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1-Ethoxyethylidene, a New Group for the Stepwise SPPS of Aminooxyacetic Acid Containing Peptides

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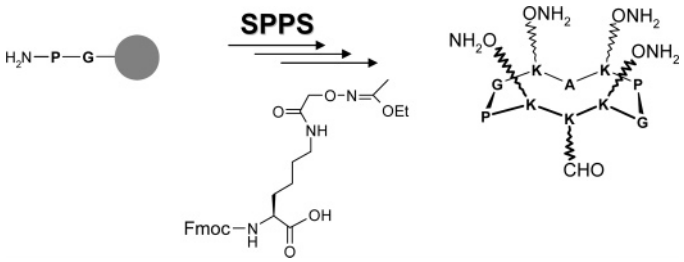

For more than a decade, the oxime ether ligation has proven to be one of the most efficient technique for the preparation of various peptide conjugates. However, despite numerous reports, the preparation of aminooxy-containing peptides is still hampered by *N*-overacylation of the NH−O function either during its incorporation or through the peptide-chain elongation. This restricts the introduction of protected-NH−O function at the last acylation step and prevents the use of standard solid-phase peptide synthesis (SPPS) procedures for the preparation of more complex aminooxy-peptides. We have studied the coupling of modified Fmoc-lysine containing either *N*-Boc- or *N*,*N*′-bis-Boc-protected aminooxyacetic acids (Aoa) during the elongation of the peptide chain and found that none of them is adequate. To circumvent this limitation, we propose to protect the Aoa moiety with a 1-ethoxyethylidene group (Eei) to provide 2-(1-ethoxyethylideneaminooxy)acetic acid building block. We showed that the Eei group is fully compatible with standard SPPS conditions and safely allows the multiple incorporation of the aminooxy functionality into the growing peptide. Since Eei-protected Aoa remains as flexible as normal amino acids in peptide synthesis, it may become the rule for the straightforward preparation of aminooxy peptides.

Introduction

Recent progress in biomolecule assembling using chemoselective ligations has paved the way to novel engineered molecules. These conjugates exploit the intrinsic properties of peptides, nucleic acids, or carbohydrates for various applications, notably targeted therapy, tumor imaging, and synthetic vaccines.[1] Among the chemoselective methods, the oxime ligation is becoming increasingly useful for the preparation of bioconjugates[2] and small proteins.[3] The oxime bond formation benefits from the high chemoselectivity and reactivity between the aminooxy function and the carbonyl group,[4] since the nitrogen atom behaves as a weak base and as an excellent nucleophile due to its well-known α-effect.[5]

Aminooxy-containing peptides are usually obtained by inserting the classical *N*-Boc-protected 2-(aminooxy)acetic acid (Aoa) at the last stage of peptide synthesis either at *N*-terminus or side-chain position.[6−8] Although not clearly delineated in the

