Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron
Tetrahedron 63 (2007) 11952–11958

Ethoxyethylidene protecting group prevents N-overacylation in aminooxy peptide synthesis

Vincent Duléry, Olivier Renaudet* and Pascal Dumy*

Département de Chimie Moléculaire, UMR-CNRS 5250 ICMG FR 2607, Université Joseph Fourier, 38041 Grenoble Cedex 9, France

Received 24 July 2007; revised 29 August 2007; accepted 7 September 2007
Available online 12 September 2007

Abstract—We report herein an improved synthetic route for the preparation of homogenous aminooxy peptides suitable for oxime ligation. Aminooxyacetic acid (Aoa) was protected with 1-ethoxyethylidene group (Eei) then incorporated either using PyBOP or as *N*-hydroxysuccinimidyl ester at N-terminal end or at a lysine side chain into model peptides in solution and on solid support. Due to the Eei protecting group, these new reagents prevent the N-overacylation side reaction in comparison with Boc–Aoa derivatives. Subsequent deprotection under mild acidic conditions gave the corresponding pure aminooxylated peptides.

1. Introduction

The synthesis of relevant functional macromolecules remains a crucial challenge for the investigation of biological processes. Among the large number of chemical ligation methods developed for this purpose,[1–5] chemoselective oxime bond formation represents one of the most efficient synthetic strategies. First developed by Rose for the preparation of homogenous artificial proteins,[6] the oxime-based strategy has found increasing applications in the last few years, going from the conjugation of peptides,[7–9] carbohydrates,[10–12] and oligonucleotides,[13,14] to the preparation of combinatorial libraries,[15] the on-chips immobilization of biomolecules,[16–19] or the chemical engineering.[20,21]

The oxime ligation requires the convergent preparation of each counterpart bearing either an aldehyde or a keto group and a super nucleophile aminooxy moiety, which react mutually with high chemoselectivity under mild acidic conditions to form a stable oxime linkage.[22] Whereas the methods to generate aldehydes in solid phase peptide synthesis (SPPS) are well documented,[23] and the incorporation of aminooxy at N-terminal end or at a lysine side chain of peptide remains tricky. Indeed, in most cases aminooxyacetic acid (Aoa) building block bearing a single carbamate protecting group (e.g., Boc or Aloc) is introduced as *N*-hydroxysuccinimidyl ester (OSu) or using in situ activation. However, the mono-protection of Aoa does not ensure the complete protection of the nitrogen, therefore providing *N*-overacylated Aoa–Aoa-peptide as an undesirable side product (Fig. 1, route A).[24] Several methods have been reported to overcome this side reaction. For example, it was shown recently that N-overacylation can be minimized either by controlling the pH of the coupling reaction mixture containing Aloc–Aoa–OH and uronium coupling reagents or by using DCC, which does not require basic conditions for activation.[25,26] However, incomplete reaction or traces of bis-acylated side product can still be observed even when a large excess of reagents, repeated coupling reactions, or different reagents are used. Since mono-protection of Aoa is not satisfactory to prevent N-overacylation, an alternative strategy would consist in using protecting groups, which mask completely the nucleophilicity of the nitrogen atom such as phthaloyl protected Aoa.[27] The bis-protected derivative Boc_2–Aoa–OH[24] constitutes also an attractive building block, but its instability under Fmoc deprotection conditions precludes, however, the use of this reagent in SPPS, except at the end of the peptide elongation.[28]

As part of our program related to the construction of synthetic multiepitopic vaccine candidates, which requires a highly clean chemistry to ensure the formation of well-defined molecules,[29] we focused recently our investigations in using a new reagent providing aminooxy peptides without trace of *N*-overacylated side products. We presumed that an Aoa derivative protected with 1-ethoxyethylidene group (Eei), which ensures the full protection of aminooxy function might assume this purpose (Fig. 1, route B). In this study, we thus investigated the incorporation of Aoa in different peptides by varying conditions and reagents both in solution and on solid support (Fig. 1). We prepared Aoa derivatives protected with a Boc or an Eei then we compared the outcome of acylation of different model peptides. The Aoa building block was either incorporated as active ester or by using in situ

* Corresponding authors. Tel.: +33 04 76 51 48 63; fax: +33 04 76 51 49 46; e-mail: pascal.dumy@ujf-grenoble.fr

