

AWARD NUMBER: W81XWH-18-1-0116

TITLE: Targeting Shiga Toxins for Treatment-resistant Bacterial Gut Infections

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REPORT DATE: July 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE July 2019		2. REPORT TYPE Annual		3. DATES COVERED 1 Jul 2018 - 30 Jun 2019	
4. TITLE AND SUBTITLE Targeting Shiga Toxins for Treatment-resistant Bacterial Gut Infections				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-18-1-0116	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Michael S. Kay, MD/PhD E-Mail:				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Utah, The Gary Gledhill 201 S President Circle RM 408 Salt Lake City, UT 84112-9023				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The purpose of this research is to identify a protease-resistant D-peptide inhibitor of Shiga toxin for use as an orally delivered prevention or therapeutic agent to treat severe bacterial gut infections caused by <i>Shigella</i> or Shiga toxin-producing <i>E. coli</i> (STEC). During this period, we successfully synthesized the Shiga toxin B-subunit using solid-phase peptide synthesis and native chemical ligation. The N-terminal half of the protein suffers from poor solubility, so we developed a new "helping hand" solubilizing tag to enable isolation of this peptide and its ligation to the C-terminal half. This synthetic protein was folded into pentamers and validated using LC/MS, circular dichroism, size-exclusion chromatography, and analytical ultracentrifugation. To overcome problems with instability of the pentamer, we developed a "cork" peptide that mimics the C-terminal tail of the natural Shiga A subunit. This peptide will stabilize the B-subunit pentamer by filling its central cavity. This stabilized pentamer will be used in mirror-image phage display to identify D-peptide inhibitors.					
15. SUBJECT TERMS D-peptides, drug discovery, antibiotics, drug resistance, colitis, Shiga toxin, Shigella, E. coli, mirror-image phage display					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 28	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION:

The goal of this project is to develop a non-digestible D-peptide inhibitor of the Shiga toxin B-subunit (StxB) for the prevention and treatment of Shiga-mediated hemorrhagic colitis (caused by *Shigella* and Shiga toxin-producing *E. coli*). These infections are challenging to treat using conventional antibiotics, but an orally delivered D-peptide inhibitor that would not be broken down in the gut could specifically block this toxin to prevent or treat infections without affecting healthy gut flora. This project involves synthesizing a mirror-image StxB target for mirror-image phage display screening, following by optimization and functional/structural characterization of the most promising hits.

2. KEYWORDS:

D-peptides, drug discovery, antibiotics, drug resistance, colitis, Shiga toxin, *Shigella*, *E. coli*, mirror-image phage display

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**
 - chemical synthesis and validation of D-StxB – 50% complete
 - identify D-peptide Shiga toxin inhibitors using mirror-image phage display – 10% complete
 - affinity-mature and multimerize D-peptide hits – 0% complete
 - evaluate candidate D-peptide inhibitor binding, structure, and efficacy – 0% complete
- **What was accomplished under these goals?**
 - Synthesis of L- and D-StxB was completed, but the synthetic quality of the full-length protein was not sufficient for phage display due to micro-contaminants and pentamer instability.
 - A new synthesis route for StxB was developed using a solubilizing “helping hand” linker to produce higher quality full-length protein (as described in our ChemRxiv manuscript). The synthesis of this new linker (called Ddap) is shown in Fig. 1 and its performance is described in Fig. 2. The new synthesis route is shown in Fig. 3, and the dramatic improvement in solubility of the N-terminal half of the StxB protein is shown in Fig. 4 (40-fold higher solubility).
 - This new synthetic L-StxB was thoroughly characterized and found to behave similarly to a recombinant L-StxB control (validated by size-exclusion chromatography, circular dichroism spectroscopy, HPLC/mass spectrometry, and analytical ultracentrifugation, as shown in Fig. 6 of our ChemRxiv manuscript).

- Although our synthetic L-StxB behaves very similarly to recombinant protein (Fig. 6), the recombinant pentamer is unstable, making it less than optimal for phage display screening (especially at the lower concentrations used in later rounds of phage display). See Fig. A below for a dilution series monitored by size-exclusion chromatography, which shows how pentamer stability declines with dilution. This instability is exacerbated by biotinylation, as monitored by ELISA (see Fig. B).
- To overcome this stability issue, we developed a novel “cork” peptide (derived from StxA) to “plug” the open cavity in the StxB pentamer and stabilize the pentamer.
- The higher quality synthesis scheme for StxB is now being repeated using D-amino acids. This protein will be stabilized using a D-cork peptide and used in mirror-image phage display.

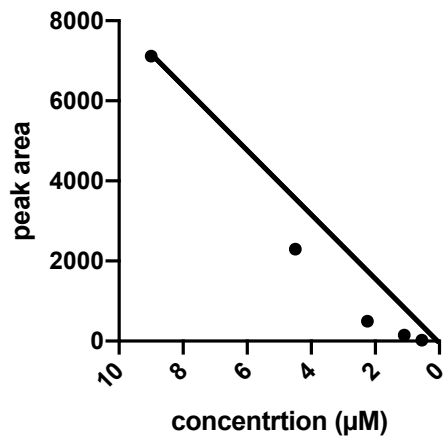


Figure A. Plot of StxB pentamer peak areas (measured by HPLC) vs. concentration. A stable pentamer would show a linear relationship between concentration and peak area (as depicted by the line). The peak areas for more dilute samples are well below this line, indicating decomposition of the pentamer.

