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Synthesis of a Selenocysteine-Containing Peptide by Native Chemical Ligation

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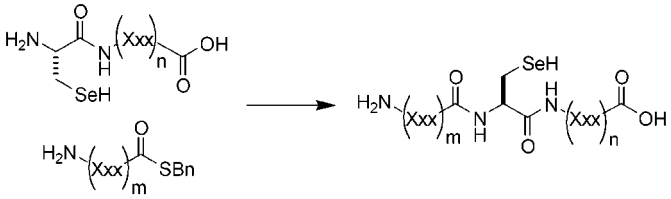
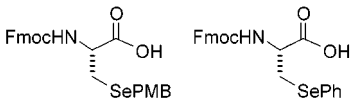
ABSTRACT



A new method for the synthesis of selenocysteine derivatives and selenocysteine-containing peptides is described. Fmoc-*Se*-*p*-methoxybenzylselenocysteine (1) was prepared and used for solid-phase synthesis of peptides with an *N*-terminal unprotected selenocysteine. Subsequent native chemical ligation with a peptide thioester provided a 17-mer that corresponds to the C-terminus of ribonucleotide reductase with selenocysteine in place of cysteine.

The synthesis of peptides containing selenocysteine (Sec) is rapidly gaining interest with the discovery of an increasing number of proteins containing this amino acid.[1] In addition, selenocysteine derivatives can serve as convenient precursors to dehydroamino acids,[2] which are useful electrophilic handles for the chemoselective preparation of peptide conjugates.[3] The Fmoc-protected amino acids **1** and **2** have been used as precursors for solid-phase peptide synthesis (SPPS) of selenocysteine-containing peptides.[2a,4] Compound **1** allows the incorporation of free selenocysteine into peptides or the selenocystine derivatives thereof,[4] whereas **2** is used for the mild oxidative introduction of dehydroalanines.[2a] Unfortunately, the reported syntheses of both compounds are not very suitable for gram-scale preparations. *Se*-*p*-Methoxybenzyl-protected selenocysteine, **1**, has been previously prepared from Boc-serine in eight steps in 24% overall yield.[4a,5] Fmoc-*Se*-phenylselenocysteine **2** was obtained from Boc-protected serine β-lactone,[2a,c] the preparation of which is well-known to suffer in yield upon scale-up.[6] We describe



1 **2**

here two new synthetic routes to these compounds that can be carried out in multigram quantities. Notably, **2** has been prepared on a 15-g scale without the need for chromatographic purification steps. We also show the utility of **1** for the synthesis of selenocysteine-containing peptides and their use in native chemical ligations.[7] Specifically, we have used

(1) (a) Stadtman, T. C. *J. Biol. Chem.* **1991**, *266*, 16257−60. (b) Stadtman, T. C. *Annu. Rev. Biochem.* **1996**, *65*, 83−100.

(2) (a) Okeley, N. M.; Zhu, Y.; van der Donk, W. A. *Org. Lett.* **2000**, *2*, 3603−3606. (b) Hashimoto, K.; Sakai, M.; Okuno, T.; Shirahama, H. *Chem. Commun.* **1996**, 1139−1140. (c) Sakai, M.; Hashimoto, K.; Shirahama, H. *Heterocycles* **1997**, *44*, 319−324.

(3) Zhu, Y.; van der Donk, W. A. *Org Lett.* **2001**, *3*, in press.

(4) (a) Koide, T.; Itoh, H.; Otaka, A.; Yasui, H.; Kuroda, M. *Chem. Pharm. Bull.* **1993**, *41*, 502−506. (b) Koide, T.; Itoh, H.; Otaka, A.; Furaya, M.; Kitajima, Y.; Fuji, N. *Chem. Pharm. Bull.* **1993**, *41*, 1596−1600 (c) Moroder, L.; Besse, D.; Musiol, H.-J.; Rudolph-Bohner, S.; Siedler, F. *Biopolymers* **1996**, *40*, 207−234.