0040–4020/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2007.09.015

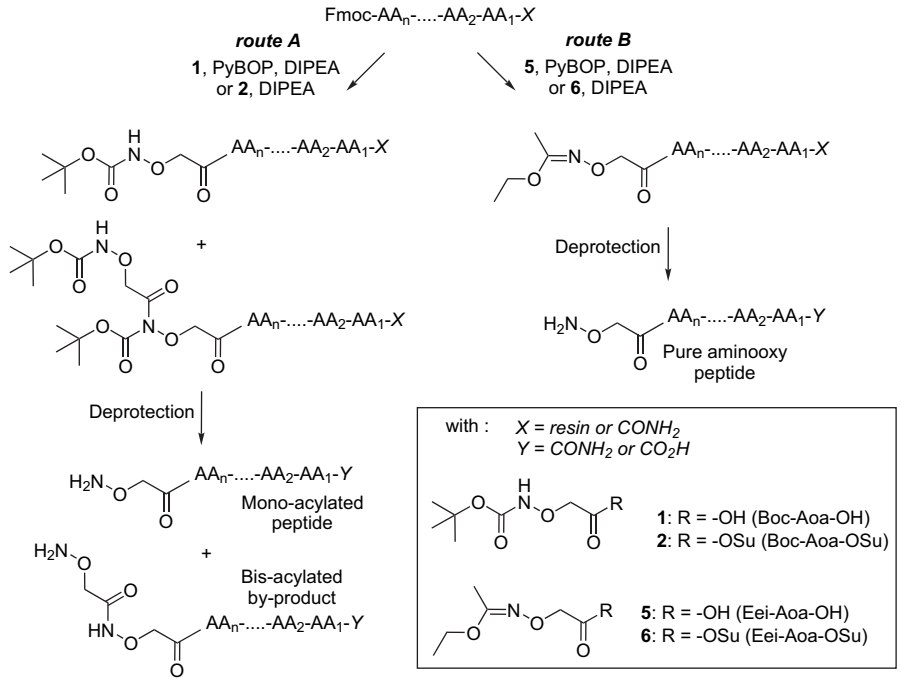


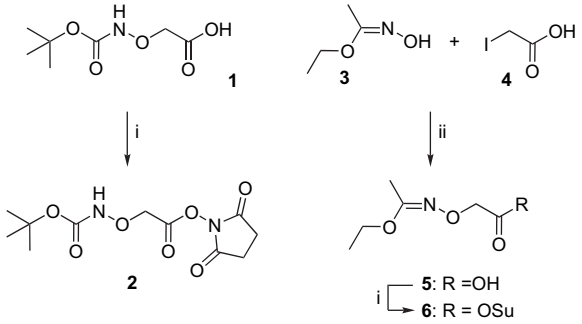
Figure 1. General strategy for the functionalization of peptides with aminooxy moiety in solution and on solid support using Boc protected Aoa (route A) or ethoxyethylidene (Eei) derivatives (route B).

activation to provide pendant and N-terminal aminooxy moiety on linear, bridged, and branched peptides.

2. Results and discussion

2.1. Synthesis of Aoa derivatives

The commercially available aminooxyacetic acid **1** protected with a Boc was activated using *N*-hydroxysuccinimide and dicyclocarbodiimide (DCC) in a mixture of ethyl acetate and dioxane. After removal of DCU by filtration and precipitation, the active ester Boc–Aoa–OSu **2**[30] was obtained in 83% yield and was used without further purification for the coupling reaction with peptides (Scheme 1).



Scheme 1. Reagents and conditions: (i) DCC, NHS, AcOEt/dioxane, 83% for **2**, 68% for **6**; (ii) NaOH 40%, 80 °C, 69%.

The synthesis of Aoa derivatives protected with 1-ethoxyethylidene starts with the condensation between ethyl *N*-hydroxyacetimidate **3** and iodoacetic acid **4** in sodium hydroxide, which provides *N*-(1-ethoxyethylidene)2-aminooxyacetic acid **5** in good yield.[31] The subsequent activation was performed using the procedure described previously for **2**. The pure imidate Eei–Aoa–OSu **6** was obtained after flash chromatography and recrystallization as single crystals, which were analyzed by X-ray diffraction (Fig. 2).[32] Both reagents **5** and **6** were easily prepared in gram scale and were proved completely stable at room temperature and under storage.

2.2. Stability of Eei protecting group

We were next interested in studying the stability of the Eei protecting group in various conditions used in the Fmoc/^{t}Bu strategy (Table 1).

No deprotection was detected by RP-HPLC under the standard basic conditions used for coupling reaction and Fmoc removal, therefore confirming the compatibility of Eei–Aoa–OH or Eei–Aoa–OSu derivatives with peptide elongation. Interestingly, Eei was stable by treatment with mild acidic solution such as 1% of TFA in CH_2Cl_2 or 50% aqueous solution of acetic acid, whereas the complete removal of Eei was observed in 3 or 1 h with aqueous solution of 1% or

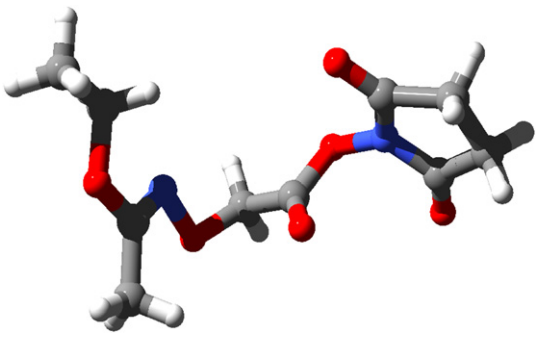


Figure 2. X-ray structure of Eei–Aoa–OSu **6**.

