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Divinylsulfonamides as Specific Linkers for Stapling Disulfide Bonds in Peptides

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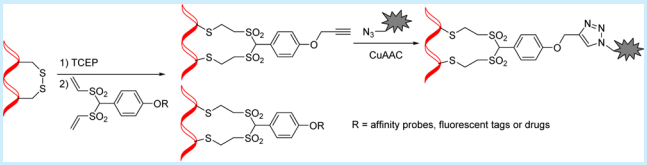
Supporting Information

ABSTRACT: A new class of *N*-phenyl-divinylsulfonamides which can be easily prepared have been successfully developed and utilized as efficient linkers in the field of disulfide bond modification. Functional divinylsulfonamides provide opportunities for the specific introduction of various functionalities, including affinity probes, fluorescent tags, and drugs, into peptides.



Peptide stapling has attracted much attention due to its biological applications,[1] including improvement of binding affinity, development of inhibitors of protein−protein interactions, and creation of novel therapeutics. During the past several decades, there has been widespread investment into efforts to target specific amino acids, such as tryptophan, histidine, tyrosine, lysine, or cysteine, in native peptides and proteins.[2] Also, the modification of disulfide bonds via functional rebridging has received considerable development in recent years.[3] Accessible disulfide bonds are easily found in most biologically relevant peptides and proteins. The direct reduction of disulfide bonds under mild reaction conditions followed by immediately covalent rebridging of the two resulting thiols permits the production of stapled peptides that retained their tertiary structure and stability. Shaunak et al. pioneered a useful methodology in which α,β-unsaturated bis-thiol alkylating reagents were developed for the PEGylation of protein disulfide bonds.[4] Subsequently, a series of bissulfones or allyl sulfones was reported.[5] Dibromomaleimide,[6] dithiophenolmaleimides,[7] and aryloxymaleimides[8] are other typical bis-alkylating reagents[9] for the in situ rebridging of disulfide bonds in peptides and proteins. Recently, a thiol−ene coupling approach,[10] dibromopyridazinediones,[11] alkynes,[12] organic arsenicals,[13] s-tetrazine,[14] perfluoroaryl,[15] and acetone[16] have also been employed as efficient linkers for disulfide rebridging.

As highly reactive conjugate receptors, vinylsulfonamides can be reacted effectively with thiol and amino compounds, and they are widely used for covalent cross-linking with cysteine[17] or lysine[18] in peptides and proteins. Cysteine is generally the easiest amino acid to selectively conjugate with chemical linkers because of its highly nucleophilic characteristic. As important Michael receptors, maleimides and vinylsulfonamides have been extensively investigated for the selective modification of peptides and proteins, with the objective of taking advantage of the most easily targetable characteristic of cysteine. Moreover, a series of substituted maleimides has been successfully developed and utilized for the specific rebridging of disulfide bonds in peptides and proteins. However, no reports have described the discovery and utilization of divinylsulfonamides as efficient linkers in the field of disulfide bond modification.

Here, we report a new class of divinylsulfonamides, the *N*-phenyl-divinylsulfonamides, which can be inserted into disulfide bonds of peptides. Our first goal was the preparation of a new range of *N*-phenyl-divinylsulfonamides. We then investigated the reactivity and selectivity of these reagents and chose the optimal reagent to conjugate with peptides via the rebridging of disulfide bonds. Furthermore, a functional moiety could also be introduced to the phenyl group, and this capability provides a point of attachment for chemical or biological entities of interest, such as affinity probes, fluorophores, and drugs.

A series of *N*-phenyl-divinylsulfonamides (Figure 1) with different substituents at the *p*-position were easily obtained via reaction with phenyl amines and 2-chloroethanesulfonyl chloride. Oxytocin (**2**) was chosen as a model peptide for evaluation of these compounds' ability to rebridge disulfide

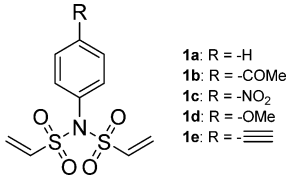


Figure 1. Chemical structures of differently substituted *N*-phenyl-divinylsulfonamides.

