EurJOC European Journal of Organic Chemistry

FULL PAPER

DOI: 10.1002/ejoc.201500033

A Photocleavable Auxiliary for Extended Native Chemical Ligation

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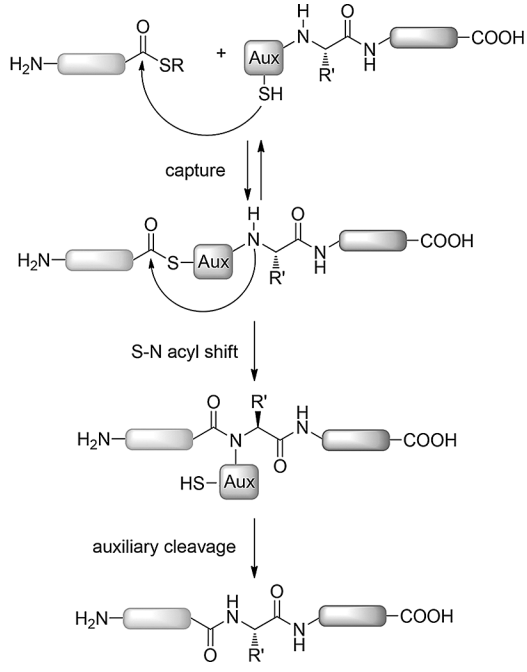
Keywords: Peptides / Photochemistry / Cleavage reactions / Chemical ligations / Sulfur

To extend the scope of native chemical ligations beyond X-Cys connections, auxiliaries that contain thiol moieties were developed to mimic the function of the Cys residue in the ligation reaction/capture step. Auxiliaries known so far feature complicated attachment protocols and generally need harsh conditions for their cleavage. Herein, we present the development of a new photoremovable ligation auxiliary, which is easily synthesized and attached to peptides that contain a variety of N-terminal amino acids by a reductive amination reaction. Ligations at Gly-Gly and Ala-Gly junctions were carried out with high conversion, and the auxiliary can be cleaved from the final product by using a mild photolysis protocol. This methodological advancement is an important step forward in peptide ligation chemistry.

Introduction

Since its introduction in 1994, native chemical ligation (NCL) has been the standard technique to synthesize or synthetically modify proteins and large peptides by connecting two fragments with a native peptide bond.[1,2] The technique crucially relies on rapid reversible thiol exchange reactions that thioesters undergo whenever free thiols are present. This reaction constitutes the first step of a ligation sequence, connecting a peptide fragment that contains a C-terminal thioester with the thiol group of an N-terminal cysteine from a second fragment. In the second step, an irreversible intramolecular rearrangement through an S–N acyl shift takes place resulting in the formation of the native peptide bond. The reaction is chemoselective and can also be used in the semisynthesis of proteins by using intein-mediated protein splicing to form recombinant thioester peptides (expressed protein ligation, EPL).[3] Despite its extensive use in the synthesis of proteins, the applicability of NCL is restricted by the need for a cysteine residue at the N-terminus of the C-terminal peptide or protein fragment. As Cys is one of the least prevalent amino acids in protein sequences, this restriction gravely limits the use of NCL.[4]

Different methods have been developed to allow ligation at sites other than X-Cys, such as post-ligational desulfurization,[5–8] alkylation,[9] and the use of different chemoselective reactions in the capture step (i.e., the Staudinger ligation).[10–13] Although these techniques have proven to be of great synthetic value, they also have their inherent limitations that still need to be addressed by further development. Arguably, the most promising approach in this regard is the application of ligation auxiliaries positioned at the N-terminal end of the C-terminal fragment, which then undergo an NCL-like ligation reaction (Scheme 1). These auxiliaries contain a thiol moiety, which mimics the function of the cysteine residue in NCL, and some can be cleaved after ligation to thus generate the native peptide bond.[14,15] So far, a number of different auxiliaries have been introduced to allow ligation reactions at sites other



Scheme 1. Concept of auxiliary-mediated ligation. In the first step (capture), the thioester function on one peptide undergoes a thiol exchange with the thiol group of the auxiliary. After rearrangement through an S–N acyl shift, the auxiliary can be cleaved to yield the product, which contains the natural peptide bond.

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejoc.201500033.

than X-Cys. However, this impressive synthetic feat comes with a caveat. Many of these auxiliaries cannot be cleaved from the ligation product or require lengthy synthetic preparations and attachment protocols.[15,16] Furthermore, cleavage conditions are often harsh, which thus complicates the synthesis of relatively labile compounds such as glycopeptides. To address the latter problem, a photocleavable auxiliary was developed that enables ligations at Gly-Gly and Ala-Gly ligation sites, but this method still requires a lengthy synthesis and features a complicated attachment protocol.[17,18] Ideally, an NCL auxiliary should be photocleavable and accessible by a short synthesis. Its attachment to the N-terminus of resin-bound peptides should be straightforward, and it should be stable under acidic peptide cleavage and deprotection conditions. Herein, we report the synthesis and application of a photocleavable NCL auxiliary, which meets the above-mentioned criteria.

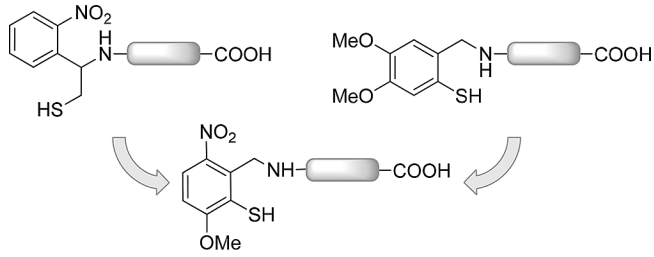


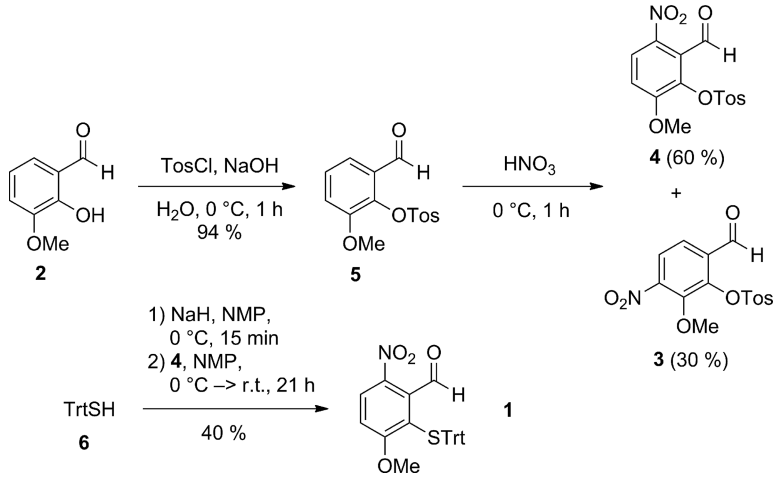
Figure 1. Peptide that contains new auxiliary **1** (bottom); conceptually the auxiliary combines the photocleavable 2-mercapto-1-(2-nitrophenyl)ethyl moiety (top left) and the acid-labile 2-mercaptobenzyl auxiliary (top right).

