2.47 – 2.38 (m, 4H), 2.18 (t, $J = 12.3$ Hz, 1H), 2.07 (ddt, $J = 14.2, 10.5, 6.0$ Hz, 5H), 1.89 (t, $J = 7.3$ Hz, 2H), 1.75 (dq, $J = 8.5, 6.5$ Hz, 2H), 1.54 – 1.49 (m, 9H), 1.22 (q, $J = 7.4$ Hz, 2H), 0.95 (p, $J = 7.5$ Hz, 2H), 0.67 (d, $J = 11.6$ Hz, 1H), 0.41 (dd, $J = 12.3, 6.9$ Hz, 1H). Note: the structure of AlexaFluor 647 NHS ester was assumed to be consistent with that reported in reference 7. (ES-LCMS) m/z 813.5 ($M+4H$)⁴⁺; Compound decomposed on HRMS

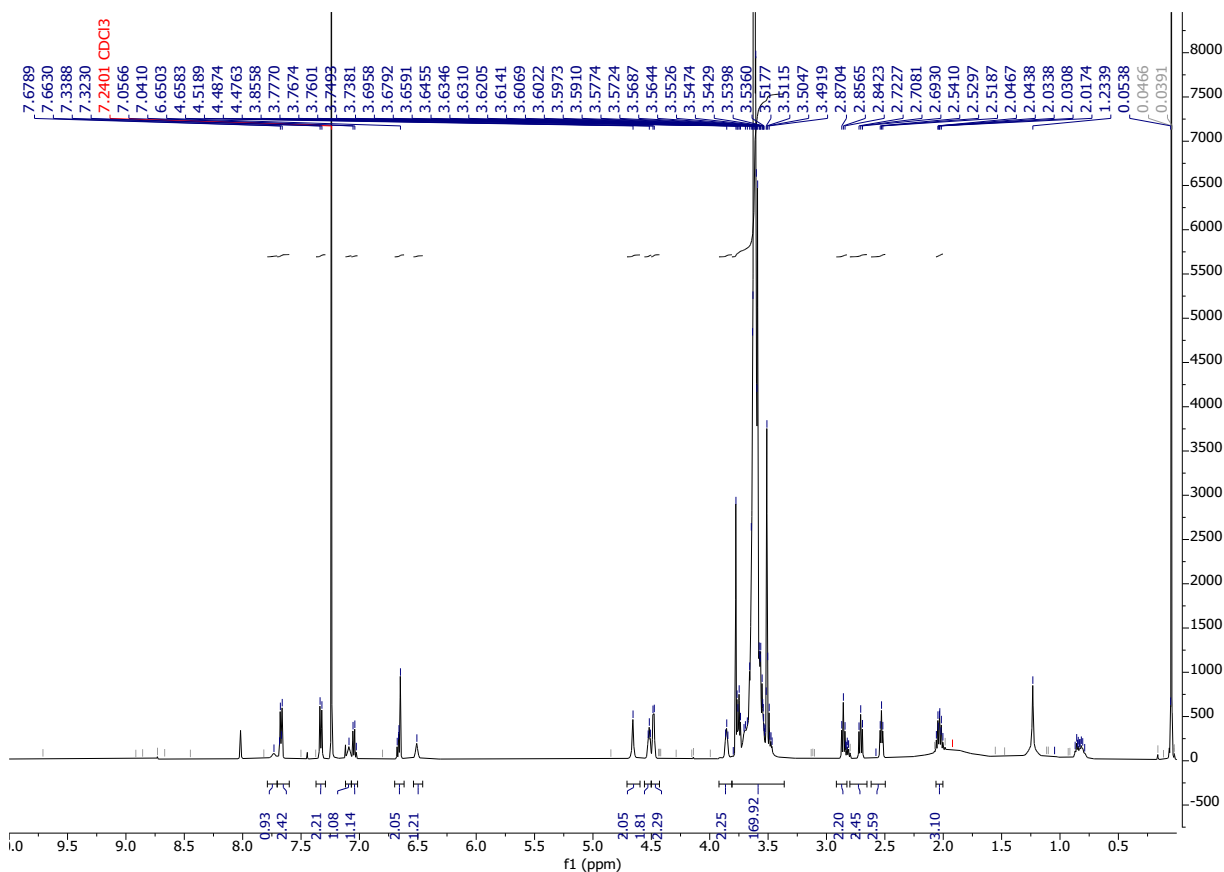


¹H NMR of Alexa Fluor-647.1^{DART.2} (R11) in D₂O (water suppression)

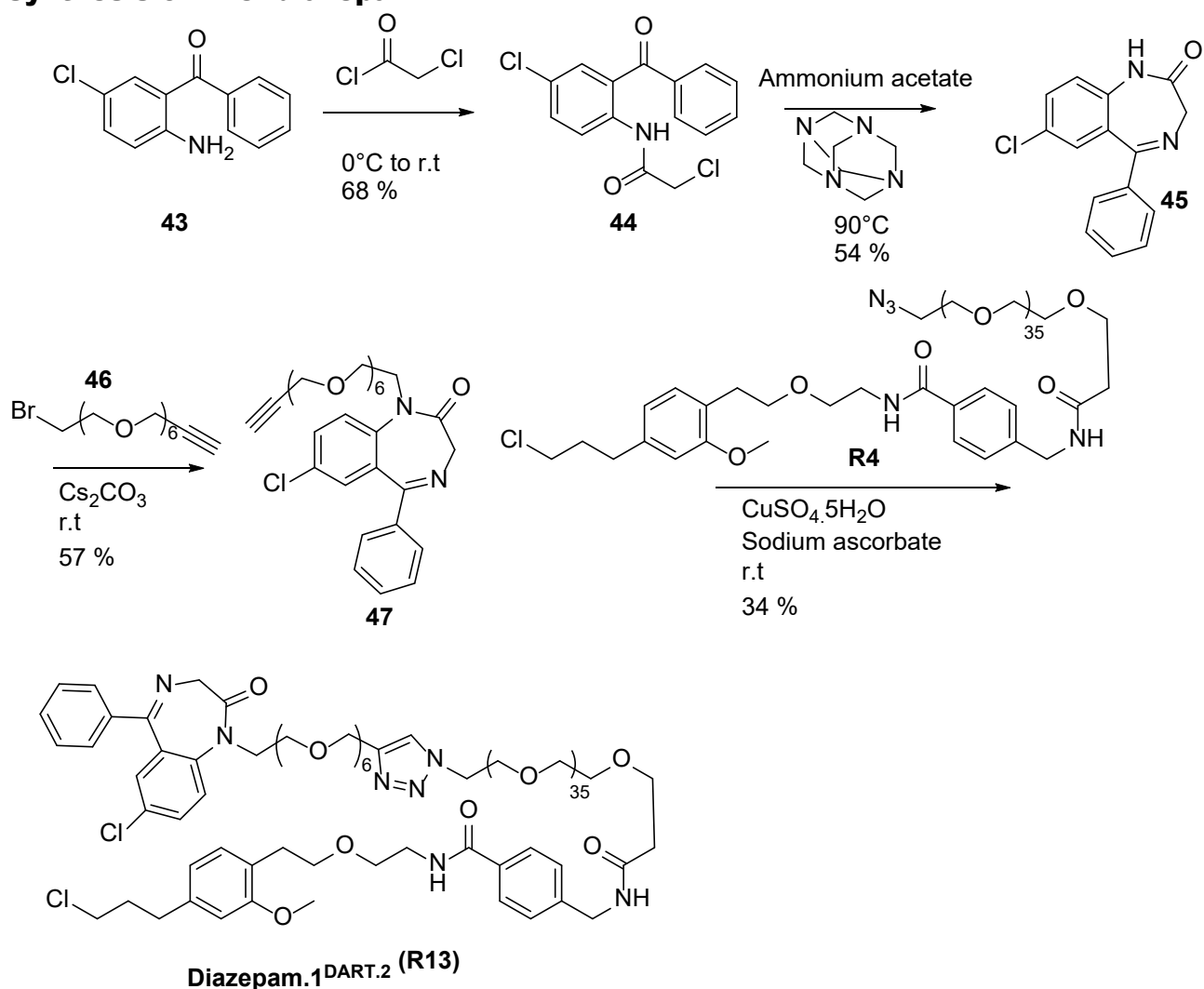
Synthesis of R12–blank.1^{DART.2}



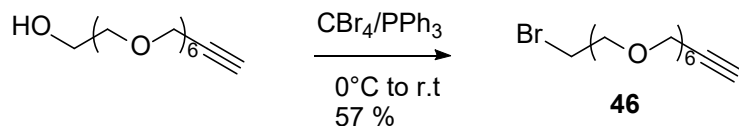
To 3,6,9,12,15,18-hexaoxahenicos-20-yn-1-ol (4.0 mg, 12.5 μmol) was added EtOH:t-BuOH:water (2:1:1, 0.8 mL) followed by azido-PEG36-HTL2.0, **R4** (26.1 mg, 12.5 μmol). Then, copper (II) sulfate pentahydrate (0.16 mg, 0.62 μmol) and sodium ascorbate (0.2 mg, 1.25 μmol) were added and the reaction was stirred overnight at room temperature after which it was purified by reverse phase prep HPLC eluting with 25 to 90 % acetonitrile in water (0.1 % formic acid, 220 nm wavelength for collection and the parameters as described in the general chemical synthesis section). The fraction was lyophilized to give **Blank.1^{DART.2}, R12** (11.4 mg, 38 %). ^1H NMR (500 MHz, CDCl_3) δ 7.73 (br.s, 1H), 7.69-7.66 (m, 2H), 7.34(d, J = 7.9Hz, 2H), 7.09 (br.s, 1H), 7.03(d, J = 7.8Hz, 1H), 6.71 – 6.61 (m, 2H), 6.51 (br, 1H), 4.66 (s, 2H), 4.52 (t, J = 5.0 Hz, 2H), 4.48 (dd, J = 5.0 Hz, 2H), 3.86 (t, J = 4.9 Hz, 2H), 3.79 – 3.47 (m, 170H), 2.88 (t, J = 7.0Hz, 2H), 2.71 (t, J = 7.4 Hz, 2H), 2.53 (t, J = 5.6 Hz, 2H), 2.03 (dq, J = 8.3, 6.6 Hz, 2H). HRMS (ESI⁺): m/z calcd for $\text{C}_{112}\text{H}_{205}\text{ClNaO}_{47}$: 1215.6707 $[\text{M}+\text{Na}+\text{H}]^{2+}$; found: 1215.6749, $[\text{M}+\text{Na}+\text{H}]^{2+}$



Synthesis of R13—diazepam.1^{DART.2}

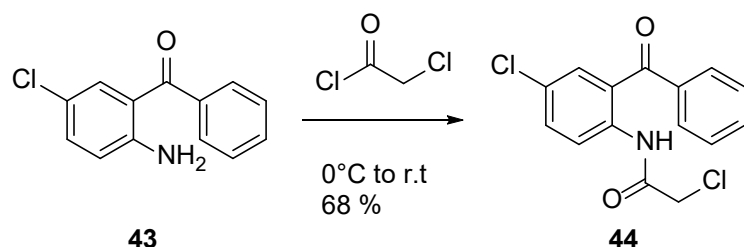


Synthesis of 1-bromo-3,6,9,12,15,18-hexaoxaheptacos-20-yne, **46**



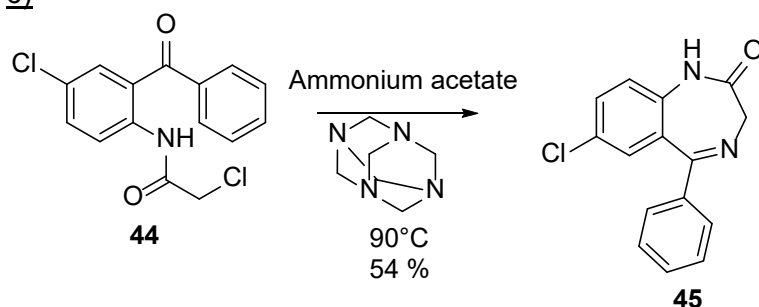
To 3,6,9,12,15,18-hexaoxaheptacos-20-yn-1-ol (1.00 g, 1 equiv., 3.12 mmol) in THF (20 mL) in a cooled ice/water bath was added CBr_4 (1.55 g, 1.50 equiv., 4.68 mmol) and triphenylphosphine (1.23 g, 1.50 equiv., 4.68 mmol) and the reaction mixture was stirred as the ice bath expired for 4 h after which hexanes (15 mL) were added. The mixture was filtered and the solids were discarded. The filtrate was concentrated, triturated in a mixture of hexanes/THF (~ 1:1), filtered and concentrated again to remove more triphenylphosphine oxide. The yellow oil was purified by silica gel chromatography eluting with 0 to 100 % ethyl acetate in hexanes to give 1-bromo-3,6,9,12,15,18-hexaoxaheptacos-20-yne, **46** (688 mg, 1.80 mmol, 57.5 %) as a yellow oil. ^1H NMR (500 MHz, CDCl_3 , contains a small amount of $\text{PPh}_3=\text{O}$) δ 4.19 (d, $J = 2.4$ Hz, 2H), 3.79 (t, $J = 6.4$ Hz, 2H), 3.70 – 3.59 (m, 20H), 3.45 (t, $J = 6.4$ Hz, 2H), 2.41 (t, $J = 2.4$ Hz, 1H). (ES-LCMS) m/z 385.3 ($\text{M}+\text{H}$)⁺.

