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Development of a Chemical Methodology for the Preparation of Peptide Thioesters Applicable to Naturally Occurring Peptides Using a Sequential Quadruple Acyl Transfer System

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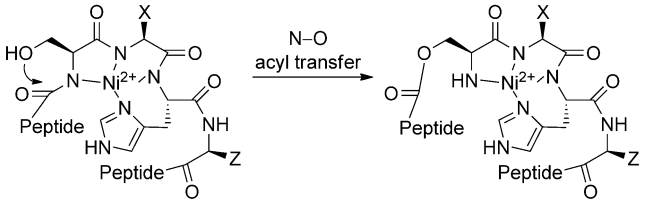
Peptide thioesters are very useful in protein chemistry, and chemistry- and biochemistry-based protocols are used for the preparation of thioesters. Among such protocols, only a few biochemistry-based approaches have been use for naturally occurring peptide sequences. The development of chemistry-based protocols applicable to natural sequences remains a challenge, and the development of such methods would be a major contribution to protein science. Here, we describe the preparation of peptide thioesters using innovative methodology that features nickel(II)-mediated alcoholysis of a naturally occurring peptide sequence, followed by O–N and N–S acyl transfers. This protocol involves sequential quadruple acyl transfer, termed SQAT. Notably, the SQAT system consists of sequential chemical reactions that allow naturally occurring peptide sequences to be converted to thioesters without requiring an artificial chemical unit.

There is increasing interest in the challenge of preparing peptide/protein thioesters, through chemical or biochemical means, because of their great utility in protein synthesis using native chemical ligation (NCL).[1] Protocols using chemical devices for the synthesis of peptide thioesters have been extensively reported in the literature.[2–4] However, one major shortcoming of the use of chemical devices is incompatibility with expressed proteins. Engineered intein[5,6] or sortase-mediated[7,8] methodologies have enabled the preparation of thioesters from expressed proteins, in which thioester formation relies on the enzyme or enzyme-like activity of sortase or intein, respectively. Although such biochemical processes have gradually become popular, the development of chemistry-based protocols applicable to expressed proteins remains necessary. Reported chemical methods include the N–S acyl-transfer-mediated conversion of specific cysteinyl sequences to thioesters[9,10] and the S-thiocarbonylation of a cysteinyl residue followed by acyl-guanidine-mediated cleavage;[11] however, these pioneering attempts still require further investigation to enable their practical use. During the progress of the present work, Macmillan and co-workers developed a chemical method based on N–S acyl-transfer chemistry.[12] However, an innovative protocol for thioester preparation applicable to a naturally occurring sequence is still required because the present protocols do not always afford satisfactory results. Thus, we have approached the subject from a different perspective and report herein a chemical approach to peptide thioesters production that can be applied to naturally occurring peptide sequences.

We began our research by evaluating the applicability of the nickel(II)-assisted peptide bond hydrolysis developed by Bal and co-workers[13] to thioester synthesis. In nickel(II)-assisted hydrolysis, the peptide bond preceding the serine or threonine in the sequence S/T-X-H-Z (where X and Z can be any amino acid residues except proline) is hydrolyzed. Mechanistic investigations of the hydrolysis indicated that two crucial steps are involved in the reaction: 1) formation of a square-planer nickel(II)-bound active complex consisting of the imidazole nitrogen and the three preceding amide nitrogens; 2) formation of *O*-peptidyl intermediates resulting from N–O acyl transfer of the amide bond preceding the serine or threonine residue and subsequent hydrolysis of the *O*-acyl intermediate (Scheme 1). A similar N–O acyl transfer is also seen in intein-mediated protein splicing, and this prompted us to investigate the nickel(II)-mediated hydrolysis in the preparation of thioesters.

Initially, we examined the applicability of the nickel(II)-mediated hydrolysis to the peptide sequence: H-



Scheme 1. Formation of *O*-acyl peptide intermediates during nickel(II)-mediated hydrolysis of peptide bond preceding Ser/Thr-X-His-Z sequence.

