

# ChemComm

Accepted Manuscript



This article can be cited before page numbers have been issued, to do this please use: H. E. Elashal and M. Raj, *Chem. Commun.*, 2016, DOI: 10.1039/C6CC01509C.



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

*Accepted Manuscripts* are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

Journal Name

## ARTICLE

## Site-Selective Chemical Cleavage of Peptide Bonds

Hader E. Elashal and Monika Raj\*

Received 00th January 20xx,  
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org

Site-selective cleavage of extremely unreactive peptide bonds is a very important chemical modification that provides invaluable information regarding protein sequence, and it acts as a modulator of protein structure and function for therapeutic applications. For controlled and selective cleavage, a daunting task, chemical reagents must selectively recognize or bind to one or more amino acid residues in the peptide chain and selectively cleave a peptide bond. Building on this principle, we have developed an approach that utilizes a chemical reagent to selectively modify the serine residue in a peptide chain and leads to the cleavage of a peptide backbone at the N-terminus of the serine residue. After cleavage, modified residues can be converted back to the original fragments. This method exhibits broad substrate scope and selectively cleaves various bioactive peptides with post-translational modifications (e.g. N-acetylation and -methylation) and mutations (D- and  $\beta$ -amino acids), which are a known cause of age related diseases.

Site-selective cleavage of a peptide backbone is an important chemical modification which is used for protein sequencing and has various applications in bioanalytical, biotherapeutic, and bioengineering techniques.<sup>1</sup> For the selective hydrolysis of peptide bonds, enzymatic cleavage is commonly employed. Proteases that are commonly used in protein hydrolysis include: trypsin (cleaves at Arg and Lys), chymotrypsin (Phe, Trp, and Tyr), and pepsin (Phe and Leu). While proteases cleave proteins with high precision and specificity, they have several disadvantages. They are sometimes limited because they contaminate the protein digests and they require narrow ranges of temperature and pH for optimal activity. Moreover, proteases often have high site-selectivity that can limit their use for proteins containing unnatural amino acid residues and post-translational modifications. Therefore, chemical cleavage methods have been developed as a complementary approach to enzymes due to their applicability to a wide range of substrates. Peptide bonds, nonetheless, are highly unreactive towards hydrolysis; even nonselective cleavage is difficult to achieve. The cleavage of peptides by water at neutral pH occurs with a half-life of 250-600 years.<sup>2</sup> This extreme stability of peptide bonds limits the range of appropriate cleavage reagents.<sup>3</sup> The most common chemical cleavage method used in protein sequencing is Edman's degradation,<sup>4</sup> which utilizes phenyl isothiocyanate for non-selective N-terminal cleavage of peptides. Other methods

for N-terminal amino acid determination include the use of Sanger's method<sup>5</sup> and dansyl chloride.<sup>6</sup> However, due to the non-selectivity of the abovementioned chemical methods, site-selective methods are more desirable in peptide cleavage. Those currently used for selective cleavage include cyanogen bromide for Met,<sup>7</sup> 2-nitro-5-thio-cyanobenzoic acid,<sup>8</sup> dehydroalanine<sup>9, 10</sup> for Cys, and 2-iodosylbenzoic acid for Trp and Tyr.<sup>11</sup> However, these chemical reagents, sometimes, are inadequate in providing site-selectivity, often require harsh reaction conditions, and result in low yields.<sup>12</sup> For example, cyanogen bromide uses toxic conditions and has a limited scope due to side reactions, which prevent cleavage in the presence of methionyl-serine and methionyl-threonine.<sup>13</sup> Recently, diacetoxiodobenzene (DIB) has been reported for asparagine-selective cleavage of peptide bonds but oxidative modifications were observed at several reactive amino acid side chains.<sup>14</sup> Metals have been intensively studied for hydrolysis of peptide bonds, but their practical use in structure determination of proteins is still in infancy.<sup>15-22</sup>

Here, we report a chemical methodology that selectively cleaves the peptide bond at serine residues with high efficiency. Selective cleavage was achieved by chemical modification of the amide backbone chain to the cyclic urethane moiety (**B**, Scheme 1) that is susceptible to hydrolysis under neutral, aqueous conditions. Most importantly, the reactive side chains of amino acids remained unmodified under the reaction conditions, preferred over existing cleavage methods which yield irreversibly modified fragments.

