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An *o*-nitrobenzyl scaffold for peptide ligation: synthesis and applications

Chiara Marinzi,[a,b,*] John Offer,[c] Renato Longhi[a] and Philip E. Dawson[c]

[a]*Istituto di Chimica del Riconoscimento Molecolare, C. N. R., Via M. Bianco 9, 20131 Milano, Italy*
[b]*Dipartimento di Chimica Organica e Industriale, Università di Milano, 20133 Milano, Italy*
[c]*Departments of Chemistry and Cell Biology and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 92037 La Jolla CA, USA*

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Abstract—Chemical ligation approaches facilitate the chemoselective assembly of unprotected peptides in aqueous solution. Here, two photolabile auxiliaries are described that enlarge the applicability of native chemical ligation to non-cysteine targets. The auxiliaries, designed to allow reaction with thioester peptides, generate an amide bond between the two initial fragments. The *o*-nitrobenzyl tertiary benzylamide that is formed at the ligation junction can be transformed into a native amide group under mild photolysis conditions.

The veratryl auxiliary was found to be excessively labile during peptide purification and ligation. However, the auxiliary based on the *o*-nitrobenzyl group shows all the necessary properties for a general application in routine peptide and protein synthesis. In addition, the auxiliary linked to the N-terminus can be efficiently photolyzed, suggesting a new approach for the generation of photocaged amines.

Synthesis, solid phase introduction onto peptide chains, ligation properties and photolysis in water are described, and a careful study of compatibility of the method with potentially fragile peptide side chains is reported.

1. Introduction

Chemical ligation approaches for the assembly of polypeptides using unprotected peptides of either synthetic or biological origin is an established method for small protein synthesis (Refs. 1–3 and references cited therein). The general utility of the native chemical ligation approach,[4] in which a C-terminal thioester peptide undergoes thiol exchange with a peptide bearing an N-terminal Cys residue followed by an irreversible acyl shift to yield an amide bond at the ligation site, has stimulated several studies that aim to broaden the scope of the reaction to non-cysteine peptides. One general approach has been the use of an auxiliary positioned at the N-terminus of a peptide that mimics the ligation properties of cysteine (thioester exchange and acyl migration).[5–11]

In order to be generally useful for synthetic purposes, the auxiliary must be synthesized and introduced onto the N-terminus of a peptide with high yields in a simple and general way. It also must allow reaction with a thioester peptide, and finally it has to be removed from the newly formed amide bond in a way that is fully compatible with multifunctional polypeptide chains.

It has been reported that ligation auxiliaries based on a 1-amino-1-phenyl ethane-2-thiol group efficiently react with thioester peptides and enable chemoselective amide bond formation between peptides.[8] To satisfy the last stringent requirement of post-ligation lability, substituents can be introduced onto the aromatic ring of the original scaffold. This purpose has been achieved with the introduction of electron donating (methoxy) groups that allow removal of the auxiliary from the amide bond by treatment with strong acids (HF, TFMSA or TFA).[11] Alternatively, as suggested in our preliminary work[8] and recently described by Kawakami and Aimoto,[12] an *o*-nitro group can be introduced, that renders the scaffold labile upon mild photolytic treatment. Such system should maintain the nucleophilic properties of the amine

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* Corresponding author. Tel.: +39-02-28500026; fax: +39-02-285000-36; e-mail: chiara.marinzi@icrm.cnr.it

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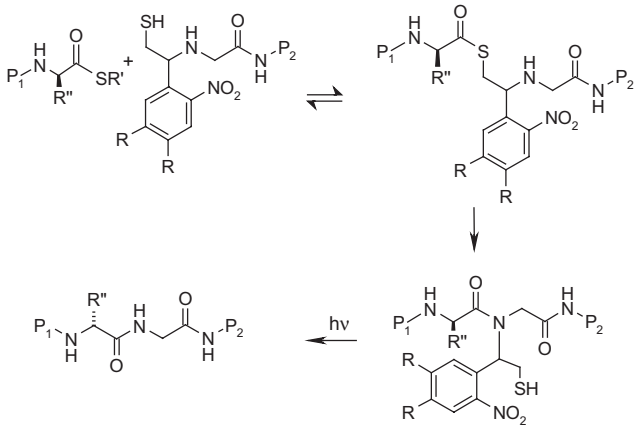


Scheme 1. Peptide ligation mediated by *o*-nitrobenzyl auxiliaries. P_1 and P_2: unprotected peptide chains; R = H: auxiliary **9**; R = OCH_3: auxiliary **13**; R′ = MPAL; R″ = CH_3: Ala-Gly junction; R″ = H: Gly-Gly junction.

and thiol groups, and thus allow reaction with thioester peptides according to a two-step mechanism analogous to native chemical ligation. After ligation, the auxiliary should be removed from the newly formed amide bond by photolysis (Scheme 1). This possibility is particularly attractive if the method is to be applied to the formation of amide bonds in more fragile compounds, such as glycopeptides or acid labile proteins.[13]

ortho-Nitrobenzyl groups have been extensively used in organic chemistry as linkers and protecting groups for many functionalities, including amines and amides, because they can be removed under mild conditions upon irradiation.[14–20] Efficiency of the photolytic process is enhanced in case of veratryl compounds, whose chromogenic properties allow photolysis at higher wavelengths, reducing potential side reactions.[21] Compatibility with amino acid side chains and peptide chains is very well documented, but it is also well known that fragile residues such as tryptophan and methionine undergo oxidation when long irradiation times (over 24 h) are required.[22,23]

In the present study we report the synthesis of the two *o*-nitrobenzyl scaffolds **9** and **15** (Fig. 1) and their incorporation at the peptide N-terminus on the solid phase with high yields. In order to evaluate the scope and utility of the reaction, ligation properties are described for model peptide systems at different peptide ligation junctions. Photolysis yields at 310 nm for different peptides, including Trp and Met containing peptides, in aqueous systems are reported and compatibility of the method with the presence of those residues is demonstrated. Furthermore, a straightforward procedure for the whole process is reported, in which removal of the auxiliary from the ligation product can be performed directly on the ligation mixture, without purification of the product from the phosphine-containing buffer.