Results and Discussion

Synthesis of Auxiliary 1

The design of 3-methoxy-6-nitro-2-mercaptobenzyl auxiliary **1** is based on previously reported acid-labile 2-mercaptobenzyl auxiliaries (Figure 1), which enable ligation reactions at numerous ligation junctions (e.g., Gly-Gly, Ala-Gly, Lys-Gly).[19,20] The introduction of a nitro group at the *ortho* position of the benzene ring should render the auxiliary photocleavable, as *o*-nitrobenzyl groups have been widely used as photoremovable protecting groups.[21] The only photocleavable ligation auxiliary known so far also contains an *o*-nitrobenzyl moiety, but its structure is otherwise based on the 2-mercapto-1-phenylethyl auxiliaries, which feature a complicated attachment protocol.[17,18]

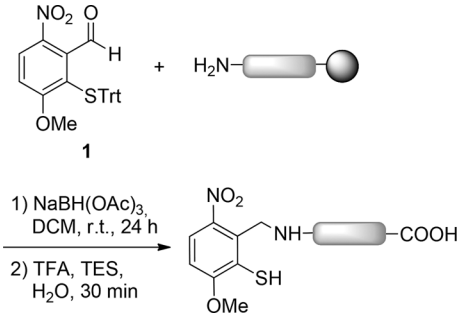
The use of a benzaldehyde scaffold for this new auxiliary allows for it to be easily attached to the N-terminus of peptides that contain different terminal amino acids by reductive amination.[22,23] The natural product *o*-vanillin (**2**) was used as an inexpensive starting material for the brief, three-step synthesis of auxiliary **1**. The first two steps were carried out by following the reported synthesis of the corresponding phenylsulfonyl compound.[24] The free hydroxy group was transformed into the corresponding tosylate **5** in high yield (Scheme 2). The nitro group was subsequently introduced by using concentrated HNO_3 to give a mixture of regioisomers, which were easily separated by recrystallization from acetone. The undesired 4-nitro-substituted side product **3** was insoluble in the acetone, whereas target compound **4** dissolved. The thiol group was introduced by a nucleophilic aromatic substitution using trityl thiol, which allowed for the isolation of the final product **1** in moderate yields, presumably because of the steric demands of the bulky trityl group. The trityl group was chosen as a protecting group for the thiol function because it is acid-labile and easily cleaved under standard acidic peptide cleavage and deprotection conditions, thus eliminating the need for a dedicated deprotection step for the auxiliary.



Scheme 2. Synthesis of auxiliary **1** (Tos = 4-tolylsulfonyl, Trt = triphenylmethyl, NMP = *N*-methyl-2-pyrrolidone).

Attachment of Auxiliary 1 to Peptides

By using a reductive amination reaction, auxiliary **1** was attached to a number of peptides that contain different N-terminal amino acids (Scheme 3). To prevent side reactions, all reactions were conducted with the exclusion of light under an inert atmosphere and using activated molecular sieves to ensure complete removal of the residual water. Peptides were synthesized by a standard 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) on preloaded Wang resins. After removal of the final Fmoc protecting group from the peptide, the resin was swollen in anhydrous dichloromethane (DCM) for 1 h, and auxiliary **1** and $NaBH(OAc)_3$ were added in excess. The suspension was agitated for 24 h and diluted with an excess amount of DCM. Because of its low density, the resin could be removed by pipette from the liquid surface. Afterwards, the peptide was cleaved from the resin by using a mixture of trifluoroacetic acid (TFA), triethylsilane (TES), and water (95:2.5:2.5) simultaneously cleaving all the other protecting groups including the trityl group on the thiol function of the auxiliary. Cleavage times were limited to no more than 30 min, after which time the TFA was quickly removed by evaporation to avoid decomposition of the auxiliary.



Scheme 3. Attachment of auxiliary **1** to peptides.

Apart from the expected product, the analysis of the obtained peptides also showed the formation of a molecule with a mass of two hydrogen atoms less than expected, which corresponded to a formal elimination of H_2. This oxidized form accumulated upon prolonged storage. However, this oxidation turned out to be irrelevant to the actual ligation, as the reducing conditions for the ligation reaction led to nearly complete regeneration of the reactive reduced form directly upon dissolution (Figure 2). Thus, the auxiliary peptides were isolated and characterized in their oxidized form and reduced in situ for the ligation reactions.

With regard to the nature of the oxidized form of the auxiliary peptides, the most obvious explanation would be the formation of a dimeric compound with a disulfide bridge. However, this was ruled out by mass spectrometric analysis (see Supporting Information). To investigate the structure of the oxidized species further, a small molecule analogue (**S2**) of the auxiliary peptides was synthesized by coupling auxiliary **1** to glycine methyl ester. NMR

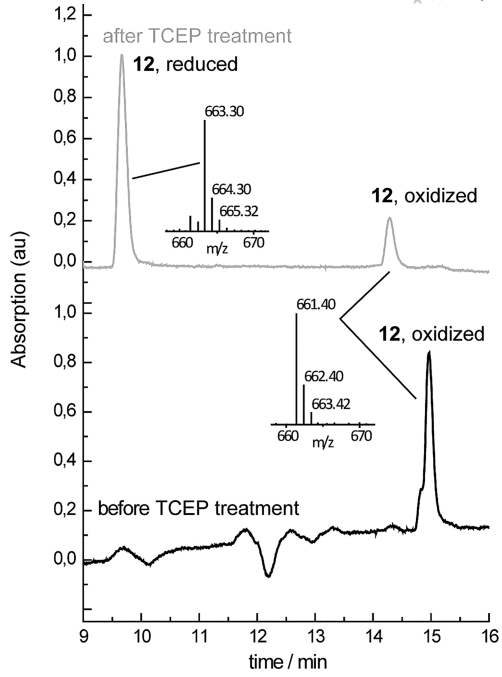


Figure 2. LC–MS traces of isolated auxiliary peptide **12** before and after dissolution in a buffer solution that contained the reducing agent tris(2-carboxyethyl)phosphine (TCEP), which indicates that the oxidized form of the auxiliary peptide is readily reduced under NCL conditions. Absorption was measured at 400 nm.

analysis of the reduced and oxidized forms revealed that a cyclization reaction that involved the NH and SH moieties was the most compelling explanation for the observed formal H_2 elimination (see Supporting Information).