We envisioned serine-selective cleavage of peptide bonds by the reaction of their side chain hydroxymethyl group with N,N'-disuccinimidyl carbonate (DSC) to generate an activated intermediate (**A**) (Scheme 1). Intramolecular nucleophilic attack

Department of Chemistry, Seton Hall University, 400 South Orange Ave, South Orange, NJ 07079, United States

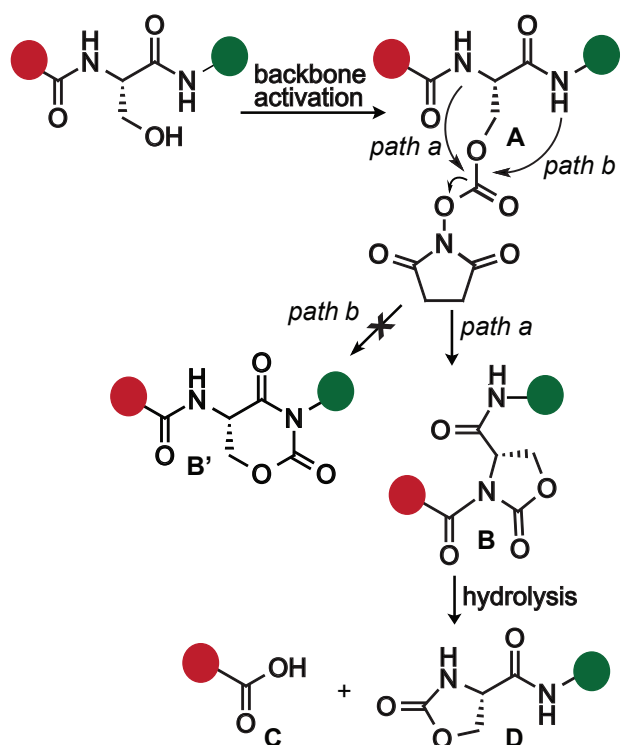
E-mail: [monika.raj@shu.edu](mailto:monika.raj@shu.edu)

\* Electronic supplementary information (ESI) available: Experimental procedures and analysis data for new compounds. For ESI and other electronic format see DOI: 10.1039/b000000x/

## ARTICLE

## Journal Name

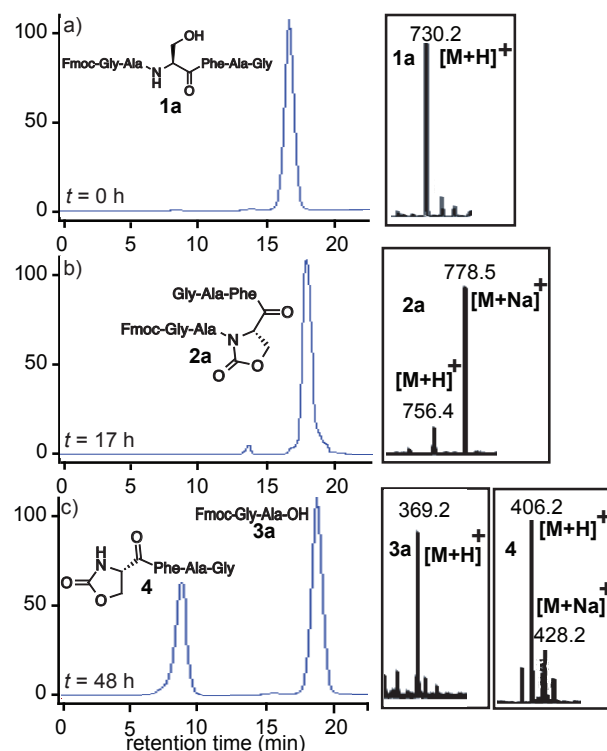
of the amide nitrogen of the peptide backbone on intermediate (A) could produce a five-membered cyclic urethane ring (B) through *path a* or a six-membered ring (B') by following *path b* (Scheme 1). Our studies suggest the high specificity for the formation of the kinetically favorable five-membered cyclic urethane ring (B), which is supported by Nuclear Magnetic Resonance (NMR) analysis (Supporting Information). Earlier attempts of utilizing DSC for the formation of the cyclic urethane moiety on Fmoc/Boc peptides were unsuccessful and led to the formation of dehydroalanine.<sup>23</sup> Hydrolysis of the peptide bond at the cyclic urethane moiety (B) produces an N-terminal peptide fragment (C) and the cyclic urethane modified C-terminal fragment (D).