Table 1. Stability of Eei protecting group under standard conditions of SPPS

Conditions	Deprotection[b]
DIPEA/DMF (pH 8)[a]	No deprotection after 45 min
Piperidine/DMF (1:4)[a]	No deprotection after 30 min
TFA/CH_2Cl_2 (1:99)[a]	No deprotection after 1 h
AcOH/H_2O (1:1)	No deprotection after 3 h
TFA/CH_3CN/H_2O (1:49.5:49.5)	Complete deprotection after 3 h
TFA/CH_3CN/H_2O (5:47.5:47.5)	Complete deprotection after 1 h
TFA/TIS/H_2O (95:2.5:2.5)	Complete deprotection after 1 h
AcOH/TFE/CH_2Cl_2 (1:1:8)[a]	No deprotection after 2 h

[a] Dry solvents were used.
[b] The stability of Eei was followed by analytical RP-HPLC of reaction mixture.

5% of TFA in acetonitrile, respectively. This result denotes a useful orthogonality between Eei and acid labile protecting groups such as Boc or ^{t}Bu. Moreover, the standard cleavage cocktail containing TFA/TIS/H_2O ensured the complete deprotection of peptide, in contrast with cocktail containing AcOH/TFE/CH_2Cl_2, which preserved the Eei and other acid labile protecting groups of the peptide.

2.3. Functionalization using active ester derivatives

We first studied the acylation of different peptides with these protected Aoa–OSu reagents. For this purpose, linear, bridged, and branched peptides were chosen as model peptides to incorporate aminooxy function either at the N-terminal end of peptides **A** and **B** or at the side chain of Lys residue of peptide **C** after removal of Aloc protecting group. All the peptides were synthesized manually on solid phase using the standard Fmoc/^{t}Bu strategy. The peptide **B** was obtained after the formation of a disulfide bridge between two Cys(*S*-Trt) by an iodine-mediated oxidation.[33]

The coupling reactions of Aoa were performed at room temperature both in solid phase and in solution using 1 or 2 equiv of Aoa derivative and DIPEA (pH adjusted at 8) in

Table 2. Acylation of peptides **A**, **B**, and **C** on solid support and of peptides **A′**, **B′**, and **C′** in solution using protected Aoa–OSu **2** or **6**

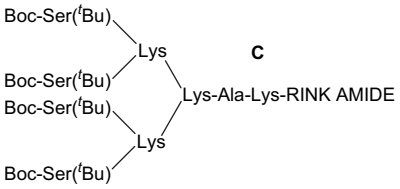
Linear peptides

A H-Gly-Leu-Ser(^{t}Bu)-Asp(^{t}Bu)-Val-Gly-SASRIN

A' H-Gly-Leu-Ser(^{t}Bu)-Asp(^{t}Bu)-Val-Gly-OH

A" H_2NOCH_2CO-Gly-Leu-Ser-Asp-Val-Gly-OH

Branched peptides

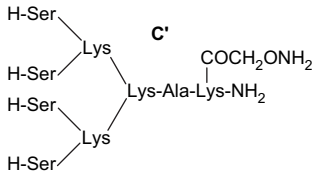


Bridged peptides

B H-Gly-Gly-Cys-Asp(^{t}Bu)-Gly-Asp(^{t}Bu)-Gly-Cys-SIEBER AMIDE

B' H-Gly-Gly-Cys-Asp(^{t}Bu)-Gly-Asp(^{t}Bu)-Gly-Cys-NH_2

B" H_2NOCH_2CO-Gly-Gly-Cys-Asp-Gly-Asp-Gly-Cys-NH_2



Peptide	Conditions[a]	H_2N-peptide		Mono-acylated peptide		Bis-acylated peptide	
		%[b]	MS found[c]	%[b]	MS found[c]	%[b]	MS found[c]
A	1 equiv of **2**	20	547.11 (547.27)	80	620.11 (620.29)	0	—
A	2 equiv of **2**	0	—	91	620.08	9	693.10 (693.31)
A	2 equiv of **6**	0	—	100	620.08	0	—
B	1 equiv of **2**	50	792.28 (792.30)	50	965.09 (965.35)	0	—
B	2 equiv of **2**	41	792.28	55	965.13	4	1138.22 (1138.44)
	+2 equiv of **2**	0	—	88	965.03	12	1138.34
B	2 equiv of **6**	40	792.23	60	935.08 (935.34)	0	—
	+2 equiv of **6**	0	—	100	934.91	0	—
C	1 equiv of **2**	d.[d]	949.11 (949.58)	d.	1022.08 (1022.59)	n.d.	1095.2 (1095.61)
C	2 equiv of **2**	d.	949.02	d.	1022.07	d.	1095.21
	+2 equiv of **2**	n.d.[d]	—	d.	1022.17	d.	1095.31
C	2 equiv of **6**	d.	949.07	d.	1022.09	n.d.	—
	+2 equiv of **6**	n.d.	—	d.	1022.14	n.d.	—
A′	1 equiv of **2**	30	547.06	70	620.12	0	—
A′	2 equiv of **2**	0	—	91	620.08	9	693.12
A′	2 equiv of **6**	0	—	100	620.12	0	—
B′	1 equiv of **2**	7	792.34	86	965.36	7	1138.78 (1138.44)
B′	2 equiv of **2**	0	792.28	80	965.89	20	1138.89
B′	2 equiv of **6**	0	—	100	934.75 (935.34)	0	—

[a] Reactions were performed in DMF in the presence of DIPEA (2 equiv, pH 8) for 45 min at room temperature.
[b] Percentage of each compound is determined by analytical RP-HPLC by the integration of each peak after cleavage (peptides **A**, **B**, and **C**) or by analyzing the reaction mixture (peptides **A′** and **B′**).
[c] Mass spectrometry analysis was performed by electrospray ionization method in positive mode (ESI-MS). The mass found is given for either protected (**B**) or fully deprotected peptides (**A** and **C**). Calculated mass is given in brackets.
[d] The high polarity of both mono- and bis-acylated peptide **C** prevents the identification of the corresponding peaks in our HPLC analysis conditions. Thus, no percentage can be calculated from HPLC profile and only a qualitative result obtained by MS analysis is given as detection (d.) or no detection (n.d.).