(5) Stocking, E. M.; Schwarz, J. N.; Senn, H.; Salzmann, M.; Silks, L. A. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2443−2447.

(6) Pansare, S. V.; Arnold, L. D.; Vederas, J. C. *Org. Synth.* **1991**, *70*, 10−17.

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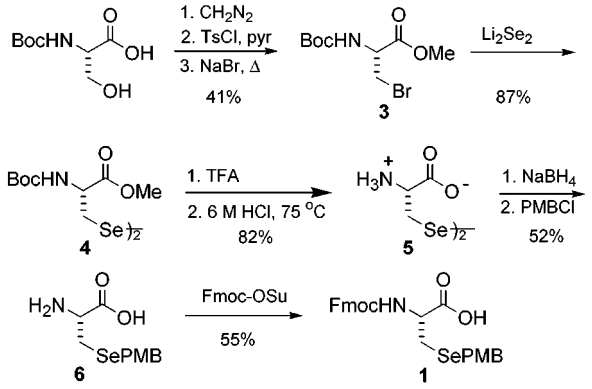
this technique for the preparation of a selenocysteine-containing analogue of the *C*-terminal peptide of ribonucleotide reductase.

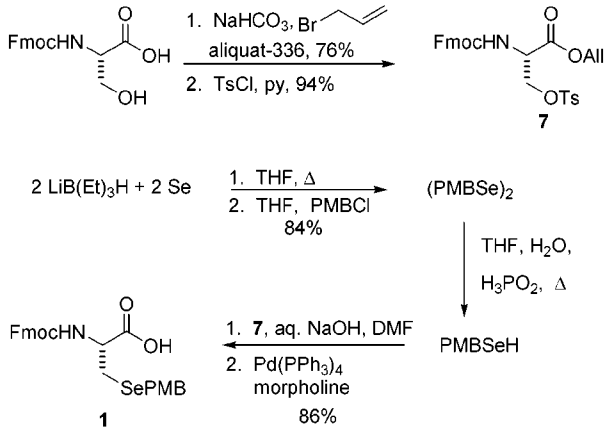
Our initial route to **1** focused on modifications to literature preparations of selenocystine[5] and *Se*-PMB-Sec.[4a] Boc-protected serine was converted into bromoalanine methyl ester **3**, which was transformed with dilithium diselenide to the selenocystine derivative **4**. Deprotection of both the amino and carboxylate functionalities under acidic conditions, followed by recrystallization and cation exchange chromatography, provided selenocystine **5**. Reduction of the diselenide and protection of the selenol with *p*-methoxybenzyl chloride gave **6**, which was converted into the desired product in 55% unoptimized yield. Although literature yields for some of the individual steps in Scheme 1 are somewhat

Scheme 1



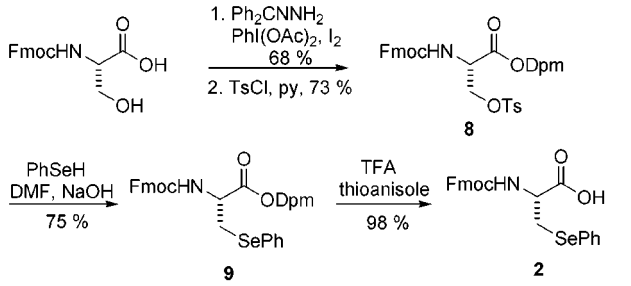
higher than in our hands, the route is lengthy and initial attempts toward scale-up did not look promising. In light of these results, we decided to evaluate an alternative synthetic route to **1** that would require fewer steps and be amenable to scale-up. Scheme 2 depicts the strategy that we ultimately