**Scheme 1.** Rationale for the serine-selective modification and peptide bond cleavage in neutral aqueous solution.

To achieve cyclization at serine, reaction conditions were optimized using varying amounts of DSC, 4-dimethylaminopyridine (DMAP), and N,N-diisopropylethylamine (DIEA) on a model hexapeptide, Fmoc-Gly-Ala-Ser-Phe-Ala-Gly **1a** (retention time ( $t_R$ ) = 16.9 min, Figure 1, Table S1, Figure S1 Supporting Information). After 17 h, one sharp peak **2a**, with retention time  $t_R$  = 17.9 min was observed, indicating serine cyclization as analyzed by a mass spectrometer (MS) (b, Figure 1). After incubating the resulting sample in phosphate buffer (pH 7.5) for 31 h, the sharp peak **2a** at 17.9 min disappeared and two new peaks appeared with retention times  $t_R$  = 8.9 min and  $t_R$  = 18.6 min (c, Figure 1). MS analysis of these peaks corresponded to the cleavage products at the N-terminal side of the serine residue, with N-terminal fragment **3a** at 18.6 min, and the modified C-terminal fragment, **4** at 8.9 min (c, Figure 1). The

modified C-terminal fragment can undergo ring opening under basic conditions reverting back to unmodified serine at the terminus (see Supporting Information). With the original fragment in hand, it is possible to proceed with semi-synthesis by further coupling with another peptide or protein.



**Fig. 1** (a) HPLC charts of the reaction mixture of **1a** and DSC (10 equiv), DIEA (10 equiv), and a crystal of DMAP in DMF at  $t = 0$  h (top), at  $t = 17$  h (middle), and after addition of 0.1 M phosphate buffer (pH 7.5) at  $t = 48$  h (bottom) at 37 °C. HPLC conditions: 0.1 % FA (v/v) in water, 0.1 % FA in  $\text{CH}_3\text{CN}$ , gradient: 0–80 % in 25 min, 1.0 mL min<sup>-1</sup> flow rate, detected at 254 nm. (b) Insets show the LCMS spectrum corresponding to  $t_R$  at 16.9 min (**1a**, top), 17.9 min (**2a**, middle), 18.6 min (**3a**, bottom) and 8.9 min (**4**, bottom) (Supporting Information).

To determine the effect of the side chain functionality of the residue preceding serine on the cyclization and cleavage of the peptides, various hexapeptides, Fmoc-Gly-Xaa-Ser-Phe-Ala-Gly (**1a–1j**) with a different residue at the Xaa position were screened (Table 1). Thus, after incubation in buffer (pH 7.5) for 48 h, C-terminal fragment, **Oxd**-Phe-Ala-Gly **4** was obtained in all the cases with varying HPLC conversion (70 - >99%). The results indicate that peptides with Xaa = Gly (**1b**), Met (**1c**), His (**1d**), Tyr (**1e**), Trp (**1f**), and Asp (**1i**) (entries 2-6 and 9, Table 1) underwent smooth conversion to cleavage products after 48 h similar to **1a** with Xaa = Ala (entry 1, Table 1). This occurs in contrast to known chemical reagents, which result in over-oxidation in Tyr, Trp, and Met containing peptides.<sup>14, 22</sup> The peptide with a bulky amino acid adjacent to serine, **1g** (Xaa = Val), gave moderate yield after four days in phosphate buffer (pH 7.8) (entry 7, Table 1). Peptide **1h** (Xaa = Lys) with a free side chain amine, also reacted with DSC and upon treatment with buffer underwent desired cleavage at the **Lys-Ser** bond and generated the N-terminal carboxylate lysine derivative and the C-terminal