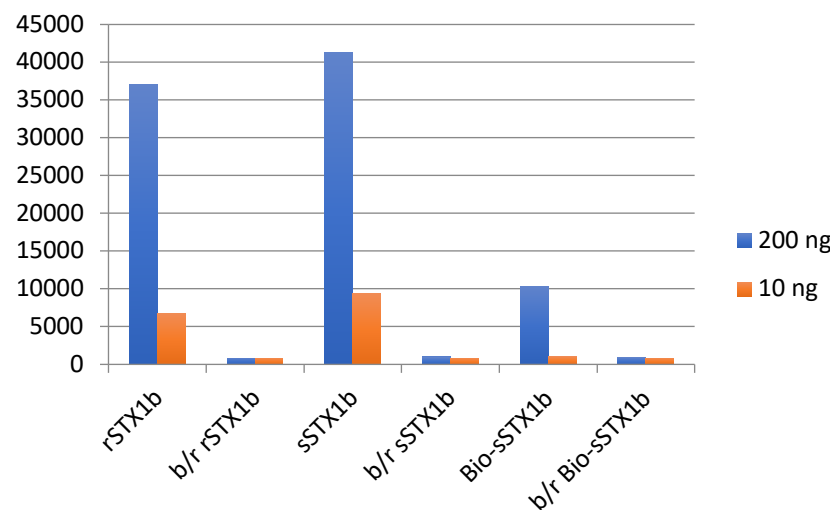


Figure B. Detection of folded StxB via ELISA. Bar heights indicate level of folded (pentameric) StxB present in each well. Legend: r=recombinant (positive control), s=synthetic, b/r=boiled and reduced (negative control), Bio-s=biotinylated synthetic. Recombinant and synthetic protein show similar activity, but the biotinylated protein shows significantly reduced ELISA signal at both concentrations.

- **What opportunities for training and professional development has the project provided?**
 - This project has provided substantial training opportunities for graduate students Zachary Cruz, Sarah Apple, and Patrick Erickson. These students have received extensive training in solid-phase peptide synthesis, native chemical ligation, biophysical characterization of synthetic proteins, and troubleshooting challenging syntheses.
 - Zachary Cruz and Patrick Erickson attended the 2019 American Peptide Symposium to present (in part) their work on this project. This conference also provided valuable networking opportunities, as well as broad exposure to the chemical protein synthesis field.
- **How were the results disseminated to communities of interest?**
 - A pre-print describing much of the early work on this grant has been posted on ChemRxiv (<https://doi.org/10.26434/chemrxiv.9759314.v2>) and has been accepted with very minor revisions at Organic and Biomolecular Chemistry.
- **What do you plan to do during the next reporting period to accomplish the goals?**
 - We will use our improved synthesis route to produce purer D-StxB and stabilize it using the D-cork peptide. Biotinylation has also been shown to destabilize StxB, but is required for immobilization during phage display. To avoid this issue, we will incorporate biotin onto the cork peptide to avoid perturbing the fragile StxB structure.
 - We will characterize mirror-image phage display hits as described in the original proposal (phage binding assays, SPR, crystallography, inhibition assays)
 - We will optimize the most promising hits via multimerization (into pentamers) to maximize avidity, as described in the original proposal.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
 - The 2nd-generation helping hand solubilizing tag (Ddap) was developed in part under this grant to overcome solubility problems with the StxB N-terminal segment (as described in the attached manuscript, Fig. 4). This tool will find wide use among peptide/protein synthesis groups, as it is a novel easy-to-use tool that overcomes one of the most common problems in chemical peptide/protein synthesis – insolubility of peptide segments or assembly intermediates. This tool is fully compatible with all common conditions employed in chemical protein synthesis by native chemical ligation (including desulfurization).
- **What was the impact on other disciplines?**
 - Nothing to Report
- **What was the impact on technology transfer?**

- Nothing to Report
 - **What was the impact on society beyond science and technology?**
 - Nothing to Report
- 5. **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*
 - **Changes in approach and reasons for change**
 - As described above, the stability of the pentameric StxB protein was determined to be insufficient for use in mirror-image phage display screening. We therefore developed a new synthesis strategy (described above and in our manuscript) to produce higher quality material with improved stability.
 - **Actual or anticipated problems or delays and actions or plans to resolve them**
 - Synthesis of stable and high-quality StxB has taken longer than expected due to the issues described above, though our new synthesis route and cork peptide are expected to resolve these issues.
 - **Changes that had a significant impact on expenditures**
 - Nothing to Report
 - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
 - Nothing to Report
- 6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*
 - **Publications, conference papers, and presentations**
Report only the major publication(s) resulting from the work under this award.
 - **Journal publications.** A manuscript describing our improved StxB synthesis method entitled "Chemical synthesis of Shiga toxin subunit B using a next-generation traceless "helping hand" solubilizing tag" has been posted on ChemRxiv and submitted for publication in Organic and Biomolecular Chemistry. The author list is: James M Fulcher, Mark E Petersen, Riley J Giesler, Zachary S Cruz, Debra M Eckert, J Nicholas Francis, Eric M Kawamoto, Michael T Jacobsen, and Michael S Kay. Federal support from this grant was acknowledged in this manuscript.
 - **Books or other non-periodical, one-time publications.** Nothing to Report.

- **Other publications, conference papers, and presentations.** A poster presentation was given at the American Peptide Symposium in June 2019 entitled: *"Chemical Synthesis of Shiga Toxin B-Subunit".

- **Website(s) or other Internet site(s)**
Nothing to Report
- **Technologies or techniques**
The 2nd-generation helping hand reagent (Addp) described in our manuscript is available upon request. We are also working with chemical vendors to make this non-patented reagent available to the research community. A full description of methods for making and using this reagent is available in our manuscript.
- **Inventions, patent applications, and/or licenses**
Nothing to Report
- **Other Products**
Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Michael Kay
Project Role:	Principle Investigator
Researcher Identifier:	orcid.org/0000-0003-3186-9684
Nearest person months worked:	2.4 CM
Contribution to Project:	Dr. Kay has provided guidance and oversight over this project as well as training of graduate students, data interpretation and troubleshooting, and writing of manuscripts/reports.
Funding Support:	NIH: R01GM128507 (Co-I) & P50AI150464 (Co-I)
Name:	Eckert, Debra
Project Role:	Research Professor
Researcher Identifier:	orcid.org/0000-0001-7159-8351
Nearest person months worked:	2.4 CM
Contribution to Project:	Dr. Eckert has supervised and trained lab personnel, particularly with biophysical assays such as analytical ultracentrifugation.
Funding Support:	NIH: P50GM82545 (Co-I)
Name:	Patrick Erickson