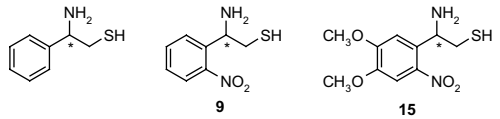


Figure 1. 1-Amino-1-phenyl ethane-2-thiol auxiliaries for peptide ligation.

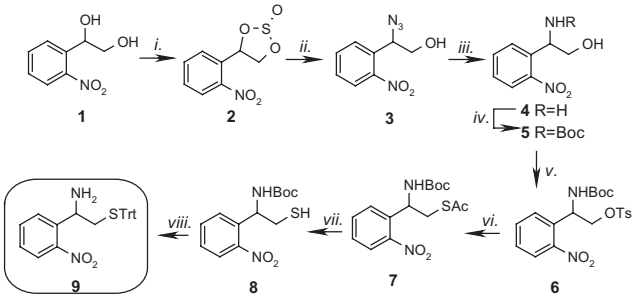
2. Results and discussion

2.1. Synthesis of *o*-nitrobenzyl auxiliaries 9 and 15

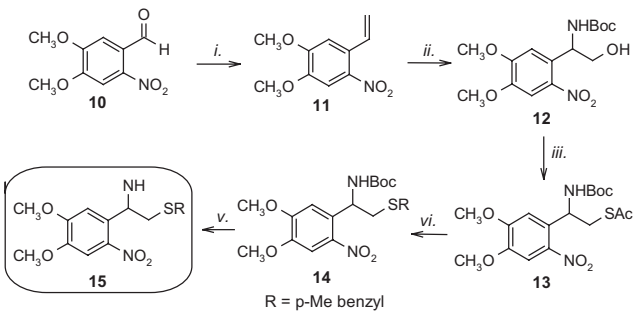
In our previous report we have already demonstrated that the stereocentre of the auxiliary has no significant influence on the rate of thioester exchange and intramolecular acyl transfer. No effort has therefore been made in searching enantiodivergent routes and both auxiliaries have been prepared as mixtures of enantiomers.

Auxiliary **9** was synthesized starting from the commercially available diol **1** (racemate) that was converted into the benzyl-azido alcohol **3** by regioselective ring opening of the cyclic sulfite **2**.[24,25] Functional group interconversion of alcohol into thiol and reduction of azide to amine gave facile and high yielding access to *S*-trityl protected aminothiol **9** (Scheme 2).[12,26,27]

Veratryl auxiliary **15** was synthesized according to the pathway of Scheme 3. *N*-Boc-benzylamine **12** was prepared from the corresponding styrene by Sharpless' regioselective aminohydroxylation.[28] The thiol group was then introduced by Mitsunobu reaction with thiol-



Scheme 2. (i) $SOCl_2$, Et_3N, CH_2Cl_2 (98%); (ii) NaN_3, DMF, 60 °C (93%); (iii) PPh_3, THF, H_2O (91%); (iv) Boc_2O, Et_3N, MeOH (quant.); (v) TsCl, py (98%); (vi) $CsCO_3$, AcSH, DMF (98%); (vii) NH_2OH, MeOH; (viii) TrtOH, TFA (95%) (overall yield 76%).



Scheme 3. (i) sodium hexamethyldisilazide, Ph_3PCH_2Br, THF (65%); (ii) $K_2OsO_2(OH)_4$, $(DHQ)_2PHAL$, *n*-prOH, *t*-Bu carbamate, *t*-Bu hypochlorite, NaOH, H_2O (53%); (iii) AcSH, Ph_3P, DIAD, THF (98%); (iv) MeONa, *p*-Me benzylbromide (97%); (v) TFA (quant.) (overall yield 32%).

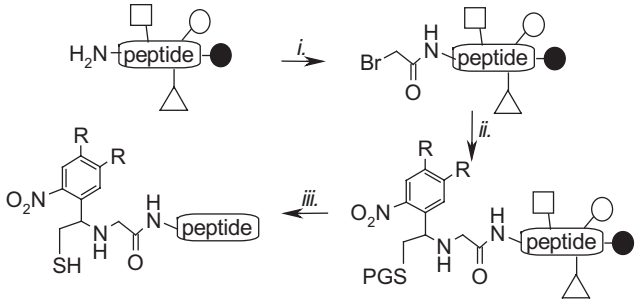
acetic acid.[29] Basic hydrolysis of the acetyl group, S-alkylation and amine deprotection afforded the desired product in good overall yield.

2.2. Introduction of auxiliaries onto peptide chain

The auxiliaries were introduced at the N-terminus of a peptide on the solid phase following the submonomer approach (Scheme 4):[30] the terminal glycine residue was assembled by first coupling α-bromo acetic acid, followed by reaction of the peptide resin with the racemic amine auxiliary **9** or **15**. Protection of the thiol functionality is required to avoid S-alkylation. Standard acidolytic cleavage afforded the desired, fully unprotected peptide as a mixture of diastereomers (>90% chromatographic yield, >60% recovered yield after HPLC purification).