fragment, **Oxd**-Phe-Ala-Gly-NH<sub>2</sub> **4**. N-terminal carboxylated lysine derivative **3h** eventually underwent decarboxylation to generate N-terminal fragment, Fmoc-Gly-Lys-OH **3h'** (entry 8, Table 1, Figure S2 see Supporting Information). In the case of peptide (**1j**) with a Pro residue next to serine, easy cyclization and cleavage at the **Pro-Ser** bond was observed, which is in contrast to enzymatic degradation, where Pro at a subsequent position nearly blocks the cleavage completely independent of the amino acid residue.<sup>21</sup>

**Table 1** Serine-selective amide bond cleavage of Fmoc-Gly-Xaa-Ser-Phe-Ala-Gly (**1a-j**).<sup>a</sup>

Entry	Substrate	Xaa	Conv <sup>b</sup> (%)
1	<b>1a</b>	Ala	>99
2	<b>1b</b>	Gly	>99
3	<b>1c</b>	Met	98
4	<b>1d</b>	His	98
5	<b>1e</b>	Tyr	97
6	<b>1f</b>	Trp	97
7	<b>1g<sup>c</sup></b>	Val	70
8	<b>1h<sup>c</sup></b>	Lys	80
9	<b>1i</b>	Asp	95
10	<b>1j</b>	Pro	98

<sup>a</sup>Reaction conditions: <sup>a</sup> Peptide (1 equiv) was reacted with DSC (5-20 equiv), DIEA (5-20 equiv) and a crystal of DMAP in DMF followed by cleavage with 0.1 M phosphate buffer (pH 7.5) at 37 °C for 48 h. <sup>b</sup>Conversion to N-terminal fragment, Fmoc-Gly-Xaa-OH (**3a-j**), was calculated from the absorbance at 254 nm using HPLC. <sup>c</sup>Cleavage with 0.1 M phosphate buffer (pH 7.8) at 37 °C for 4 days. <sup>d</sup>Acylation of the side chain of the lysine with DSC generated acylated lysine fragment **2h** and after buffer treatment, generated fragments **3h'** and **4** (Figure S2, see Supporting Information).

The substrate scope of this reaction was further evaluated (Table 2). The reaction was also applicable to substrates containing threonine **5** and **6** (entries 1 and 2, Table 2). After treatment with buffer, cleavage was observed at the N-terminal side of both serine and threonine, which is expected due to the similar side group functionality exhibited by both residues. Threonine side chain cyclization required a higher amount of DSC than the cyclization at serine residue (entry 2, Table 2). Longer 11-mer and 12-mer peptides, **7** and **8** with multiple serine residues gave comparable results (75-79%) (entries 3 and 4, Table 2, see Supporting Information). Peptides **9** and **10**, which contain D-amino acid residues and are unsuitable for enzymatic degradation, were successfully cleaved under the reaction conditions (entries 5 and 6, Table 2). Peptide **11**, comprising an intramolecular disulfide bridge, afforded the cleavage product at the Ser with an intact disulfide bond (entry 7, Table 2). Thus, this methodology allows for determination of disulfide pairing positions in a peptide chain, which is in contrast to other chemical reagents.<sup>4, 24</sup> As expected, peptide Fmoc-Gly-Ala-Cys-Phe-Arg-Phe-Gly-NH<sub>2</sub> with a free cysteine residue underwent cleavage at the N-terminus of cysteine and generated a thiazolidinone modified C-terminal fragment **Thz**-Phe-Arg-Phe-Gly-NH<sub>2</sub> and the N-terminal fragment Fmoc-Gly-Ala-OH (Figure S3, Supporting Information).