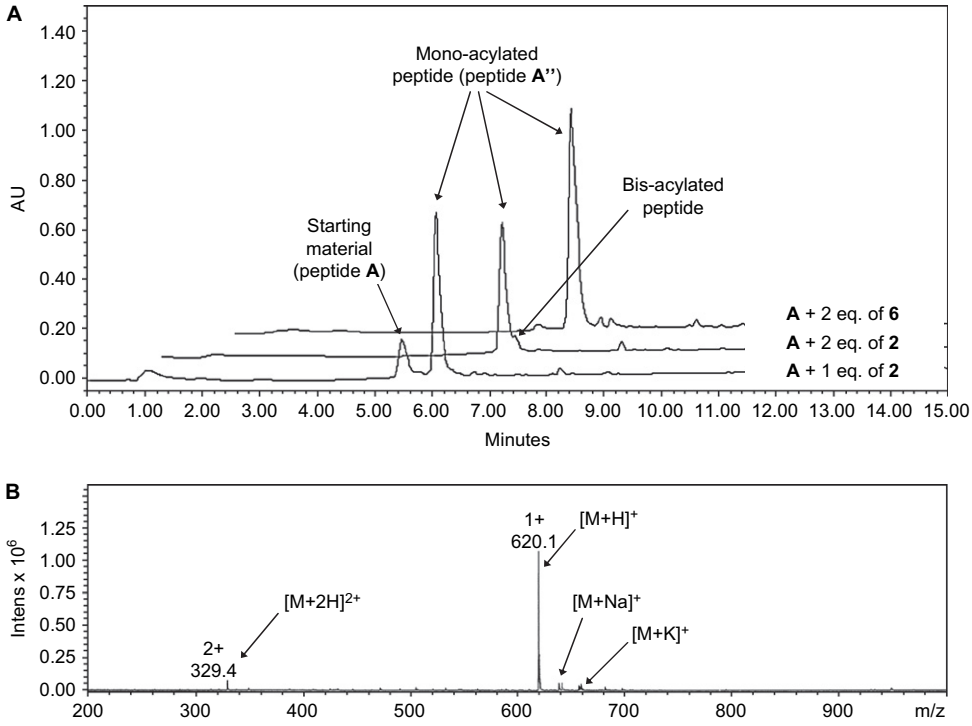


Figure 3. (A) Analytical RP-HPLC chromatogram (C_{18} column, linear gradient 5–60% B in 15 min, detection: λ=214 nm) of crude peptide **A**″ obtained after solid phase functionalization of peptide **A** with Boc–Aoa–OSu **2** or Eei–Aoa–OSu **6** followed by deprotection. (B) Mass spectrum (electrospray analysis, positive mode) of crude peptide **A**″ obtained after solid phase functionalization of peptide **A** with Eei–Aoa–OSu **6** followed by deprotection.

DMF. The outcome of functionalization given in Table 2 was determined by analytical RP-HPLC by integrating the peaks corresponding to the mono- or bis-acylated peptide as well as by electrospray ionization mass spectrometry (ESI-MS) as illustrated in Figure 3.

On solid phase, we first observed that 1 equiv of Boc–Aoa–OSu **2** did not ensure a complete conversion since starting peptide **A**, **B**, or **C** was still observed after 45 min, therefore suggesting that an excess of reagent might be required. RP-HPLC chromatogram analysis of crude reaction mixtures (Fig. 3A) shows that 2 equiv of **2** was necessary to complete the reaction with peptide **A**, however, a significant quantity of overacylated compound (9%) was detected as previously reported.[25,26] Presumably due to the low accessibility of the amino anchoring site, one additional cycle was even necessary to get full reaction with peptides **B** and **C**, affording also the desired mono-acylated peptides **B**′ and **C**′ contaminated with an increased quantity of undesired bis-acylated side products. In contrast, the use of excess of imidate Eei–Aoa–OSu **6** (2 or 4 equiv) ensured the complete conversion of peptides **A**, **B**, and **C** into the corresponding mono-acylated peptide. As expected, this new reagent allows the efficient preparation of pure aminooxy derivatives **A**′, **B**′, and **C**′ after deprotection since no trace of starting material and overacylated side product was detected by RP-HPLC and mass analysis (Fig. 3). The results obtained in solution with peptides **A**′ and **B**′ were very similar. As 2 equiv of Boc–Aoa–OSu **2** was necessary for the total conversion of starting material, 9% and 20% of the corresponding bis-acylated side products were obtained. On the other hand, the coupling reaction with Eei–Aoa–OSu **6** provided the desired aminooxy peptides **A**′ and **B**′ quantitatively.