Step 1: Synthesis of N-(2-benzoyl-4-chlorophenyl)-2-chloroacetamide, **44** (reported in reference 5)



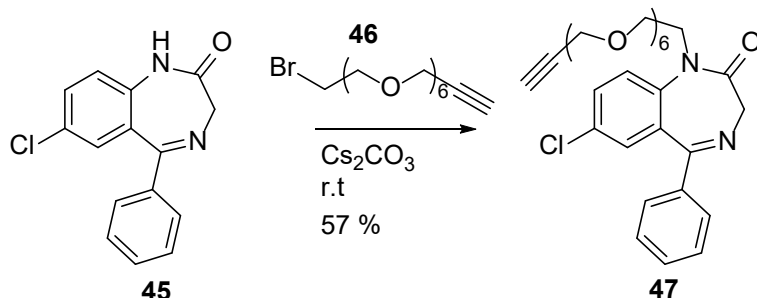
To a cooled (ice/water bath) solution of (2-amino-5-chlorophenyl)(phenyl)methanone, **43** (1 g, 4.32 mmol) in dichloromethane (14.39 mL) under nitrogen flow was added triethylamine (0.902 ml, 6.47 mmol) and DMAP (5.27 mg, 0.043 mmol) after which chloroacetyl chloride (0.380 ml, 4.75 mmol) was added dropwise. Additional chloroacetyl chloride (0.2 ml, 2.497 mmol) was added and the ice bath was removed. The reaction was stirred for another 2 h at room temperature after which the reaction was filtered to remove solids, which were washed with DCM. The organic layer was washed with saturated NaHCO_3 solution (2 x 5 mL), water (5 mL), brine (5 mL), and filtered through an isolate phase separator. The filtrate was concentrated to a brown solid which was crystallized from EtOH to give N-(2-benzoyl-4-chlorophenyl)-2-chloroacetamide, **44** (0.9 g, 2.92 mmol, 67.7 % yield) as a light brown/light yellow solid. ^1H NMR (500 MHz, CDCl_3) δ 11.44 (s, 1H), 8.61 – 8.55 (m, 1H), 7.76 – 7.67 (m, 2H), 7.66 – 7.58 (m, 1H), 7.58 – 7.46 (m, 4H), 4.17 (s, 2H). (ES-LCMS) m/z 310.1 ($\text{M}+\text{H}$) $^+$.

Step 2: Synthesis of 7-chloro-5-phenyl-1H-benzo[e][1,4]diazepin-2(3H)-one, **45** (reported in references 5 and 6)



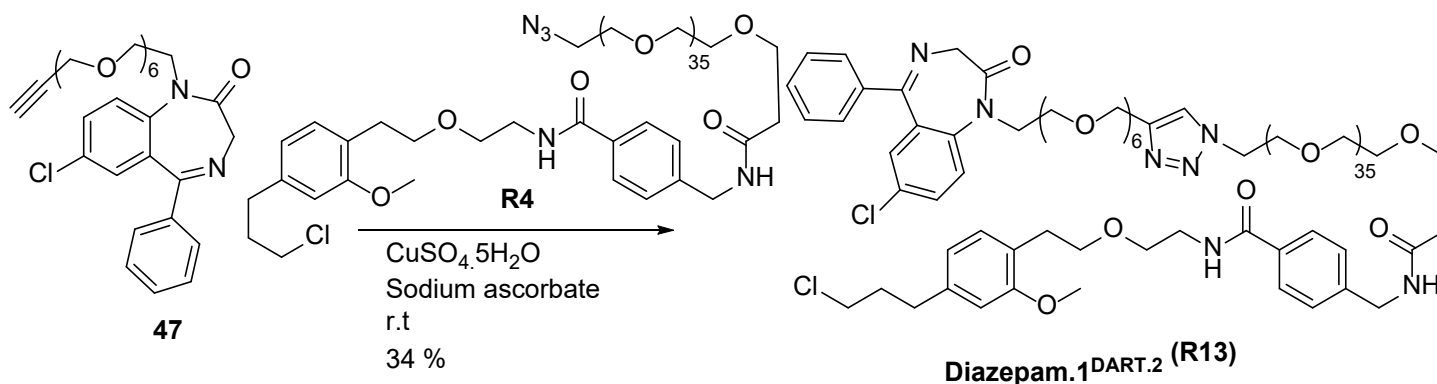
To N-(2-benzoyl-4-chlorophenyl)-2-chloroacetamide, **44** (0.9 g, 2.92 mmol) in ethanol (58.4 mL) was added ammonium acetate (0.45 g, 5.84 mmol) and hexamethylenetetramine (0.82 g, 5.84 mmol) after which the reaction was heated to 90°C for 1 h. Additional ammonium acetate (120 mg, 1.56 mmol) and hexamethylenetetramine (240 mg, 1.7 mmol) were added and the reaction was heated at 75°C overnight after which the reaction was diluted with water (240 mL). The suspension was extracted with ethyl acetate (2 x 100 mL), brine (30 mL), and filtered through an isolate phase separator after which the filtrate was concentrated. The residue was triturated in hexanes/acetone and filtered to give 7-chloro-5-phenyl-1H-benzo[e][1,4]diazepin-2(3H)-one, **45** (0.43 g, 1.588 mmol, 54.4 % yield) as a tan solid. ^1H NMR (500 MHz, Chloroform- d) δ 9.05 (s, 1H), 7.54 – 7.49 (m, 2H), 7.49 – 7.35 (m, 4H), 7.29 (d, J = 2.4 Hz, 1H), 7.10 (d, J = 8.7 Hz, 1H), 4.57 – 4.02 (m, 2H). (ES-LCMS) m/z 271.2 ($\text{M}+\text{H}$) $^+$.

Step 3: Synthesis of 7-chloro-1-(3,6,9,12,15,18-hexaoxahenicos-20-yn-1-yl)-5-phenyl-1H-benzo[e][1,4]diazepin-2(3H)-one



To a vial was added 7-chloro-5-phenyl-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one, **45** (100 mg, 1 equiv., 369 μmol) and DMF (3 mL) after which 1-bromo-3,6,9,12,15,18-hexaoxahenicos-20-yne, **46** (142 mg, 1 equiv., 369 μmol) was added followed by cesium carbonate (181 mg, 1.5 Eq, 554 μmol) and the reaction mixture was allowed to stir overnight. The reaction mixture was diluted with water (5 mL) and ethyl acetate (20 mL). The aqueous layer was extracted once with ethyl acetate (20 mL). Combined organic layers were washed with brine, filtered through an isolate phase separator, concentrated and purified by reverse phase prep HPLC eluting with 20 to 80 % acetonitrile in water (0.1 % formic acid and other conditions as reported in the general chemical synthesis section) to give 7-chloro-1-(3,6,9,12,15,18-hexaoxahenicos-20-yn-1-yl)-5-phenyl-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one, **47** (121 mg, 211 μmol , 57.2 %) as a yellow oil. ^1H NMR (500 MHz, CDCl_3) δ 7.63 (d, J = 8.8 Hz, 1H), 7.61 – 7.57 (m, 2H), 7.49 – 7.43 (m, 2H), 7.42 – 7.36 (m, 2H), 7.22 (d, J = 2.5 Hz, 1H), 4.77 (d, J = 10.4 Hz, 1H), 4.17 (d, J = 2.4 Hz, 2H), 4.16 – 4.09 (m, 1H), 3.89 (ddd, J = 14.3, 5.4, 4.0 Hz, 1H), 3.79 – 3.71 (m, 2H), 3.70 – 3.38 (m, 21H), 2.40 (t, J = 2.4 Hz, 1H). (ES-LCMS) m/z 573.4 ($\text{M}+\text{H}$) $^+$.

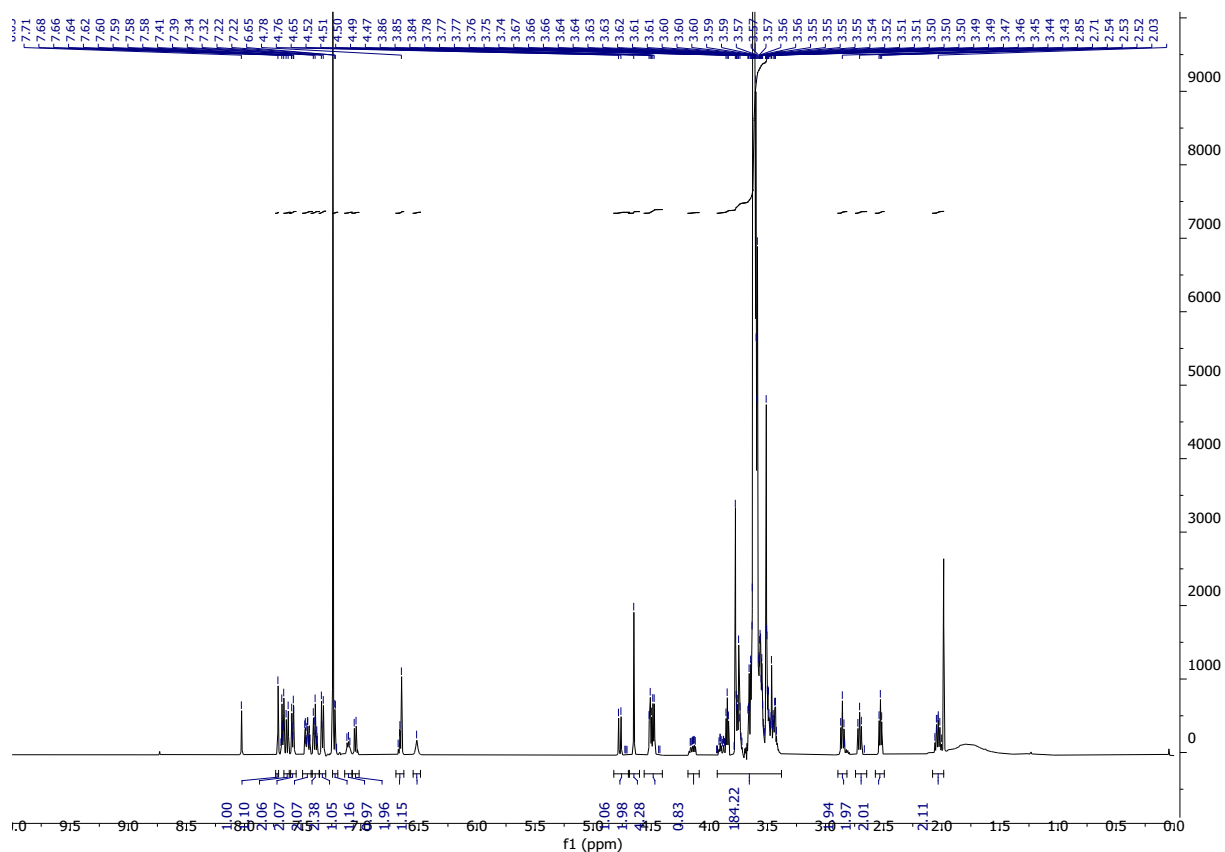
Step 4: Synthesis of Diazepam.1^{DART.2} (R13)



To 7-chloro-1-(3,6,9,12,15,18-hexaoxahenicos-20-yn-1-yl)-5-phenyl-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (5 mg, 8.7 μmol), **47** was added a solution azido-PEG36-HTL2.0, **R4** (18 mg, 8.7 μmol) in EtOH (0.4 mL)/t-BuOH (0.2 mL)/Water (0.2 mL). Then, copper (II) sulfate pentahydrate (0.1 mg, 0.44 μmol) and sodium ascorbate (0.15 mg, 0.87 μmol) were added and the reaction mixture was stirred overnight after which it was purified by reverse phase prep HPLC eluting with 5 to 95 % acetonitrile in water (0.1 % formic acid, 220 nm wavelength for collection and other conditions as reported in the general chemical synthesis methods section) to give 1-(4-(19-(7-chloro-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-1-yl)-2,5,8,11,14,17-hexaoxanonadecyl)-1H-1,2,3-triazol-1-yl)-N-(4-((2-(4-(3-chloropropyl)-2-methoxyphenethoxy)ethyl)carbamoyl)benzyl)-