Scheme 2



used for this purpose. Three orthogonal protecting groups were used for the amino, carboxylate, and selenol functionalities that allowed their independent manipulation. The free carboxylate of Fmoc-Ser was converted to the allyl ester, followed by activation of the alcohol with *p*-toluenesulfonyl chloride to provide **7**. *p*-Methoxybenzyldiselenide $(PMBSe)_2$, obtained by treating selenium powder with super hydride followed by PMBCl, was reduced to the selenol with hypophosphoric acid. Without further purification, the resulting selenol was added to **7** under basic conditions to yield the fully protected selenocysteine derivative. Selective deprotection of the carboxylate with catalytic palladium and morpholine gave **1** in 61% yield over four steps, a marked improvement over the existing route. The optical rotation of the final product after recrystallization compared well with the previously reported value[4a] and that obtained for the same compound produced via Scheme 1. This suggests that the introduction of the selenide occurred without any extensive racemization in agreement with a previous account of a similar reaction.[8] The preparation of **2** followed a similar route except that the carboxylate protecting group was changed to a diphenylmethyl ester (Scheme 3). The four-

Scheme 3



step sequence provided **2** in 37% overall yield on a 15-g scale, which would not have been achievable with our previously reported procedure.[2a]

Selenocysteine is often referred to as the 21st natural amino acid as it is ribosomally incorporated into several important redox enzymes.[1,9] Replacement of cysteine by selenocysteine in proteins and peptides can impose interesting properties[10,11] because of the higher acidity of the selenol,[4a,12] the lower redox potential,[11,13] and the greater nucleophilicity of the

(7) (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science* **1994**, *266*, 776−779. (b) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923−960.

(8) Theodoropoulos, D.; Schwartz, I. L.; Walter, R. *Biochemistry* **1967**, *6*, 3927−3932.

(9) (a) Leinfelder, W.; Zehelein, E.; Mandrand-Berthelot, M. A.; Böck, A. *Nature* **1988**, *331*, 723−5. (b) Leinfelder, W.; Forchhammer, K.; Veprek, B.; Zehelein, E.; Böck, A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 543−547 (c) Low, S. C.; Berry, M. J. *TIBS* **1996**, 203−208.

(10) Selenosubtilisin is an interesting example of altered properties of an enzyme by selenocysteine substitution; see (a) Wu, Z. P.; Hilvert, D. *J. Am. Chem. Soc.* **1990**, *112*, 5647−8. (b) Syed, R.; Wu, Z. P.; Hogle, J. M.; Hilvert, D. *Biochemistry* **1993**, *32*, 6157−64.

(11) Besse, D.; Siedler, F.; Diercks, T.; Kessler, H.; Moroder, L. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 883−885.

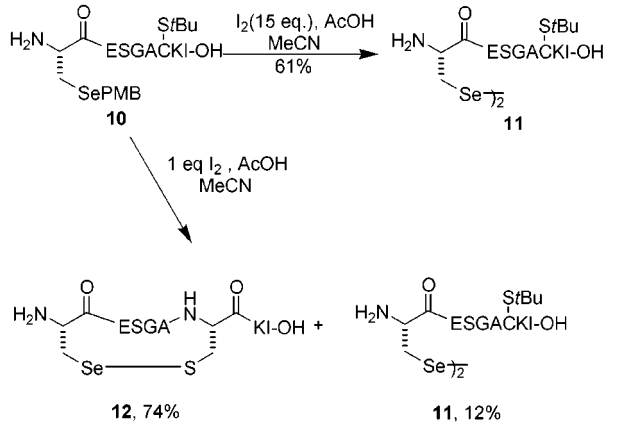
(12) (a) Nygard, B. *Ark. Kemi* **1967**, *27*, 341−361. (b) Arnold, A. P.; Tan, K. S.; Rabenstein, D. L. *Inorg. Chem.* **1986**, *25*, 2433−2437.