2.4. Functionalization using PyBOP coupling reagent

We further investigated the convenience of Eei protection for the functionalization of peptide with aminooxy moiety on solid phase using PyBOP coupling reagent. Eei–Aoa–OH **5** was introduced at the N-terminal end of peptide **A** and the outcome of N-overacylation was compared with the mono-protected Boc–Aoa–OH **1** (Table 3).

Table 3. Acylation of peptide **A** (H-Gly-Leu-Ser(^{t}Bu)-Asp(^{t}Bu)-Val-Gly-SASRIN) with protected-Aoa–OH and PyBOP

Conditions[a]	H_2N-peptide		Mono-acylated peptide		Bis-acylated peptide	
	%[b]	MS found[c]	%[b]	MS found[c]	%[b]	MS found[c]
1 equiv of **1**	7	547.10 (547.27)	60	620.08 (620.29)	33	693.07 (693.31)
2 equiv of **1**	0	—	59	620.11	41	693.08
2 equiv of **5**	0	—	100	620.08	0	—

[a] Reactions were performed using 1 or 2 equiv of PyBOP in DMF in the presence of DIPEA (2 equiv, pH 8) for 45 min at room temperature.
[b] See Table 2.
[c] Mass spectrometry analysis was performed by ESI in positive mode. The mass found is given for fully deprotected peptide. Calculated mass is given in brackets.

We observed that 1 equiv of **2** led to a mixture of compounds corresponding to the starting material, the mono-, and bis-acylated peptides with 7%, 60%, and 33% ratio, whereas the use of 2 equiv of **2** dramatically increased the quantity of bis-acylated side product (41%). As expected, the efficiency of the Eei protection group was confirmed by the reaction with excess of Eei–Aoa–OH **5** and PyBOP since the aminooxy functionalized peptide **A′** was obtained cleanly. This interesting result suggests that the imidate **5** could be incorporated manually or automatically during the solid phase peptide elongation.

In summary, we have investigated the use of 1-ethoxyethylidene protected aminooxy acetic acid for the synthesis of aminooxy peptides suitable for oxime ligation. Two stable reagents prepared in one or two steps were incorporated into peptide sequence either using PyBOP coupling reagent or as *N*-hydroxysuccinimidyl ester (Eei–Aoa–OH **5** and Eei–Aoa–OSu **6**) and were easily deprotected under mild aqueous acidic conditions. In contrast with the corresponding Boc protected Aoa, the use of Eei derivatives prevents the formation of *N*-overacylated side product. Indeed, we have demonstrated that these new reagents ensure the efficient preparation of homogenous linear, bridged, or branched peptides bearing N-terminal or pendent oxyamine both in solution and on solid support. These results might find broad interests for bio-organic chemists who are interested in the synthesis of functional macromolecules by chemoselective methods. Particularly, this strategy is currently used in our laboratory for the preparation of multiepitopic anticancer vaccines[29] and molecular conjugate vectors.[34]

3. Experimental

3.1. General

Protected amino acids, Sasrin, Sieber Amide, and Rink Amide MBHA resin were obtained from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Voisins-Les-Bretonneux, France), and France Biochem S.A. (Meudon, France). PyBOP was purchased from France Biochem and other reagents were obtained from either Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France). RP-HPLC was performed on Waters equipment equipped with a 600 controller and a Waters 2487 Dual Absorbance Detector. The purity of peptide derivatives were analyzed on an analytical column (Macherey–Nagel Nucleosil 120 Å 3 μm C_{18} particles, 30×4.6 mm, flow rate of 1.3 mL min^{-1} for 15 min gradient, Nucleosil 100 Å 5 μm C_{18} particles, 250×4.6 mm, flow rate of 1 mL min^{-1} for 30 min gradient) using linear gradient and the following solvent system: solvent A, water containing 0.09% TFA; solvent B, acetonitrile containing 0.09% TFA and 9.91% H_2O. UV absorbance was monitored at 214 and 250 nm simultaneously. Semi-preparative column (Delta-Pak™ 100 Å 15 μm C_{18} particles, 200×2.5 mm) was used to purify crude peptides (when necessary) by using an identical solvent system at a flow rate of 22 mL min^{-1}. The direct chemical ionization (DCI) mass spectra were obtained on a Thermo Filligan PolarisQ with NH_3/isobutane as the reagent gas. The electrospray ionization mass spectrometry (ESI-MS) was recorded on a VG Platform II (Micromass). The analysis was performed in the positive mode for peptide derivatives using 50% aqueous acetonitrile as an eluent.

3.2. Synthesis of Aoa derivatives

3.2.1. *N′*-Boc-aminooxyacetyl *N*-hydroxysuccinimide ester (2). To a stirred solution of *N*-Boc 2-aminooxyacetic acid **1** (0.500 g, 2.6 mmol) in EtOAc/dioxane (1:1, 10 mL) at 0 °C were added *N*-hydroxysuccinimide (0.310 g, 2.7 mmol) and DCC (0.563 g, 2.7 mmol). The mixture was stirred at room temperature for 5 h and then filtered through a pad of Celite. After evaporation of the solvent, the residue was dissolved in EtOAc and washed with aqueous $NaHCO_3$, water, and brine. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The crude solid was recrystallized from CH_2Cl_2/ether/pentane thereby providing pure and white solid (0.618 g, 2.14 mmol, 83%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.66 (s, 1H), 4.78 (s, 2H), 2.87 (s, 4H), 1.49 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.8, 171.1, 168.7, 165.1, 70.9, 28.1, 25.6, 25.4; DCI-MS calcd for $C_{11}H_{20}N_3O_7$: 306.1; found: *m*/*z* 305.8 $[M+NH_4]^+$.