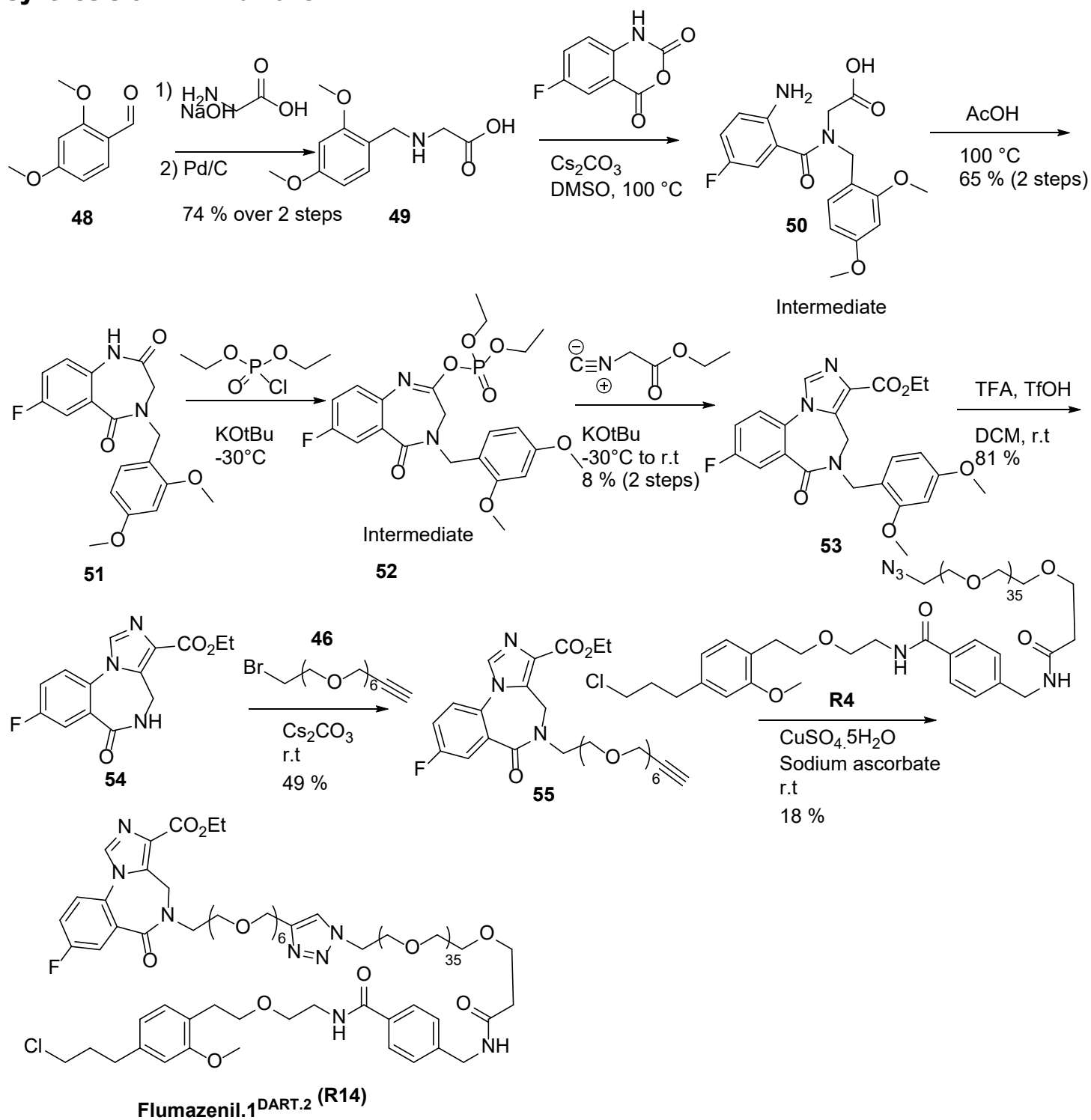
3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108-hexatriacontaoxaundecahectan-111-amide, Diazepam.1^{DART.2}, **R13** (8 mg, 34 %). ^1H NMR (500 MHz, CDCl_3) δ 7.71 (s, 1H), 7.69-7.66 (m, 2H), 7.63 (d, J = 8.9 Hz, 1H), 7.60 – 7.57 (m, 2H), 7.48 – 7.44 (m, 2H), 7.41-7.37 (m, 2H), 7.33 (d, J = 8.2 Hz, 2H), 7.22 (d, J = 2.5 Hz, 1H), 7.10 (t, J = 6.4 Hz, 1H), 7.05 (d, J = 7.8 Hz, 1H), 6.66 (d, J = 6.4 Hz, 2H), 6.52 (br.s, 1H), 4.77 (d, J = 10.5 Hz, 1H), 4.65 (s, 2H), 4.51 (t, J = 5.1 Hz, 2H),

HRMS (ESI+): m/z calcd for $C_{127}H_{214}Cl_2N_7NaO_{47}$: 1341.6934 $[M+Na+H]^{2+}$; found: 1341.6982 $[M+Na+H]^{2+}$



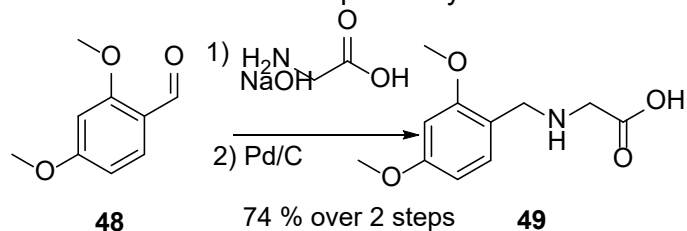
¹H NMR of Diazepam.¹DART.² (R13) in CDCl₃

Synthesis of R14—flumazenil.1^{DART.2}



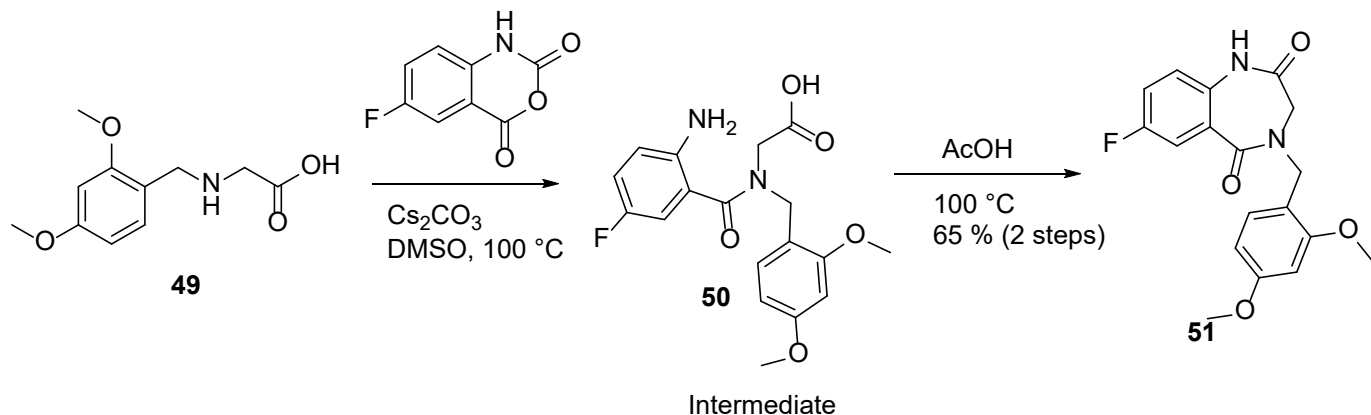
Step 1: Synthesis of 2-((2,4-dimethoxybenzyl)amino)acetic acid, **49**

See reference 8 for the reported synthesis until the debenzylated analog.



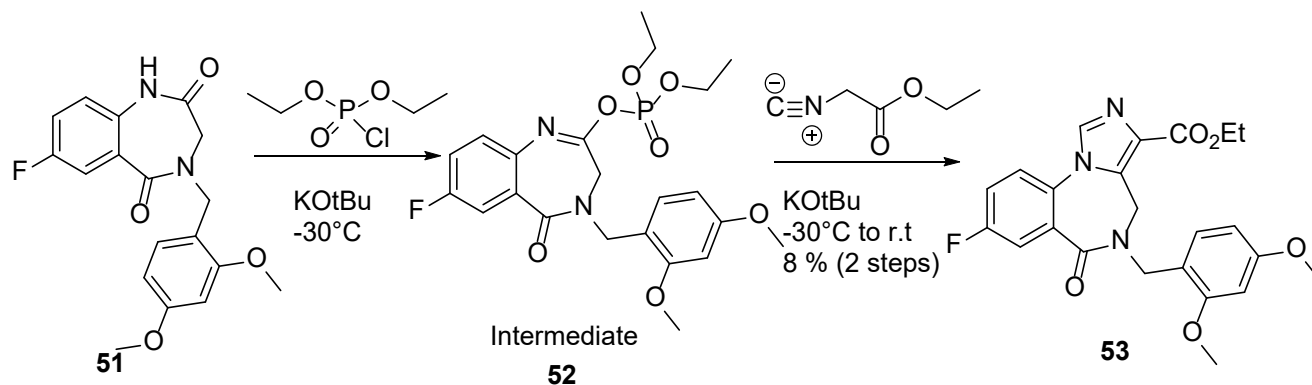
To a round bottom flask was added glycine (2 g, 26.6 mmol) to which 1M NaOH (32 mL) was added. In a separate vial was added 2,4-dimethoxybenzaldehyde, **48** (3.98 g, 23.98 mmol) in methanol (16 mL) and this stirred suspension was added portionwise to the round bottom flask containing glycine/NaOH. A white solid precipitated and the suspension was stirred for 10 min at room temperature after which Pd/C (0.8 g, 0.752 mmol) was added under a stream of nitrogen. The reaction was stirred overnight under an atmosphere of hydrogen after which it was filtered through a pad of celite, washing with hot methanol. The filtrate was concentrated on the rotary evaporator to give a yellow aqueous solution which was cooled in an ice bath. The mixture was acidified (3 M HCl) until pH = 4. The mixture was concentrated to remove water, taken up in methanol and filtered. The filtrate was concentrated then triturated from acetone to give 2-((2,4-dimethoxybenzyl)amino)acetic acid, **49** (4.45 g, 19.76 mmol, 74.2 % yield) as a white solid. ^1H NMR (500 MHz, DMSO-d_6) δ 13.71 (br.s, 1H), 9.26 (br.s, 2H), 7.36 (d, J = 8.4 Hz, 1H), 6.62 (d, J = 2.4 Hz, 1H), 6.57 (dd, J = 8.4, 2.4 Hz, 1H), 4.08 (s, 2H), 3.82 (s, 3H), 3.79 (s, 3H), 3.72 (s, 2H). (ES-LCMS) m/z 226.1 ($\text{M}+\text{H}$) $^+$.

Step 2/3: Synthesis of 4-(2,4-dimethoxybenzyl)-7-fluoro-3,4-dihydro-1H-benzo[e][1,4]diazepine-2,5-dione, **51**



To a vial was added 2-((2,4-dimethoxybenzyl)amino)acetic acid, **49** (2 g, 8.88 mmol) to which DMSO (11 mL) was added. Then, 6-fluoro-1H-benzodioxazine-2,4-dione (1.59 g, 8.78 mmol) was added and the mixture was stirred until all solids dissolved. Cesium carbonate (2.89 g, 8.88 mmol) was added and the reaction mixture was heated at 100 °C for 10 min after the intermediate **50** was observed on LCMS. Pressure buildup was observed during this reaction. Acetic acid (2.033 mL, 35.5 mmol) was added and the reaction mixture was heated at 100 °C overnight. To this mixture was added water and the suspension was thoroughly stirred, then filtered. The collected tan solid was washed generously with water and dried overnight to give 4-(2,4-dimethoxybenzyl)-7-fluoro-3,4-dihydro-1H-benzo[e][1,4]diazepine-2,5-dione, **51** (2 g, 5.81 mmol, 65.4 % yield) as a tan solid. ^1H NMR (500 MHz, Chloroform-d) δ 8.38 (s, 1H), 7.67 (dd, J = 9.0, 3.1 Hz, 1H), 7.27 (d, J = 8.1 Hz, 1H), 7.15 (ddd, J = 8.8, 7.3, 3.1 Hz, 1H), 6.90 (dd, J = 8.8, 4.6 Hz, 1H), 6.44 (d, J = 7.6 Hz, 2H), 4.77 (s, 2H), 3.88 (s, 2H), 3.80 (s, 3H), 3.77 (s, 3H). (ES-LCMS) m/z 345.0 ($\text{M}+\text{H}$) $^+$.

Step 4/5: Synthesis of ethyl 5-(2,4-dimethoxybenzyl)-8-fluoro-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate, **53**



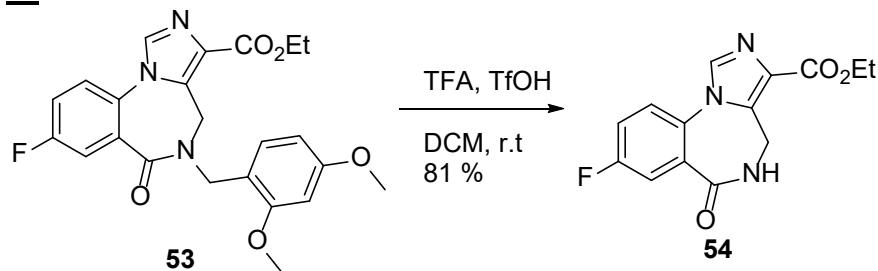
4-(2,4-dimethoxybenzyl)-7-fluoro-3,4-dihydro-1H-benzo[e][1,4]diazepine-2,5-dione, **51** (2 g, 5.81 mmol) was added to a vial to which dry DMF (8 mL) was added followed by potassium tert-butoxide (0.782 g, 6.97 mmol). After stirring for 15 min under nitrogen, the reaction was placed in an acetonitrile/dry ice bath at -30 °C and diethyl chlorophosphate (0.919 mL, 6.39 mmol) was added dropwise over 2 minutes. Upon addition, the reaction was stirred for 20 min and maintained in the cold bath during this time.

In a separate vial, potassium tert-butoxide (0.782 g, 6.97 mmol) was dissolved in anhydrous DMF (3 mL) and after stirring for 5 min at room temperature, this solution was cooled in an acetonitrile/dry ice bath at -50 °C and placed under nitrogen after which ethyl 2-isocyanoacetate (0.698 mL, 6.39 mmol) in DMF (1 mL) was added dropwise. This dark brown solution was immediately added dropwise over 5 min to the solution (which at this point has warmed to -20 °C) containing the intermediate described in the previous paragraph. The resulting mixture was stirred at room temperature for 2 hours after which it was quenched with acetic acid (0.499 mL, 8.71 mmol). Saturated aqueous NH₄Cl solution (5 mL) was added. The reaction mixture was diluted with ethyl acetate (40 mL) and the aqueous layer was extracted with EtOAc (2x30 mL). Combined organic extracts were washed with brine and dried (sodium sulfate). The organic layer was decanted off and concentrated to give a black oil (~ 3 g) with a stench odor.

The mixture was purified by silica gel chromatography eluting with 5 to 50 % ethylacetate in dichloromethane to give 600 mg of a slightly unclean product. This solid was recrystallized from ethyl acetate to give desired product, **53** as a white powder (200 mg, 8 %). ¹H NMR (500 MHz, Chloroform-d) δ 7.86 - 7.75 (m, 2H), 7.38 (dd, J = 8.8, 4.5 Hz, 1H), 7.31 (ddd, J = 8.8, 7.1, 3.0 Hz, 1H), 7.19 (d, J = 8.2 Hz, 1H), 6.52 - 6.28 (m, 2H), 5.36 (d, J = 15.9 Hz, 1H), 5.00 - 4.55 (m, 2H), 4.31 (q, J = 7.1 Hz, 2H), 4.15 (d, J = 15.9 Hz, 1H), 3.77 (d, J = 17.1 Hz, 6H), 1.37 (t, J = 7.1 Hz, 3H). (ES-LCMS) *m/z* 440.3 (M+H)⁺.