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literature,[9−11] there was reported evidence that this constraint results from the high reactivity of the *N*-protected aminooxy function exhibited during peptide coupling condition. Indeed, significant *N*-overacylation was observed during the insertion of the Aoa building block which led to heterogeneous peptides. Only very recently, this pitfall was carefully studied and addressed by different means based on the modulation of the nitrogen atom reactivity. On the one hand, *N*-overacylation can be limited by a very careful control of the nature and the quantity of the used base.[8] On the other hand, carbodiimide-mediated one-pot acylation without base also provided much homogeneous compounds in harmony with previous work employing carbodiimide-prepared Aoa-OSu activated ester as acylating reagent.[8] Much less is documented on the behavior of protected-Aoa group during acylation conditions subsequent to its insertion in peptides: *N*-Boc-protected aminooxy-containing amino acids such as L-homocanaline,[12] *O*-aminoserine (Ams),[13] or *N*-trityl-protected aminooxy-containing diaminopropionic acid[9] were incorporated manually during solid-phase peptide synthesis (SPPS) without reported *N*-overacylation. As a matter of fact, the Aoa moiety cannot be incorporated into peptide sequences as a normal *N*-protected amino-acids. It is consequently mandatory to incorporate the protected Aoa group at the last stage of peptide synthesis. If this is not a great disadvantage for the preparation of *N*-terminal Aoa peptide, it makes the synthesis of the side-chain aminooxy containing-peptides much more complex and tedious since orthogonal protecting group manipulation is required. Despite the obvious interest in these aminooxy-containing compounds, these drawbacks restrict their scope of application. In this paper, we studied for the first time the behavior of differently protected Aoa groups upon acylation conditions when incorporated in the peptide chain. It aimed to set up a new *N*-protected Aoa group and allow its stepwise incorporation into a peptide sequence under conventional SPPS conditions. We have focused the study on cyclopeptide compounds containing several Aoa groups[14] as models since the problem is exacerbated because even when minor *N*-overacylation side reaction occurs it results in an inextricably complex mixture. Comparison of Fmoc-lysine side chains containing either *N*-Boc-protected or *N*,*N*′-bis-Boc-protected Aoa moiety **1** and **2** demonstrated that none of them is compatible with their stepwise incorporation under conventional SPPS conditions. Consequently, we propose 1-ethoxyethylidene (Eei) as new total *N*-overacylation free and stepwise adapted protecting group of aminooxy function. 2-(1-Ethoxyethylideneaminooxy)acetic acid was incorporated in the *N*-Fmoc-lysine side chain to provide key building block **3**. The latter can be introduced cleanly and proved compatible with the subsequent growth of the peptide chain as a normal protected amino acid, thus making its use general and safe for the synthesis of Aoa-containing peptides. In sharp contrast with previous reported strategy of multiple Aoa containing peptides,[14] the building block **3** makes the synthesis of such interesting compounds straightforward. Consequently, the Eei protecting group may become the rule for the introduction of the aminooxy function in peptides and other polyfunctional molecules. To study the multiple incorporation of these building blocks under stepwise SPPS conditions, we opted to construct CAT peptides containing several aminooxy functions. For instance, we selected the cyclodecapeptide **10** or **23** named CAT (chemoselectively addressable template) (Figure 1)[14] because we use them as key compounds to prepare by oxime combination of peptides, carbohydrates, and oligonucleotides. These functional systems are successfully exploited for diverse applications such as tumor imaging,[15,16] tumor therapy,[17] synthetic vaccines,[18] or β-sheet structures.[19]

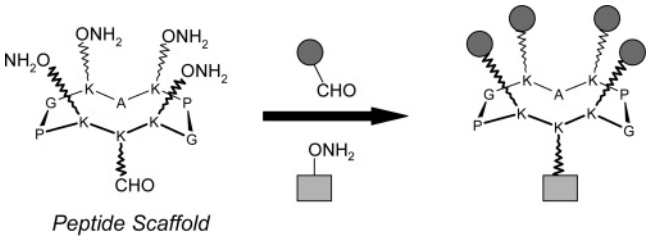

FIGURE 1. Chemoselectively addressable template. Rectangles and circles denote different types of multifunctional molecules assembled through successive oxime ligations.

Results and Discussion

To incorporate aminooxy functions in the peptide chain, we designed several building blocks **1**−**3** (Scheme 1) which correspond to *N*-Fmoc-lysine acylated by the Aoa moiety. Boc-Aoa-OH was activated as the *N*-hydroxysuccinimide (OSu) ester to prevent side reaction with the lysine free-acid function. In addition, this activation was known and was reported recently to be free of *N*-overacylation problem.[12] Compound **5** was first obtained in 83% yield following the procedure reported by Kubo.[20] It was reacted with Fmoc-Lys-OH to provide the corresponding building block **1** in 84% yields free of *N*-overacylation (Scheme 1) as inferred from careful NMR and mass spectrometry analyses. Using the same route with the commercial available *N*,*N*′-bis-Boc-Aoa acid, the building block **2** was readily obtained in 49% overall yield.

To study building blocks **1** and **2** under stepwise SPPS conditions, we opted to construct CAT peptides containing several aminooxy functions (Figure 1). These molecules are typically obtained by successive protection−deprotection of orthogonal protecting groups such as Boc/Alloc residues.[14]

We therefore addressed the peptide synthesis manually using in parallel the building block **1** and **2** (Scheme 2). The syntheses were carried out following rigorously the standard Fmoc/*t*-Bu SPPS procedure using PyBOP as coupling reagent on 2-chlorotrityl resin. Reactions were carefully monitored by RP-HPLC