3.2.2. *N*-(1-Ethoxyethylidene)2-aminooxyacetic acid (5). To a stirred solution of iodoacetic acid **4** (5.00 g, 26.9 mmol) in water (10 mL) was added an aqueous solution of NaOH (1.6 mL, 40%) at 0 °C. After reaching room temperature, ethyl *N*-hydroxyacetimidate **3** (4.16 g, 40.3 mmol) was added followed by an aqueous solution of NaOH (2.5 mL, 40%) and water (10 mL) to get a basic pH (>12). The mixture was stirred for 5 h at 80 °C and cooled at room temperature. Water was then added (50 mL) and the aqueous solution was extracted successively with Et_2O and CH_2Cl_2. The aqueous phase was brought to pH 2–3 with concentrated hydrochloric acid. The acidified aqueous phase was then extracted with AcOEt and these combined organic layers were washed with brine (50 mL), dried over Na_2SO_4, and concentrated in vacuum. The compound **5** was obtained as a colorless oil (2.99 g, 18.6 mmol, 69%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.48 (s, 2H), 4.00 (q, 2H, *J*=7.2 Hz), 2.01 (s, 3H), 1.27 (t, 3H, *J*=7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.5, 70.2, 62.8, 14.2, 14.0; ESI-MS calcd for $C_6H_{12}NO_4$: 162.2; found: *m*/*z* 162.0 $[M+H]^+$.

3.2.3. *N′*-(1-Ethoxyethylidene)2-aminooxyacetyl *N*-hydroxysuccinimide ester (6). The activated ester **6** was obtained following the procedure described for **2**. After filtration of DCU and evaporation, the residue was purified by a flash chromatography on silica gel with AcOEt/hexane (1:1) and recrystallized in CH_2Cl_2/pentane to get **6** as single crystals (3.3 g, 12.7 mmol, 68%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.78 (s, 2H), 4.01 (q, 2H, *J*=7.2 Hz), 2.84 (s, 4H), 1.98 (s, 3H), 1.28 (t, 3H, *J*=7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.8, 165.7, 164.8, 68.5, 62.8, 25.6, 14.2, 13.9; DCI-MS (positive mode) calcd for $C_{10}H_{14}N_2O_6$: 259.1; found: *m*/*z* 259.2 $[M+H]^+$.

3.3. Peptides synthesis

3.3.1. Standard procedures for solid phase synthesis of peptides. The synthesis of all protected peptides was carried out manually using Fmoc/^{t}Bu strategy in a glass reaction vessel fitted with a sintered glass frit. The coupling reactions were performed using, relative to the resin loading,

2 equiv of *N*-α-Fmoc protecting amino acids activated in situ with 2 equiv of PyBOP and 4 equiv of DIPEA in DMF (10 mL/g of resin) for 30 min. After washing with DMF (4×1 min) and CH_2Cl_2 (2×1 min), the completeness of the coupling reactions was controlled by TNBS test. *N*-α-Fmoc protection was removed by treatment with a piperidine/DMF solution (1:4, 10 mL/g of resin) for 10 min. The process was repeated two times. After washing the resin with DMF (6×1 min), the loading of the resin was quantified by measuring the absorption of washing solutions at 299 nm.

3.3.2. H-Gly-Leu-Ser(^{t}Bu)-Asp(^{t}Bu)-Val-Gly-OH (A′). The linear protected peptide **A** was synthesized using the Sasrin resin (450 mg, loading: 0.68 mmol/g) following the standard procedure. One part of the resin was used for the solid phase functionalization with Aoa derivatives (peptide **A**). With the second part of the resin, the peptide was cleaved by successive treatments with a solution of 1% TFA in CH_2Cl_2 until the resin became dark purple. The combined washings were concentrated under reduced pressure and white solid peptides were obtained by precipitation from ether (135 mg, 0.2 mmol, 68%). The protected linear peptide **A′** was used for functionalization with Aoa derivatives without further purification. Analytical RP-HPLC: t_R=6.1 min (5–60% B in 15 min); ESI-MS (positive mode) calcd for $C_{30}H_{55}N_6O_{10}$ 659.39; found: *m/z* 659.11 $[M+H]^+$.