Project Role:	Graduate Student
Researcher Identifier:	https://orcid.org/0000-0002-4913-2317
Nearest person months worked:	4 CM
Contribution to Project:	Mr. Erickson has assisted in the training and supervision of students on solid-phase peptide synthesis and native chemical ligation techniques (including associated analytical methods like LC-MS and circular dichroism).
Funding Support:	No direct other funding support
Name:	Zachary Cruz
Project Role:	Graduate Student
Researcher Identifier:	orcid.org/0000-0001-6319-287X
Nearest person months worked:	5 CM
Contribution to Project:	Mr. Cruz has synthesized StxB using solid-phase peptide synthesis and native chemical ligation, as well as validating synthetic proteins. He is also performing all phage display screens and synthesis of peptide hits.
Funding Support:	No other direct funding support
Name:	Sarah Apple
Project Role:	Graduate Student
Researcher Identifier:	https://orcid.org/0000-0002-7000-247X
Nearest person months worked:	4 CM
Contribution to Project:	Ms. Apple has assisted with the chemical synthesis of L- and D-StxB with a particular focus on preventing aspartimide formation (a common synthetic peptide side product).
Funding Support:	No other direct funding support

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
 - Nothing to Report
- **What other organizations were involved as partners?**
 - Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

- **QUAD CHARTS:** Nothing to Report.

9. **APPENDICES:** Our submitted manuscript “Chemical synthesis of Shiga toxin subunit B using a next-generation traceless “helping hand” solubilizing tag” is attached as an appendix.

Chemical synthesis of Shiga toxin subunit B using a next-generation traceless “helping
hand” solubilizing tag

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Abstract

The application of solid-phase peptide synthesis and native chemical ligation in chemical protein synthesis (CPS) has enabled access to synthetic proteins that cannot be produced recombinantly, such as site-specific post-translationally modified or mirror-image(D-) proteins. However, CPS is commonly hampered by aggregation and insolubility of peptide segments and assembly intermediates. Installation of a solubilizing tag consisting of basic Lys or Arg amino acids can overcome these issues. Through the introduction of a traceless cleavable linker, the solubilizing tag can be selectively removed to generate native peptide. Here we describe the synthesis of a next-generation amine-reactive linker *N*-Fmoc-2-(7-amino-1-hydroxyheptylidene)-5,5-dimethylcyclohexane-1,3-dione (Fmoc-Ddap-OH) that can be used to selectively introduce semipermanent solubilizing tags (“helping hands”) onto Lys side chains of difficult peptides. This linker is more stable than its predecessor, a property that can increase yields for multi-step syntheses with longer handling times. We also introduce a linker cleavage protocol using hydroxylamine that greatly accelerates removal of the linker. The utility of this linker in CPS was demonstrated by preparing the synthetically challenging Shiga toxin subunit B (StxB) protein. This robust and easy-to-use linker is a valuable addition to the CPS toolbox for the production of challenging synthetic proteins.

Introduction

Total chemical synthesis of proteins enables techniques such as racemic protein crystallography¹ and mirror-image phage display,² as well as structure/function studies of post-translationally modified proteins.³ Through the use of solid-phase peptide synthesis (SPPS)⁴ and native chemical ligation (NCL),⁵⁻⁷ chemical protein synthesis (CPS)^{8,9} permits the routine synthesis of proteins up to ~200 amino acids. However, challenges with peptide insolubility are commonly encountered during the assembly of synthetic proteins and can limit the scope of CPS.¹⁰ Ambitious synthesis projects are often hindered by peptide segments that are too insoluble to be purified by HPLC or dissolved at high concentrations (typically mM) needed for efficient NCL.^{11,12}

To address and overcome issues encountered with insoluble peptides, several groups have devised strategies that incorporate two main components: 1) a solubilizing tag composed of multiple basic amino acids, and 2) a linker between the tag and insoluble peptide that can be removed to restore the native peptide sequence (referred to as a “semipermanent tag”).¹³ For example, work by Kent¹⁴ and Aimoto¹⁵ detailed a thioester linker combined with a poly-Arg tag to increase the solubility of hydrophobic peptide segments. After HPLC purification, this tag can be removed through transthioesterification during NCL. Although this direct thioester linker is restricted to Boc-SPPS, several Fmoc-compatible strategies have been developed.¹⁶⁻¹⁸ The main disadvantage to these strategies is that they generally cannot survive more than one NCL reaction.¹⁹ Recently, several NCL-compatible strategies introducing semipermanent solubilizing tags have been presented. Liu's group developed a salicylaldehyde-derived linker and Arg-tag for the introduction of solubilizing removable backbone modifications (RBMs).^{20,21} Several Cys-based linkers/solubilizing tags have also been developed recently including the phenylacetamidomethyl (Phacm) linker by Brik's group, the Arg-tagged ACM^R by Danishefsky's group, and an Arg-tagged trityl linker from the Yoshiya group.²²⁻²⁶ Additionally, the Yoshiya group recently introduced a self-cleavable canaline linker.²⁷

The introduction of these linkers and solubilizing tags has expanded the scope of CPS, but significant barriers to their broader use remain. These barriers include complex

linker synthesis, limited availability of sites for attachment of linkers, or lability under certain reaction conditions. Building on previous work with the Dde protecting group,²⁸⁻³⁰ we recently described a linker (*N*-Fmoc-1-(4,4-dimethyl-2,6-dioxocyclo-hexylidene)-3-[2-(2-aminoethoxy)ethoxy]-propan-1-ol, or Fmoc-Ddae-OH) that aimed to address these limitations with its ease of use and compatibility with common conditions employed during Fmoc-SPPS and NCL.³¹ The Ddae linker could easily be incorporated at various Lys sites within a peptide and tracelessly removed to generate the target of interest. This Ddae linker met all of our initial design requirements; however, we sought to improve its stability and handling properties, as well as reduce the cost of synthesis to increase its utility and accessibility.