Step 6: Synthesis of ethyl 8-fluoro-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate,

54

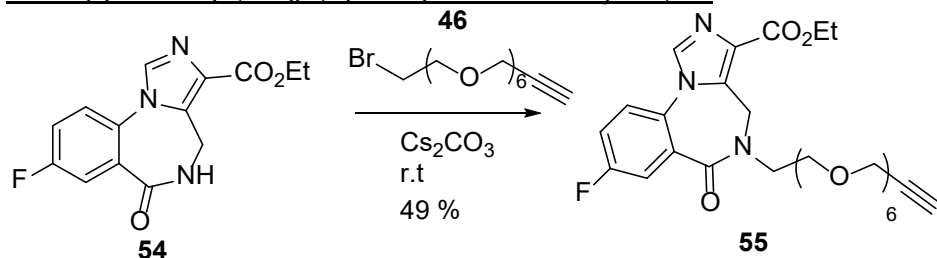


To a vial containing ethyl 5-(2,4-dimethoxybenzyl)-8-fluoro-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate, **53** (0.15 g, 0.341 mmol) was added DCM (1 mL) and the mixture was placed in an ice/water bath after which TFA (1 mL, 12.98 mmol) and trifluoromethanesulfonic acid (69 μL, 0.777 mmol) were added. The reaction mixture turned from light pink to dark purple over the course of 90 minutes. The reaction mixture was concentrated and diluted with DCM/saturated aqueous sodium bicarbonate solution. The solution was stirred and the color changed from purple to a very light pink, and the aqueous layer was slightly basic (pH=8). The aqueous layer was extracted with DCM and all combined organic extracts were washed with brine, filtered through an isolate phase separator, and concentrated to give a white solid which was triturated from hot ethyl

acetate and diethyl ether to give ethyl 8-fluoro-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate, **54** (80 mg, 0.277 mmol, 81 % yield) as a white solid.

^1H NMR (500 MHz, CDCl_3) δ 7.85 – 7.77 (m, 2H), 7.44 (dd, J = 8.8, 4.5 Hz, 1H), 7.37 (ddd, J = 8.9, 7.0, 2.9 Hz, 1H), 6.52 (t, J = 6.3 Hz, 1H), 4.41 (q, J = 7.1 Hz, 2H), 3.84 – 3.63 (m, 1H), 1.42 (t, J = 7.1 Hz, 3H). (ES-LCMS) m/z 290.1 ($\text{M}+\text{H}$) $^+$.

Step 7: Synthesis of ethyl 8-fluoro-5-(3,6,9,12,15,18-hexaoxahenicos-20-yn-1-yl)-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate, **55**

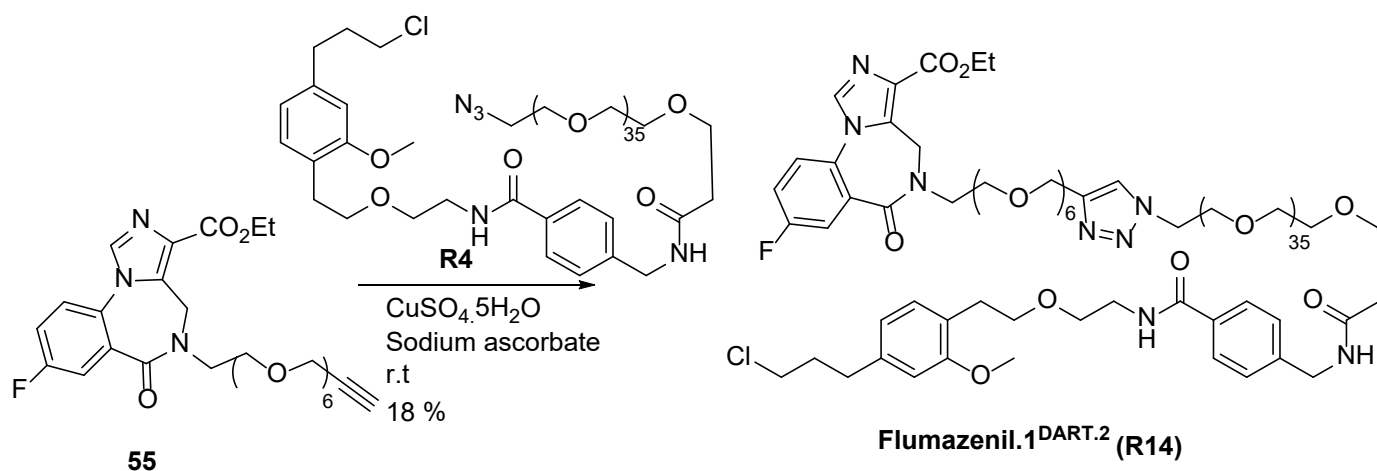


In a vial was added ethyl 8-fluoro-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate, **54** (10 mg, 0.035 mmol) followed by anhydrous DMF (700 μL) and 1-bromo-3,6,9,12,15,18-hexaoxahenicos-20-yne, **46** (13.25 mg, 0.035 mmol) followed by anhydrous cesium carbonate (22.53 mg, 0.069 mmol) and the reaction was stirred at room temperature overnight after which additional alkyl bromide (5 mg) was added. After stirring for several hours, no change was detected by LCMS (still some SM remaining).

The reaction was diluted with ethyl acetate (20 mL) and water (5 mL). The organic layer was washed with brine (5 mL), filtered through an isolate phase separator, concentrated and purified by RP HPLC eluting with 5 to 70 % acetonitrile in water (0.1 % FA and the rest of conditions as described in the general chemistry synthesis section) to give ethyl 8-fluoro-5-(3,6,9,12,15,18-hexaoxahenicos-20-yn-1-yl)-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate, **55** (10 mg, 0.017 mmol, 48.9 % yield) as a brown oil.

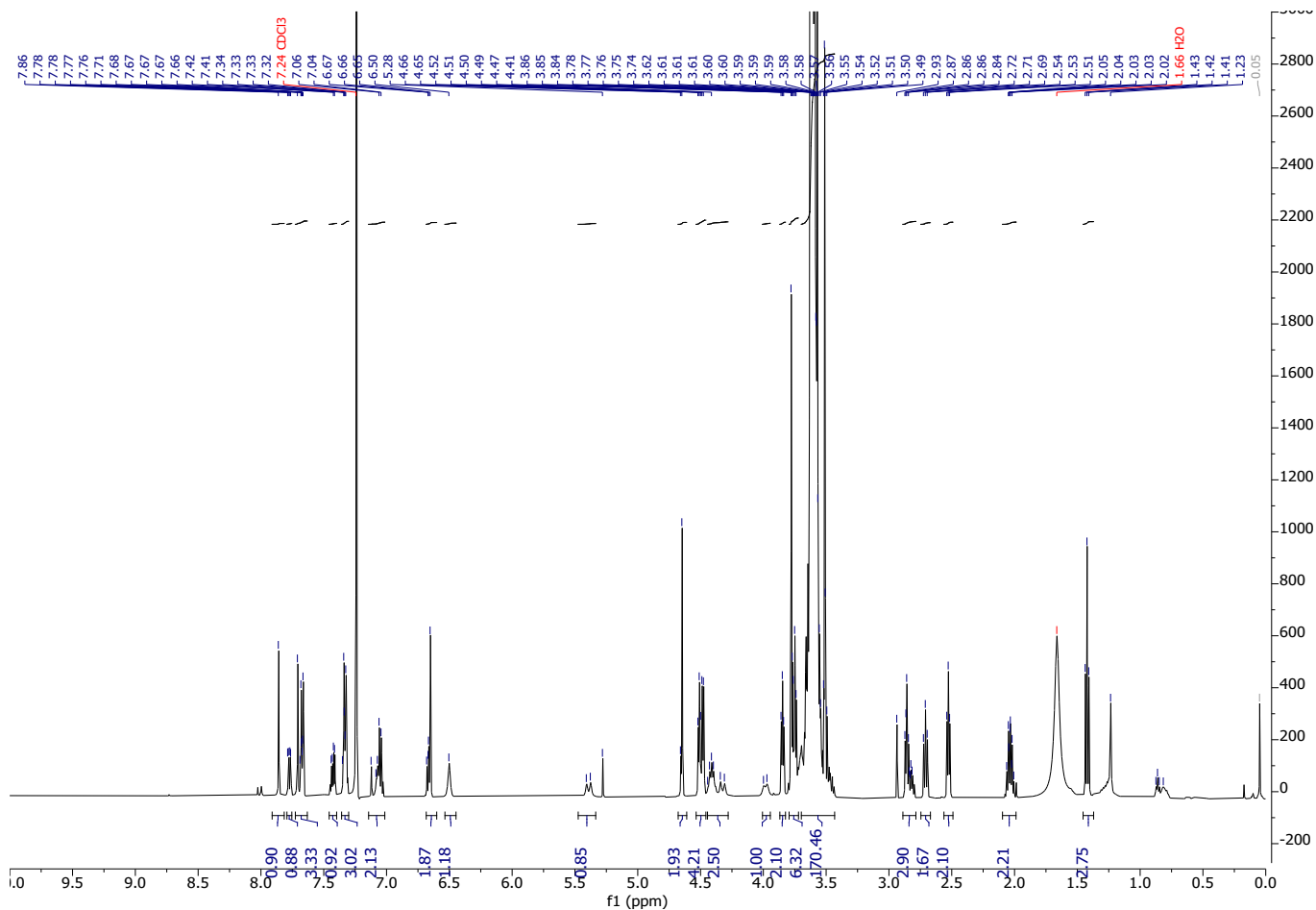
^1H NMR (500 MHz, CDCl_3) δ 7.85 (s, 1H), 7.77 (d, J = 8.8 Hz, 1H), 7.42 (dd, J = 9.2, 4.4 Hz, 1H), 7.33 (t, J = 8.2 Hz, 1H), 5.39 (d, J = 16.1 Hz, 1H), 4.48 – 4.23 (m, 4H), 4.17 (d, J = 5.7 Hz, 4H), 3.98 (d, J = 13.6 Hz, 1H), 3.84 – 3.37 (m, 45H), 2.40 (s, 2H), 1.42 (t, J = 7.2 Hz, 3H). (ES-LCMS) m/z 592.1 ($\text{M}+\text{H}$) $^+$.

Step 8: Synthesis of Flumazenil.1^{DART.2} (R14)



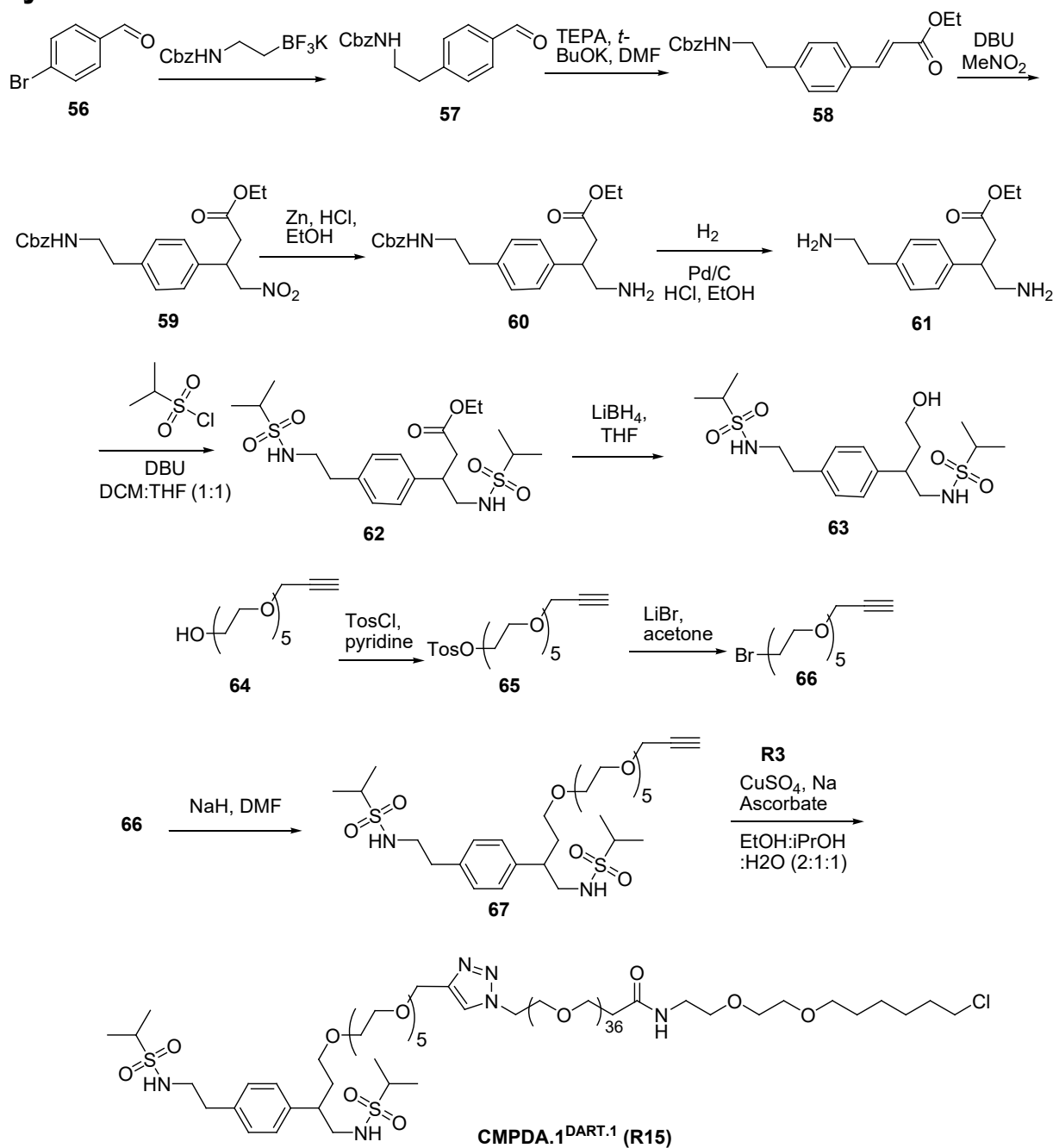
To a vial containing ethyl 8-fluoro-5-(3,6,9,12,15,18-hexaoxahenicos-20-yn-1-yl)-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate, **55** (5 mg, 8.5 μmol) was added a solution of azido-PEG36 HTL2.0, **R4** (18 mg, 8.5 μmol) in EtOH:t-BuOH:water (2:1:1, 0.8 mL). Then, copper (II) sulfate pentahydrate (0.1 mg, 0.42 μmol) and sodium ascorbate (0.15 mg, 0.85 μmol) were added and the reaction mixture was stirred overnight after which it was purified by RP HPLC eluting with 10 to 80 % acetonitrile in water (0.1 % FA, 220 nm wavelength) and other conditions as described in the general chemistry synthesis section to give **Flumazenil.1^{DART.2}, R14** (4 mg, 18 %).