3.3.3. H-Gly-Gly-c[Cys-Asp(^{t}Bu)-Gly-Asp(^{t}Bu)-Cys]-NH_2 (B′). The protected cyclic peptide **B** was synthesized on Sieber Amide resin (1 g, loading: 0.72 mmol/g) following the standard procedure. A solution of iodine (1.97 mg, 7.7 mmol) in DMF (10 mL) was added to the resin and stirred for 4 h. The solution was filtered and the resin was washed with DMF (10×1 min), CH_2Cl_2 (10×1 min), and DMF until washing solution became colorless. One part of the resin was used for the solid phase functionalization with Aoa derivatives (peptide **B**). With the second part of the resin, the peptide was cleaved by successive treatments with a solution of 1% TFA in CH_2Cl_2 until the resin became dark purple. The combined washings were concentrated under reduced pressure and white solid peptides were obtained by precipitation from ether (402 mg, 0.52 mmol, 71%). This protected cyclic peptide **B′** was used for functionalization with Aoa derivatives after semi-preparative RP-HPLC purification (5–100% B in 30 min). Analytical RP-HPLC: t_R=6.3 min (5–100% B in 15 min); ESI-MS (positive mode) calcd for $C_{30}H_{50}N_9O_{12}S_2$: 792.28; found: *m/z* 792.30 $[M+H]^+$.

3.3.4. BocSer(^{t}Bu)-Lys[BocSer(^{t}Bu)]-Lys{BocSer(^{t}Bu)-Lys[BocSer(^{t}Bu)]}-Ala-Lys-Rink amide (C). Peptide **C** protected with an Alloc on the C-terminal lysine side chain was synthesized on Rink Amide MBHA resin (450 mg, loading: 0.69 mmol/g). The Alloc protecting group was removed by adding a solution of phenylsilane (3.9 mL, 31.5 mmol) and tetrakis trisphenyl palladium (91 mg, 0.08 mmol) in dry CH_2Cl_2 (10 mL) to the resin, and the solution was mixed under argon for 20 min. The resin was washed with CH_2Cl_2 (2×1 min), dioxane/water (9:1, 2×1 min), DMF (2×1 min) and CH_2Cl_2 (2×1 min). For analysis an aliquot of the resin was cleaved with a solution of TFA/TIS/H_2O (95:2.5:2.5) for 2 h. ESI-MS (positive mode) of fully deprotected peptide calcd for $C_{39}H_{77}N_{14}O_{13}$: 949.57; found: *m/z* 949.23 $[M+H]^+$.

3.4. Standard procedure for functionalization of peptides with Aoa derivatives

3.4.1. Solid phase functionalization with Boc–Aoa–OH 1 and Eei–Aoa–OSu 5 with PyBOP. A solution of 1 equiv of Boc–Aoa–OH **1** or 2 equiv of Eei–Aoa–OH **5** (relative to the resin loading of peptide **A**), 1 equiv of PyBOP (or 2 equiv for **5**) and 2 equiv of DIPEA (or 2 equiv for **5**, pH 8) in dry DMF was added to the resin. After shaking for 45 min at room temperature, the solution was filtered and the resin was washed with DMF (5×1 min) and CH_2Cl_2 (2×1 min). The completeness of the coupling reaction was controlled by TNBS test. Each peptide was finally obtained upon acidic cleavage and deprotection by treatment of the resin with a mixture of TFA/TIS/H_2O (95:2.5:2.5) for 2 h. After evaporation of the cleavage solution, the peptides were precipitated with diethyl ether and analyzed by RP-HPLC and ES-MS. Deprotected aminooxy peptide **A′**: analytical RP-HPLC: t_R=5.9 min (5–60% B in 15 min); ESI-MS (positive mode) calcd for $C_{24}H_{42}N_7O_{12}$: 620.29; found: *m/z* 620.11 $[M+H]^+$.

3.4.2. Solid phase functionalization with Boc–Aoa–OSu 2 and Eei–Aoa–OSu 6. A solution of 1 equiv of Boc–Aoa–OSu **2** or 2 equiv of Eei–Aoa–OSu **6** (relative to the resin loading of peptide **A**, **B**, or **C**) and 1 equiv of DIPEA (pH 8) in dry DMF was added to the resin. After shaking for 45 min at room temperature, the solution was filtered and the resin was washed with DMF (5×1 min) and CH_2Cl_2 (2×1 min). The completeness of the coupling reaction was controlled by TNBS test. If necessary, this process is repeated once. Each peptide was finally obtained upon acidic cleavage and deprotection by treatment of the resin with a mixture of TFA/TIS/H_2O (95:2.5:2.5) for 2 h. After evaporation of the cleavage solution, the peptides were precipitated with diethyl ether and analyzed by ES-MS. Deprotected aminooxy peptide **A′**: see Section 3.4.1; deprotected aminooxy peptide **B′**: analytical RP-HPLC: t_R=12.6 min (0–30% B in 30 min); ES-MS (positive mode) calcd for $C_{24}H_{37}N_{10}O_{14}S_2$: 753.19; found: *m/z* 753.01 $[M+H]^+$. Deprotected aminooxy peptide **C′**: analytical RP-HPLC: t_R= 7.5 min (5–40% B in 30 min); ESI-MS (positive mode) calcd for $C_{41}H_{80}N_{15}O_{15}$: 1022.59; found: *m/z* 1022.07 $[M+H]^+$.

3.4.3. Functionalization with Boc–Aoa–OSu 2 and Eei–Aoa–OSu 6 in solution. The functionalization reaction was realized using 1 equiv of Boc–Aoa–OSu **2** (relative to quantity of peptide **A′** or **B′**), 1 equiv of DIPEA (pH 8) in dry DMF. The reaction was monitored by RP-HPLC. After 45 min, the solution was evaporated and the peptide precipitated in diethyl ether. The peptides were finally deprotected by treatment with a solution of TFA/TIS/H_2O (95:2.5:2.5) for 2 h. After evaporation of the cleavage solution, the peptides were precipitated with diethyl ether and analyzed by RP-HPLC and ESI-MS (see Section 3.4.2).