To investigate the scope of the attachment protocol, we examined the functionalization of a variety of peptides with different N-terminal amino acids. Gly, Ala, Leu, Lys, and Val were chosen as the N-terminal amino acids of peptides to be functionalized with **1**, as they exhibited different grades of polarity and steric hindrance at the ligation junction in the following experiments. Auxiliary **1** was attached to peptides that contained an N-terminal Gly, Ala, Leu, or Lys in good overall yields with regard to the original resin loading. The attachment of **1** to a peptide with an N-terminal Val residue was possible, but only in low yields, which indicates that auxiliary **1** is better suited for the functionalization of non-β-branched N-terminal amino acids.

Ligation Reactions

Peptide thioesters for ligation reactions were synthesized on safety-catch sulfamylbutyryl resin by following known literature protocols.[25,26] Thioester peptides with a Gly or Ala as the C-terminal amino acid were prepared as alkyl thioesters by using ethyl 3-mercaptopropionate and as aryl thioesters by using (4-mercaptophenyl)acetic acid (MPAA). Alkyl thioesters are known to be less reactive in thiol exchanges, and therefore in ligation reactions, than aryl thioesters.[1,27,28] In agreement with this, the ligation reactions between alkyl thioesters and peptides that contained auxil-

iary **1** did not yield any product, whereas the formation of product was possible by using aryl thioesters. As aryl thioesters are more prone to hydrolysis, even at low temperatures, and thus can only be stored for a limited amount of time, they were typically generated in situ by using the more stable alkyl thioesters and an excess amount of the aryl thiol (MPAA) as an additive during the ligation reactions.

A variety of ligation junctions were selected to show the applicability and scope of auxiliary **1** (Table 1). Ligation was possible at all chosen junctions, albeit with different turnover. The ligation reactions were performed under argon and with the exclusion of light to prevent the oxidation of the auxiliary peptides as well as any light-induced side reactions and premature cleavage of the auxiliary. Ligation buffer solutions contained 20 mM TCEP as a reducing agent and were degassed prior to use. By following the reactions with LC–MS at specific times, the conversion rate was determined by comparing the LC peak areas of the product to the auxiliary peptide at 400 nm (Figure 3). The auxiliary chromophore absorbs at this wavelength to allow for the visualization of both the auxiliary peptide and auxiliary-containing product. All other components of the reaction mixture were not visible at a wavelength of 400 nm.

Table 1. Overview of ligation reactions with auxiliary **1**.

Ligation junction	Conditions	Thioester peptide[a]	Auxiliary peptide[b]	Conv. [%]
Gly-Gly	pH = 7.6, r.t., 24 h	ALYRG-SR′ (**7**)	Aux-GFKV (**13**)	91
Gly-Ala	pH = 7.0, r.t., 24 h	ARVTLYGG-SR′ (**8**)	Aux-AAFIKA (**9**)	13
Gly-Leu	pH = 7.7, 50 °C, MPAA, 8 h	ARVTLYGG-SR (**10**)	Aux-LAFIKA (**11**)	6
Ala-Gly	pH = 7.1, 35 °C, MPAA, 49 h	ARVTLYGA-SR (**14**)	Aux-GYRA (**12**)	82
Ala-Ala	pH = 7.0, 35 °C, MPAA, 32 h	ARVTLYGA-SR (**14**)	Aux-AAFIKA (**9**)	5

[a] R = ethyl 3-mercaptopropionate ester, R′ = (4-mercaptophenyl)-acetic acid. [b] Aux = auxiliary **1**.

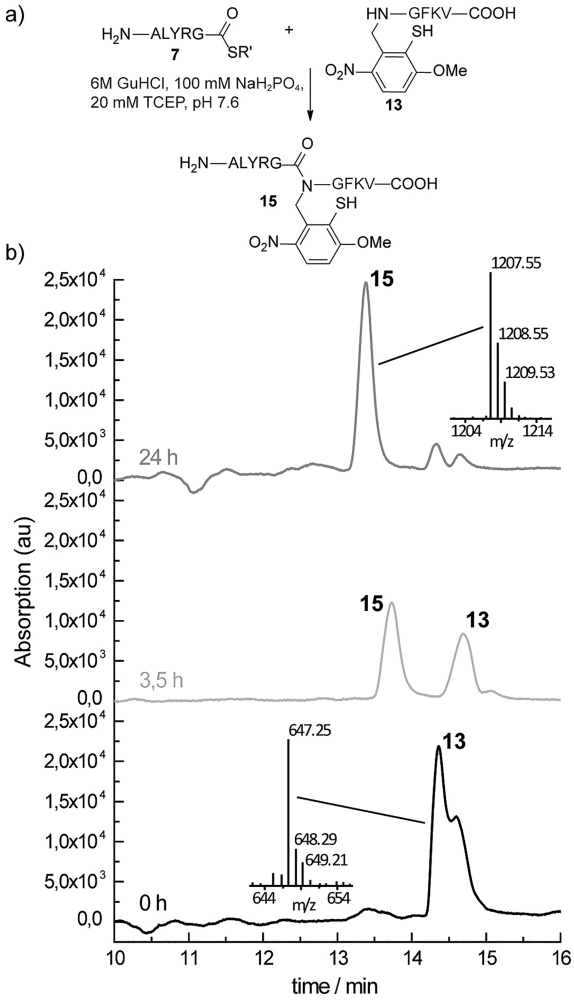


Figure 3. (a) Ligation reaction at a Gly-Gly site between auxiliary peptide **13** and thioester peptide **7**. (b) LC–MS traces of the reaction showing 91% conversion into product **15** after 24 h. Absorption was measured at 400 nm (GuHCl = guanidinium hydrochloride).