^1H NMR (500 MHz, CDCl_3) δ 7.86 (s, 1H), 7.77 (dd, J = 8.8, 2.9 Hz, 1H), 7.72 – 7.63 (m, 3H), 7.43 (dt, J = 8.8, 4.5 Hz, 1H), 7.36 – 7.30 (m, 3H), 7.14 – 7.01 (m, 2H), 6.68 – 6.60 (m, 2H), 6.50 (s, 1H), 5.39 (d, J = 16.0 Hz, 1H), 4.65 (d, J = 5.5 Hz, 2H), 4.53 – 4.46 (m, 4H), 4.44 – 4.28 (m, 2H), 3.98 (d, J = 13.7 Hz, 1H), 3.85 (t, J = 5.2 Hz, 2H), 3.79 – 3.72 (m, 6H), 3.70 – 3.43 (m, 170H), 2.89 – 2.78 (m, 3H), 2.71 (t, J = 7.4 Hz, 2H), 2.53 (t, J = 5.6 Hz, 2H), 2.03 (dq, J = 8.3, 6.6 Hz, 2H), 1.42 (t, J = 7.1 Hz, 3H). HRMS (ESI $^+$): m/z calcd for $\text{C}_{126}\text{H}_{214}\text{ClFN}_8\text{Na}_2\text{O}_{49}$: 1362.1995 $[\text{M}+2\text{Na}]^{2+}$; found: 1362.1700 $[\text{M}+2\text{Na}]^{2+}$

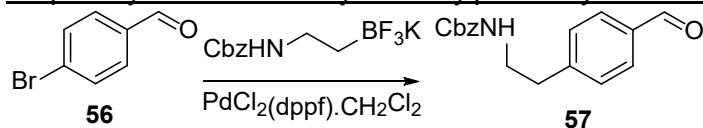


^1H NMR of Flumazenil.1 $^{\text{DART.2}}$ (R14) in CDCl_3

Synthesis of R15–CMPDA.1^{DART.1}

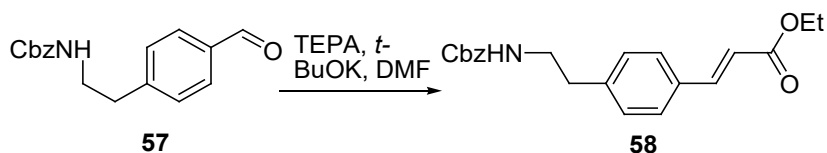


Step 1: Synthesis of benzyl 4-formylphenethylcarbamate, 57



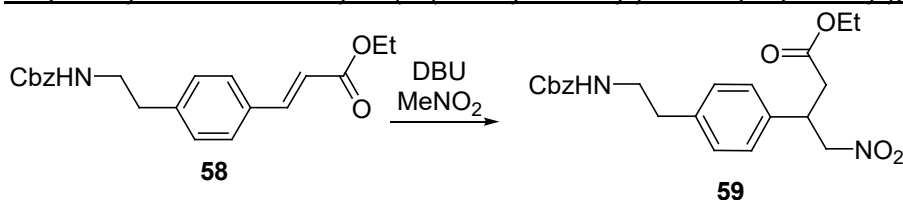
To solution of 4-bromobenzaldehyde (2.36 g, 12.7 mmol) in toluene (75 mL) in a sealed tube were added potassium potassium benzyl N-[2-(trifluoroboranuidyl)ethyl]carbamate (4.0 g, 14.0 mmol), Cs_2CO_3 (12.4 g, 38.2 mmol), $\text{PdCl}_2(\text{dppf})\cdot\text{CH}_2\text{Cl}_2$ (0.52 g, 0.64 mmol) and water (25 mL). The reaction mixture was purged with nitrogen. The tube was sealed and heated to 80 °C for 18 h. The reaction was allowed to cool to room temperature and saturated solution of NH_4Cl was added. The suspension was extracted with CH_2Cl_2 (30 mL X 3 times). The organic extracts were combined, dried (Na_2SO_4), and concentrated under reduced pressure to provide crude compound. Purification by flash chromatography (24g silica cartridge, EtOAc/Hexane) gave the title compound (2.0 g, 56% yield).

Step 2: Synthesis of benzyl-4-((E/Z)-2-(ethoxycarbonyl)vinyl)phenethylcarbamate, **58**



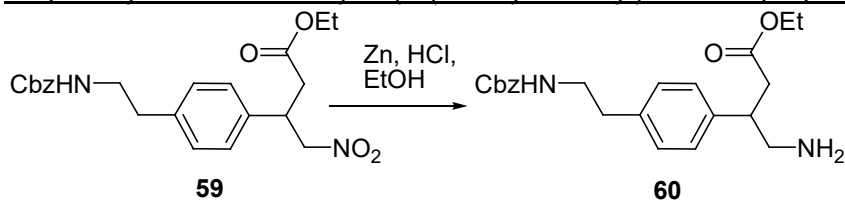
Triethylphosphonoacetate (9.6 g, 2.4 mmol) and potassium *t*-butoxide (1.1 g, 27.1 mmol) were dissolved in DMF (10 mL) at 0°C and stirred 30 minutes. Benzyl-4-formylphenethylcarbamate (3.3 g, 13.5 mmol) dissolved in DMF (15 mL) was then added over 5 minutes. Reaction stirred overnight. The reaction was diluted with water and then extracted thrice (EtOAc). The organic extracts were combined, dried (Na₂SO₄), and concentrated under reduced pressure to provide crude compound. Purification by flash chromatography (24g silica cartridge, EtOAc/Hexane) gave the title compound (1.96 g, 78% yield). (ESI) *m/z* 354 (M+1)⁺.

Step 3: Synthesis of benzyl 4-(1-(ethoxycarbonyl)-3-nitropropan-2-yl)phenethylcarbamate, **59**



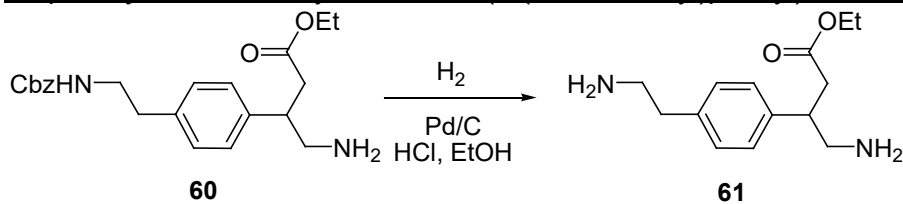
The starting material benzyl-4-((E/Z)-2-(ethoxycarbonyl)vinyl)phenethylcarbamate (1.96 g, 5.55 mmol) was dissolved in nitromethane (20 mL) and 1,8-diazabicyclo 5.4.0 undec-7-ene (1.5 g, 10 mmol) was then added. The reaction mixture was stirred at room temperature for 48h. The reaction mixture was diluted with ethyl acetate and washed twice with aqueous 1N HCl. The organic layer was dried (Na₂SO₄), and concentrated under reduced pressure to provide crude product. Purification by flash chromatography (EtOAc/Hexane) gave the title compound (2.34 g, quant. yield). ¹H NMR (300 MHz, CHLOROFORM-*d*) δ = 7.36 (s, 5H), 7.16 (s, 4H), 5.10 (s, 2H), 4.82 - 4.54 (m, 3H), 4.17 - 3.91 (m, 4H), 3.51 - 3.37 (m, 2H), 2.83 - 2.69 (m, 4H), 2.06 (s, 1H), 1.42 - 1.07 (m, 3H). (ESI) *m/z* 415 (M+1)⁺.

Step 4: Synthesis of benzyl 4-(1-(ethoxycarbonyl)-3-aminopropan-2-yl)phenethylcarbamate, **60**



To benzyl 4-(1-(ethoxycarbonyl)-3-nitropropan-2-yl)phenethylcarbamate (3.35 g, 8.1 mmol) in ethanol (100 mL) was added zinc (5.25 g, 81 mmol) and 4N HCl in dioxane (24 mL, 97 mmol). After 1 hour, the reaction was filtered through celite and the filtrate concentrated. The residue was dissolved in ethyl acetate and washed with aqueous sodium bicarbonate. The aqueous layer was extracted twice (EtOAc). The organic extracts were combined, dried (Na₂SO₄), and concentrated under reduced pressure to provide crude compound. Purification by flash chromatography (24g silica cartridge, EtOAc/Hexane) gave the title compound (3.11 g, quant. yield). (ESI) *m/z* 385 (M+1)⁺.

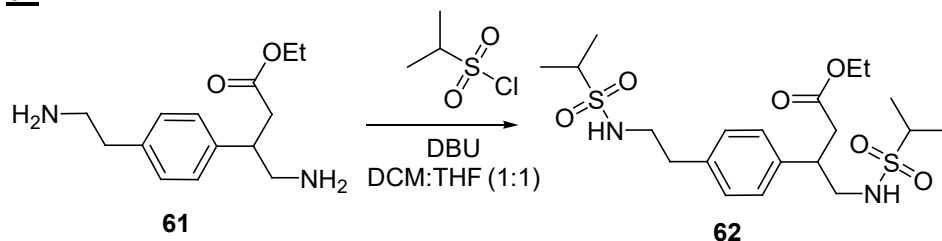
Step 5: Synthesis of ethyl 4-amino-3-(4-(2-aminoethyl)phenyl)butanoate, **61**



To benzyl-4-(1-(ethoxycarbonyl)-3-aminopropan-2-yl)phenethylcarbamate (3.11 g, 8.1 mmol) in ethanol (100 mL) in a Parr bottle was added 10% Pd/C (350 mg) and 4N HCl in dioxane (6.1 mL). The sample was hydrogenated (50 psi) for 20 hours. Methanol was added to solubilize the product and the reaction was then

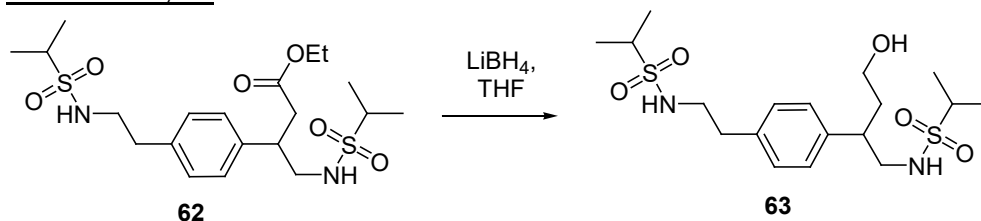
filtered through celite and concentrated to crude product used without further purification. ^1H NMR (300 MHz, CHLOROFORM- d) δ = 7.19 (br s, 4H), 4.08 (br s, 3H), 3.40 (br s, 3H), 3.10 (br d, J = 5.9 Hz, 2H), 2.86 (br s, 1H), 2.81 - 2.71 (m, 1H), 2.70 - 2.59 (m, 1H), 1.39 - 1.25 (m, 5H), 1.20 (br s, 2H). (ESI) m/z 251 ($M+1$) $^+$.

Step 6: Synthesis of ethyl 4-(propane-2-sulfonamido)-3-{4-[2-(propane-2-sulfonamido)ethyl]phenyl}butanoate, 62



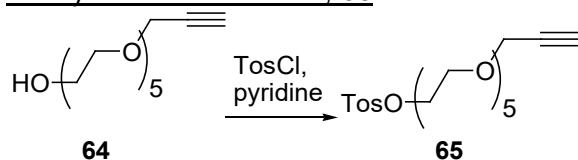
Ethyl 4-amino-3-(4-(2-aminoethyl)phenyl)butanoate (2.07 g, 6.4 mmol) in CH_2Cl_2 :THF (60 mL, 1:1) was cooled to 0 °C. 1,8-Diazabicyclo 5.4.0 undec-7-ene (5.7 mL, 38.4 mmol), and isopropyl sulfonyl chloride (2.73 g, 19.2 mmol) were then added. The reaction was stirred at room temperature for 22 h. The reaction was poured into 1N HCl and the solid collected by filtration to yield title compound (1.73 g, 58%).