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bonds. Oxytocin is a neurohypophyseal uterotonic nonapeptide that is used as a medication to facilitate childbirth. A disulfide bridge is formed between the Cys^1 and Cys^6 of oxytocin in the sequence (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2). A variety of modifications at this disulfide bond have previously been evaluated, including the insertion of a disulfide bridge via treatment with dithiophenol maleimide.[19] After oxytocin (1.20 mM) was treated with tris(2-carboxyethyl)phosphine (TCEP) (1.7 equiv) for 1.5 h, each of the five *N*-phenyl-divinylsulfonamides referenced above (3.0 equiv) was reacted with the reduced oxytocin, and these compounds' rebridging activities were examined and compared. Reaction products were analyzed by HPLC (Supporting Information (SI), Figure S1), and we found that the stapling of disulfide bonds was difficult when *N*-phenyl-divinylsulfonamide derivatives with electron-withdrawing groups at the *p*-position (**1b** and **1c**) were utilized. This result is likely attributable to the instability of these compounds in the reaction mixture. For compounds **1a** and **1e**, they resulted in complex products. Fortunately, the *p*-OCH_3-substituted compound (**1d**) showed good stability in the reaction and it resulted a relatively clean product in 47% yield. However, the ESI-HRMS spectrum showed that the product **2b** was dilabeled with signals at 1615.5253 $[M + H]^+$ and 808.2667 $[M + 2H]^{2+}$. The amino residue at the N-terminus of oxytocin was also involved in the Micheal addition with the divinylsulfonamide **1d** rather than the addition of two divinylsulfonamides to the two free thiol groups, based on the experiment that when the product **2b** was treated with excess ethyl thioglycolate, the sole vinylsulfonamide residue was captured to generate compound **2d**. Further experiments in which oxytocin directly reacted with compound **1d** was performed. The *N*-residue-attached compound **2c** was produced in 85% yield (Scheme 1). This result confirmed that amino residues also had high reactivity with compound **1d** under the current reaction conditions.

Based on our results, we next tried to improve the reaction selectivity of thiols and amino residues so as to obtain the monodisulfide modified product **2a**. We began to optimize the reaction conditions with respect to pH, the ratio between the substrates, and the concentration of the substrates. In a typical reaction, after the reduction of oxytocin (at a final concentration of 1.20 mM) with TCEP (1.7 equiv) in CH_3CN and 50 mM PBS buffer (2:3) for 1.5 h at room temperature (rt), **1d** (3.0 equiv) was added to the mixture, and the resulting reaction mixture was shaken for 2 h. PBS buffers with pH values ranging from 6 to 10 were screened, and reaction outcomes were analyzed using HPLC (SI, Figure S2). The results revealed no obvious effects of pH on the yield when the reaction was performed in PBS at pH 7−9 (Table 1, entries 1−4). We then investigated the effect of the ratio between **1d** and oxytocin at pH = 7 (SI, Figure S3). Unsurprisingly, the byproduct **2b** decreased as **1d** was reduced. Moreover **2a** could even become the major product when less than 2 equiv of **1d** were used. When 1 equiv of **1d** was used, the byproduct **2b** was not detected, but the yield of **2a** was abundant (Table 1, entries 2 and 5−8). It has been reported that appropriate reactant concentrations are critical for successful conjugation.[20] Encouraged by this finding, we attempted to improve the yield of **2a** by adjusting concentrations of the reaction mixture (SI, Figure S4). To our delight, a change in the concentration of **2** led to satisfactory results (Table 1, entries 8−13). The yield of the target compound **2a** increased, when the concentration of the peptide **2** was reduced, and the rebridged disulfide product **2a** was obtained in 74% yield (64% isolated yield) when the final concentration of **2** was decreased to 0.05 mM (Table 1, entry 13). In addition, when the reaction was performed at 37 °C, compound **2a** was also obtained in a good yield of 77% (Table 1, entry 14), suggesting that our disulfide rebridging strategy has potential applications under physiological conditions.