Several reaction parameters (temperature, reaction times, pH, concentration, use of preformed or in situ generated aryl thioester) were adjusted to each specific ligation reaction, as the reactivity varied at different ligation junctions. The employed conditions are specified in Table 1.

Reactions at the Gly-Gly ligation junctions proceeded smoothly with a high conversion of 91% within 24 h (Figure 3). These findings were consistent with preformed aryl thioester **7** and in situ generated thioester by using MPAA as an additive to the reaction mixture. To investigate the scope of the auxiliary **1** mediated ligation reactions, the amino acid at the N-terminal end of the auxiliary peptide was varied with more sterically demanding amino acids than glycine. Ligations at a Gly-Ala site by employing thioester peptide **8** and auxiliary peptide **9** were possible, but the reaction had a lower conversion of 13% after 24 h. The ligation reaction of glycine thioester peptide **10** with an auxiliary peptide that contained an even more sterically demanding N-terminal amino acid such as Leu (**11**) provided minimal product formation when conducted at room temperature. When the reaction temperature was increased to 50 °C, a conversion of 5% was detected after 8 h. However, the thioester was completely hydrolyzed at this higher temperature and length of time, and a higher conversion could not be attained. The reaction that featured sterically demanding Ala residues on both sides of the ligation site yielded a conversion of 5% after 32 h at an elevated temperature of 35 °C, which demonstrates that auxiliary **1** can enable ligations at difficult junctions that were so far not accessible by using auxiliary-based ligation reactions.[15]

In addition, we examined the exchange of the C-terminal Gly of the thioester peptide for the sterically more demanding Ala. In the ligation reaction with Gly auxiliary peptide **12**, a conversion of 82% was obtained after 49 h at

35 °C. This longer reaction time is in line with the observation that Ala thioester peptides generally undergo slower reactions than Gly thioester peptides in Native Chemical Ligation reactions.[29]

Cleavage of the auxiliary was carried out by irradiation with UV light (365 nm). Complete cleavage of auxiliary **1** was achieved after 40 min. The reaction did not produce any side products, and the respective products could be obtained with remarkably clean conversion (Figure 4). Photolysis was possible by using samples taken directly from the reaction mixture as well as purified samples. As the auxiliary could be cleaved in all cases without any side products, the limiting step seems to be the transthioesterification and not the S–N acyl shift, as the cleavage of the auxiliary from the nonrearranged intermediate would yield the respective starting materials. A reason for this might be the steric hindrance of the auxiliary as well as the competition between the auxiliary thiol and the excess amount of MPAA during transthioesterification. Increasing the nucleophilicity of the thiol group of the auxiliary by modifying its substitution pattern may be a solution to improve ligation rates.

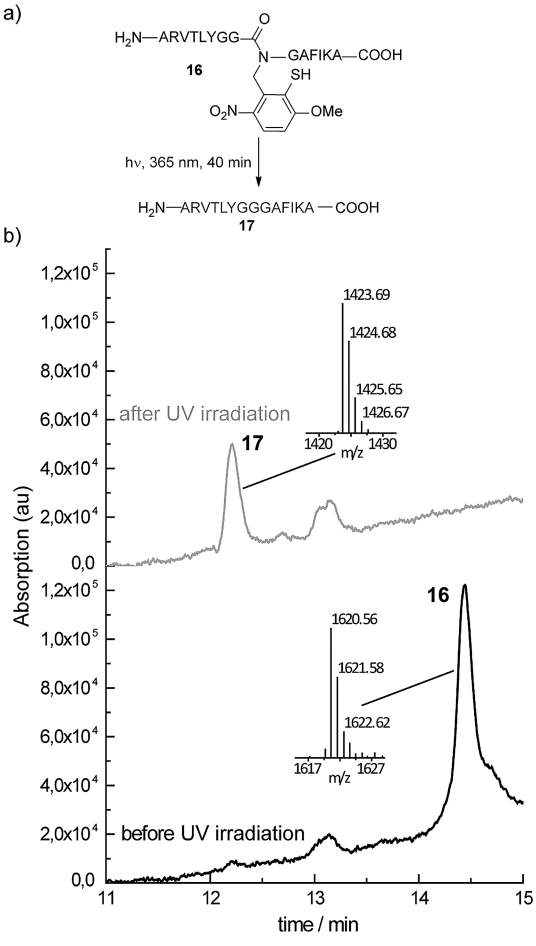


Figure 4. (a) Cleavage of auxiliary **1** from ligation product **16** by irradiation with UV light (365 nm) for 40 min to form peptide **17**. (b) LC–MS traces of the cleavage reaction. UV absorption was measured at 215 nm.

Conclusions

A new photocleavable ligation auxiliary **1** was synthesized and employed in NCL-like reactions at a variety of ligation junctions. Auxiliary **1** was readily synthesized in a three-step procedure and successfully introduced into peptides that contain a wide array of different N-terminal amino acids by reductive amination directly on the resin. This straightforward protocol has several advantages compared to the attachment protocols of previously known ligation auxiliaries. It is a short, one-step procedure that is fully compatible with acidic cleavage conditions and can be easily applied to different N-terminal amino acids without the need for different amino acid building blocks. Furthermore, the auxiliary peptides can be generated in good yields.

Auxiliary **1** enables ligation at Gly-Gly and Ala-Gly ligation sites with high conversion. After the reaction, auxiliary **1** was readily cleaved from the final ligation product by mild photolysis using UV light (365 nm). In contrast to the previously reported photoremovable 2-mercapto-1-(2-nitrophenyl)ethyl auxiliary,[17] ligations with sterically more demanding amino acids at the N-terminus of the auxiliary peptide and even with sterically demanding amino acids on both sides of the ligation junction were possible. However, the conversion was generally low (5–13%). This hints at significant potential for the further development of this methodology by generating derivatives with different substitution patterns at the aromatic ring. Modifying the nucleophilicity of the auxiliary thiol would be a solution to increase ligation rates. This could be achieved, for example, by introducing additional methoxy groups on the phenyl ring, as those also increase the nucleophilicity of the thiol group of 2-mercaptobenzyl auxiliaries.[20,30] Continuous development of more sophisticated peptide and protein ligation strategies is crucial to be able to address challenging total syntheses or semisyntheses of protein molecules. This is especially important when the synthetic target features labile posttranslational modifications, and homogeneous material is required to establish the precise role of an investigated protein in biochemical experiments. The newly developed auxiliary **1** is an important step in this direction, as it enables traceless NCL-like reactions at ligation sites other than X-Cys and can be removed by brief illumination with UV light, thus eliminating the need for harsh deprotection conditions.