2.3. Stability of derivatized peptides

Peptides bearing the two auxiliaries at their N-terminus are potentially photolabile and therefore their stability under ligation conditions had to be tested. To this purpose, different peptides bearing auxiliaries **9** and **15** at the N-terminus were dissolved in HPLC buffers or in 0.2 M phosphate buffer, pH 7, in the presence of 35 mM TCEP, at different peptide concentrations. Aliquots of these solutions were injected on analytical HPLC at different times. In the case of peptides derivatized with auxiliary **9**, no significant degradation was detected even after 24 h after dissolution. In the case of auxiliary **15**, protection of the reaction vessel from light was necessary to prevent the spontaneous formation of a variety of degradation products.[31]



Scheme 4. Introduction of the auxiliary onto a glycine terminal peptide according to the submonomer approach. (i). $BrCH_2COOH$, DIC, DCM; (ii) **9** (R = H) or **15** (R = OCH_3), diea, DMF; (iii) HF or TFA, scavengers. PG = protecting group for thiol functionality.

2.4. Peptide ligations

Ligation properties of the two scaffolds were tested in a series of model reactions with thioesters peptides at Gly-Gly and Ala-Gly junctions in conditions similar to those employed for ligations of larger polypeptides. These two junctions were chosen as representative of an interesting set of differently sterically demanding junctions, as previously described (Refs. 7,8 and references cited therein).

In the case of ligations mediated by auxiliary **9**, reactions proceeded efficiently to generate the expected amide products as confirmed by mass spectrometry analysis and treatment with hydroxylamine of the product (Table 1).[32] Two diastereoisomeric products are formed in each ligation (Fig. 2). For ligations at Gly-Gly

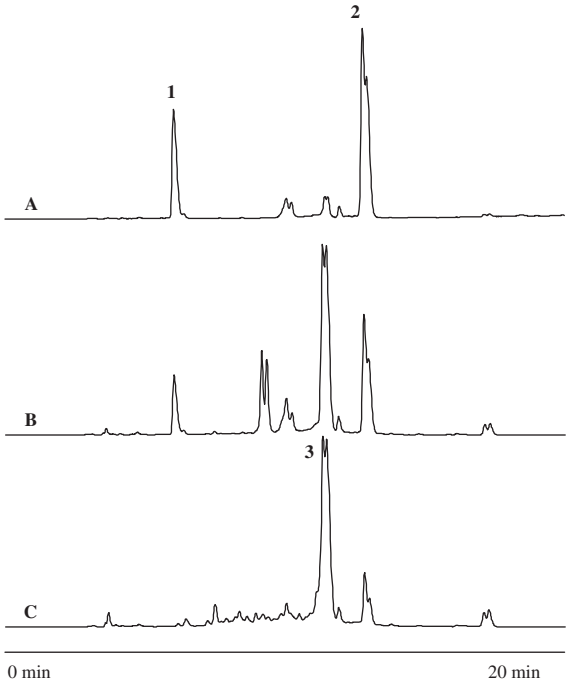


Figure 2. Analytical HPLC trace for the ligation of thioester *H*-KLAPYRAG-*MPAL* (**1**) with **9**-GLLRPFYRK-NH_2 (**2**) to give *H*-KLAPYRAG-(**9**)-GLLRPFYRK-NH_2 (**3**). (A) $t = 0$; (B) $t = 60$ min; (C) $t = 16$ h. Analytical HPLC conditions: Waters Symmetry 300 C18 column (5 μm, 4.6×250 mm); 20–50% B in A linear gradient for 20 min (1 cm^3/min, 214 nm UV monitor detection) (A = 0.1% aq TFA; B = 0.1% TFA in CH_3CN).

Table 1. Ligations between peptide thioesters and peptides bearing auxiliary **9** at the N-terminus

Thioester[b]	C-t fragment	Junction	$t_{1/2}$ (h)[a]	t_{over} (h)[a]
KLAPYRAG-*MPAL*	**9**-GLLRPFYRKnh_2	Gly-Gly	0.5	16
KLAPYRAG-*MPAL*	**9**-GLLRPFHRKnh_2	Gly-Gly	0.5	16
KLAPYRAG-*MPAL*	**9**-GLLRPY**M**RKnh_2	Gly-Gly	0.5	16
L**W**APYRAG-*MPAL*	**9**-GLLRPFHRKnh_2	Gly-Gly	0.5	16
L**W**APYRAA-*MPAL*	**9**-GLLRPY**M**RKnh_2	Ala-Gly	2.5	22
L**W**APYRAA-*MPAL*	**9**-GLLRPFHRKnh_2	Ala-Gly	2.5	22
L**W**APYRAA-*MPAL*	**9**-GLLRPFYRKnh_2	Ala-Gly	2.5	22

[a] $t_{1/2}$ = Half lives of starting peptides; t_{over} = time of completion. Values were determined by inspection of analytical HPLC traces for time course of each ligation. Ligation conditions: 0.2 M sodium phosphate buffer, 35 mM TCEP, pH 7.5, peptide concentration: 5.5–8 mM.
[b] MPAL = β-mercaptopropionic acid leucine.

junctions, the reactions were complete in 16 h, while longer times (22 h) were necessary for the more sterically demanding Ala-Gly junction. It has already been shown that 1-amino-1-phenyl ethane-2-thiol scaffolds do not allow the required intramolecular acyl shift to take place when the auxiliary is attached to a C-α-substituted residue. Therefore Gly-Ala or, more generally, Gly-X, X≠Gly, junctions were not considered in the present study.

Reaction is considered to proceed in an analogous manner to native chemical ligation, with an initial bimolecular thioester exchange, followed by intramolecular rearrangement. Half-lives of starting peptides were similar to those observed in the case of ligation mediated by the non-substituted phenylethane thiol auxiliary.[8] As expected, the introduction of the nitro group on the aromatic ring has little effect on the nucleophilicity of the thiol and amine groups involved in the reaction with thioesters.

A completely different scenario is observed when the veratryl auxiliary **15** was used. As soon as the two reacting peptides **15**-GLLRPFFRK-NH_2 and *H*-KLAPYRAG-*MPAL* (β-mercaptopropionic acid–leucine) were put together under ligation conditions, HPLC analysis showed the gradual disappearance of the peaks relative to the two starting peptides, with concomitant formation of many different species.