As a further demonstration of our exciting results, an experiment in which oxytocin was directly treated with **1d** under the optimized conditions was performed. No reaction occurred at low oxytocin concentrations (Table 2, entry 2). Our explanation for this finding is that, at a low oxytocin

Scheme 1. Treatment of Oxytocin with *p*-CH_3O-*N*-Phenyldivinylsulfonamide under Different Conditions

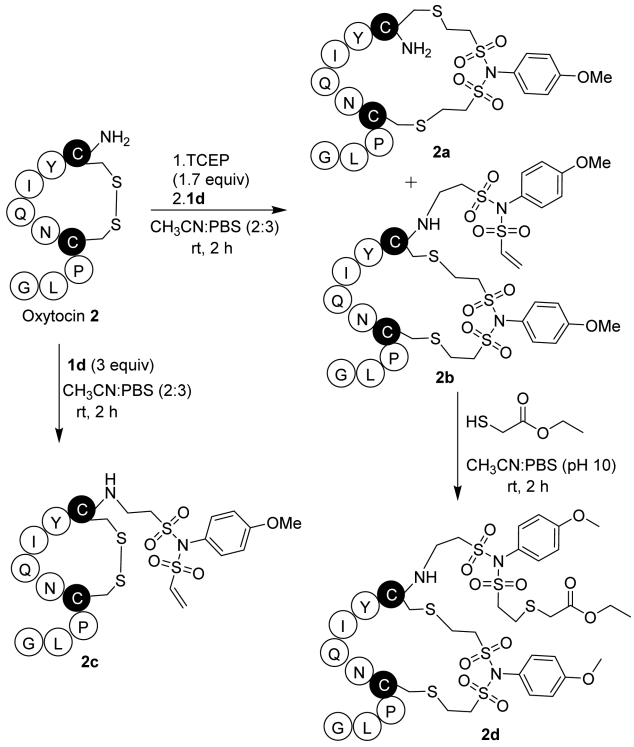

Table 1. Conditions Tested during Optimization[a]

entry	PBS (pH)	**1d** (equiv)	concn[b] (mM)	**2a**[c] (%)	**2b**[c,e] (%)
1	5.99	3.0	1.20	minority	36
2	7.07	3.0	1.20	trace	47
3	7.98	3.0	1.20	trace	44
4	9.03	3.0	1.20	trace	43
5	7.07	2.5	1.20	18	33
6	7.07	2.0	1.20	25	23
7	7.07	1.5	1.20	37	minority
8	7.07	1.0	1.20	42	trace
9	7.07	1.0	1.00	43	none
10	7.07	1.0	0.50	49	none
11	7.07	1.0	0.25	51	none
12	7.07	1.0	0.10	59	none
13	7.07	1.0	0.05	74 (64[d])	none
14[f]	7.07	1.0	0.05	77	none

[a]Reaction conditions: 12 μL of oxytocin solution (1−25 mM) in water and 20 μL of TCEP solution (1−25 mM) in water were diluted with 120 μL of PBS and 80 μL of CH_3CN at room temperature for 1.5 h; subsequently, **1d** (1−3 equiv) was added and the reaction was incubated for 2 h. [b]The final concentration of oxytocin in the mixture. [c]Yield was determined using HPLC. [d]Isolated yield. [f]Conducted at 37 °C. [e]Dilabeled product.