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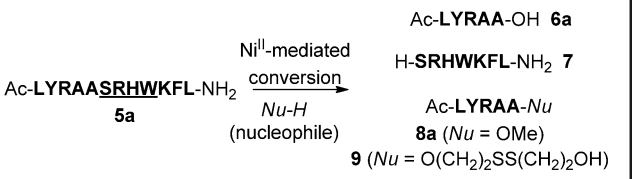
AKLRFGCPSRHWKFL-NH_2 (**1**),[14] with the anticipated one-step formation of a thioester: H-**AKLRFG**-SR (**2**). The reasons for selecting peptide **1** were based on Aimoto's experimental results, in which a cysteinyl prolyl ester afforded peptide thioesters.[9] However, subjecting peptide **1** to the nickel(II)-mediated reaction failed to afford thioester **2**.[15]

We speculated that one possible reason for the failure was related to the presence of the cysteinyl residue close to the nickel(II)-bound active complex. We therefore examined the hydrolysis of a peptide, H-**AKLRFGAPSRHWKFL**-NH_2 (**3**), with alanine substituted for cysteine. Although the nickel(II)-mediated hydrolysis of **3** (100 mM $NiCl_2$, 50 °C, pH 8.2) did not go to completion even after 48 h, we found that the reaction in the presence of 0.2 M tris(hydroxymethyl)aminomethane (Tris) afforded a small amount of a Tris adduct, H-**AKLRFGAP**-Tris (**4**), on the processed N-peptide, the N-terminal half of the cleaved peptide (see Figure S1 in the Supporting Information). It is worth noting that an appropriate oxygen nucleophile has a good chance of being involved in the nucleophilic conversion of the serine-isopeptide intermediate to the corresponding oxyester via O–O intermolecular acyl transfer. This suggested the feasibility of a stepwise conversion of an **SRHW**-containing peptide to a thioester through an oxyester. We therefore next used Ac-**LYRAASRHWKFL**-NH_2 (**5a**), which has a more scissile alanyl–serine linkage, to examine the conversion to an oxyester.

Standard hydrolysis reaction of **5a** in 0.2 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (pH 8.2) in the presence of 10 mM nickel(II) chloride at 37 °C went to completion within 12 hours to give the corresponding hydrolyzed peptides, Ac-**LYRAA**-OH (**6a**) and H-**SRHWKFL**-NH_2 (**7**). Based on these hydrolysis conditions, the nickel(II)-mediated conversion of **5a** to the corresponding oxyester in the presence of alcohols was examined (Table 1). The use of trifluoroethanol (TFE) or propan-2-ol as a nucleophile failed to yield the corresponding oxyester. However, methanol participated in the nucleophilic attack to yield the methyl ester peptide, Ac-**LYRAA**-OMe (**8a**). The fraction converted increased with increasing methanol concentration (entries 3–5 Table 1). Across the nickel(II) concentration range (1–20 mM), no significant differences were observed (entries 4, 6 and 7 in Table 1). Methanolysis at pH 8.2 gave the best result (entries 4, 8 and 9 in Table 1).[16] The nickel(II)-mediated reaction tolerated the presence of guanidine hydrochloride (Gn·HCl), although the fraction converted decreased (entry 10 in Table 1). Conversion to a dithiodiethyl (DTDE; $HOCH_2CH_2S$-SCH_2CH_2OH) oxyester, Ac-**LYRAA**-OCH_2CH_2S-SCH_2CH_2OH (**9**), also proceeded (entries 11 and 12 in Table 1). Here, DTDE was selected due to the anticipation of the O–S acyl-transfer-mediated conversion of the DTDE oxyester to a thioester.[4] Among several attempts to achieve conversion to the thioesters,[17] the use of 0.1% (*v*/*v*) trifluoromethanesulfonic acid/5% (*v*/*v*) *para*-thiocresol in trifluoroacetic acid successfully converted **9** to the corresponding methylphenyl thioester, Ac-**LYRAA**-SPh(4-Me) (**10**), via a tandem thiol switch.[18,19] However, this procedure is accompanied by the formation of a considerable amount of alanine-epimerized peptide. The origin of this was shown to be an O–S acyl-transfer step under acidic conditions using an alternatively synthesized L-alanine-containing peptide.[20]

Table 1. Nickel(II)-mediated conversion of **5a** to oxyesters.