Characterization of the products by mass spectrometry was not possible, presumably due to further degradation of those compounds by going through the HPLC detector. This behavior was observed even when the reaction vessel was maintained away from light. Because of stability of the auxiliary bearing peptide under ligation conditions (see above), we reasonably hypothesize that the two initial fragments do react with one another, probably undergoing thioester exchange, but the intermediate or the amide product that may be formed are irreversibly degraded by photochemical processes, and the desired amide products cannot be isolated.

2.5. Photolysis experiments

In order to evaluate the photochemical behavior of auxiliary **9**, a preliminary experiment was performed by dissolving the peptide **9**-GLLRPFHRK-NH_2 (2 mg) in water (2 mL) and irradiating the solution in a Ryonet photoreactor at 310 nm. Analytical HPLC inspection of the reaction on time course showed complete photolysis of the *o*-nitrobenzyl-amine-peptide after 20 min of exposure to 310 nm light, generating the expected peptide *H*-GLLRPFHRK-NH_2.

The well-known photolytic properties of *o*-nitrobenzyl groups are thus conserved when the auxiliary is incorporated in an amine compound. Potential competitive intramolecular radical reaction of the thiol with the nitro group or with the photolysis intermediates, that could not be estimated in an 'a priori' theoretical examination, does not occur.[33]

Ligation products bearing auxiliary **9** were dissolved in water or water/acetonitrile mixtures and subjected to photolysis in a Ryonet photoreactor at 310 nm. Photolysis reactions were performed in aqueous systems for the double advantage of facilitating dissolution of unprotected peptides and favouring the photochemical reaction. Reactions were monitored by analytical HPLC at different times.

After 20 min of exposure to 310 nm light all tested peptides were completely photolyzed, generating the desired, enantiomerically pure, amide products (>90% chromatographic yield) along with compounds derived by degradation of the *o*-nitrobenzyl group, that can be removed from the mixture by simple ether extraction or HPLC purification. No difference in the chromatographic profile was observed for longer reaction times.

In order to find the best practical conditions for the method, photolysis reactions were also performed in mixtures of HPLC buffers and no difference was observed, demonstrating that the presence of TFA does not affect the photolytic process (Fig. 3). This implies that photolysis can be performed simply by irradiating the peptide solutions obtained from semipreparative HPLC.

In case of the ligation between *H*-LWAPYRAG-*MPAL* and peptide **9**-GLLRPFHRK-NH_2, a third practically favourable set of conditions was tested: when ligation was over, the mixture was diluted with HPLC buffers and the resulting mixture was irradiated without prior chromatographic purification of the ligation product. After 20 min of exposure to 310 nm light, HPLC and mass spectrometry analysis showed the complete transformation of the ligation product into the desired peptide (Fig. 4).

Noteworthy, no side chain oxidation was observed when peptides bearing Trp or Met were irradiated, demonstrating that the method is fully compatible with the presence of these residues in the sequence.

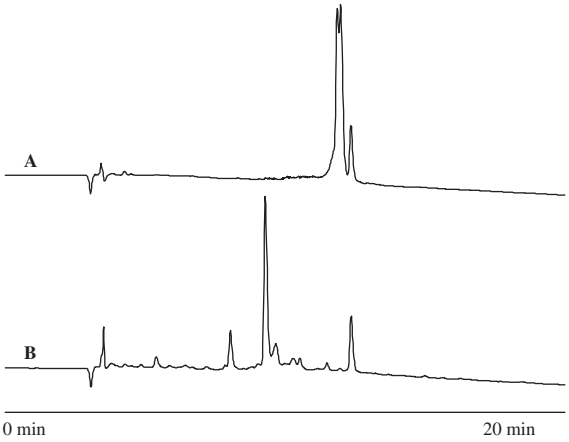


Figure 3. Analytical HPLC trace for the photolysis of purified *H*-KLAPYRAG-(**9**)-GLLRPFYRK-NH_2. (A) $t = 0$; (B) $t = 20$ min. Analytical HPLC conditions: Waters Symmetry 300 C18 column (5 μm, 4.6×250 mm); 20–50% B in A linear gradient for 20 min (1 cm^3/min, 214 nm UV monitor detection) (A = 0.1% aq TFA; B = 0.1% TFA in CH_3CN).

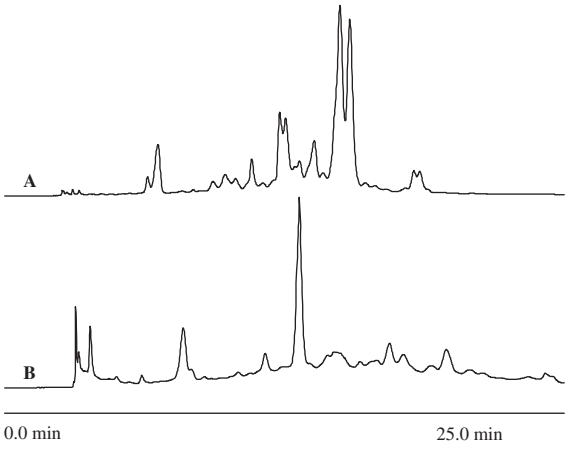


Figure 4. Analytical HPLC trace of photolysis of crude *H*-LWAPYRAG-(**9**)-GLLRPFHRK-NH_2 after 16 h of ligation (A) and of crude *H*-LWAPYRAGGLLRPFHRK-NH_2 after 20 min of irradiation (B). Analytical HPLC conditions: Waters Symmetry 300 C18 column (5 μm, 4.6×250 mm); 20–35% B in A linear gradient for 20 min (1 cm^3/min, 214 nm UV monitor detection) (A = 0.1% aq TFA; B = 0.1% TFA in CH_3CN).