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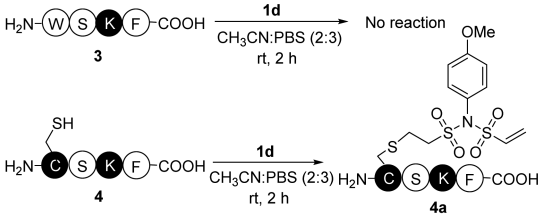
Table 2. Investigation of Site Selectivity

entry	peptide	concn.[a] (mM)	**1d** (equiv)	product (%)[b]
1	oxytocin	1.20	3.0	**2c**, 85
2	oxytocin	0.05	1.0	N.R.[c]
3	WSKF	0.05	1.0	N.R.
4	CSKF	0.05	1.0	**4a**, 75

[a]The final peptide concentration in the mixture. [b]Relative yield was determined by HPLC. [c]No reaction.

concentration, the reaction with the *N*-residue was restrained but the thiols remained sufficiently reactive to produce the rebridged disulfide product. To confirm that **1d** had the ability to site-selectively react with thiol under the optimal method, peptides **3** and **4** were used to validate the site specificity under the same reaction conditions (Scheme 2). Our results showed that no

Scheme 2. Site-Selective Reactivity of *p*-CH_3O-*N*-Phenyldivinylsulfonamide with Thiol at Low Concentration

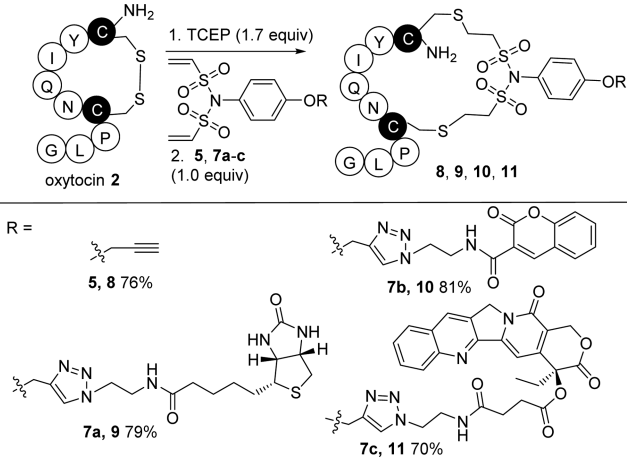


entry	peptide	concn.[a] (mM)	**1d** (equiv)	product (%)[b]
1	oxytocin	1.20	3.0	**2c**, 85
2	oxytocin	0.05	1.0	N.R[c]
3	WSKF	0.05	1.0	N.R
4	CSKF	0.05	1.0	**4a**, 75

reaction occurred for **3** (Trp-Ser-Lys-Phe) but that the thiol of **4** (Cys-Ser-Lys-Phe) was modified with **1d** (Table 2, entries 3−4), which was in keeping with these results (Table 2, entries 1−2).

To evaluate the viability of divinylsulfonamides as chemoselective ligation handles, a versatile toolbox of such compounds that included **5** and **7a**−**7c** was synthesized (Scheme 3) to offer multiple functionalities associated with the stapling of disulfide

Scheme 3. One-Pot Disulfide Rebridging of Oxytocin with Functional Divinylsulfonamides