Here we describe the synthesis of a next generation linker, Fmoc-Ddap-OH (*N*-Fmoc-2-(7-amino-1-hydroxyheptylidene)-5,5-dimethylcyclohexane-1,3-dione), that is more stable in aqueous solvents and easier to handle compared to the Ddae linker. Incorporation of the Ddap linker follows the same protocol as Ddae and is achieved through direct addition onto a free amine, typically a Lys side chain, present on an otherwise protected peptide. Following Fmoc removal, the solubilizing sequence can be built through standard Fmoc-SPPS. After cleavage using TFA-containing standard scavengers, the Ddap linker is stable to several commonly used buffers in chemical protein synthesis. Once the handling steps that require enhanced solubilization are complete, the linker can be cleaved using an α -nucleophile, such as hydrazine or hydroxylamine.³² We demonstrate the versatility of this new linker in the synthetically challenging Shiga toxin subunit B (StxB), a 69-amino acid protein essential for the pathogenesis of *Shigella* and Shiga Toxin-Producing *E. coli* (STEC).³³ Synthetic StxB and a recombinant StxB control were compared using high-resolution mass spectrometry (HRMS), circular dichroism (CD), size exclusion chromatography (SEC) and analytical ultracentrifugation (AUC) to validate the synthetic approach.

Results and Discussion

Synthesis and Characterization of Linkers using the Model Peptide C20

We began by substituting the PEG₂ moiety present in our original Ddae linker with 6, 7, or 8-carbon alkyl chains (termed Ddax, Ddap, and Ddac, respectively; Fig. 1 and S1-16) as the starting materials are commercially available and relatively inexpensive (Table S1). After flash purification and lyophilization, we observed that the Ddap and Ddac linkers are solids at room temperature, unlike the Ddax and Ddae linkers, which are viscous oils (Fig. S17). These linkers were then compared throughout several stages of SPPS assembly using the model peptide C20 (Ac-DWTKNITDK(**Dde**)IDQIIHDFVDK-NH₂, Fig. S18). This model peptide was selected due to its diverse peptide sequence (including a Lys residue), high crude purity (>70%), and previous use in the characterization of the Ddae linker.³¹ After synthesis of C20 at 30 μ mol scale, the Dde group was removed using 5% hydrazine in DMF to reveal an unprotected primary amine at Lys9. The coupling (attachment of the linker to amine) was performed by adding 1 mL of 200 mM linker in N-methylpyrrolidine (NMP) to the resin at 37°C. Attachment of alkyl chain linkers reached completion in <15 min compared to 60 min required for Ddae coupling (Fig. S19). As no other additives are needed for coupling, the excess linker can be recycled by flash chromatography and reused. After coupling of the linkers, we performed standard Fmoc-SPPS to build a Lys₆ solubilizing tag (referred to as a “helping hand” or HH) for each C20 linker variant. All peptides were cleaved from solid supports using standard TFA cleavage conditions (95% TFA, 2.5% TIS, 2.5% H₂O) and purified by RP-HPLC (Fig. S20-23).

With these purified peptides in hand, we next tested the cleavage kinetics of each linker using 1 M hydrazine in denaturing buffer (6 M GnHCl, 100 mM NaPO₄, pH 7.5) with C20(HH) peptides at 0.5 mM. Timepoints were analyzed using analytical HPLC monitoring at 214 nm, and product formation was calculated based on relative peak areas with a correction factor to account for the UV absorbance of the cleaved linker (Fig. S24 and S25). All alkyl chain linkers were cleaved within 8 h, compared to 4 h for the PEG₂-based Ddae linker (Fig. 2A). Comparison of C20 with Lys₆-Ddap and Lys₆-Ddae in several common reaction conditions used in CPS demonstrates the improved stability of the Ddap linker

over Ddae as well (Table 1 and Fig. S26). Although cleavage kinetics are similar between the alkyl chain linkers, we picked the Ddap linker as the most favorable compound due to its lower-cost starting material compared to Ddac and solid physical state compared to Ddax. Therefore, we continued our characterization using the Ddap linker as our lead candidate.

Ddap Cleavage Kinetics Using Hydroxylamine

The greater stability of Ddap, though advantageous for minimizing dissociation during multiple handling steps, led us to wonder if the cleavage time could be reduced by using a different α -nucleophile. Considering the pKa of the conjugate acid of hydroxylamine (~ 6) allows for a higher proportion of nucleophilic species at lower pH than hydrazine (pKa ~ 8),³⁴ we rationalized that hydroxylamine at pH 6.75 could potentially be much faster than our standard hydrazine cleavage conditions (1 M hydrazine in denaturing buffer: 6 M GnHCl, 100 mM NaPO₄, pH 7.5).^{32, 35} The lower pH of 6.75 was chosen to more closely match NCL conditions and reduce the potential for hydroxylamine-induced cleavage of peptide bonds.³⁶ The rate of cleavage with 1 M hydroxylamine at pH 6.75 in denaturing buffer was $\sim 19\times$ faster than our previous cleavage protocol using 1 M hydrazine at pH 7.5 (k of 225 vs. $11.6 \times 10^{-3} \text{ s}^{-1}$), reaching completion within 30 min (Fig. 2B and S27). The reaction also proceeded cleanly without formation of any significant side products (Fig. 2C and Fig. S28-29). We extended both cleavage reactions for 24 h to investigate the potential for side reactions. Under these exceptionally harsh conditions, the majority of the C20 peptide remained unmodified by LC-MS, however several hydrazide and hydroxamate modifications were observed (Fig. S30 and S31).³⁷

UV Absorbance of Ddap linker

One characteristic of the Ddap linker we observed in our initial characterization was significant 280 nm absorbance (A_{280}). As A_{280} from Trp or Tyr residues is a convenient method for determining peptide concentration, we determined the molar extinction coefficient (ϵ , $\text{M}^{-1}\cdot\text{cm}^{-1}$) of our linker so that it could be utilized as a UV tag and would not interfere with peptide concentration measurements. Utilizing our C20 test peptide modified with an N-terminal carboxyfluorescein, we compared the A_{280} of the peptide with

and without the Ddap linker at equal concentrations as determined using the A_{495} of fluorescein (Fig. S32 and S33).³⁸ The difference in A_{280} was found to correspond to an ϵ of $\sim 14,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in denaturing buffer (6 M GnHCl, 200 mM NaPO₄, pH 8), similar to the ϵ of the related *N*-4,4-Dimethyl-2,6-dioxocyclohexylidenemethyl (Dcm) protecting group ($15,020 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 360 nm).³⁹ It is worth noting the ϵ of Ddap is considerably higher than Trp and Tyr, which have ϵ of 5,500 and 1,490 $\text{M}^{-1} \cdot \text{cm}^{-1}$, respectively.⁴⁰ Therefore, this property of Ddap would be particularly useful for peptides lacking Tyr or Trp, allowing it to be used as a tag for UV monitoring or concentration measurements via A_{280} .