(13) Müller, S.; Senn, H.; Gsell, B.; Vetter, W.; Baron, C.; Böck, A. *Biochemistry* **1994**, *33*, 3404−3412.

selenolate compared to the thiolate.[14] Unfortunately, the conditions necessary for selenocysteine incorporation by the cell translational machinery are very specific. As a result, preparation of non-natural selenocysteine-containing peptides and proteins by molecular biology methods is difficult, although some recent successful approaches have been documented.[15] We envisioned that the practical synthesis of **1** in combination with the recently developed native chemical ligation technique[7] could overcome this limitation. In principle, two different approaches can be used to substitute a cysteine with a selenocysteine in a protein or peptide using the native ligation strategy. One could either position the selenocysteine in the interior of one of the ligation partners or at the site of ligation itself. In the former method, an additional cysteine is required at the junction site,[16] limiting the applicability. The latter method would be more powerful as it does not require a second nearby cysteine. To the best of our knowledge, native chemical ligations using selenocysteine have not been reported previously. Therefore, we selected a test peptide to investigate the feasibility of the approach.

The C-terminus of class Ia ribonucleotide reductase (RNR) contains two cysteine residues (Cys754 and Cys759, *E. coli* numbering) that are involved in shuttling reducing equivalents into the active site of the protein.[17] During ribonucleotide reduction a disulfide linkage is formed by two cysteines in the active site. This disulfide is subsequently reduced via a dithiol−disulfide interchange with the two C-terminal cysteines (C754 and 759). The disulfide so formed at the C-terminus is reduced in turn by thioredoxin, preparing RNR for another turnover. Substitution of one or both of the cysteines at the C-terminus with selenocysteines would introduce altered redox properties[11] that may be used to investigate the two dithiol−disulfide interchange reactions.[18] Two peptide segments were synthesized that correspond to residues 745−753 and 754−761 of the C-terminus of RNR. Peptide **10** was synthesized by Fmoc-based SPPS on Wang resin. It contains the protected selenocysteine in place of Cys754 at its *N*-terminus and a *t*Bu disulfide protected cysteine at position 759 (Sec(PMB)ESGAC(S-*t*Bu)KI-OH) (Scheme 4). After cleavage from the resin, performed under conditions that maintained the PMB protecting group, and HPLC purification the desired peptide was obtained in 58% yield.

Scheme 4



Oxidative deprotection of the selenocysteine derivative yielded two different products depending on the amount of iodine used (Scheme 4). Treatment of **10** with 15 equiv of I_2 in AcOH/MeCN/H_2O[4c,19] gave the diselenide **11** in 61% yield, in which the cysteine retained the *tert*-butyl disulfide protecting group. Alternatively, when only 1 equiv of I_2 was used, **11** and **12** were recovered in 86% combined yield. Product **12** is presumably formed when the free selenol liberated at the *N*-terminus reacts with the disulfide at Cys759.[20] Peptide **13** (Ac-LVPSIQDDG-SBn, residues 745−753) containing a *C*-terminal benzyl thioester was synthesized using a safety catch resin[21] in 61% yield.

In parallel reactions, peptides **11** and **12** were ligated with the thioester **13** in sodium phosphate buffer (0.1 M)

(14) Huber, R. E.; Criddle, R. S. *Arch. Biochem. Biophys.* **1967**, *122*, 164−173.

(15) (a) Boschi-Muller, S.; Muller, S.; Van Dorsselaer, A.; Böck, A.; Branlant, G. *FEBS Lett.* **1998**, *439*, 241−245. (b) Arner, E. S.; Sarioglu, H.; Lottspeich, F.; Holmgren, A.; Böck, A. *J. Mol. Biol.* **1999**, *292*, 1003−1016 (c) Sandman, K. E.; Benner, J. S.; Noren, C. J. *J. Am. Chem. Soc.* **2000**, *122*, 960−961.

(16) More recent techniques have been developed where the presence of a cysteine at ligation sites is not required; see (a) Canne, L. E.; Bark, S. J.; Kent, S. B. H. *J. Am. Chem. Soc.* **1996**, *118*, 5891−5896. (b) Beligere, G. S.; Dawson, P. E. *J. Am. Chem. Soc.* **1999**, *121*, 6332−6333. (c) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R. *Org. Lett.* **2000**, *2*, 2141−2143. (d) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2000**, *2*, 1939−1941. (e) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2001**, *3*, 9−12. (f) Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526−533.