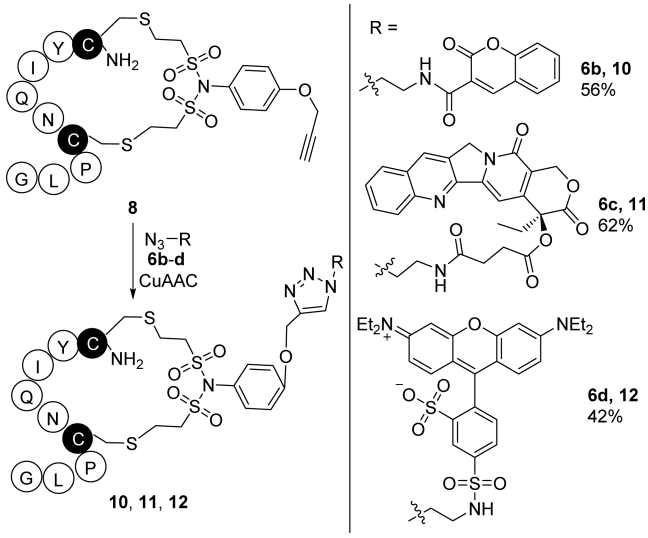


bonds and thereby introduce particular functional elements at specific positions of peptides, including a functional group (**5**), an affinity probe (**7a**), a fluorescent tag (**7b**), and a drug (**7c**). After these functional divinylsulfonamides were successfully obtained, their abilities to staple the disulfide bonds in oxytocin were examined. Under the previously determined optimized conditions, after complete reduction of the disulfide bond, oxytocin was treated with each of these functional divinylsulfonamides. To our delight, all of those reagents afforded the desired products (**8**−**11**) in good yields (SI, Figures S7 and S8).

In addition, the fluorescent character of the conjugated compound **10** was compared with those of oxytocin and the fluorescent probe **7b**. This comparison indicated that **10** had almost the same strong fluorescence as **7b** (SI, Figure S13).

Since we successfully inserted an alkynyl group into a peptide (**8**), our method offered an attractive tool for the selective chemical modification of peptides in a manner that required less energy and expense relative to methods that have been widely used to incorporate functional groups such as cysteine or an unnatural amino acid into peptides to provide a privileged site for conjugation with other probes or peptides. We evaluated the reactivity of our functional modified oxytocin (**8**). As a unique functional group, the alkyne in **8** was coupled with azide derivatives to perform typical click chemistry (Scheme 4), and the desired products were obtained smoothly (SI, Figures S8, S9, and S10).

Scheme 4. Functional Modified Oxytocin (8) Was Conjugated with Azide Tags via Click Chemistry



We also applied this approach to sCT, a 32-amino acid hormonal peptide with an important disulfide bridge (Cys^1-Cys^7) and that had been used to demonstrate the efficiency of disulfide bond rebridging with maleimides[6b,21] and organic arsenicals.[13] After the complete reduction of the disulfide in sCT with TCEP, the representative divinylsulfonamides **1d**, **5**, and **7a** were used for rebridging conjugation (Scheme 5). As expected, satisfactory yields of the desired products were obtained (SI, Figures S11 and S12).

We have demonstrated a new type of divinylsulfonamide that can be used for the selective modification of disulfide bonds in the peptides oxytocin and sCT with an efficiency comparable to that of α,β-unsaturated-β'-monosulfone or maleimides, the currently popular reagents used for disulfide bridging. Moreover,

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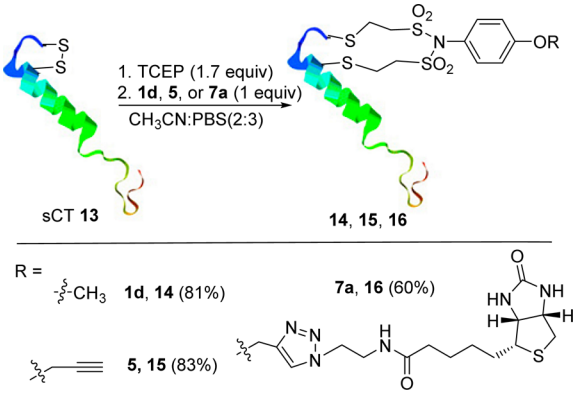
Scheme 5. One-Pot Disulfide Rebridging of sCT with Functional Divinylsulfonamides



functional divinylsulfonamides provide opportunities for the specific introduction of various functionalities, including affinity probes, fluorescent tags, and drugs, into peptides. In addition, given the successful insertion of a linkable group, such as an alkyne at a specific position, our method also provides an attractive tool for selectively modifying peptides. We will report on further developments and applications involving this powerful new class of reagents.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.7b02464.

Experimental details, characterization data, and NMR spectra for all new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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