3. Conclusions

When introduced at the N-terminus of a peptide, the *o*-nitrobenzyl scaffold **9** allows efficient ligation with thioester peptides, generating a tertiary *o*-nitrobenzyl amide in full analogy to what already described for its unsubstituted 2-phenylethane analogue. Efficient removal of the auxiliary is possible by mild photolysis in water of the crude ligation product, affording the native peptide in high yields. The use of the proposed auxiliary is compatible with the presence of potentially fragile residues such as tryptophan and methionine, and thus it is general with respect to amino acid side chains.

The described methodology meets all the requirements for extended amide ligation at glycine junctions. Considering the natural abundance of this residue in natural products,[34] and the simplicity and efficiency of the method, we believe it can and will find many applications in the preparation of proteins and amide bearing compounds. It is our intent to apply this new methodology for the synthesis of amide bioconjugates and acid-labile fragile compounds of interest.

Moreover, the synthetic approach can be considered an elegant way of synthesizing protected amide bonds that can be released by photochemical control, and thus it can find use in phototriggered protein folding and activity applications.[35,36]

4. Experimental

4.1. Synthesis of the auxiliaries. General methods

All reagents and solvents were purchased at the highest commercial purity grade and used without further purification. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials. Reactions were monitored to completion by thin layer chromatography (TLC) on E. Merck silica gel plates (60F254) using UV light as a visualizing agent and 3% ethanolic ninhydrin or 2% methanolic *p*-hydroxybenzaldehyde solution and heat as developing agent. E. Merck silica gel (60, particle size 0.040–0.063 mm) was used for flash column chromatography.

NMR spectra were recorded on a Varian Mercury 400 or on a Varian Mercury 200 instrument and calibrated using residual undeuterated solvents as an internal reference. The following abbreviations are used to describe multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, bs = broad singlet.

4.2. Synthetic procedures. Synthesis of auxiliary 9. 4-(*o*-nitrophenyl)-1,2,3-dioxathiolane-2-oxide (2)

Thionyl chloride (1.2 mL, 43.68 mmol) was added dropwise over 10 min to an ice-cold, stirred solution of diol **1** (2 g, 10.93 mmol) and triethylamine (6.2 mL, 16.4 mmol) in dichloromethane (25 mL) and stirred for 30 min. The solution was diluted with cold diethyl ether (200 mL) and water (200 mL), separated, washed with water (2×200 mL), dried (Na_2SO_4) and concentrated in vacuo. Flash column chromatography of the residue (silica gel, 20% ethyl acetate in petroleum ether) afforded **2** as a mixture of diastereoisomers (2.45 g, 98.2%).[37] Compound $\mathbf{2_A}$: $R_f = 0.52$ (silica gel, 20% ethyl acetate in petroleum ether);^{1}H NMR (400 MHz, $CDCl_3$): δ 7.5–8.2 (m, 4H, H_{ar}), 6.5 (dd, $J_1 = 7.6$, $J_2 = 5.3$ Hz, 1H, Ar*CH*), 5.35 (dd, $J_1 = 9.1$, $J_2 = 7.6$, 1H, CH_2), 4.38 (dd, $J_1 = 9.1$, $J_2 = 5.3$, 1H, CH_2). Compound $\mathbf{2_B}$: $R_f = 0.40$ (silica gel, 20% ethyl acetate in petroleum ether); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.5–8.1 (m, 4H, H_{ar}), 6.0 (dd, $J_1 = 7.6$, $J_2 = 6.8$ Hz, 1H, Ar*CH*), 5.25 (dd, $J_1 = 7.6$, $J_2 = 6.8$, 1H, CH_2), 4.38 (t, $J = 7.6$, 1H, CH_2).

4.3. 2-Azido-2-(*o*-nitrophenyl) ethanol (3)

Sodium azide (1.39 g, 21.4 mmol) was added to a stirred solution of the cyclic sulfite **2** (2.45 g, 10.7 mmol) in DMF (25 mL) and the resulting mixture was heated at 60 °C for 2 h. The solvent was evaporated and the crude product was dissolved in ethyl acetate. The organic layer was washed with brine (3×200 mL), dried (Na_2SO_4) and concentrated in vacuo. Flash column chromatography of the residue (silica gel, 20% ethyl acetate in petroleum ether) afforded **3** (2.05 g, 92.5%).[38] Compound **3**: $R_f = 0.20$ (silica gel, 20% ethyl acetate in petroleum ether); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42–7.95 (m, 4H, H_{ar}), 5.34 (dd, $J_1 = 7.3$, $J_2 = 3.6$ Hz, 1H, Ar*CH*), 3.92 (dd, $J_1 = 11.8$, $J_2 = 3.6$ Hz, 1H, CH_2), 3.68 (dd, $J_1 = 11.8$, $J_2 = 7.3$ Hz, 1H, CH_2).

4.4. 2-Amino-2-(*o*-nitrophenyl) ethanol (4)

A solution of azido alcohol **3** (1.96 g, 9.45 mmol) and triphenylphosphine (2.7 g, 10.4 mmol) in THF (25 mL) and water (8 mL) was heated at 70 °C for 1 h and then

concentrated in vacuo. Flash column chromatography of the residue (silica gel, 20% methanol in ethyl acetate) afforded **4** (1.56 g, 91%). Compound **4**: $R_f = 0.22$ (silica gel, 20% methanol in ethyl acetate); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40–7.86 (m, 4H, H_{ar}), 4.65 (dd, $J_1 = 7.3$, $J_2 = 4.4$ Hz, 1H, Ar*CH*), 3.92 (dd, $J_1 = 11.1$, $J_2 = 4.4$ Hz, 1H, CH_2), 3.68 (dd, $J_1 = 11.1$, $J_2 = 7.3$ Hz, 1H, CH_2), 2.1 (bs, 3H, NH_2, *OH*).