Experimental Section

General Methods: All of the employed reagents were of analytical grade. Solvents were of the highest grade available. Dry solvents were stored over molecular sieves (4 Å). All air- and water-sensitive reactions were conducted under an inert atmosphere. For large-scale reactions, the glassware was flame-dried, and for small-scale reactions, which were conducted in tubes that were not heat resistant, a purge and refill technique without heat was applied. Purification by flash chromatography was conducted by using Merck silica gel 60. The NMR spectroscopic data were recorded with Varian instruments (Mercury 300, Unity 300, INOVA-500,

and INOVA-600). Chemical shifts are reported in ppm (for TMS, δ = 0 ppm), and coupling constants $^{n}J_{X,X}$ are reported in Hz. ESI mass spectra were recorded with Finnigan LCQ 7000 and Bruker micrOTOF spectrometers. High resolution ESI spectra were obtained with a Bruker APEX-Q IV 7T or Bruker micrOTOF spectrometer. LC–MS measurements were conducted on a Thermo Finnigan LCQ ion trap mass spectrometer connected to a JASCO 851 autosampler with a Rheos 4000 pump and a Thermo Finnigan Surveyor PDA (photodiode array) detector. Reactions were analyzed on a Phenomenex Synergi LC Column, RP-C12 (150 × 2.0 mm, 4 μm, 80 Å) or a MN Nucleodur EC column, RP-C18, (100 × 2.0 mm, 3 μm, 110 Å) with a linear gradient of eluent A (H_2O, 0.05% formic acid) to B (MeOH, 0.05% formic acid) in 15 min. The conversions of the ligation reactions were calculated by comparing the LC peak areas of the auxiliary peptide (starting material) and the product that contained the auxiliary at 400 nm. Origin[32] was used for all calculations employing a polynomial baseline.

3-Methoxy-2-*p*-tolylsulfonylbenzaldehyde (5): *o*-Vanillin (**2**, 10.0 g, 65.8 mmol, 1.00 equiv.) and KOH (4.00 g, 70.1 mmol, 1.07 equiv.) were dissolved in H_2O (70 mL). Tosyl chloride (12.5 g, 65.8 mmol, 1.00 equiv.) was added, and the mixture was stirred at room temperature for 1 h. DCM (5 mL) was added, and the precipitate was removed by filtration, washed with H_2O (3 × 100 mL), and dried under reduced pressure to yield the crude product (19.0 g, 62.0 mmol, 94%), which was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$, 29 °C): δ = 10.09 (s, 1 H, CHO), 7.84–7.71 (m, 2 H, Tos-H_{Ar}), 7.55–7.44 (m, 1 H, 5/6-H), 7.44–7.24 (m, 3 H, Tos-H_{Ar} and 5/6-H), 7.11 (dd, $^3J_{H,H}$ = 8.2 Hz, $^4J_{H,H}$ = 1.5 Hz, 1 H, 4-H), 3.59 (s, 3 H, OCH_3), 2.47 (s, 3 H, Tos-CH_3) ppm. ^{13}C NMR (126 MHz, $CDCl_3$, 27 °C): δ = 188.10 (C=O), 152.72 (C-3), 145.85 (C-2), 132.79 (C_{Tos}-4), 131.34 (C_{Tos}-1), 129.79 (C_{Tos}-2/6), 128.74 (C_{Tos}-3/5), 128.00 (C-6), 119.54 (C-5), 118.08 (C-4), 56.06 (OCH_3), 21.86 (Tos-CH_3) ppm. MS (ESI): *m*/*z* (rel. %) = 307.1 (6) $[M + H]^+$, 329.1 (100) $[M + Na]^+$, 635.1 (56) $[2M + Na]^+$. HRMS (ESI): calcd. for $[C_{15}H_{15}O_5S]^+$ $[M + H]^+$ 307.0635; found 307.0638; calcd. for $[C_{15}H_{14}O_5SNa]^+$ $[M + Na]^+$ 329.0454; found 329.0455; calcd. for $[C_{15}H_{13}O_5S]^-$ $[M - H]^-$ 305.0489; found 305.0485.

3-Methoxy-6-nitro-2-(*p*-tolylsulfonyl)benzaldehyde (4): Benzaldehyde **4** was synthesized according to a reported protocol for the corresponding phenylsulfonyl derivative.[24] 3-Methoxy-2-(*p*-tolylsulfonyl)benzaldehyde (**5**, 18.1 g, 59.2 mmol, 1.00 equiv.) was added to 90% HNO_3 (42 mL) at 0 °C over the course of 30 min. After additional stirring for 30 min, the mixture was poured onto ice (240 g), and the mixture was warmed to room temperature. The resulting precipitate was collected by filtration and washed with H_2O (50 mL). The solid was suspended in acetone, and was kept at 4 °C for 20 h. The solvent was removed by filtration and the crude product purified by flash chromatography (pentane/EtOAc, 4:1) to yield the product (12.6 g, 35.9 mmol, 60%) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$, 29 °C): δ = 10.08 (s, 1 H, CHO), 8.15 (d, $^3J_{H,H}$ = 9.2 Hz, 1 H, 5-H), 7.86–7.75 (m, 2 H, Tos-H_{Ar}), 7.44–7.33 (m, 2 H, Tos-H_{Ar}), 7.07 (d, $^3J_{H,H}$ = 9.3 Hz, 1 H, 4-H), 3.78 (s, 3 H, OCH_3), 2.49 (s, 3 H, Tos-CH_3) ppm. ^{13}C NMR (126 MHz, $CDCl_3$, 27 °C): δ = 185.89 (CHO), 157.96 (C-3), 146.14 (C_{Tos}-4), 139.45 (C-6), 135.15 (C-1), 132.49 (C_{Tos}-1), 131.93 (C-2), 129.81 (C_{Tos}-2/6), 128.64 (C_{Tos}-3/5), 124.98 (C-4), 113.16 (C-5), 56.85 (OCH_3), 21.96 (Tos-CH_3) ppm. MS (ESI): *m*/*z* (rel. %) = 352.1 (37) $[M + H]^+$, 374.1 (95) $[M + Na]^+$, 725.1 (100) $[2M + Na]^+$. HRMS (ESI): calcd. for $[C_{15}H_{14}NO_5S]^+$ $[M + H]^+$ 352.0485; found 352.0483; calcd. for $[C_{15}H_{13}NO_5SNa]^+$ $[M + Na]^+$ 374.0305; found 374.0305; calcd. for: $[C_{15}H_{12}NO_5S]^-$ $[M - H]^-$ 350.0340; found 350.0328.