Step 7: Synthesis of N-(2-{4-[4-hydroxy-1-(propane-2-sulfonamido)butan-2-yl]phenyl}ethyl)propane-2-sulfonamide, 63



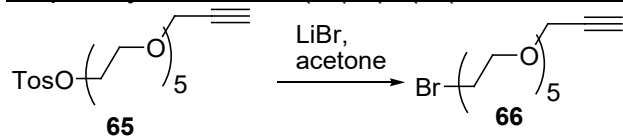
To ethyl 4-(propane-2-sulfonamido)-3-{4-[2-(propane-2-sulfonamido)ethyl]phenyl}butanoate (200 mg, 0.45 mmol) in THF (15 mL) was added lithium borohydride (4M in THF, 110 μL , 0.45 mmol) and the reaction heated to reflux for 2 hours. After cooling, the reaction was poured into aqueous buffer (pH=7) and extracted thrice with ethyl acetate. The organic extracts were combined, dried (Na_2SO_4), and concentrated under reduced pressure. Purification by flash chromatography (EtOAc/Hexane) afforded the title compound (0.16 g, 83 yield). (ESI) m/z 421 ($M+1$) $^+$.

Step 8: Synthesis of 2-(2-(2-(2-(2-(prop-2-ynoxy)ethoxy)ethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate, 65



To 2-(2-(2-(2-(2-(prop-2-ynoxy)ethoxy)ethoxy)ethoxy)ethoxy)ethanol (250 mg, 0.91 mmol) in pyridine (3 mL) at 0°C was added tosyl chloride (345 mg, 1.81 mmol). The reaction warmed to room temperature while stirring overnight. The reaction was then concentrated and purified by flash chromatography (EtOAc/Hexane) to yield the title compound (57 mg, 14% yield). ^1H NMR (300 MHz, CHLOROFORM- d) δ = 7.72 (br d, J = 6.7 Hz, 2H), 7.27 - 6.99 (m, 2H), 4.24 - 3.99 (m, 4H), 3.64 - 3.50 (m, 18H), 2.38 (br s, 4H).

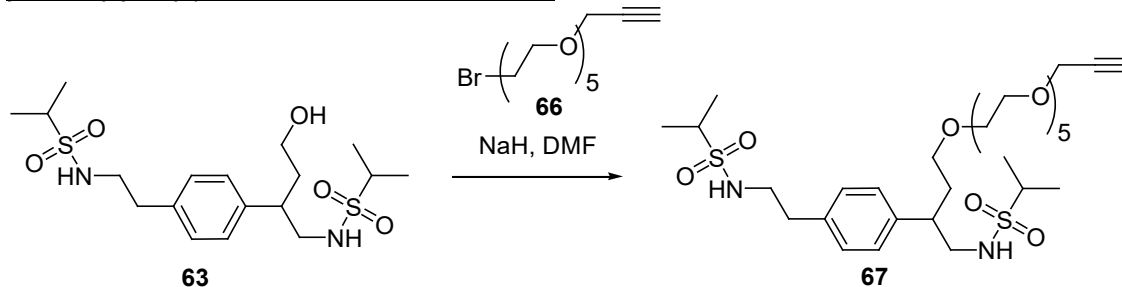
Step 9: Synthesis of 3-(2-(2-(2-(2-(2-bromoethoxy)ethoxy)ethoxy)ethoxy)ethoxy)prop-1-yne, 66



2-(2-(2-(2-(2-(Prop-2-ynoxy)ethoxy)ethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (57 mg, 0.132 mmol), and lithium bromide (35 mg, 0.397 mmol) were refluxed in acetone (5 mL) for 3 hours. The reaction was concentrated and residue purified by flash chromatography (EtOAc/Hexane) to yield the title compound

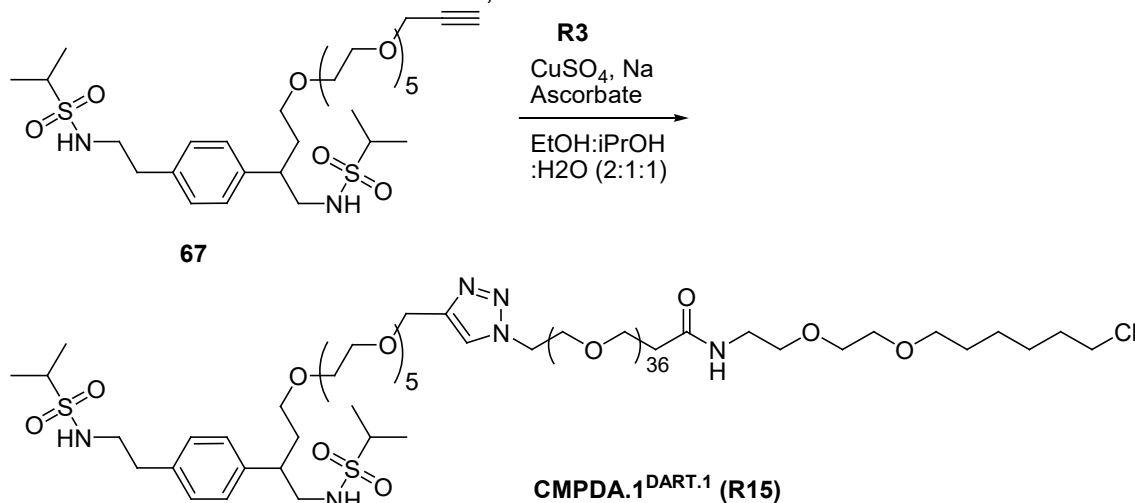
(45 mg, quant. yield). ^1H NMR (300 MHz, CHCl_3 - d) δ = 4.21 (br s, 2H), 3.86 - 3.76 (m, 2H), 3.67 (br s, 16H), 3.59 - 3.38 (m, 2H), 2.44 (br s, 1H).

Step 10: Synthesis of N-(2-{4-[23-(propane-2-sulfonamido)-4,7,10,13,16,19-hexaoxatricos-1-yn-22-yl]phenyl}ethyl)propane-2-sulfonamide, **67**



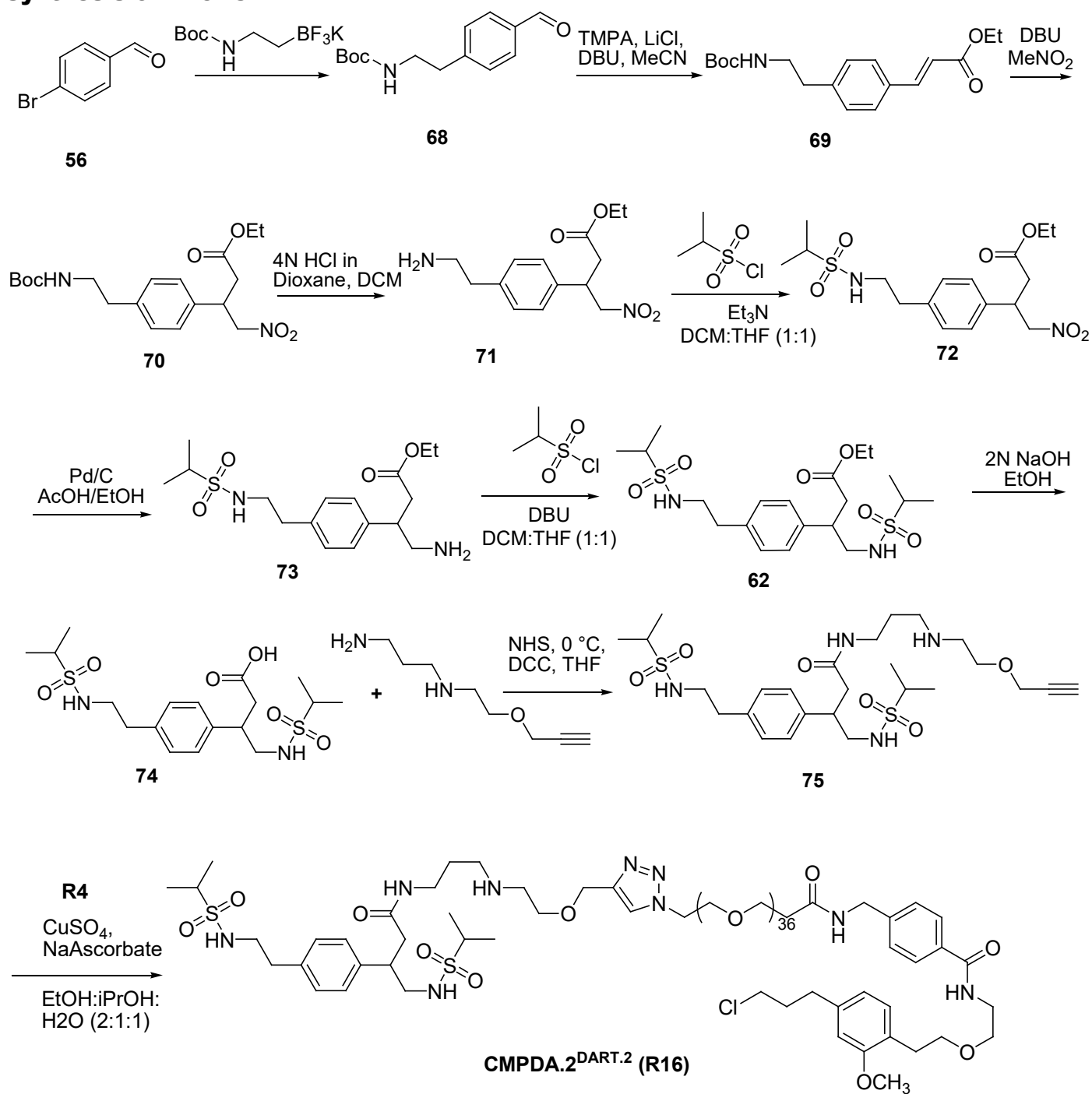
To N-(2-{4-[4-hydroxy-1-(propane-2-sulfonamido)butan-2-yl]phenyl}ethyl)propane-2-sulfonamide (**63**, 51 mg, 0.12 mmol) in DMF (1 mL) was added 60% sodium hydride in mineral oil (15 mg, 0.37 mmol) and the reaction stirred at 0°C for 30 minutes. 3-(2-(2-(2-(2-(2-bromoethoxy)ethoxy)ethoxy)ethoxy)ethoxy)prop-1-yne (**66**, 45 mg, 0.133 mmol) was added and the reaction stirred at ambient temperature overnight. The reaction was purified via reverse phase chromatography to yield the title compound (19 mg, 23%). (ESI) m/z 679 ($M+1$)⁺.

Step 11: Synthesis of N-(2-{2-[(6-chlorohexyl)oxy]ethoxy}ethyl)-1-{4-[21-(propane-2-sulfonamido)-20-{4-[2-(propane-2-sulfonamido)ethyl]phenyl}-2,5,8,11,14,17-hexaoxahenicosan-1-yl]-1H-1,2,3-triazol-1-yl}-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108-hexatriacontaoxa111n-111-amide, **CMPDA.1^{DART.1}**

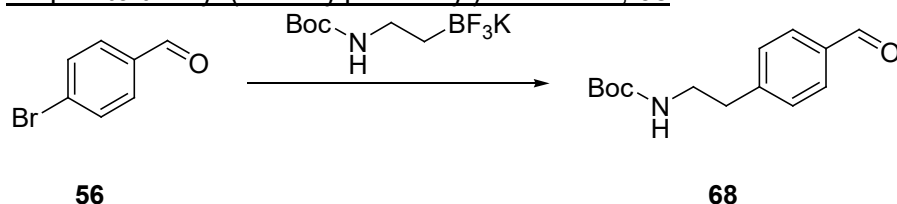


To a vial containing azido-PEG₃₆-HTL.1 (^{azido}DART.1, **R3**) (37 mg, 0.019 mmol), N-(2-{4-[23-(propane-2-sulfonamido)-4,7,10,13,16,19-hexaoxatricos-1-yn-22-yl]phenyl}ethyl)propane-2-sulfonamide (**67**, 13 mg, 0.19 mmol), and the solvents EtOH:*i*-PrOH:H₂O (2:1:1) (1 mL) was added CuSO₄·5H₂O (0.3 mg, 0.001 mmol) and (+)-sodium L-ascorbate (0.4 mg, 0.002 mmol) pre-dissolved in solutions of EtOH:*i*-PrOH:H₂O (2:1:1) (1 mL). The reaction was stirred for 20h at room temperature. The reaction mixture was then filtered, concentrated, and purified by reverse phase HPLC to afford the title compound, **R15** (36 mg, 72% yield). (ESI) m/z 1293 ($^{1/2}M+1$)⁺.

Synthesis of R16—CMPDA.2^{DART.2}

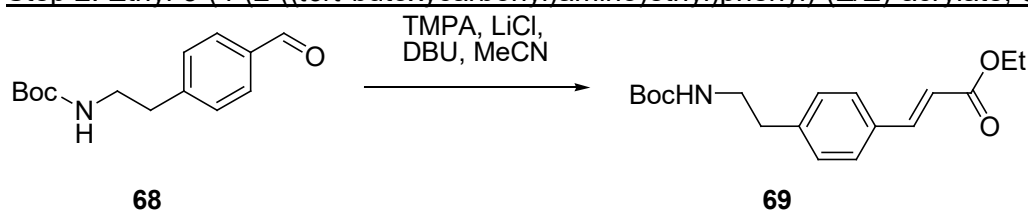


Step 1: *tert*-butyl (4-formylphenethyl)carbamate, **68**



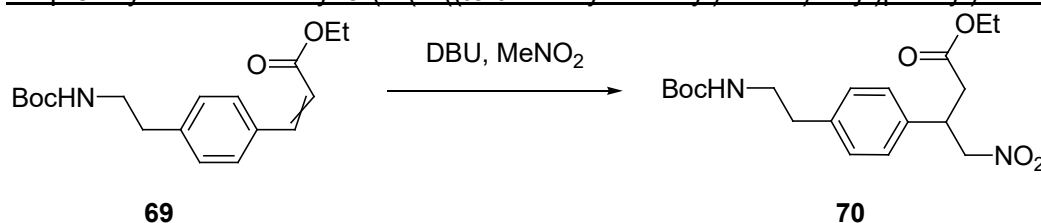
To solution of 4-bromobenzaldehyde (3.0 g, 16.3 mmol) in toluene (25 mL) in a sealed tube were added potassium *tert*-butyl *N*-[2-(trifluoroboranyl)ethyl]carbamate (4.9 g, 19.6 mmol), Cs₂CO₃ (15.9 g, 48.9 mmol), PdCl₂(dppf).CH₂Cl₂ (2.6 g, 3.2 mmol) and water (5 mL). The reaction mixture was degassed under vacuum and filled with nitrogen gas, the process repeated three times. The tube with reaction mixture was sealed and heated to 80 °C for 18 h. The reaction was allowed to cool to room temperature, saturated solution of NH₄Cl (20 mL) was added, and the suspension was extracted with CH₂Cl₂ (30 mL X 3 times). The organic extracts were combined, dried (Na₂SO₄), and concentrated under reduced pressure to provide crude compound. Purification by flash chromatography (24g silica cartridge, EtOAc/Hexane) gave the title compound (3.3g, 73% yield). ¹H NMR (CHLOROFORM-*d*, 300MHz): δ = 9.93 - 10.04 (m, 1 H), 7.74 - 7.90 (m, 2 H), 7.32 - 7.42 (m, 2 H), 4.39 - 4.63 (m, 1 H), 3.23 - 3.54 (m, 2 H), 2.73 - 3.02 (m, 2 H), 1.52 - 1.60 (m, 1 H), 1.43 ppm (s, 9 H). (ESI) *m/z* 273 (M+23)⁺.