4.5. (*N-tert*-Butyloxycarbonyl)-2-amino-2-(*o*-nitrophenyl) ethanol (5)

Aminoalcohol **4** (1 g, 5.49 mmol) was dissolved in methanol (20 mL) and di-*tert*-butylcarbonate (1.32 g, 6.04 mmol) and triethylamine (1.54 mL, 10.98 mmol) were added at 0 °C. The mixture was stirred for 2 h. The solvent was evaporated and the residue was dissolved in chloroform (150 mL). The organic layer was washed with HCl (5% water solution, 2×200 mL), brine (2×200 mL), dried (Na_2SO_4) and evaporated. Flash column chromatography of the residue (silica gel, 50% ethyl acetate in petroleum ether) afforded **5** (1.71 g, 99.6%). Compound **5**: $R_f = 0.5$ (silica gel, 50% ethyl acetate in petroleum ether); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40–7.90 (m, 4H, H_{ar}), 3.65 (m, 1H, Ar*CH*), 2.05–2.30 (m, 2H, CH_2OH), 1.40 (s, 9H, *t*-Bu), 3.30 (s, 1H, O*H*).

4.6. (*N-tert*-Butyloxycarbonyl)-2-amino-2-(*o*-nitrophenyl) ethyl p-toluenesulfonate (6)

To a stirred solution of **5** (1.54 g, 5.47 mmol) in pyridine (7 mL), tosylchloride (1.3 g, 6.5 mmol) was added at 0 °C and the mixture was stirred for 8 h at room temperature. Dichloromethane (200 mL) was added and the resulting mixture was washed with HCl (5% aqueous solution, 4×200 mL), brine (2×200 mL) and evaporated. Crystallization of the residue (10% ethyl acetate in petroleum ether) afforded **6** (2.4 g, 98%). Compound **6**: $R_f = 0.3$ (silica gel, 20% ethyl acetate in toluene); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.4–8.0 (m, 8H, H_{ar}), 5.6 (m, 1H, *NH*), 5.45 (bs, 1H, Ar*CH*NH), 4.3–4.5 (m, 2H, CH_2OTs), 2.45 (s, 3H, CH_3), 1.4 (s, 9H, *t*-Bu).

4.7. (*N-tert*-Butyloxycarbonyl)-2-amino-2-(*o*-nitrophenyl) (*S*-acetyl) ethanethiol (7)

A solution of caesium thiolacetate was prepared by adding thiolacetic acid (1 mL, 14 mmol) to a suspension of caesium carbonate (2.28 g, 7 mmol) in DMF (8 mL). This solution was added dropwise to a solution of **6** (1.54 g, 3.54 mmol) in DMF (5 mL). After stirring for 24 h, the mixture was diluted with chloroform (200 mL) and the organic layer was washed with brine (3×200 mL), dried and evaporated. Flash column chromatography of the residue (silica gel, 10% ethyl acetate in toluene) afforded **7** (1.2 g, 98%). Compound **7**: $R_f = 0.25$ (silica gel, 10% ethyl acetate in toluene); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35–7.95 (m, 4H, H_{ar}), 5.55 (bs, 1H, Ar*CH*NH), 5.25 (bs, 1H, *NH*), 3.20–3.40 (m, 2H, CH_2SAc), 2.33 (s, 3H, CH_3), 1.30 (s, 9H, *t*-Bu).

4.8. (*N-tert*-Butyloxycarbonyl)-2-amino-2-(*o*-nitrophenyl) ethanethiol (8)

A solution of hydroxylamine (1 g, 14.4 mmol) in 50% aqueous methanol (15 mL), pH 6.5, was added to a solution of derivative **7** and the resulting mixture was stirred for 2 h. Water (200 mL) was added and the mixture was extracted with dichloromethane (2×200 mL). The combined organic extracts were washed with brine (400 mL), dried (Na_2SO_4) and evaporated. The crude product was used without further purification. Compound **8**: $R_f = 0.38$ (silica gel, 10% ethyl acetate in toluene).

4.9. 2-Amino-2-(*o*-nitrophenyl) (*S*-trityl) ethanethiol (9)

N-Boc protected derivative **8** (313 mg, 1.05 mmol) was dissolved in neat TFA (1 mL) and tritylalcohol (290 mg, 1.1 mmol) was added. After stirring for 30 min, the solvent was evaporated and the residue was dissolved in chloroform (200 mL). Aqueous NaOH (0.2 M, 200 mL) was added and the mixture was extracted with chloroform (4×200 mL). The combined organic extracts were washed with brine (2×200 mL), dried (Na_2SO_4) and concentrated in vacuo. Flash column chromatography of the residue (silica gel, 0.1% methanol in chloroform) afforded **9** (425 mg, 95%). Compound **9**: $R_f = 0.5$ (silica gel, 0.25% methanol in chloroform); (**9**) ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.20–7.80 (m, 19H, H_{ar}), 4.25 (dd, $J_1 = 5.1$, $J_2 = 7.7$ Hz, 1H, Ar*CH*NH_2), 2.75 (dd, $J_1 = 11.3$, $J_2 = 5.1$ Hz, 1H, CH_2STrt), 2.55 (dd, $J_1 = 11.3$, $J_2 = 7.7$ Hz, 1H, CH_2STrt), 1.70 (bs, 2H, NH_2).