Synthesis of Shiga Toxin Subunit B using the Helping Hand

We tested the utility of our next-generation helping hand by incorporating it into the synthesis of Shiga toxin subunit B (StxB; note StxB contains an N-terminal signal peptide that is cleaved to form the mature protein, Fig. 3A).⁴¹ Our initial synthesis attempts to make the full-length 69-amino acid mature protein via SPPS were hampered by poor crude quality and insolubility in HPLC conditions. To improve the quality of the crude peptide produced by SPPS, StxB was divided into two segments (StxB-N and StxB-C) for NCL, and several pseudoproline dipeptides were used in the synthesis of StxB-N.^{42, 43} To address insolubility of StxB-N, we installed the Ddap helping hand at Lys47 (Fig. 3B and 3C). StxB represents a good test for our new linker as it not only displays insolubility in aqueous conditions but also contains an Asn-Gly in its sequence, a reported hydroxylamine cleavage site (though under much harsher conditions, such as 2 M hydroxylamine at pH 9).^{36, 44, 45}

StxB-N was synthesized as a C-terminal hydrazide for NCL.⁴⁶⁻⁴⁸ The C-terminal hydrazide was utilized as a thioester surrogate due to its convenience and compatibility with Fmoc-SPPS. StxB-N was prepared with Boc- protection at the N-terminus and an orthogonally protected Lys(Dde) for incorporation of the helping hand (Fig. 3B). As a control to evaluate improvement in solubility, StxB-N was also produced without a solubilizing tag. StxB-C was synthesized with a C-terminal acid using standard Fmoc-SPPS. The crude peptides (StxB-N and StxB-N(HH)) were dissolved in HPLC buffer (20% ACN 0.1% TFA) until saturation and centrifuged at 5,000g for 20 min, and the supernatants were lyophilized to determine the soluble peptide fraction. StxB-N without the solubilizing

tag was only soluble to 0.4 mg/mL and was not studied further, while StxB-N(HH) with the solubilizing tag was 40-fold more soluble (16.0 mg/mL). This increased solubility considerably sharpened the analytical HPLC trace (Fig. 4). Although the HPLC purification of StxB-N(HH) was considerably easier with the helping hand due to the improved solubility, we could not resolve material containing a Val deletion (-99 Da) from the correct product. StxB-C was purified without issues using HPLC. With the purified peptides in hand (Fig. S34 and S35), we proceeded with NCL. StxB-N(HH) (0.5 mM) was converted in situ to an MPAA thioester and combined with 3 equiv. of StxB-C (1.5 mM) in denaturing buffer. NCL between StxB-N(HH) and StxB-C was complete within 30 min, with minimal loss of product due to hydrolysis of the thioester on StxB-N(HH) (Fig. 5 and S36). Cleavage of the helping hand was performed in one pot by equal volume addition of 2 M hydroxylamine, pH 6.75 in denaturing buffer. As anticipated, cleavage proceeded rapidly and was complete within 30 min, producing full-length StxB after a final HPLC purification (Fig. 5 and S37).

Importantly, we did not observe any side products resulting from cleavage at the Asn-Gly bond in StxB, suggesting that treatment with 1 M hydroxylamine is relatively mild. A final step-wise dialysis under oxidizing conditions was performed to allow for disulfide bond formation followed by folding of the synthetic material. The deletion products that carried over from the initial, challenging HPLC purification of StxB-N(HH) did not appear to fold correctly and were not found in the final, post-dialysis clarified material (Fig. 6A and S38). After folding, synthetic StxB was compared to a recombinant StxB control using high-resolution mass spectrometry (HRMS), circular dichroism (CD), size-exclusion chromatography (SEC), and analytical ultracentrifugation (AUC). Comparison data between the recombinant and synthetic StxB from all four techniques closely agree, suggesting similar chemical structure (mass spectra in Fig. 6A), secondary structure (CD spectra in Fig. 6B), and the expected pentameric quaternary structure (SEC in Fig. 6C and AUC in ESI Fig. S39).

Conclusions

In this study, we describe the one-step synthesis of a next-generation amine-reactive linker, Fmoc-Ddap-OH, using inexpensive and accessible starting materials. Like the first-generation Fmoc-Ddae-OH, this linker can be used to address insolubility of peptides through the addition of basic Lys/Arg amino acids (referred to as “helping hands”). An added convenience is that this new linker is a solid powder at room temperature unlike the previous viscous oil. We also found the alkyl chain linker conferred two-fold greater stability in various common reaction conditions used in the assembly of synthetic proteins. For large protein syntheses that require a solubilizing tag and have numerous handling steps, the greater stability of this linker in aqueous conditions should prevent helping hand leakage and provide higher final yields. This added stability, though advantageous for multiple handling steps, increased the time needed to cleave the Ddap linker using hydrazine. To this end, we demonstrated a new method for cleaving the linker with hydroxylamine that greatly accelerated the rate of cleavage, allowing complete removal of the linker within 30 min. We expect these accelerated cleavage conditions to be particularly advantageous for removal of multiple Ddap linkers from a single peptide or protein.

The synthesis of StxB not only presents an ideal opportunity to test the new Ddap linker in a challenging real-world CPS problem but also results in a relevant target for mirror-image phage display (MIPD).² Shiga toxins (Stx), classical AB₅ toxins, are produced by various *Shigella* bacterial species and are important virulence factors in the development of hemorrhagic colitis/shigellosis.^{33, 49} StxB mediates the introduction of the ribotoxic StxA by binding to host glycosphingolipid Gb₃.³³ Currently there are no approved treatments for the prevention or reduction of disease symptoms, and treatment with traditional antibiotics can increase the risk of developing the potentially fatal hemolytic uremic syndrome.⁵⁰ A D-peptide therapeutic identified using MIPD that blocks the interaction of StxB with Gb₃ directly at the site of binding would be of substantial clinical benefit. A requirement for MIPD, however, is the synthesis of the target in the opposite (D-) chirality. With a synthesis strategy for L-StxB now established, synthesis of the mirror-image D-StxB can be performed following the same steps described here. In conclusion, this

convenient Ddap linker with solubilizing Lys₆ tag is a widely accessible and easy-to-use tool that enables the synthesis of insoluble peptides and proteins.