(17) (a) Mao, S. S.; Holler, T. P.; Yu, G. X.; Bollinger, J. M.; Booker, S.; Johnston, M. I.; Stubbe, J. *Biochemistry* **1992**, *31*, 9733−9743. (b) Stubbe, J.; van der Donk, W. A. *Chem. Biol.* **1995**, *2*, 793−801.

(18) A redox active cysteine−selenocysteine pair is actually present in mammalian thioredoxin reductase. This pair cycles between the free amino acids and the selenosulfide oxidation states; see (a) Lee, S. R.; Bar-Noy, S.; Kwon, J.; Levine, R. L.; Stadtman, T. C.; Rhee, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2521−2526. (b) Zhong, L.; Arnér, E. S.; Holmgren, A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5854−5859.

(19) Kamber, B.; Hartmann, A.; Eisler, K.; Riniker, B.; Rink, H.; Sieber, P.; Rittel, W. *Helv. Chim. Acta* **1980**, *63*, 899−915.

(20) Thermodynamically, selenosulfide **12** is expected to be less stable than diselenide **11**; see ref 11.

(21) (a) Kenner, G. W.; McDermott, J. R.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1971**, 636−637. (b) Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684−11689 (c) Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369−11374.

(22) All yields are after purification by preparative RP-HPLC and are based on thioester starting material.

(23) See, for instance, (a) Dawson, P. E.; Churchill, M. J.; Ghadiri, M. R.; Kent, S. B. H. *J. Am. Chem. Soc.* **1997**, *119*, 4325−4329. (b) Miranda, L. P.; Alewood, P. F. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1181−1186. (c) Kochendoerfer, G. G.; Salom, D.; Lear, J. D.; Wilk-Orescan, R.; Kent, S. B.; DeGrado, W. F. *Biochemistry* **1999**, *38*, 11905−11913.

(24) Singh, R.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 2332−7. Thiophenol may generate low but sufficient concentrations of the free selenol for the ligation reaction to proceed. Selenosulfides such as **12** are reported to be more easily reduced than diselenides (ref 11).

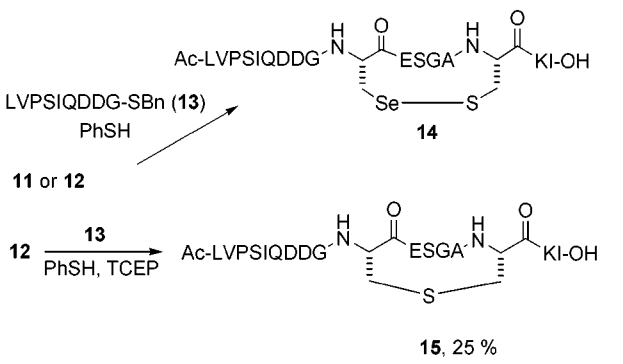
(25) Vedejs, E.; Diver, S. T. *J. Am. Chem. Soc.* **1993**, *115*, 5.

(26) A dehydroalanine-containing ligation product would have identical mass as **15**.

(27) (a) Harpp, D. N.; Gleason, J. G.; Snyder, J. P. *J. Am. Chem. Soc.* **1968**, *90*, 4181−4182. (b) Harpp, D. N.; Gleason, J. G. *J. Org. Chem.* **1971**, *36*, 73−80. (c) Harpp, D. N.; Gleason, J. G. *J. Am. Chem. Soc.* **1971**, *93*, 2437−2445. (d) Wakamiya, T.; Shimbo, K.; Sano, A.; Fukase, K.; Shiba, T. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 2044−2049. (e) Lai, Y. H.; Soo, T. B.

containing 6 M Gn·HCl (pH 7.5) and thiophenol (4%) (Scheme 5). The ligation product **14** was isolated as the