4.10. Synthesis of auxiliary 15. 2-Nitro-4,5-dimethoxystyrene (11)

To a suspension of triphenylphosphonium bromide (22.1 g, 61.6 mmol) in THF (100 mL), a solution of sodium hexamethyldisilazide (31 mL, 2 M in THF, 62 mmol) was added dropwise over 30 min at 0 °C and stirred for 1 h. A solution of *o*-nitroveratraldehyde (10 g, 47.4 mmol) in THF (120 mL) was then added dropwise and the mixture was stirred at room temperature for an additional 12 h. The solvent was evaporated and the residue was dissolved in chloroform (300 mL). NH_4Cl (saturated aqueous solution, 300 mL) was added and the layers were separated. The organic layer was washed with NH_4Cl (saturated aqueous solution, 2×300 mL), brine (2×300 mL) dried (Na_2SO_4) and concentrated in vacuo. Flash column chromatography of the residue (silica gel, 10% ethyl acetate in petroleum ether) afforded **11** (6.2 g, 65%). Compound **11**: $R_f = 0.42$ (silica gel, 30% ethyl acetate in petroleum ether); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.58 (s, 1H, H_{ar}), 7.30 (dd, $J = 16.0$, 8.9 Hz, 1H, H_2), 6.97 (s, 1H, H_{ar}), 5.63 (d, $J = 15.7$ Hz, 1H,

H_{1b}), 5.42 (d, $J = 8.9$ Hz, 1H, H_{1a}), 3.99 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3).

4.11. (*N-tert*-Butyloxycarbonyl)-2-amino-2-(2′-nitro-4′,5′-dimethoxyphenyl) ethanol (12)

To a stirred solution of *tert*-butylcarbamate (843 mg, 7.2 mmol) in *n*-propanol (10 mL), sodium hydroxide (9.6 mL, 0.5 M in water, 4.8 mL) and *tert*-butyl hypochlorite (0.5 mL)[39] were sequentially added and the resulting mixture was stirred for 5 min. The flask was put in a ice slurry and a $(DHQ)_2$ PHAL (112 mg, 0.14 mmol, in 10 mL of *n*-propanol), styrene **11** (500 mg, 2.4 mmol, in 40 mL of *n*-propanol) and $K_2OsO_2(OH)_4$ (35 mg, 0.1 mmol) were sequentially added. The resulting mixture was stirred for 45 h at 4 °C and quenched with addition of Na_2S (saturated aqueous solution, 50 mL). The layers were separated, the aqueous layer was extracted with ethyl acetate (2 × 100 mL), the combined organic extracts were washed with brine (2 × 500 mL), dried (Na_2SO_4) and evaporated. Flash column chromatography of the residue (silica gel, 30% ethyl acetate in petroleum ether) afforded **12** (434 mg, 1.3 mmol, 53%) as well as the unreacted styrene **11** (190 mg, 38%). Compound **12**: $R_f = 0.45$ (silica gel, 50% ethyl acetate in petroleum ether); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.62 (s, 1H, H_{ar}), 7.40 (s, 1H, H_{ar}), 5.48 (t, $J = 4.0$ Hz, 1H, *CH*NH), 3.99 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 3.51 (m, 2H, CH_2OH), 1.45 (s, 9H, *t*-Bu).

4.12. (*N-tert*-Butyloxycarbonyl)-2-amino-2-(2′-nitro-4′, 5′-dimethoxyphenyl) (*S*-acetyl) ethane thiol (13)

To an ice-cold, stirred solution of triphenylphosphine (680 mg, 2.6 mmol) in THF (20 mL), di-*iso*-propylazadicarboxylate (0.56 mL, 2.6 mmol) was added. The mixture was stirred for 30 min, during which time a white precipitate was formed. A solution of alcohol **12** (410 mg, 1.2 mmol) in THF (25 mL) and thiolacetic acid (0.19 mL, 2.6 mmol) were sequentially added dropwise and the mixture stirred for an additional 10 h at room temperature. The solvent was evaporated and the residue was dissolved in toluene (150 mL) and left standing at 0 °C for 10 h. The precipitate was filtered and the solvent evaporated. Flash column chromatography of the residue (silica gel, 40% ethyl acetate in petroleum ether) afforded **13** (490 mg, 98%). Compound **13**: $R_f = 0.43$ (50% ethyl acetate in petroleum ether); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.52 (s, 1H, H_{ar}), 7.25 (s, 1H, H_{ar}), 5.42 (t, $J = 4$ Hz, 1H, *CH*NH), 3.98 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 3.70 (m, 2H, CH_2S), 2.31 (s, 3H, $SCOCH_3$), 1.38 (s, 9H, *t*-Bu).

4.13. (*N-tert*-Butyloxycarbonyl)-2-amino-2-(2′-nitro-4′,5′-dimethoxyphenyl) (*S-p*-methylbenzyl) ethanethiol (14)

To a stirred solution of derivative **13** in absolute ethanol (100 mL), sodium methoxide (1.32 mL, 1 M in methanol, 1.3 mmol) was added and the resulting mixture was stirred for 5 min. *p*-Methylbenzylbromide (245 mg, 1.3 mmol) was added and the mixture was stirred for an additional 20 min. The pH was adjusted to 4 by adding Dowex H^+. The resin was filtered and the solvent evaporated. Flash column chromatography of the residue (silica gel, 5% ethyl acetate in petroleum ether) afforded **14** (500 mg, 97%). Compound **14**: $R_f = 0.8$ (40% ethyl acetate in petroleum ether); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.0–7.42 (m, 6H, H_{ar}), 4.68 (t, $J = 5$ Hz, 1H, *CH*NH), 3.98 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 3.58 (d, $J = 5$ Hz, 2H, $CHCH_2S$), 2.36 (s, 2H), 1.60 (s, 3H, p-CH_3Bzl), 1.38 (s, 9H, *t*-Bu).

4.14. 2-Amino-2-(2′-nitro-4′,5′-dimethoxyphenyl) (*S-p*-methylbenzyl) ethanethiol (15)

Boc-amine **14** was dissolved in neat TFA and the mixture was stirred for 5 min. The solvent was evaporated in vacuo and the crude product was used without further purification for reaction with the bromoacetyl peptide.