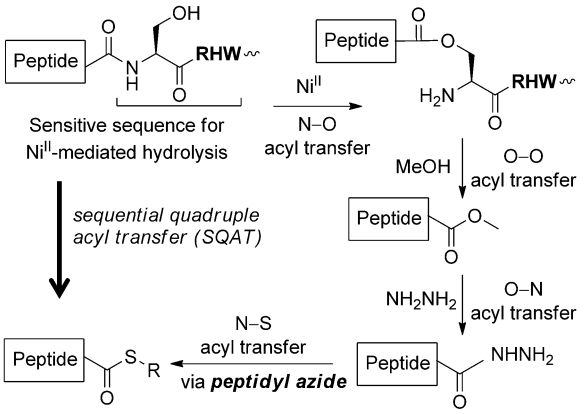
Entry	Conditions[a] $NiCl_2$ [mM]	pH	Nucleophile	FC[b]
1	10	8.2	50% (*v*/*v*) TFE	–[d]
2	10	8.2	50% (*v*/*v*) *i*PrOH	–[d]
3	10	8.2	10% (*v*/*v*) MeOH	0.44
4	10	8.2	30% (*v*/*v*) MeOH	0.70
5	10	8.2	50% (*v*/*v*) MeOH	0.72
6	1	8.2	30% (*v*/*v*) MeOH	0.69
7	20	8.2	30% (*v*/*v*) MeOH	0.68
8	10	7.8	30% (*v*/*v*) MeOH	0.61
9	10	8.6	30% (*v*/*v*) MeOH	0.53
10[c]	10	8.2	30% (*v*/*v*) MeOH	0.43
11	1	8.2	30% (*v*/*v*) MeOH	0.59
12	10	8.2	30% (*v*/*v*) MeOH	0.65

[a] Reactions were performed in 0.2 M HEPES buffer at 37 °C for 12 h in the presence of 1 mM of **5a**. [b] The fraction converted (FC) was determined by HPLC separation and integration (integ.) of **8a** (or **9**) as a fraction of the sum of the integration of unreacted **5a** + hydrolyzed **6a** + **8a** (or **9**). [c] In the presence of 6 M Gn·HCl. [d] Oxyesters were not obtained.

To develop an alternative methodology to the O–S acyl-transfer step, we focused on an innovative protocol involving a peptide hydrazide/azide, reported by Liu and co-workers.[21] As such, we next examined thioester synthesis from methyl ester **8a** using hydrazide/azide. The nickel(II)-mediated alcoholysis of **5a** (0.2 M HEPES, 10 mM $NiCl_2$, 50% (*v*/*v*) MeOH, pH 8.2, 37 °C, for 12 h), followed by addition of hydrazine monohydrate ($NH_2NH_2{\cdot}H_2O$) to the reaction mixture (final concentration: 5% (*v*/*v*) $NH_2NH_2{\cdot}H_2O$) with additional reaction for 1 hour at 25 °C, gave the peptide hydrazide, Ac-**LYRAA**-$NHNH_2$ (**11a**), in 80% isolated yield.[22] Furthermore, the resulting **11a** was converted to the corresponding sodium mercaptoethanesulfonate (MESNa) thioester, Ac-**LYRAA**-$SCH_2CH_2SO_3Na$ (**13a**), using Liu's conditions via the peptide azide, Ac-**LYRAA**-N_3 (**12a**), and no epimerization was observed in the sequence of reactions.[23] We named this thioesterification system SQAT, because the thioesters were produced by sequential quadruple acyl transfer (N–O, O–O, O–N, and N–S acyl transfers) as shown in Scheme 2.

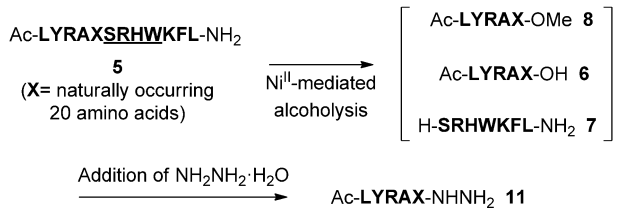
These results encouraged us to examine the applicability of the protocol to the 20 other naturally occurring amino acids (X)–serine junctions in Ac-**LYRAXSRHWKFL**-NH_2 (**5**) (Table 2). Several **X-SRHW** sequences were proven to be potential sites for thioester synthesis.