Acknowledgements

This work was supported by the Centre National pour la Recherche Scientifique (CNRS), the Université Joseph Fourier

(UJF), and COST D-34. We also acknowledge the Ministère de la Recherche for ACI-2003 'Molécules et Cibles Thérapeutiques' and for Grant No. 15432-2004 to V.D.

References and notes

1. Kimmerlin, T.; Seebach, D. *J. Pept. Res.* **2005**, *65*, 229.
2. Lemieux, G. A.; Bertozzi, C. R. *Trends Biotechnol.* **1998**, *16*, 506.
3. Langenhan, J. M.; Thorson, J. S. *Curr. Org. Synth.* **2005**, *2*, 59.
4. Koehn, M.; Breinbauer, R. *Angew. Chem., Int. Ed.* **2004**, *4*, 3106.
5. Kolb, H. C.; Sharpless, K. B. *Drug Discov. Today* **2003**, *8*, 1128.
6. Rose, K. *J. Am. Chem. Soc.* **1994**, *116*, 30.
7. Canne, L. E.; Ferre-D'Amare, A. R.; Adrian, R.; Burley, S. K.; Kent, S. B. H. *J. Am. Chem. Soc.* **1995**, *117*, 1998.
8. Shao, J.; Tam, J. P. *J. Am. Chem. Soc.* **1995**, *117*, 2998.
9. Spetzler, J. C.; Hoeg-Jensen, T. *J. Pept. Sci.* **2001**, *7*, 537.
10. Rodriguez, E. C.; Winans, K. A.; King, D. S.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1997**, *119*, 9905.
11. Cervigni, S. E.; Dumy, P.; Mutter, M. *Angew. Chem., Int. Ed.* **1996**, *35*, 1230.
12. Renaudet, O.; Dumy, P. *Org. Lett.* **2003**, *5*, 243.
13. Forget, D.; Renaudet, O.; Defrancq, E.; Dumy, P. *Tetrahedron Lett.* **2001**, *42*, 7829.
14. Forget, D.; Boturyn, D.; Defrancq, E.; Lhomme, J.; Dumy, P. *Chem.—Eur. J.* **2001**, *7*, 3976.
15. Renaudet, O.; Reymond, J.-L. *Org. Lett.* **2003**, *5*, 4693.
16. Zhou, X.; Zhou, J. *Biosens. Bioelectron.* **2006**, *21*, 1451.
17. Falsey, J. R.; Renil, R.; Park, S.; Li, S.; Lam, K. S. *Bioconjugate Chem.* **2001**, *12*, 346.
18. Liu, Y.; Chai, W.; Childs, R. A.; Feizi, T. *Methods Enzymol.* **2006**, *415*, 326.
19. Defrancq, E.; Hoang, A.; Vinet, F.; Dumy, P. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2683.
20. Chen, X.; Lee, G. S.; Zettl, A.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2004**, *43*, 6112.
21. Prescher, J. A.; Bertozzi, C. R. *Cell* **2006**, *126*, 851.
22. Jencks, W. P. *J. Am. Chem. Soc.* **1959**, *81*, 475.
23. Melnyk, O.; Fehrentz, J. A.; Martinez, J.; Gras-Masse, H. *Biopolymers* **2000**, *55*, 165.
24. Brask, J.; Jensen, K. J. *J. Pept. Sci.* **2000**, *6*, 290.
25. Decostaire, I. P.; Lelièvre, D.; Zhang, H.; Delmas, A. F. *Tetrahedron Lett.* **2006**, *47*, 7057.
26. Jiménez-Castells, C.; de la Torre, B. G.; Gutiérrez Gallego, R.; Andreu, D. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5155.
27. Lee, M.-R.; Lee, J.; Baek, B.-H.; Shin, I. *Synlett* **2003**, 325.
28. Unpublished result.
29. Grigalevicius, S.; Chierici, S.; Renaudet, O.; Lo-Man, R.; Dériaud, E.; Leclerc, C.; Dumy, P. *Bioconjugate Chem.* **2005**, *5*, 1149.
30. Ide, H.; Akamatsu, K.; Kimura, Y.; Michiue, K.; Makino, K.; Asaeda, A.; Takamori, Y.; Kubo, K. *Biochemistry* **1993**, *32*, 8276.
31. Stanek, J.; Frei, J.; Mett, H.; Schneider, P.; Regenass, U. *J. Med. Chem.* **1992**, *30*, 1339.
32. Crystallographic data (excluding structure factors) of Eei–Aoa–OSu **6** have been deposited at the Cambridge Crystallographic Data Center as supplementary publication number CCDC-644968. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambrige CB21EZ, UK. [Fax: +44 1223 336 033 or e-mail: deposit@ccdc.cam.ac.uk.]
33. Han, Y.; Barany, G. *J. Org. Chem.* **1997**, *62*, 3841.
34. Garanger, E.; Boturyn, D.; Renaudet, O.; Defrancq, E.; Dumy, P. *J. Org. Chem.* **2006**, *71*, 2402.