Conflicts of Interest

DME and MSK are consultants and equity holders in Navigen, Inc., which is developing D-peptide drugs.

Funding Information

This research was supported by NIH grants AI150464 and AI120414 and DoD grant W81XWH1810116.

Acknowledgments

The authors would like to thank Dr. Paul Sebahar, Nai-Pin Lin, and Judah Evangelista for experimental/material assistance. We also thank Dr. Sandra Osburn-Staker of the Proteomics Core Facility (University of Utah) for high-resolution mass spectrometry analysis and Dr. Vincent Aucagne for his helpful discussions.

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Fig. 1. One-step synthetic route for all linkers used in this study (yields shown next to each linker). See ESI for synthesis details.

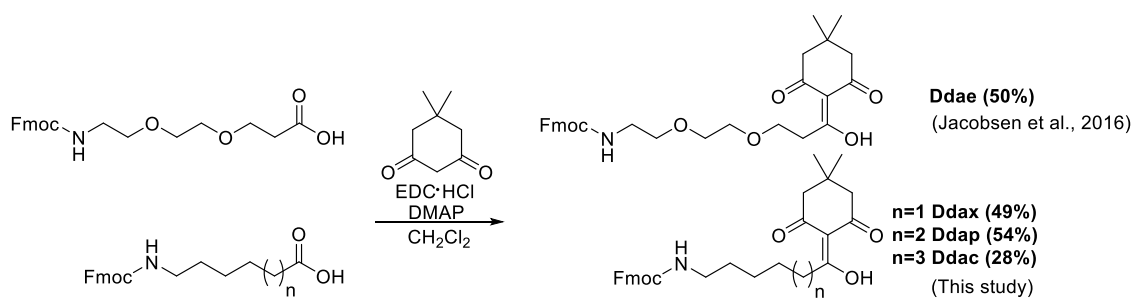


Fig. 2. Characterization of linker cleavage kinetics with different α -nucleophiles.

(A) Cleavage kinetics of C20(HH) with all linkers using 1 M hydrazine in cleavage buffer (6 M GnHCl, 100 mM NaPO₄, pH 7.5). **(B)** Cleavage kinetics of C20(Lys₆-Ddap) with 1 M hydrazine or hydroxylamine in cleavage buffer at pH 6.75 vs. 7.5. Average of 2 replicates shown with s.d. error bars. **(C)** Representative HPLC traces of C20(Lys₆-Ddap) cleavage using 1 M hydroxylamine in cleavage buffer, pH 6.75. Y-axis shows A₂₁₄ normalized to the highest peak.

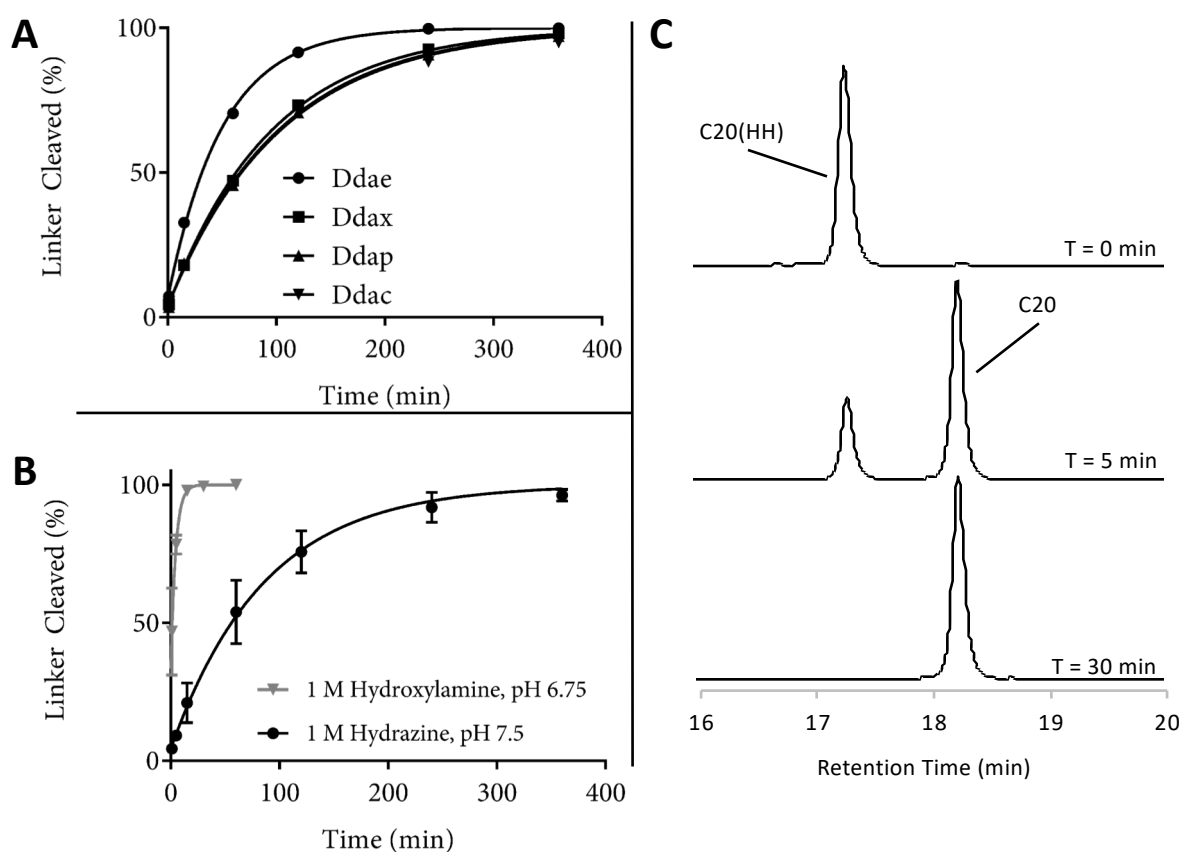


Fig. 3. StxB sequence and synthesis strategy.
(A) Sequence of mature StxB (without precursor signal peptide). N-terminal (T²¹ – A⁷⁶) and C-terminal (C⁷⁷ – R⁸⁹) segments are shown in red and blue, respectively. **(B)** Sequences of individual peptides showing position of pseudoproline dipeptides (black), helping hand (underline), and Boc-modified N-terminus. **(C)** Assembly strategy for the two segments showing one-pot NCL/helping hand cleavage to produce full-length StxB before folding through step-wise dialysis under oxidizing conditions.