3-Methoxy-6-nitro-2-(tritylthio)benzaldehyde (1): NaH (60% suspension in mineral oil, 144 mg, 3.67 mmol, 1.00 equiv.) was added to a solution of triphenylmethanethiol (1.00 g, 3.67 mmol, 1.00 equiv.) in dry NMP (20 mL) at 0 °C under argon, and the mixture was stirred at this temperature for 10 min. Compound **4** (1.29 g, 3.67 mmol, 1.00 equiv.) was added in portions, and the reaction mixture was stirred for an additional 15 min. The reaction was quenched by the addition of a saturated NH_4Cl solution, and the solution was diluted with Et_2O (200 mL) and 1% HCl solution (200 mL). The phases were separated, and the organic layer was washed with H_2O (3 × 200 mL) and brine (3 × 200 mL) and dried with Na_2SO_4. The solvent was removed under reduced pressure, and after purification of the resulting residue by flash chromatography (pentane/EtOAc, 2:1), **1** (519 mg, 1.47 mmol, 40%) was obtained as a yellow solid. ^{1}H NMR (301 MHz, $CDCl_3$, 35 °C): δ = 9.53 (s, 1 H, CHO), 7.99 (d, $^3J_{H,H}$ = 9.2 Hz, 1 H, 5-H), 7.41–7.30 (m, 6 H, Trt-H), 7.30–7.12 (m, 9 H, Trt-H), 6.63 (d, $^3J_{H,H}$ = 9.2 Hz, 1 H, 4-H), 3.44 (s, 3 H, OCH_3) ppm. ^{13}CNMR (126 MHz, $CDCl_3$, 27 °C): δ = 190.14 (CHO), 165.76 (C-3), 144.04 (C-6), 143.84 (Trt-C_q), 139.14 (C-1), 130.16 (Trt-CH), 127.92 (C-5), 127.41 (Trt-CH), 127.10 (Trt-CH), 122.02 (C-2), 110.50 (C-4), 73.57 ($SCPh_3$), 55.78 (OCH_3) ppm. MS (ESI): *m*/*z* (rel. %) = 478.1 (100) $[M + Na]^+$, 933 (93) $[2M + Na]^+$. HRMS (ESI): calcd. for: $[C_{27}H_{21}NO_4SNa]^+$ $[M + Na]^+$ 478.1083; found 478.1073; calcd. for $[C_{27}H_{20}NO_4S]^-$ $[M - H]^-$ 454.1119; found 454.1114. UV (MeCN): λ_{max} = 229, 303 nm.

Peptide Synthesis: Except for the peptide thioesters, all peptides were synthesized by manual Fmoc solid-phase peptide synthesis on preloaded Wang resins by using *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU)/ 1-hydroxybenzotriazole (HOBt)/*N*,*N*-diisopropylethylamine (DIPEA) as the coupling agents and applying a fivefold excess amount of the activated Fmoc-protected amino acids in NMP. The last Fmoc group was retained on the peptide for storage purposes and removed by the standard protocol shortly before functionalization. The resins were dried in vacuo overnight before functionalization.

Attachment of Auxiliary 1 to Peptides: The resin (20.0 mg, resin load: 0.2–0.4 mmol g^{-1}) was suspended in anhydrous DCM (2 mL) under argon over activated molecular sieves (3 Å). The suspension was stirred for 1 h to allow the resin to swell. Auxiliary **1** (10.0 equiv.) was dissolved in anhydrous DCM (1 mL) under argon and transferred to the suspension. After the addition of $NaBH(OAc)_3$ (25.0 equiv.), the suspension was stirred at room temperature for 24 h with the exclusion of light. The suspension was subsequently transferred into a test tube and diluted with DCM (25 mL). Molecular sieves and the boron compounds were separated from the resin by sedimentation (15 min). The resin, which rose to the surface, was pipetted into a BD syringe, washed with DCM (5 × 1 mL), and dried in vacuo overnight. A mixture of TFA/TES/H_2O (95:2.5:2.5, 1 mL) was added to the dry resin, and the mixture was agitated at room temperature for 30 min with the exclusion of light. The solution was transferred into a lightproof flask, and the resin was washed with TFA (2 × 1 mL). The solvent was removed under reduced pressure, and the resulting solid was suspended in cold Et_2O (12 mL) and precipitated by centrifugation at –20 °C and 9000 min^{-1} for 30 min. The solvent was decanted, and the crude product was dissolved in a mixture of H_2O and MeCN (1:1). The resulting solution was lyophilized and purified by reverse phase HPLC. The auxiliary peptides were obtained in isolated yields of up to 39% based on resin loading (included peptide synthesis, reductive amination, cleavage from the resin, and HPLC purification). All products were isolated in their oxidized form.

Aux-GFKV (13): MS (ESI): *m*/*z* (rel. %) = 645.27 (100) $[M_{ox} + H]^+$. HRMS (ESI): calcd. for $[C_{30}H_{41}N_6O_8S]^+$ $[M_{ox} + H]^+$ 645.2701; found 645.2707.

Aux-AAFIKA (9): MS (ESI): *m*/*z* (rel. %) = 815.14 (100) $[M_{ox} + H]^+$. HRMS (ESI): calcd. for $[C_{38}H_{54}N_8O_{10}S]^+$ $[M_{ox} + H]^+$ 815.3756; found 815.3751.

Aux-LAFIKA (11): MS (ESI): *m*/*z* (rel. %) = 857.4 (100) $[M_{ox} + H]^+$. HRMS (ESI): calcd. for $[C_{41}H_{61}N_8O_{10}S]^+$ $[M_{ox} + H]^+$ 857.4226; found 857.4222.

Aux-KAFIKA (18): MS (ESI): *m*/*z* (rel. %) = 436.7 (100) $[M_{ox} + 2H]^{2+}$, 872.4 (18) $[M_{ox} + H]^+$. HRMS (ESI): calcd. for $[C_{41}H_{62}N_9O_{10}S]^+$ $[M_{ox} + H]^+$ 872.4335; found 872.4337; calcd. for $[C_{41}H_{64}N_9O_{10}S]^{2+}$ $[M_{ox} + 2H]^{2+}$ 436.7204; found 436.7207.