**Table 2.** Substrate scope of site-selective cleavage of peptide bonds.<sup>a</sup>

Entry	Substrate	Conv <sup>b</sup> (%)
1	Fmoc-Gly-Ala-Thr-Phe-Arg-Phe-Gly-NH <sub>2</sub> ( <b>5</b> )	78
2	Fmoc-Ala-Ser-Phe-Val-Gly-Ala-Thr-Phe-Arg-Phe-Gly-NH <sub>2</sub> ( <b>6</b> ) <sup>c</sup>	75
3	Fmoc-Ala-Val-Arg-Ser-Phe-Ser-Ala-Arg-Gly-Phe-Gly-NH <sub>2</sub> ( <b>7</b> ) <sup>c</sup>	75
4	Fmoc-Arg-Ala-Gly-Ala-Ser-Val-Arg-Phe-Ala-Ser-Phe-Gly-NH <sub>2</sub> ( <b>8</b> ) <sup>c</sup>	79
5	Fmoc-D-Val-D-Arg-D-Lys-D-Ala-D-Ser-D-Arg-D-Ala-D-Ala-NH <sub>2</sub> ( <b>9</b> )	80
6	Fmoc-D-Val-D-Arg-D-Lys-D-Ala-L-Ser-D-Arg-D-Ala-D-Ala-NH <sub>2</sub> ( <b>10</b> )	82
7	Fmoc-Cys-Gly-Arg-Arg-Ala-Cys-Gly-Ser-Phe-Ala-Gly-NH <sub>2</sub> , disulfide bond ( <b>11</b> )	72
8	Fmoc-Arg-Ala-Glu-Ala-Gly-Ser-Gly-Phe-NH <sub>2</sub> ( <b>12</b> ) <sup>d</sup>	70

<sup>a</sup>Reaction conditions: <sup>a</sup>Peptide (1 equiv) was reacted with DSC (20 equiv), DIEA (20 equiv), and a crystal of DMAP in DMF followed by cleavage with 0.1 M phosphate buffer (pH 7.5) at 37 °C for 48 h unless otherwise noted. <sup>b</sup>N-terminal fragment. <sup>c</sup>Three fragments were detected in the HPLC trace for cleavage at Ser and Thr at both the Ser residues. <sup>d</sup>Three fragments were detected in the HPLC trace for cleavage at both Ser and Glu.

**Table 3.** Site-selective cleavage of mutated and post-translationally modified peptides.

Entry	Substrate	Conv <sup>a</sup> (%)
1	Fmoc-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-NH <sub>2</sub> ( <b>13</b> )	80
2	Fmoc-Leu-Asn-Asp-Arg-Leu-Ala-Ser-Tyr-Leu-NH <sub>2</sub> ( <b>14</b> )	77
3	OAc-Ala-Val-Ala-Pro-Ala-Ala-Ser-Ile-Val-Ala-NH <sub>2</sub> ( <b>15</b> )	73
4	OAc-Ala-Val-Ala-Pro-βAla-Ala-Ser-Ile-Val-Ala-NH <sub>2</sub> ( <b>16</b> )	75
5	Fmoc-Leu-Arg-Arg-Ala-Ser-(N-methyl)Leu-Gly-NH <sub>2</sub> ( <b>17</b> )	79
6	Fmoc-Ile-βAla-Gly-Lys-Asn-Ser-Gly-Val-NH <sub>2</sub> ( <b>18</b> )	70

<sup>a</sup>N-terminal fragment.

Surprisingly, peptide **12** containing Glu along with serine underwent cleavage at the N-terminal of both Glu and Ser to generate three fragments under the reaction conditions (entry 8, Table 2, Figure S4 see Supporting Information). The activation of Glu by DSC for scission takes place by the formation of a five membered pyroglutamyl imide moiety (pGlu) (Figure S4). Recently, we have reported the site-specific activation of glutamic acid for hydrolysis of a peptide bond by using PyBrop.<sup>25</sup> The cyclization of Glu under reaction conditions occurs in contrast to the peptide containing Asp **1i** (entry 9, Table 1), which also has a free carboxylic acid side chain but cleavage was observed only at serine residue (Figure S5, Supporting Information). Next, to demonstrate the compatibility of this methodology with a free main chain carboxylic group, a peptide Fmoc-Gly-Ser-Gly-Phe-OH was synthesized on Wang resin.<sup>26</sup> Under the reaction conditions, cleavage was observed only at the N-terminus of serine residue