The feasibility of preparing thioesters using the SQAT system was confirmed by NCL-mediated syntheses of C-type and A-type natriuretic peptides (CNP and ANP, 53- and 28- residue naturally occurring peptides, respectively). For the preparation



Scheme 2. Sequential quadruple acyl transfer (SQAT) system for thioester synthesis.

Table 2. Conversion of peptides **5** to peptide hydrazides **11**.[a]



Entry	Peptide **5**	X	FC to **11**[b]
1	**5a**	A	0.61 (80%[c])
2	**5b**	G	0.66
3	**5c**	D	0.40
4	**5d**	E	0.69
5	**5e**	N	–[d]
6	**5f**	Q	–[e]
7	**5g**	S	0.42
8	**5h**	T	0.48
9	**5i**	C	–[f]
10	**5j**	P	0.09
11	**5k**	V	0.12[g]
12	**5l**	M	0.58
13	**5m**	L	0.66[h]
14	**5n**	I	–[g]
15	**5o**	Y	0.40
16	**5p**	F	0.41
17	**5q**	H	0.57
18	**5r**	K	0.65
19	**5s**	R	0.59
20	**5t**	W	0.62

[a] Peptide **5** (1 mM) in 0.2 M HEPES was treated in the presence of 10 mM $NiCl_2$ and 50% (*v/v*) MeOH, pH 8.2, at 37 °C for 24 h, followed by addition of $NH_2NH_2 \cdot H_2O$ (final concentration: 5% (*v/v*) NH_2NH_2) and left to react at 25 °C for a further 3 h. [b] The fraction converted (FC) was determined by HPLC separation and integration (integ.) of **11** as a fraction of the sum of the integration of unreacted **5** + hydrolyzed **6** + **8** + **11**. [c] Under optimized conditions (see main text), **11a** was obtained in 80% isolated yield. [d] α,β-Dihydrazide peptide was obtained. [e] A mixture of α and γ-hydrazide peptides was obtained. [f] No N-processed peptides (**6i**, **8i**, and **11i**) were observed. [g] Although the initial N–O acyl shift proceeded, subsequent reactions did not proceed to completion. [h] For satisfactory HPLC purification, N-terminally extended peptide Ac-**KLYRALSRHWKFL**-NH_2 (**5m**) was used.

of CNP (**14**) and ANP (**15**), 43-residue nickel-sensitive SRHW-fused peptide **16** (CNP 53 1–36 + **SRHWKFL**-NH_2) and 29-residue peptide **17** (ANP 28 1–22 + **SRHWKFL**-NH_2) were synthesized respectively. Treatment of 43-residue CNP peptide **16** with 10 mM nickel(II) chloride/50% (*v/v*) methanol in 0.2 M HEPES, pH 8.2, at 37 °C for 6 hours, followed by hydrazine monohydrate treatment at 25 °C for 1 hour, yielded a 36-residue peptide hydrazide **18** as the main processed N-peptide via the peptide methyl ester **19** in 69% isolated yield. Treatment of the resulting hydrazide **18** with 20 mM sodium nitrite in 6 M Gn·HCl–0.2 M sodium phosphate, pH 3.0, at 0 °C for 1 hour gave the corresponding peptide azide. Without purification, the peptide azide was treated with 200 mM (4-carboxymethyl)thiophenol (MPAA) in 6 M Gn·HCl–0.2 M sodium phosphate, pH 7.0, at room temperature for 1 hour, affording the MPAA thioester, which was then subjected to NCL with the N-terminal cysteinyl CNP (37–53) fragment **20** to yield the reduced form CNP 53 **21** in 47% isolated yield after HPLC purification. Folding of the reduced material **21** in 6 M Gn·HCl–0.1 M sodium phosphate, pH 7.3/DMSO (9:1) afforded CNP **14**.