Aux-GYRA (12): MS (ESI): *m*/*z* (rel. %) = 661.2 (100) $[M_{ox} + H]^+$, 683.2 (9) $[M_{ox} + Na]^+$. HRMS (ESI): calcd. for: $[C_{28}H_{37}N_8O_9S]^+$ $[M_{ox} + H]^+$ 661.2399; found 661.2398.

Aux-GAFIKA (19): MS (ESI): *m*/*z* (rel. %) = 801.4 (100) $[M_{ox} + H]^+$. HRMS (ESI): calcd. for: $[C_{37}H_{52}N_8O_{10}S]^+$ $[M_{ox} + H]^+$ 801.3600; found 801.3596.

Synthesis of Peptide Thioesters: Peptide thioesters were synthesized on a 4-sulfamylbutyryl resin by following literature protocols.[25,26] (a) Resin loading: The resin (2.00 g, approximately 1.34 mmol) was suspended in CH_3Cl (50 mL), which had been filtered over basic allox prior to the reaction. For attachment of glycine, the resin was suspended in *N*,*N*-dimethylformamide (DMF, 30 mL). The resin was allowed to swell in the respective solvent for at least 30 min. The respective Fmoc-protected amino acid (4 equiv.) was added, and the mixture was stirred for 10 min and then cooled to –20 °C. After 20 min of stirring at this temperature, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 4 equiv.) and DIPEA (8 equiv.) were added. The mixture was stirred at –20 °C for 8 h. For glycine, the reaction mixture was warmed to room temperature overnight. For all of the other functionalizations (because of the risk of racemization), the resin was pipetted directly into a BD syringe, washed with DCM (3× 10 mL), DMF (3× 10 mL), DCM (3× 10 mL), and MeOH (3× 10 mL) and dried overnight under reduced pressure. The procedure was repeated (2 or 3 times) until sufficient resin loading was achieved. The loading density was determined by detecting the UV absorption of dibenzofulvene, which formed upon Fmoc cleavage.[31] (b) Peptide synthesis on 4-sulfamylbutyryl resin: Attachment of the Fmoc-protected amino acids was conducted by using the standard Fmoc SPPS conditions mentioned above. The first three amino acids were double coupled. For the introduction of the last amino acid, a Boc-protected (Boc = *tert*-butoxycarbonyl) derivative was used. The resin was washed with NMP and DCM and dried in vacuo. Batchsize: 0.2 mmol. (c) Activation: Iodoacetonitrile (300 equiv.), which was filtered through basic allox, was added to a solution of DIPEA (60.0 equiv.) in NMP (8 mL). The mixture was agitated at room temperature for 22 h with the exclusion of light. The solution was discarded, and the resin was washed with NMP (5× 10 mL), DMF (5× 10 mL), and DCM (5× 10 mL) and dried in vacuo overnight. (d) Cleavage from resin: The resin was swollen in DMF for 1 h. The respective thiol [MPAA (R′) or 3-mercaptopropionic acid ethyl ester (R), 50.0 equiv.] and sodium thiophenolate (0.50 equiv.) were dissolved in DMF/DCM (1:1, 10 mL), and the resulting suspension was agitated at room temperature for 24 h with the exclusion of light. The solution was collected, and the resin was washed with DMF (3× 10 mL). The solvents were removed under reduced pressure, and the residue was washed with cold ether and kept under vacuum overnight. (e) Cleavage of protecting groups: A solution of TFA, TIS, and H_2O (95:2.5:2.5, 8 mL) was added to the residue, and the mixture was stirred at room temperature for 2 h. The solvent was removed in vacuo. The crude peptide was suspended in cold Et_2O (10 mL), and the resulting solution was centrifuged (9000 rpm, –10 °C, 20 min). The ether was decanted, and the precipitation process was repeated. The crude peptide was lyophilized from H_2O and purified by reverse phase HPLC.

ALYRG-SR (20): MS (ESI): *m*/*z* (rel. %) = 348.2 (100) $[M + 2H]^{2+}$, 695.3 (64) $[M + H]^+$. HRMS (ESI): calcd. for: $[C_{31}H_{51}N_8O_8S]^+$ $[M + H]^+$ 695.3545; found 695.3541.

ALYRG-SR′ (7): MS (ESI): *m*/*z* (rel. %) = 365.2 (100) $[M + 2H]^{2+}$, 729.4 (54) $[M + H]^+$. HRMS (ESI): calcd. for: $[C_{34}H_{49}N_8O_8S]^+$ $[M + H]^+$ 729.3389; found 729.3387; calcd. for $[C_{34}H_{47}N_8O_8S]^-$ $[M - H]^-$ 727.3243; found 727.3246.

ARVTLYGG-SR′ (8): MS (ESI): *m*/*z* (rel. %) = 985.5 (100) $[M + H]^+$, 1971.2 (4) $[2M + H]^+$.

ARVTLYGG-SR (10): MS (ESI): *m*/*z* (rel. %) = 476.75 (61) $[M + 2H]^{2+}$, 952.49 (100) $[M + H]^+$. HRMS (ESI): calcd. for: $[C_{42}H_{71}N_{11}O_{12}S]^{2+}$ $[M + 2H]^{2+}$ 476.7497; found 476.7497; calcd. for: $[C_{42}H_{70}N_{11}O_{12}S]^+$ $[M + H]^+$ 952.4921; found 952.4921.

ARVTLYGA-SR (14): MS (ESI): *m*/*z* (rel. %) = 483.76 (12) $[M + 2H]^{2+}$, 966.51 (100) $[M + H]^+$. HRMS (ESI): calcd. for: $[C_{43}H_{72}N_{11}O_{12}S]^+$ $[M + H]^+$ 966.5077; found 966.5071.