Step 2: Ethyl-3-(4-(2-((*tert*-butoxycarbonyl)amino)ethyl)phenyl)-(E/Z)-acrylate, **69**



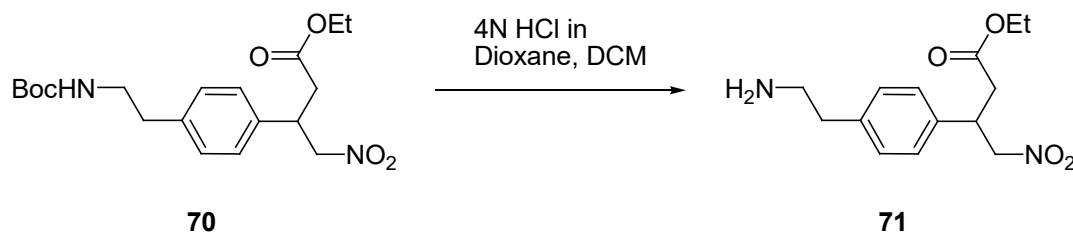
Tert-butyl (4-formylphenethyl)carbamate (3.3 g, 13.5 mmol) was dissolved in acetonitrile (15 mL) and added ethyl 2-(diethoxyphosphoryl)acetate (9.6 g, 2.4 mmol), lithium chloride (1.1 g, 27.1 mmol), 1,8-diazabicyclo 5.4.0 undec-7-ene (1.3 g, 3.9 mmol) added, the reaction mixture was maintained at room temperature for 18 h. After completion of reaction, the reaction mixture was concentrated and added CH₂Cl₂ (30 mL) and H₂O (30 mL) the suspension was extracted with CH₂Cl₂ (3 × 30 mL). The organic extracts were combined, washed with brine (20 mL), dried (Na₂SO₄), and concentrated under reduced pressure to provide crude compound. Purification by flash chromatography (40 g silica cartridge, EtOAc/Hexane) gave the title compound (2.6 g, 60% yield). ¹H NMR (CHLOROFORM-*d*, 300MHz): δ = 7.66 (d, *J*=15.8 Hz, 4 H), 7.46 (d, *J*=8.2 Hz, 7 H), 7.17 - 7.28 (m, *J*=11.1 Hz, 9 H), 6.40 (d, *J*=15.8 Hz, 4 H), 4.26 (q, *J*=7.0 Hz, 7 H), 3.38 (q, *J*=6.2 Hz, 7 H), 2.81 (t, *J*=7.0 Hz, 7 H), 1.43 (s, 32 H), 1.33 ppm (t, *J*=7.0 Hz, 12 H). (ESI) *m/z* 343 (M+23)⁺.

Step 3: Synthesis of ethyl 3-(4-(2-((*tert*-butoxycarbonyl)amino)ethyl)phenyl)-4-nitrobutanoate, **70**



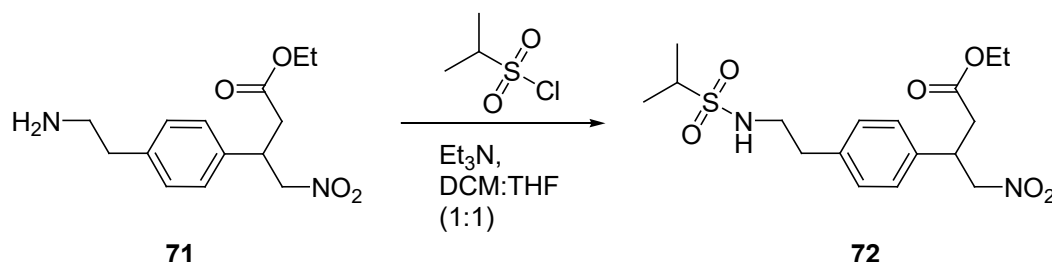
The starting material ethyl-3-(4-(2-((*tert*-butoxycarbonyl)amino)ethyl)phenyl)-(E/Z)-acrylate (1.3 g, 3.9 mmol) was dissolved in nitromethane (6.5 mL) and added 1,8-diazabicyclo 5.4.0 undec-7-ene (1.6 g, 3.9 mmol), the reaction mixture was maintained at room temperature for 18 h. After completion of reaction, the reaction mixture was diluted with ethyl acetate (20 mL) and washed with 1N HCl (15 mL X 2 times) the combined aqueous layers were reextracted with ethyl acetate (15 mL). The organic extracts were combined again washed with 1N HCl (10 mL), dried (Na₂SO₄), and concentrated under reduced pressure to provide crude. Purification by flash chromatography (40 g silica cartridge, 30% EtOAc in Hexane) gave the title compound (0.8 g, 49% yield). (ESI) *m/z* 382 (M+1)⁺.

Step 4: Synthesis of ethyl 3-(4-(2-aminoethyl)phenyl)-4-nitrobutanoate hydrochloride, **71**



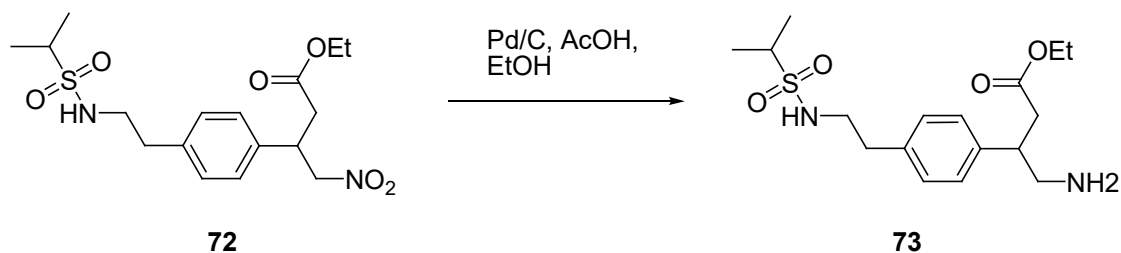
The ethyl 3-(4-(2-((*tert*-butoxycarbonyl)amino)ethyl)phenyl)-4-nitrobutanoate (1.3 g, 3.4 mmol) was dissolved in dichloromethane (10 mL) and added 4N HCl (3 mL). The reaction was maintained at room temperature overnight. Reaction was monitored by LC-MS. After completion of reaction, the reaction mixture was concentrated *in vacuo* and resultant solids were washed with ether to get desired compound in quantitative yield (0.85 g, 88.7% yield). ¹H NMR (METHANOL-*d*₄, 300MHz): δ = 7.16 - 7.42 (m, 4 H), 4.82 (s, 2 H), 3.83 - 4.13 (m, 2 H), 3.43 - 3.54 (m, 1 H), 3.09 - 3.23 (m, 2 H), 2.88 - 2.99 (m, 2 H), 2.65 - 2.87 (m, 2 H), 1.15 ppm (s, 3 H). (ESI) *m/z* 282 (M+1)⁺.

Step 5: Synthesis of ethyl 3-(4-(2-((1-methylethyl)sulfonamido)ethyl)phenyl)-4-nitrobutanoate, **72**



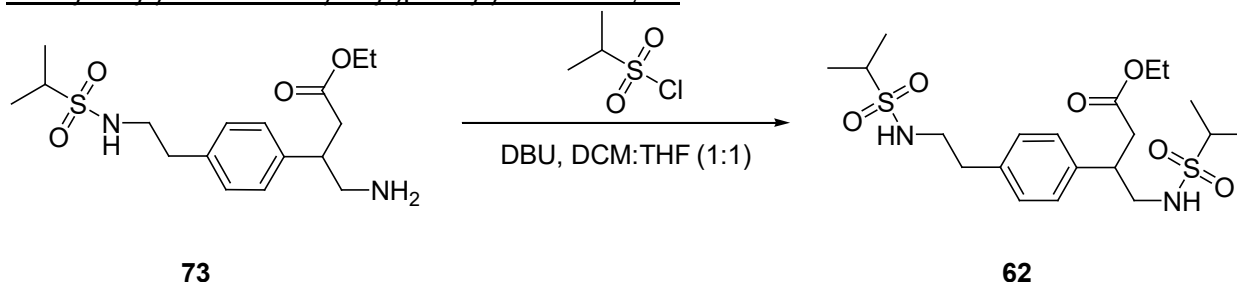
The starting material ethyl 3-(4-(2-aminoethyl)phenyl)-4-nitrobutanoate-HCl (397 mg, 1.42 mmol) in CH₂Cl₂:THF (8 mL, 1:1) was cooled to 0 °C. To reaction mixture added triethylamine (418 μ L, 3.0 mmol) and then isopropyl sulfonyl chloride (221 μ L, 1.5 mmol) drop wise. The reaction mixture progress was monitored by LC-MS. The reaction was maintained at room temperature for 18 h. To reaction mixture added EtOAc and the suspension was extracted with EtOAc (30 mL X 3 times). The organic extracts were combined, dried (Na₂SO₄), and concentrated under reduced pressure to provide crude compound. Purification by flash chromatography (24 g silica cartridge, 10% MeOH in CH₂Cl₂) gave the title compound (207 mg, 45%). (ESI) *m/z* 388 (M+1)⁺.

Step 6: Synthesis of ethyl 4-amino-3-(4-(2-((1-methylethyl)sulfonamido)ethyl)phenyl)butanoate, **73**



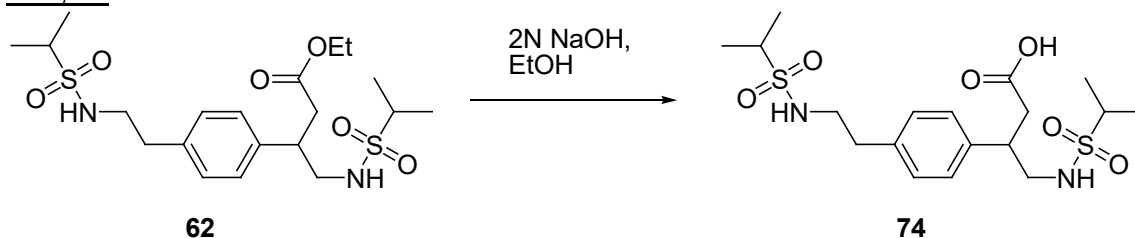
To a solution of ethyl 3-(4-(2-((1-methylethyl)sulfonamido)ethyl)phenyl)-4-nitrobutanoate (200 mg, 0.51 mmol) in ethanol, under nitrogen atmosphere added 10% Pd/C (110.6 mg, 0.15 mmol) and setup was placed on par hydrogenator and maintained under hydrogen atmosphere 50psi for 18 hours. The reaction was checked by LC-MS and after complete conversion, the reaction mixture was filtered through celite and concentrated *in vacuo* to get crude compound (82 mg, 44%). The crude product was used as is without further purification. (ESI) *m/z* 358 (M+1)⁺.

Step 7: Synthesis of ethyl 4-((1-methylethyl)sulfonamido)-3-(4-(2-((1-methylethyl)sulfonamido)ethyl)phenyl)butanoate, **62**



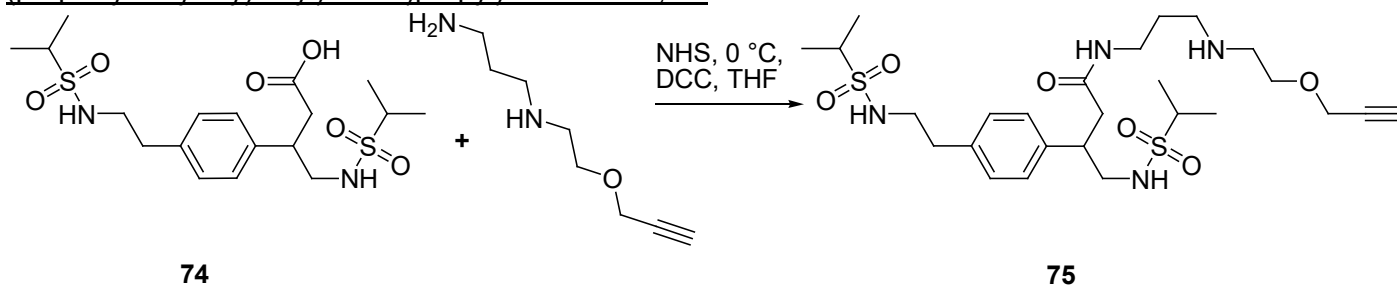
The starting material ethyl 4-amino-3-(4-(2-((1-methylethyl)sulfonamido)ethyl)phenyl)butanoate (67 mg, 0.189 mmol) in CH_2Cl_2 :THF (1 mL, 1:1) was cooled to 0 °C. 1,8-Diazabicyclo 5.4.0 undec-7-ene (68 mg, 0.45 mmol), and isopropyl sulfonyl chloride (59 mg, 0.41 mmol) were then added. The reaction was maintained at room temperature for 18 h with stirring. To the reaction mixture was added HCl (5 mL) and EtOAc (10 mL) and the suspension was extracted with EtOAc (3×10 mL). The organic extracts were combined, dried (Na_2SO_4), and concentrated under reduced pressure to provide crude compound. Purification by flash chromatography (12 g silica cartridge, 10% MeOH in CH_2Cl_2) to give the title compound (35 mg, 40%). ^1H NMR (CHLOROFORM-d , 300MHz): δ = 7.17 (s, 4 H), 4.19 - 4.33 (m, 2 H), 4.00 - 4.13 (m, 2 H), 3.27 - 3.43 (m, 5 H), 2.99 - 3.18 (m, 2 H), 2.56 - 2.88 (m, 4 H), 1.30 (d, J =7.0 Hz, 12 H), 1.14 - 1.21 ppm (m, 3 H). (ESI) m/z 464 ($\text{M}+1$)⁺.