and generated an N-terminal fragment, Fmoc-Gly and a C-terminal fragment, Oxd-Gly-Phe-OH (Figure S6, Supporting Information). This experiment in addition to Table 1 confirms that most of the reactive side chains remained unmodified under the reaction conditions, contrary to other cleavage methods which lead to the oxidation of reactive amino acid side chains.<sup>14, 22</sup>

Subsequently, bioactive peptides, Bradykinin **13**,<sup>27</sup> and type I hair keratin fragment **14**, were cleaved successfully with (77-80 %) yield (entries 1-2, Table 3, see Supporting Information). Furthermore, we extended the current conditions to the scission of bioactive peptides with various posttranslational modifications such as N-acetylated antimicrobial peptide AP00011 **15**, N-acetylated- $\beta$  amino acid containing antimicrobial peptide **16**, and N-methylated bioactive peptide **17**, which acts as a substrate for cAMP-dependent protein kinase<sup>28</sup> (entries 3-5, Table 3, see Supporting Information). All these modified peptide **15**, **16**, and **17** were cleaved selectively at the Ser with ease and high yields. This is in contradiction to proteases, where enzymes are unable to recognize and cleave these modified peptides selectively.

Next, this methodology was successfully applied on an amyloid- $\beta$  peptide, Fmoc-Ile- $\beta$ Ala-Gly-Lys-Asn-Ser-Gly-Val-NH<sub>2</sub> **18**, containing a mutated alanine  $\beta$ -amino acid residue, a well-known mutation responsible for various age related disorders such as cataracts and Alzheimer's (entry 6, Table 3, see Supporting Information Figure S7).<sup>29</sup> Finally, we carried out the reaction with fully unprotected peptide SGISGPLS, a fragment of antimicrobial Bovine  $\beta$ -defensin 13. As expected, treatment with DSC followed by hydrolysis generated the N-terminal fragment OxdGI, C-terminal fragments OxdGPL and Oxd by cleavage at the N-terminus of serine residues (Figure S8, Supporting Information).

A site-selective approach for cleavage of peptides at the N-terminal of serine residues by modification of serine using metal-free organic reagents, under mild conditions, has been developed. The reactive side chains of amino acids remained unmodified under the reaction conditions, which is in contrast to known chemical cleavage methods that generate modified fragments due to over-oxidation. Disulfide bonds are stable to the reaction conditions, enabling the application of this method for determining the position of disulfide pairings in a peptide. This method also demonstrated broad substrate scope including cleavage of a Pro-Ser bond, which is resistant to enzymatic digestion. In addition, the method was applicable to threonine cleavage due to the presence of similar side group functionality as serine, therefore mimicking protease selectivity for similar substrates. Since cleavage proceeded smoothly on mutated and post-translationally modified peptides (D-amino-acid residues and  $\beta$ -amino acids) which are unsuitable substrates for proteases, this method is potentially applicable for determining the mutations responsible for various age related disorders. Furthermore, the modified C-fragment obtained after cleavage can be converted into the original fragment with serine residue at the terminus. These results provide a firm basis for subsequent studies aimed at developing artificial chemical proteases for a target protein. Work in this direction is underway in our laboratory.

## Acknowledgements

We thank Ryan Cohen at Merck for structural analysis by NMR. M.R. thanks Paramjit S. Arora at NYU for useful discussions.