Ligation Experiments: All ligations were conducted under argon with the exclusion of light by utilizing sealed black 1.5 mL centrifuge tubes. The protocol is based on a general ligation strategy that is used in previously published extended Native Chemical Ligation protocols.[14,20] Ligation buffers were prepared by using a filtered stock solution of guanidium hydrochloride (6 M) and NaH_2PO_4 (100 mM), which could be stored at 4 °C for 1 month. The addition of the additives [MPAA (50 mM), TCEP (20 mM)] and the adjustment of the pH were performed directly before each ligation. All buffers were degassed prior to application. The thioester and auxiliary peptide were weighed into a black centrifuge tube and set under vacuum for 30 min. Afterwards, they were dissolved in the degassed buffer solution under argon. To ensure complete dissolution, the closed tubes were shaken, sonicated, and centrifuged. The reaction mixtures were stirred at room temperature or in a temperature-controlled oil bath. At consistent time intervals, samples for LC–MS measurements were removed under argon. For this, a sample of the reaction solution (10 μL) was pipetted into an opaque vial and diluted with a mixture of H_2O and MeCN (1:1) that contained 0.1% TFA (20 μL). After the indicated reaction time, the product was purified using reverse phase HPLC.

ALYRGG(Aux)FKV (15): Auxiliary peptide **13** and thioester peptide **7** at pH = 7.64 and room temp. for 24 h without MPAA yielded **15** (91%). MS (ESI): *m*/*z* (rel. %) = 604.8 (100) $[M + 2H]^{2+}$, 1207.6 (19) $[M + H]^+$.

ARVTLYGGA(Aux)AFIKA (21): Auxiliary peptide **9** and thioester peptide **8** at pH = 6.99 and room temp. for 24 h without MPAA yielded **21** (13%). MS (ESI): *m*/*z* (rel. %) = 818.3 (100) $[M + 2H]^{2+}$, 1634.6 (5) $[M + H]^+$. HRMS (ESI): calcd. for: $[C_{75}H_{117}N_{19}O_{20}S]^{2+}$ $[M + 2H]^{2+}$ 817.9216; found 817.9235; calcd. for $[C_{75}H_{116}N_{19}O_{20}S]^+$ $[M + H]^+$ 1634.8359; found 1634.8365.

ARVTLYGGL(Aux)AFIKA (22): Auxiliary peptide **11** and thioester peptide **10** at pH = 7.66 and 50 °C for 8 h with MPAA as an additive yielded **22** (6%). MS (ESI): *m*/*z* (rel. %) = 839.3 (100) $[M + 2H]^{2+}$, 1676.7 (13) $[M + H]^+$, 1698.6 (6) $[M + Na]^+$.

ARVTLYGAG(Aux)YRA (23): Auxiliary peptide **12** and thioester peptide **14** at pH = 7.10 and 35 °C for 49 h with MPAA as an additive yielded **23** (82%). MS (ESI): *m/z* (rel. %) = 748.2 (100) $[M + 2H]^{2+}$, 1494.5 (14) $[M + H]^{+}$. HRMS (ESI): calcd. for: $[C_{66}H_{101}N_{19}O_{19}S]^{2+}$ $[M + 2H]^{2+}$ 747.8615; found 747.8617; calcd. for: $[C_{66}H_{100}N_{19}O_{19}S]^{+}$ $[M + H]^{+}$ 1494.7158; found 1494.7136.

ARVTLYGAA(Aux)AFIKA (24): Auxiliary peptide **9** and thioester peptide **14** at pH = 7.00 and 35 °C for 32 h with MPAA as an additive yielded **24** (5%). MS (ESI): *m/z* (rel. %) = 824.8 (100) $[M + 2H]^{2+}$, 1648.5 (26) $[M + H]^{+}$.

ARVTLYGGG(Aux)AFIKA (16): Auxiliary peptide **19** and thioester peptide **10** at pH = 7.60 and room temp. for 72 h with MPAA as an additive yielded **16** (70%). MS (ESI): *m/z* (rel. %) = 810.9 (100) $[M + 2H]^{2+}$, 1620.8 (13) $[M + H]^{+}$. HRMS (ESI): calcd. for $[C_{74}H_{115}N_{19}O_{20}S]^{2+}$ $[M + 2H]^{2+}$ 810.9138; found 810.9151; calcd. for $[C_{74}H_{114}N_{19}O_{20}S]^{+}$ $[M + H]^{+}$ 1620.8203; found 1620.8208.

Photocleavage: Samples of the ligation product or ligation reaction mixture were pipetted onto a transparent, polystyrene six-well tissue culture test plate and illuminated by using a Biostep UV transilluminator (UST-20L-8E, 365 nm, filter size 20 × 20 cm, 100% intensity). Full cleavage was generally reached after 40 min. The reaction was analyzed by LC–MS using the same protocol as that for ligation reactions. The product was isolated by reverse phase HPLC.

ARVTLYGGAAFIKA (25): Ligation product **21** was employed to give **25**. MS (ESI): *m/z* (rel. %) = 719.7 (100) $[M_{w/o\ Aux} + 2H]^{2+}$, 1437.7 (17) $[M_{w/o\ Aux} + H]^{+}$.

ARVTLYGGLAFIKA (26): Ligation product **22** was employed to give **26**. MS (ESI): *m/z* (rel. %) = 740.7 (100) $[M_{w/o\ Aux} + 2H]^{2+}$, 1479.6 (3) $[M_{w/o\ Aux} + H]^{+}$.

ARVTLYGAGYRA (27): Ligation product **23** was employed to give **27**. HRMS (ESI): calcd. for: $[C_{58}H_{94}N_{18}O_{16}]^{2+}$ $[M_{w/o\ Aux} + 2H]^{2+}$ 649.3542; found 649.3536.

ARVTLYGGGAFIKA (17): Ligation product **16** was employed to give **17**. MS (ESI): *m/z* (rel. %) = 712.4 (100) $[M_{w/o\ Aux} + 2H]^{2+}$, 1423.8 (15) $[M_{w/o\ Aux} + H]^{+}$. HRMS (ESI): calcd. for $[C_{66}H_{108}N_{18}O_{17}]^{2+}$ $[M_{w/o\ Aux} + 2H]^{2+}$ 712.4064; found 712.4068; calcd. for $[C_{66}H_{107}N_{18}O_{17}]^{+}$ $[M_{w/o\ Aux} + H]^{+}$ 1423.8056; found 1423.8059.

Acknowledgments

Financial support from the Deutsche Forschungsgemeinschaft (DFG) (IRTG 1422, *Metal Sites in Biomolecules* and SPP 1623, *Chemoselective Reactions for the Synthesis and Application of Functional Proteins*) is gratefully acknowledged.

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Received: January 10, 2015
Published Online: March 30, 2015