4.15. Peptide synthesis. General methods

Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry was performed on an Voyager-RP Biospectrometry workstation (Perseptive Biosystem, Framingham, MA). HPLC: Analytical reversed-phase HPLC was performed on a Hewlett Packard HPLC 1050 system using a Vydac C-18 column (5 μm, 0.46 × 15 cm) or on a Waters Symmetry 300 system using a C18 column (5 μm, 0.46 × 25 cm). Semipreparative reversed-phase HPLC was performed on a Rainin HPLC system using a Vydac C-18 column (10 μm, 1.0 × 25 cm). Linear gradients of acetonitrile in water/0.1% TFA were used to elute bound peptides. The flow rates used were 1 mL/min (analytical) and 5 mL/min (semipreparative).

4.16. Reagents

N^{α}-Boc-amino acids for peptide synthesis were from Midwest Biotech (Fishers, IN), N^{α}-Fmoc-amino acids and *o*-(benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium tetrafluoroborate (TBTU) from Novabiochem (Laufingen, CH), 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluoro phosphate (HBTU) from Quantum Appligene (Carlsbad, CA), di-*iso*-propylethylamine from Applied Biosystem (Foster City, CA) or from Biosolve (Valkenswaard, The Netherlands), TFA from Halocarbon (River Edge, NJ) or from Biosolve, DMF from EM Science (Gibbstown, NJ), piperidine from Biosolve (Valkenswaard, The Netherlands).

4.17. Solid phase peptide synthesis

Peptides were prepared by manual solid phase peptide synthesis (SPPS) using the in situ neutralization/HBTU activation for Boc-chemistry or standard TBTU activation for Fmoc-chemistry as previously described[40,41]; couplings were carried out with a 5-fold excess of

activated amino acid for a minimum of 15 min and monitored by quantitative ninhydrin test.[42]

C-Terminal β-mercaptopropionic acid–leucine (MPAL) thioester peptides were synthesized according to a published procedure on a *t*-Boc-Leu-OCH_2-Pam preloaded resin (Applied Biosystems).[43] Carboxy amidated peptides were synthesized on a MBHA resin (Peninsula Laboratories Inc., San Carlos, CA) for Boc-chemistry or on a Rinkamide MHBA resin (Novabiochem) for Fmoc-chemistry. After the deprotection of the amino group of the penultimate residue, α-bromoacetic acid was coupled as the symmetric anhydride as previously described.[30]

4.18. Coupling of the auxiliary to the peptide resin

In a typical experiment, amine **9** or **15** (0.5 mmol) was dissolved in a mixture of DMF (1 mL) and DIEA (0.5 mL, 2.9 mmol) and the resulting mixture added to the bromoacetyl peptide resin (0.05 mmol). The suspension was left standing for 12 h. The peptide resin was washed with DMF, CH_2Cl_2, and dried under vacuum.[44]

After chain assembly, cleavage from the resin and side-chain deprotection was achieved as follows: *Boc-chemistry*: the peptide-resin was treated with HF (1 h, 0 °C) in the presence of 5% (v/v) *p*-cresol. After evaporation of HF, the crude peptide was precipitated in anhydrous Et_2O, dissolved in HPLC buffers and lyophilized. *Fmoc-chemistry*: the peptide-resin was treated with TFA, in the presence of phenol, water (4%), thioanisole (4%), ethandithiol (1%), tri-*iso*-propylsilane (3%) for 2 h at room temperature. The peptide was precipitated in a mixture of *tert*-butylmethylether and petroleum ether 1:1 at 0 °C, centrifuged, dissolved in HPLC buffers and lyophilized. Pure peptides were isolated by semipreparative HPLC purification. Typical yields for recovered peptides after HPLC purification and lyophylization were $\geqslant$60%.

MS values for peptides derivatized with auxiliaries: **15**-*H*-GLLRPFFRK-NH_2 1374.0 (calcd 1374.7); **9**-*H*-GLLRPFYRK-NH_2: 1332.0, 1354.2, 1370.8 (calcd 1330.6); **9**-*H*-GLLRPFHRK-NH_2: 1305.8, 1327.1 (calcd 1304.6); **9**-*H*-GLLRPFWRK-NH_2: 1354.2 (calcd 1353.7); **9**-*H*-GLLRPMYRK-NH_2: 1315.0 (calcd 1314.6). MS values for thioester peptides: *H*-KLAPYRAG-*MPAL*: 1076.1 (calcd 1076.1); *H*-LWAPYRAG-*MPAL*: 1133.9 (calcd 1134.2); *H*-LWAPYRAA-*MPAL*: 1148.7 (calcd 1148.2).

4.19. Ligation reactions

Peptides were dissolved in sodium phosphate buffer, 200 mM, pH 8.5 in the presence of 35 mM tris(2-carboxyethyl)phosphine HCl, to a final peptide concentration of 5–8 mM. The final pH of the solution after addition of peptides was 7.5. Reaction was monitored to completion by analytical HPLC. Typical chromatographic yields for ligations were >90%. At the end of the reaction, the mixture was diluted with HPLC buffer and the product was isolated by semipreparative HPLC.

4.20. Photolytic removal of the *o*-nitrobenzyl auxiliary

Peptides were dissolved in a mixture of acetonitrile/water 1:4 or in a mixture of HPLC buffers (20% B in A) to a final peptide concentration of about 0.3 mg/mL and the solution was put in a quartz tube and degassed under argon bubbling for 15 min. The tube was put in the Ryonet 310 nm photoreactor and the reaction was monitored to completion by analytical HPLC. At the end of the reaction the solution was diluted with HPLC buffer A and the product was purified by semipreparative HPLC.

H-LWAPYRAGGLLRPFHRK-NH_2: 2040.0 (calcd 2037.5); *H*-KLAPYRAG GLLRPFHRK-NH_2: 1979.0 (calcd 1979.4); *H*-KLAPYRAG GLLRPFYRK-NH_2: 2005.1 (calcd 2005.4); *H*-KLAPYRAGGLLRPMYRK-NH_2: 1990.3 (calcd 1989.2).

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