Next, applicability of the SQAT system to cysteine-containing peptides was verified by conversion of the cysteine-containing ANP precursor **17** to the corresponding peptide hydrazide **22** (Scheme 3 and Figure 1). Experimental manipulation similar to that employed for the conversion of **16** to **18** was conducted for the preparation of **22**, except for trapping of nickel(II) by the addition of ethylenediaminetetraacetic acid (EDTA) to the

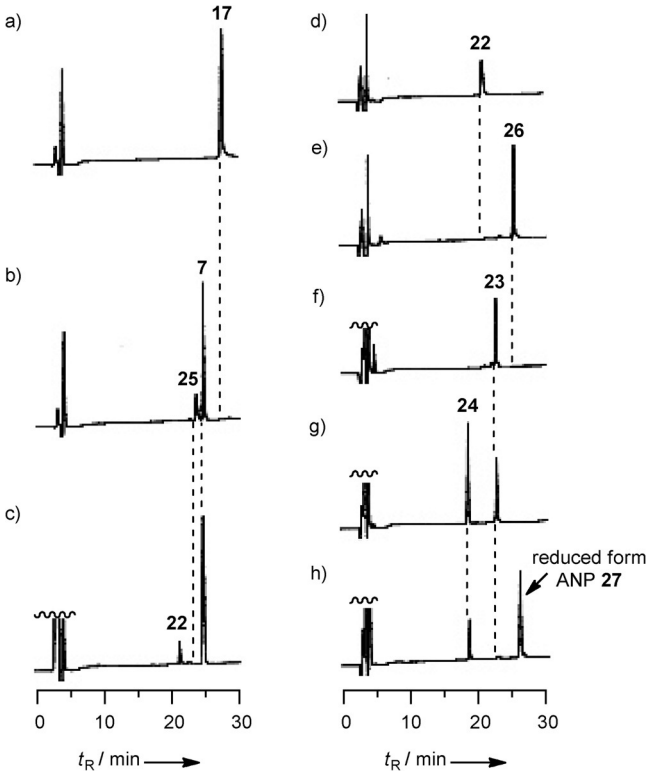
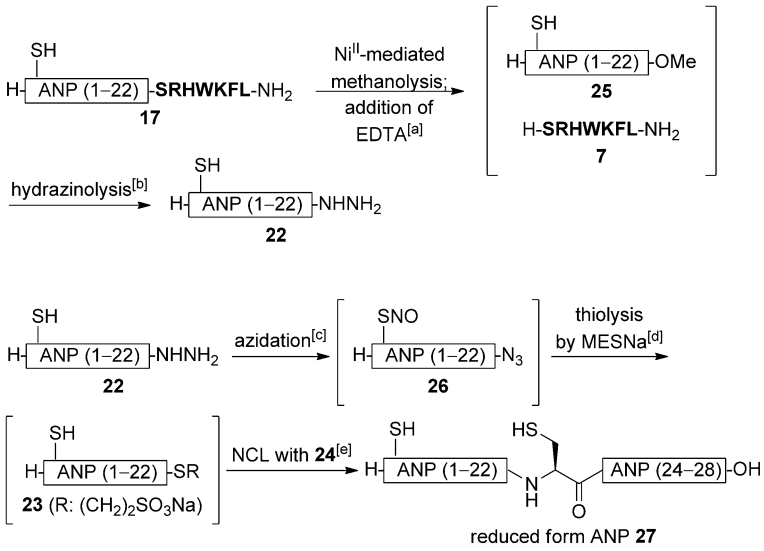


Figure 1. HPLC monitoring of reactions for the synthesis of ANP: a) nickel(II)-mediated methanolysis ($t = 0$ h); b) nickel(II)-mediated methanolysis ($t = 3$ h); c) hydrazinolysis ($t = 3$ h); d) azidation ($t = 0$ h); e) azidation ($t = 1$ h); f) Thiolysis ($t = 1$ h); g) native chemical ligation (NCL) ($t = 0$ h); h) NCL ($t = 4$ h).