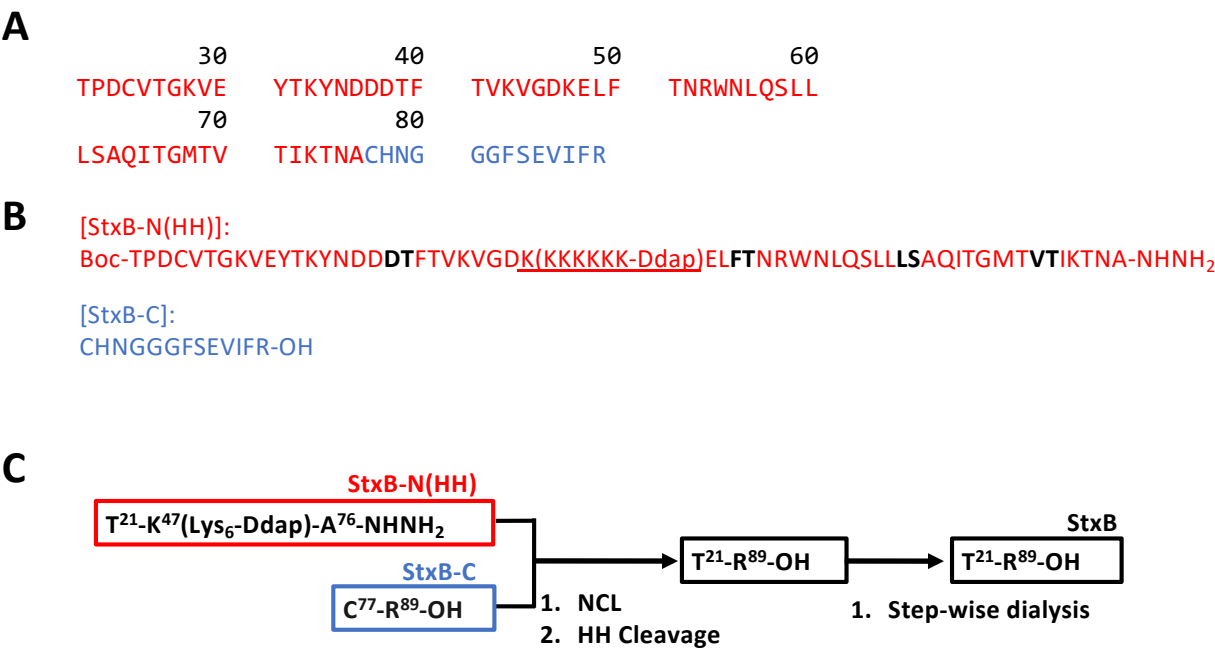


Fig. 4. Comparison of crude StxB-N with and without helping hand. StxB-N and StxB-N(HH) were dissolved in HPLC buffer (20% B, 80% A) until saturation before centrifugation at 5,000g for 20 min to remove precipitated material. HPLC traces were collected for StxB-N and StxB-N(HH) with a linear gradient of 10-60% B over 30 min.

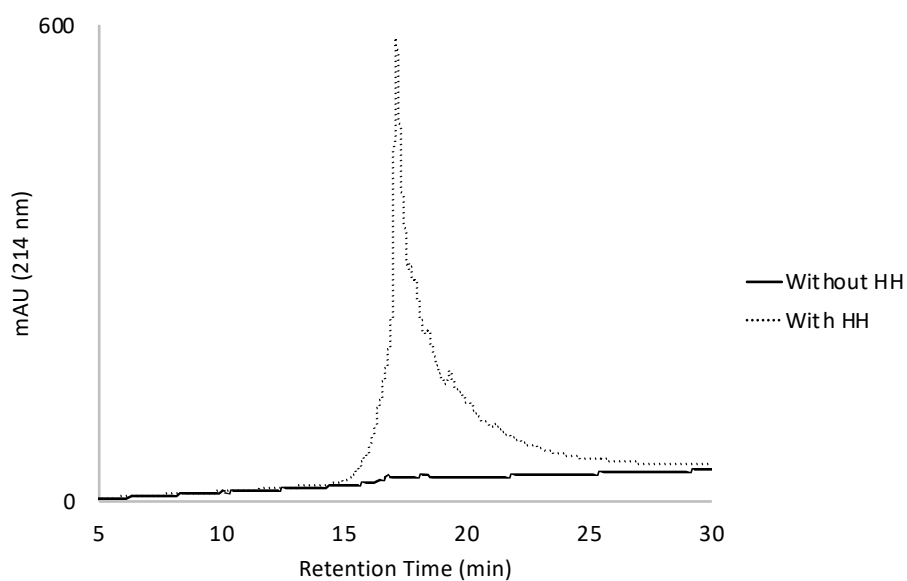


Fig. 5. HPLC traces demonstrating NCL between StxB-C and StxB-N(HH) followed by one-pot cleavage of the helping hand using 1 M hydroxylamine (pH 6.75) to produce full-length StxB. Y-axis is A_{214} and HPLC traces were collected with a linear gradient of 10 to 60% B over 30 min.