## Notes and references

View Article Online

DOI: 10.1039/C6CC01509C

1. R. L. Lundblad, *Techniques in Protein Modification*, CRC Press: Boca Raton, Florida, 1995.
2. R. M. Smith and D. E. Hansen, *J. Am. Chem. Soc.*, 1998, **120**, 8910-8913.
3. A. Radzicka and R. Wolfenden, *J. Am. Chem. Soc.*, 1996, **118**, 6105-6109.
4. P. Edman and G. Begg, *Eur. J. Biochem.*, 1967, **1**, 80-91.
5. F. Sanger, *Biochem J*, 1945, **39**, 507-515.
6. J. M. Walker, *Methods Mol Biol*, 1994, **32**, 321-328.
7. E. Gross, *Methods Enzymol.*, 1967, **11**, 238-255.
8. Y. Degani and A. Patchornik, *Biochemistry*, 1974, **13**, 1-11.
9. T. J. Holmes and R. G. Lawton, *Journal of the American Chemical Society*, 1977, **99**, 1984-1986.
10. J. M. Chalker, S. B. Gunnoo, O. Boutureira, S. C. Gerstberger, M. Fernandez-Gonzalez, G. J. L. Bernardes, L. Griffin, H. Hailu, C. J. Schofield and B. G. Davis, *Chemical Science*, 2011, **2**, 1666-1676.
11. W. C. Mahoney, P. K. Smith and M. A. Hermodson, *Biochemistry*, 1981, **20**, 443-448.
12. J. M. Walker, *The Protein Protocols Handbook*; Humana Press, Totowa, NJ, 2002.
13. R. Kaiser and L. Metzka, *Analytical Biochemistry*, 1999, **266**, 1-8.
14. K. Tanabe, A. Taniguchi, T. Matsumoto, K. Oisaki, Y. Sohma and M. Kanai, *Chem. Sci.*, 2014, **5**, 2747-2753.
15. N. V. Kaminskaia, T. W. Johnson and N. M. Kostić, *J. Am. Chem. Soc.*, 1999, **121**, 8663-8664.
16. T. N. Parac, G. M. Ullmann and N. M. Kostić, *J. Am. Chem. Soc.*, 1999, **121**, 3127-3135.
17. L. Zhu, L. Qin, T. N. Parac and N. M. Kostic, *J. Am. Chem. Soc.*, 1994, **116**, 5218-5224.
18. D. Hoyer, H. Cho and P. G. Schultz, *J. Am. Chem. Soc.*, 1990, **112**, 3249-3250.
19. A. Schepartz and B. Cuenoud, *J. Am. Chem. Soc.*, 1990, **112**, 3247-3249.
20. Y. Kita, Y. Nishii, T. Higuchi and K. Mashima, *Angew. Chem. Int. Ed.*, 2012, **51**, 5723-5726.
21. N. M. Milović and N. M. Kostić, *J. Am. Chem. Soc.*, 2003, **125**, 781-788.
22. Y. Seki, K. Tanabe, D. Sasaki, Y. Sohma, K. Oisaki and M. Kanai, *Angew. Chem.* 2014, **126**, 6619-6623; *Angew. Chem. Int. Ed.*, 2014, **53**, 6501-6505.
23. R. De Marco, A. Tolomelli, M. Campitiello, P. Rubini and L. Gentilucci, *Org. Biomol. Chem.*, 2012, **10**, 2307-2317.
24. J. J. Gorman, T. P. Wallis and J. J. Pitt, *Mass Spectrom. Rev.*, 2002, **21**, 183-216.
25. J. M. Nalbone, N. Lahankar, L. Buissereth and M. Raj, *Organic Letters*, 2016, **10.1021/acs.orglett.6b00317**.
26. M. Góngora-Benítez, J. Tulla-Puche and F. Albericio, *ACS Combinatorial Science*, 2013, **15**, 217-228.
27. C. Golias, A. Charalabopoulos, D. Stagikas, K. Charalabopoulos and A. Batistatou, *Hippokratia*, 2007, **11**, 124-128.
28. H. N. Bramson, N. E. Thomas and E. T. Kaiser, *J. Biol. Chem.*, 1985, **260**, 15452-15457.
29. H. Maeda, T. Takata, N. Fujii, H. Sakaue, S. Nirasawa, S. Takahashi, H. Sasaki and N. Fujii, *Anal. Chem.*, 2015, **87**, 561-568.