Step 8: Synthesis of 4-((1-methylethyl)sulfonamido)-3-(4-(2-((1-methylethyl)sulfonamido)ethyl)phenyl)butanoic acid, **74**



The starting material ethyl 4-((1-methylethyl)sulfonamido)-3-(4-(2-((1-methylethyl)sulfonamido)ethyl)phenyl)butanoate (33 mg, 0.09 mmol) was dissolved in ethanol (0.5 mL). To this solution, sodium hydroxide (36.8 mg, 0.87 mmol) was added and reaction stirred at room temperature for 3 h. After completion of reaction, the reaction mixture was concentrated and CH_2Cl_2 and 1N HCl (3 mL) were added. The suspension was extracted with CH_2Cl_2 (3×5 mL). The organic extracts were combined dried (Na_2SO_4), and concentrated under reduced pressure to provide crude compound, which is used as is for next step. ^1H NMR (METHANOL-d_4 , 300MHz): δ = 7.17 - 7.31 (m, 4 H), 3.35 (s, 1 H), 3.30 (br. s., 4 H), 3.07 (dq, J =13.5, 6.8 Hz, 2 H), 2.78 - 2.94 (m, J =7.0 Hz, 3 H), 2.52 - 2.69 (m, J =4.7 Hz, 1 H), 1.18 - 1.34 ppm (m, 12 H). (ESI) m/z 436 ($\text{M}+1$)⁺.

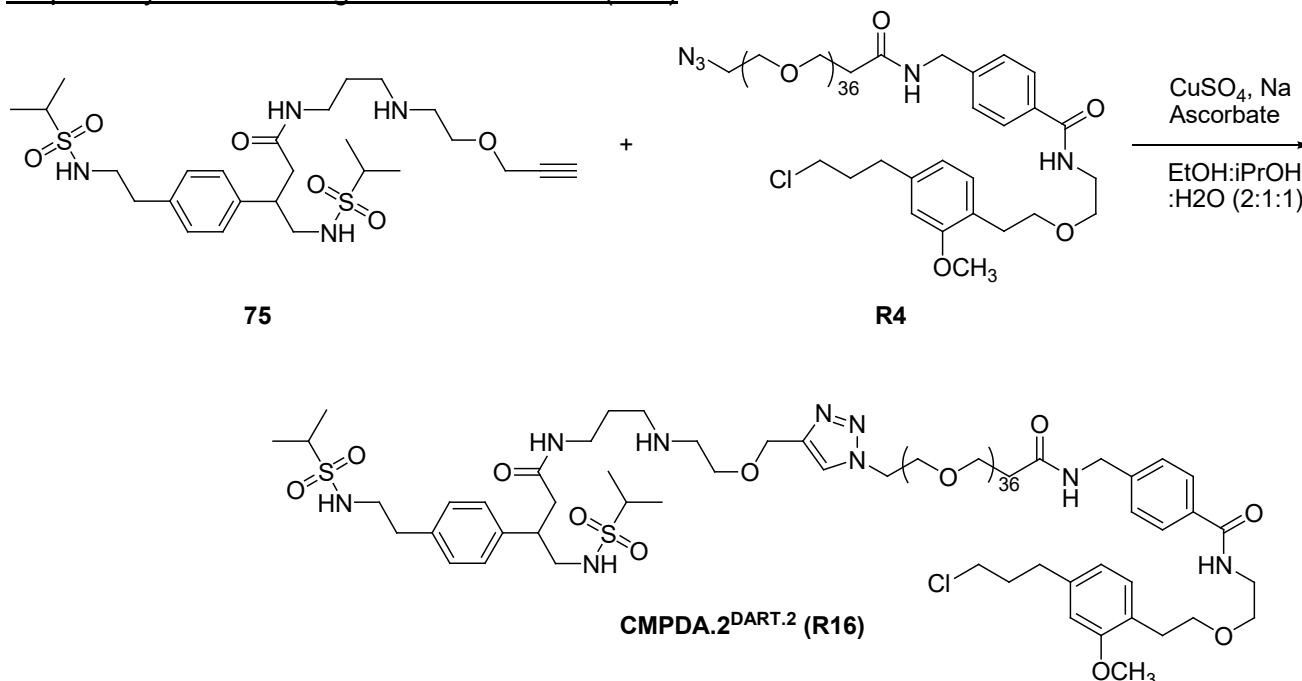
Step 9: Synthesis of 4-((1-methylethyl)sulfonamido)-3-(4-(2-((1-methylethyl)sulfonamido)ethyl)phenyl)-N-(3-((2-(prop-2-yn-1-yloxy)ethyl)amino)propyl)butanamide, **75**



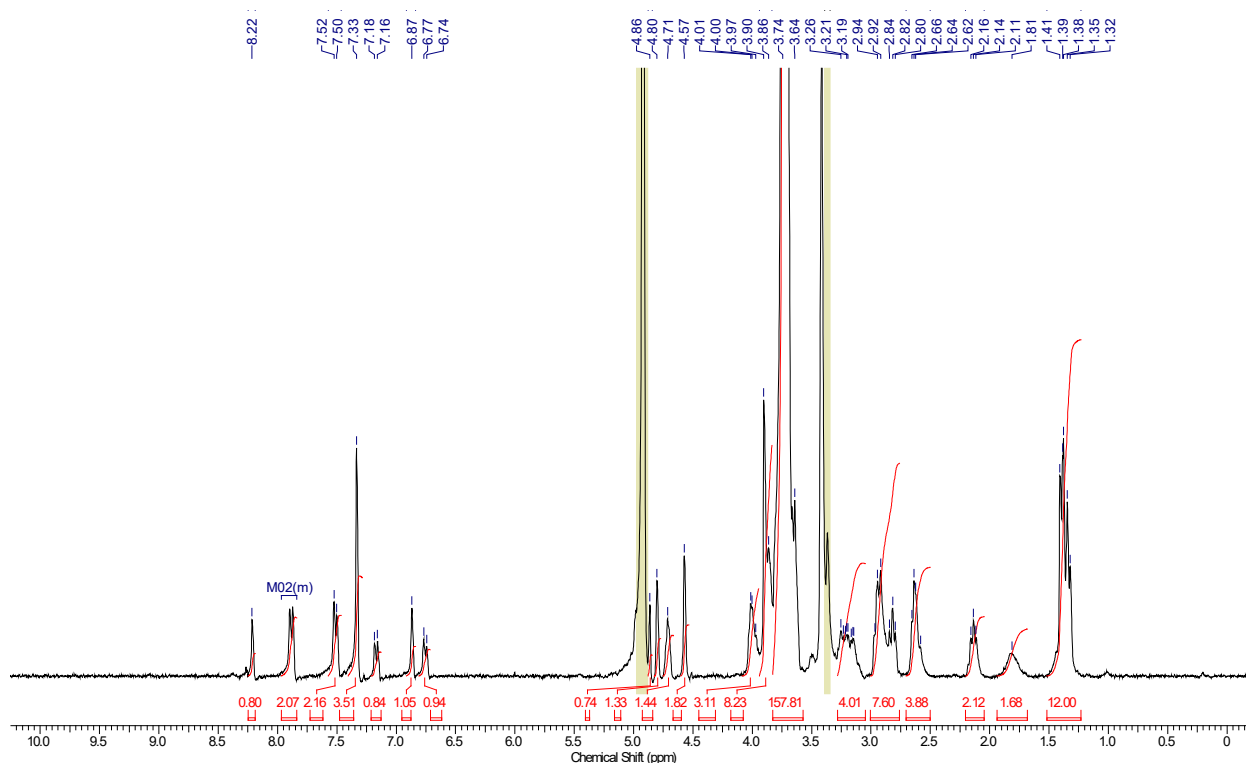
A solution of 4-((1-methylethyl)sulfonamido)-3-(4-(2-((1-methylethyl)sulfonamido)ethyl)phenyl)butanoic acid (30.2 mg, 0.07 mmol) and *N*-hydroxysuccinimide (8 mg, 0.07 mmol) in dry THF (0.3 mL) was cooled to 0 °C in an ice bath. Dicyclohexylcarbodiimide (17.9 mg, 0.09 mmol) dissolved in dry THF (0.5 mL) was added drop wise to

the mixture and the reaction stirred at room temperature for 24 h. The reaction mixture was filtered to remove dicyclohexylurea by-product formed. The filtrate was concentrated under reduced pressure. The residue was dissolved in CHCl_3 and washed consecutively with water, a saturated solution of NaHCO_3 and water. The organic layer was dried (Na_2SO_4), and concentrated under reduced pressure to obtain the *N*-hydroxysuccinimide active ester intermediate. This intermediate (37.0 mg, 0.07 mmol) and *N*'-(2-(prop-2-yn-1-yloxy)ethyl)propane-1,3-diamine (20.0 mg, 0.07 mmol) were dissolved in CHCl_3 (0.3 mL) with addition of small amount of DMF (35 μL). The reaction stirred at room temperature for 48 h. After completion of reaction, the reaction mixture was washed with H_2O , sat. NaHCO_3 , and H_2O . filtered off the desiccant, dried over Na_2SO_4 , concentrated and purified by reverse phase preparative HPLC (mobile phase: 0.1% TFA in acetonitrile and 0.1% TFA in water) to give pure compound 9.0 mg, 23% yield. ^1H NMR (METHANOL- d_4 , 300MHz): δ = 7.23 (s, 4 H), 4.22 - 4.30 (m, 2 H), 3.75 (t, J =5.0 Hz, 2 H), 3.24 (br. s., 4 H), 2.93 - 3.17 (m, 6 H), 2.71 - 2.87 (m, J =7.0, 7.0 Hz, 4 H), 2.35 - 2.58 (m, J =14.4, 9.1 Hz, 2 H), 1.57 - 1.80 (m, 2 H), 1.16 - 1.37 ppm (m, 12 H). (ESI) m/z 574 ($M+1$) $^+$.

Step 10: Synthesis of reagent CMPDA.2^{DART.2} (R16)



To a vial containing the azido-PEG₃₆-HTL.2 (azidoDART.2, **R4**) (28.4 mg, 0.013 mmol) and 4-((1-methylethyl)sulfonamido)-3-(4-(2-((1-methylethyl)sulfonamido)ethyl)phenyl)-*N*-(3-((2-(prop-2-yn-1-yloxy)ethyl)amino)propyl)butanamide (7.8 mg, 0.013 mmol), the solvent mixture EtOH:iPrOH:H₂O (2:1:1) (200 μL) was added followed by $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (2.39 mg, 0.015 mmol) and (+)-sodium L-ascorbate (3.0 mg, 0.015 mmol) was pre-dissolved in solutions of EtOH:iPrOH:H₂O (2:1:1) (400 μL). The reaction was stirred for 18 h at room temperature. After completion of reaction, reaction mixture was filtered, concentrated and purified by reverse phase preparative HPLC (mobile phase: 0.1% TFA in acetonitrile and 0.1% TFA in water) to get pure compound **R16** 17.0 mg, 47% yield. ^1H NMR (300 MHz, METHANOL- d_4) δ ppm 8.19 - 8.25 (m, 1 H) 7.84 - 7.97 (m, 2 H) 7.51 (d, J =7.03 Hz, 2 H) 7.33 (s, 4 H) 7.17 (d, J =7.03 Hz, 1 H) 6.87 (br. s., 1 H) 6.76 (d, J =7.62 Hz, 1 H) 4.86 (s, 1 H) 4.80 (br. s., 1 H) 4.71 (br. s., 1 H) 4.57 (br. s., 2 H) 3.99 (m, J =3.51 Hz, 3 H) 3.83 - 3.94 (m, 8 H) 3.57 - 3.83 (m, 158 H) 3.20 (m, J =11.72 Hz, 4 H) 2.88 (m, J =7.62 Hz, 8 H) 2.50 - 2.70 (m, 4 H) 2.14 (t, J =6.44 Hz, 2 H) 1.81 (br. s., 2 H) 1.37 (m, J =2.34 Hz, 12 H); (ESI) m/z 1332 ($M/2+1$) $^+$; HRMS (ESI): m/z calcd for $\text{C}_{123}\text{H}_{221}\text{ClN}_9\text{O}_{46}\text{S}_2$: 2659.4341 [$M+H$] $^+$; found: 2659.43551, [$M+H$] $^+$



¹H NMR of CMPDA.2^{DART.2}, R16

Chemical Synthesis References

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SUPPLEMENTARY TEXT

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LOCUS DP.X0117A_aavCAGDI 7067 bp DNA circular

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LOCUS DP.M6360D_aavCAGDI 7067 bp DNA circular

DEFINITION

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