Scheme 5



mixed selenosulfide in 56% yield when starting with **11** and 60% yield when starting with **12**.[22] Compared to typical literature procedures for native chemical ligations using peptides with *N*-terminal cysteines (~1 mM, 8−24 h),[23] the ligations with selenocysteine derivatives **11** and **12** needed a somewhat higher concentration (3 mM) for complete conversion in 24 h. Given the significantly lower pK_a of a selenol compared to a thiol[4a,12] and the higher nucleophilicity of the resulting selenolate in comparison to the thiolate,[14] it may be surprising that the ligations did not proceed faster. We speculate that the rate-limiting step in our reactions may actually be the generation of the free selenol from diselenide **11** or selenosulfide **12** by reduction with thiophenol, since diselenides are not readily reduced by monothiols.[11,24]

Interestingly, when triscarboxyethylphosphine (TCEP) was added to the ligation reaction with **12**, no selenocysteine-containing ligation product was obtained. The addition of phosphine was prompted by the possibility that it might facilitate the reduction of the selenosulfide and perhaps also act as an acyl transfer catalyst.[16d,25] However, the reaction proceeded very slowly and produced a ligation product after 7 days in poor yield (25%) that showed a molecular ion corresponding to loss of a selenium atom. The typical isotopic distribution characteristic for selenium was also absent from the molecular ion. Either this peptide may be an elimination product giving a dehydroalanine derivative, or it may consist of a lanthionine-containing peptide **15**.[26] $(Et_2N)_3P$ has been used previously for desulfurization of disulfides to provide dialkylthioethers.[27] A similar reaction may have been induced by TCEP with the selenosulfide **12**, producing a lanthionine. The formation of either an *N*-terminal dehydroalanine or a lanthionine would also explain the very slow formation of a ligated product in poor yield, as it would not be assisted by initial attack of a selenolate on the thioester of **13**. Instead, **15** might have been formed by direct attack of the *N*-terminal amine of the lanthionine derivative of **12**. Regardless of the exact structure of the product and the pathway(s) of its formation, it appears that TCEP is not a reagent of choice for native chemical ligations with selenocysteine if this residue can form a selenosulfide with a nearby cysteine.

The deselenation suggests a potential application for ligation products containing selenocysteine. Dawson recently reported on native chemical ligations combined with subsequent reductive desulfurization to provide alanines at the site of ligation.[16f] A limitation of this interesting extension of the ligation repertoire lies in the inability to control regioselective desulfurizations if other cysteines are present in the peptide or protein.[16f] We are currently exploring whether selenocysteines can be chemoselectively deselenated in the presence of cysteines.

In summary, we demonstrate high yielding short syntheses of FmocSec(PMB) (**1**) and FmocSec(Ph) (**2**). The *p*-methoxybenzyl group of the *N*-terminal selenocysteine can be removed with concomitant selenosulfide or diselenide formation by treatment with I_2. To demonstrate the utility of **1**, a C-terminal peptide of ribonucleotide reductase was synthesized by employing the first reported example of native chemical ligation with selenocysteine. We are currently investigating extension of this methodology to expressed protein ligations.[28]

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Supporting Information Available: Experimental procedures for all transformations that produced previously unknown compounds, as well as their full spectral characterization, and a general procedure for the ligation reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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Heterocycles **1985**, *23*, 1205−1214. (f) Olsen, R. K.; Kini, G. D.; Hennen, W. J. *J. Org. Chem.* **1985**, *50*, 4332−4336 (g) Fukase, K.; Kitazawa, M.; Sano, A.; Shimbo, K.; Horimoto, S.; Fujita, H.; Kubo, A.; Wakamiya, T.; Shiba, T. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 2227−2240.

(28) (a) Muir, T. W.; Sondhi, D.; Cole, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6705−6710. (b) Evans, T. C.; Benner, J.; Xu, M. Q. *Protein Sci.* **1998**, *7*, 2256−2264. (c) Cotton, G. J.; Muir, T. W. *Chem. Biol.* **1999**, *6*, R247−R256. (d) Noren, C. J.; Wang, J. M.; Perler, F. B. *Angew. Chem., Int. Ed.* **2000**, *39*, 451−466.