Scheme 3. Chemical synthesis of ANP using the sequential quadruple acyl transfer (SQAT) system. a) Peptide **17** (1 mM) was treated in 0.2 M HEPES in the presence of 10 mM $NiCl_2$ and 50% (*v*/*v*) MeOH, pH 8.2, at 37 °C for 3 h, followed by addition of EDTA into the reaction mixture. b) $NH_2NH_2{\cdot}H_2O$ was added into the reaction mixture (final concentration: 5% (*v*/*v*) NH_2NH_2) and left to react at 25 °C for a further 3 h. c) Peptide **22** was treated in 0.2 M sodium phosphate in the presence of 6 M Gn·HCl and 20 mM $NaNO_2$, pH 3.0, at −10 °C for 1 h. d) MESNa-containing buffer (6 M Gn·HCl, 0.2 M sodium phosphate, 200 mM MESNa) was added into the reaction mixture, and the pH of the solution was adjusted to pH 7.0 by using 2 M NaOH (aq). Then, the reaction mixture was stored at room temperature for 1 h. e) Peptide **24** and thiophenol were added into the reaction mixture (final concentration: peptide **23** (1.5 mM), **24** (2.0 mM), 5% (*v*/*v*) thiophenol) and left to react at 37 °C for a further 4 h.

reaction mixture before hydrazinolysis. Without treatment with EDTA, desired peptide disappeared upon HPLC analysis of reaction mixture after hydrazinolysis. This is probably attributable to formation of insoluble metal nickel species by reduction of nickel(II) with hydrazine, on which the cysteinyl peptides were adsorbed. Application of the modified SQAT system with the additional EDTA treatment successfully generated thioester **23** for ANP synthesis.[24] NCL of **23** with N-terminal cysteinyl peptide (ANP 23–28 **24**), followed by folding gave ANP **15**.

Furthermore, conversion of a 514-residue glycoprotein, β-hexosaminidase B (HexB), expressed by Chinese hamster ovary (CHO) cells,[25] possessing T-**SRHY** and L-**TRHR** sequences as potential nickel(II)-mediated processing sites, in to three possible biotinylated proteins was performed by using the SQAT system followed by NCL with a biotin peptide. Although precise mass spectrometric analyses have yet to be achieved due to inhomogeneous character of sugar moieties, a Western blotting analysis indicated that the SQAT system would be applicable to HexB protein (see Scheme S10 and Figure S18 in the Supporting Information).

In conclusion, we have developed an artificial structural-unit-free chemical methodology, termed sequential quadruple acyl transfer (SQAT), for the preparation of peptide thioesters. The SQAT system, which involves four tandem acyl-transfer steps, is applicable to naturally occurring peptide sequences. It is also worth noting that the four-residue sequence (S/T-X-H-Z) responsible for initiation of the sequential acyl transfers apparently mimics the function of intein proteins. Application of the SQAT system to recombinant proteins including full characterization of the generated protein thioesters is underway, and the results of this study will be presented in due course. Finally, we believe that the SQAT system described here will become a useful chemical procedure for thioester preparation as a complement to other available protocols including the intein-mediated procedures.

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Keywords: acyl transfer · native chemical ligation · nickel(II)-mediated alcoholysis · peptide hydrazides · peptide thioesters

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[14] SRHW is the best nickel(II)-sensitive sequence identified to date by peptide library screening. See Ref. [13a].
[15] Exposure of peptides possessing a cysteine residue close to the SRHW sequence afforded processed C-peptide, whereas no cysteine-containing N-peptides were obtained (Entry 9, Table 2).
[16] Because initial methanolysis was carried out in a mixed solvent (MeOH/H_2O), the best conversion yields were around 70%. Attempts to increase the conversion yield have been carried out. The use of MeOH as a solvent sometimes causes solubility problems for proteins.
[17] Reduction of compound **9** under neutral conditions resulted in hydrolysis of **9** rather than conversion to the corresponding thioester. See Z. P. Gates, J. R. Stephan, D. J. Lee, S. B. H. Kent, *Chem. Commun.* **2013**, *49*, 786 – 788.
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[19] As subjection of methyl ester peptide **8a** to the reaction conditions did not afford the corresponding thioester, direct reaction of **9** with thiocresol seems to be denied.
[20] Exposure of stereo-defined Ac-**LYR**(L)**A**-DTDE oxyester, alternatively synthesized by solid-phase peptide synthesis (SPPS), to the acidic tandem thiol switch conditions afforded an HPLC-separable diastereomeric mixture consisting of Ac-**LYR**(L)**A**-SPh(4-Me) and Ac-**LYR**(D)**A**-SPh(4-Me); see, Scheme S5 and Figures S5 and S6 in the Supporting Information.
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[24] During azidation, *S*-nitrosyl peptide **26** was formed; however, subsequent thiolysis regenerates the thiol group.
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