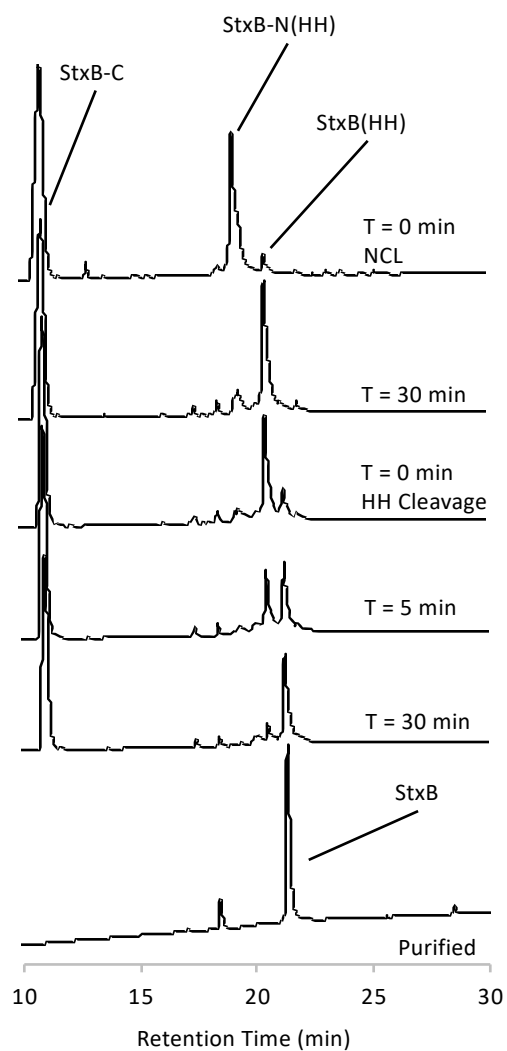


Fig. 6. Characterization of recombinant and synthetic StxB. Comparison of synthetic (blue) and recombinant StxB (green). **(A)** High-resolution mass spectrometry shows matching masses. **(B)** Circular dichroism spectra indicate matching secondary structure. **(C)** Size-exclusion chromatography suggests both synthetic and recombinant StxB form a pentamer.

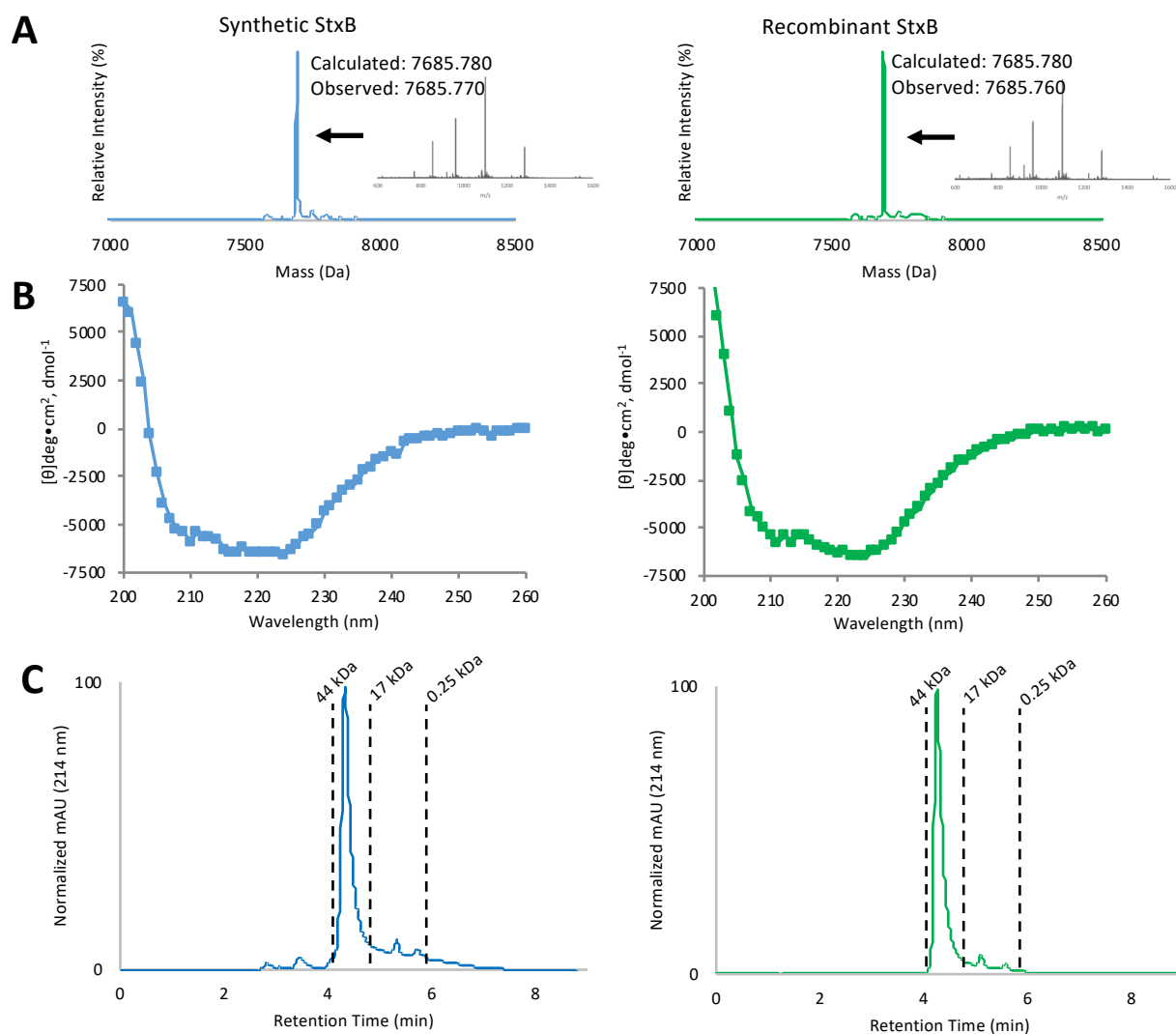


Table 1. Stability comparison between C20(Lys₆-Ddae) and C20(Lys₆-Ddap) after 48 h in several commonly used buffers in CPS. See ESI for experimental details.

Buffer	C20(Lys ₆ -Ddae) Uncleaved Linker (%) after 48 h	C20(Lys ₆ -Ddap) Uncleaved Linker (%) after 48 h
0.1% TFA in 50% ACN (HPLC Buffer)	90	95
6 M GnHCl, 100 mM NaPO ₄ , pH 3	88	90
6 M GnHCl, 200 mM NaPO ₄ , pH 7	89	92
6 M GnHCl, 5% AcOH	89	94
6 M GnHCl, 100 mM NaPO ₄ , 200 mM MeONH ₂ , pH 3 (Thz cleavage buffer)	60	87
6 M GnHCl, 200 mM NaPO ₄ , 200 mM MPAA, 50 mM TCEP, pH 7 (NCL conditions)	86	94