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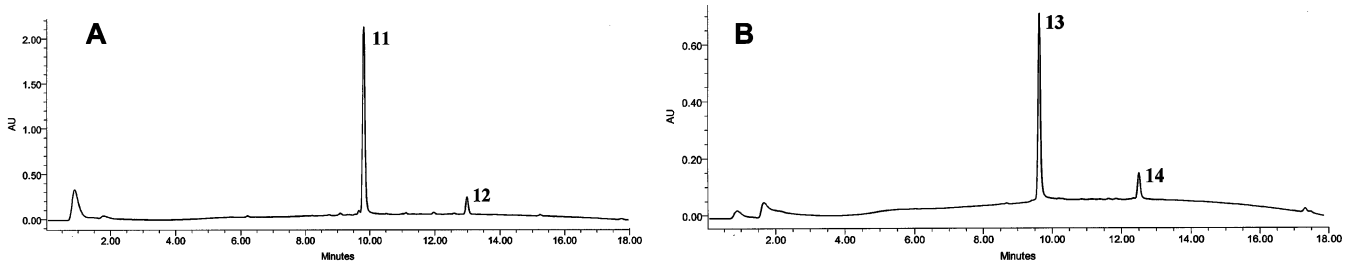
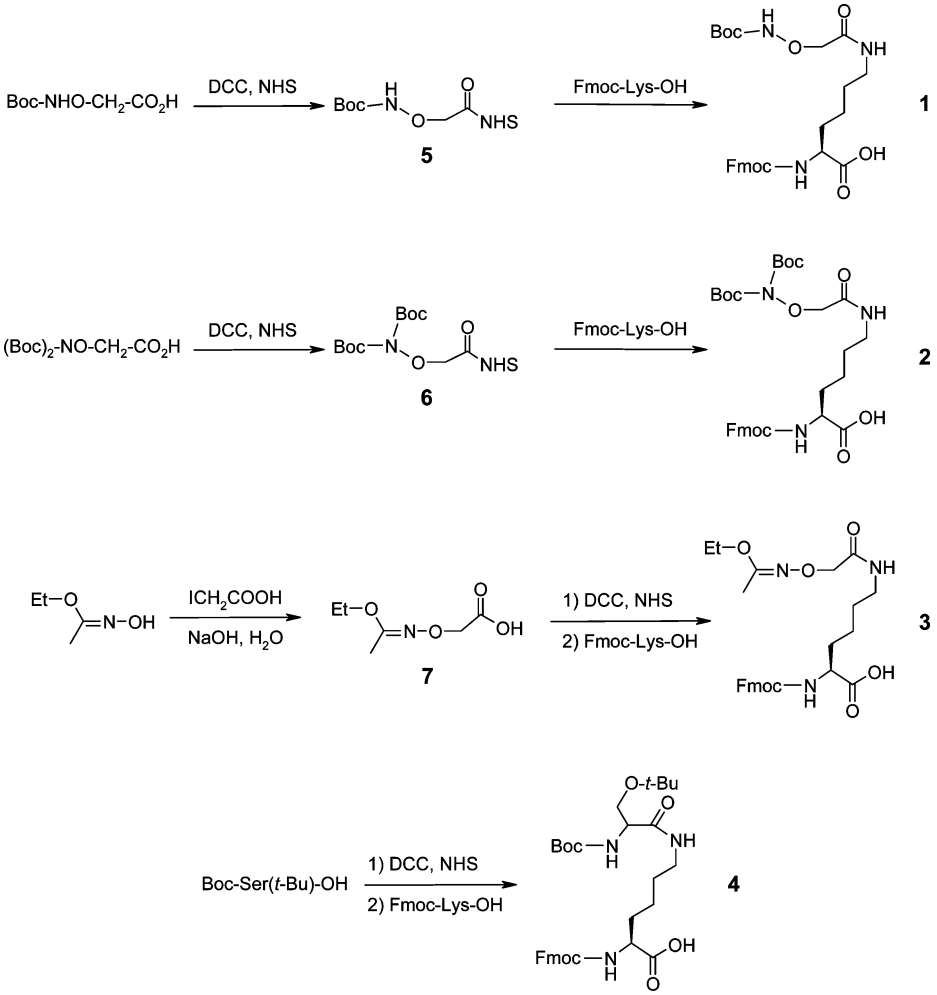


FIGURE 2. RP-HPLC profiles of (A) the coupling reaction of **1** on H-Pro-Gly resin and (B) the Fmoc-Ala-OH coupling reaction to **17** on resin.

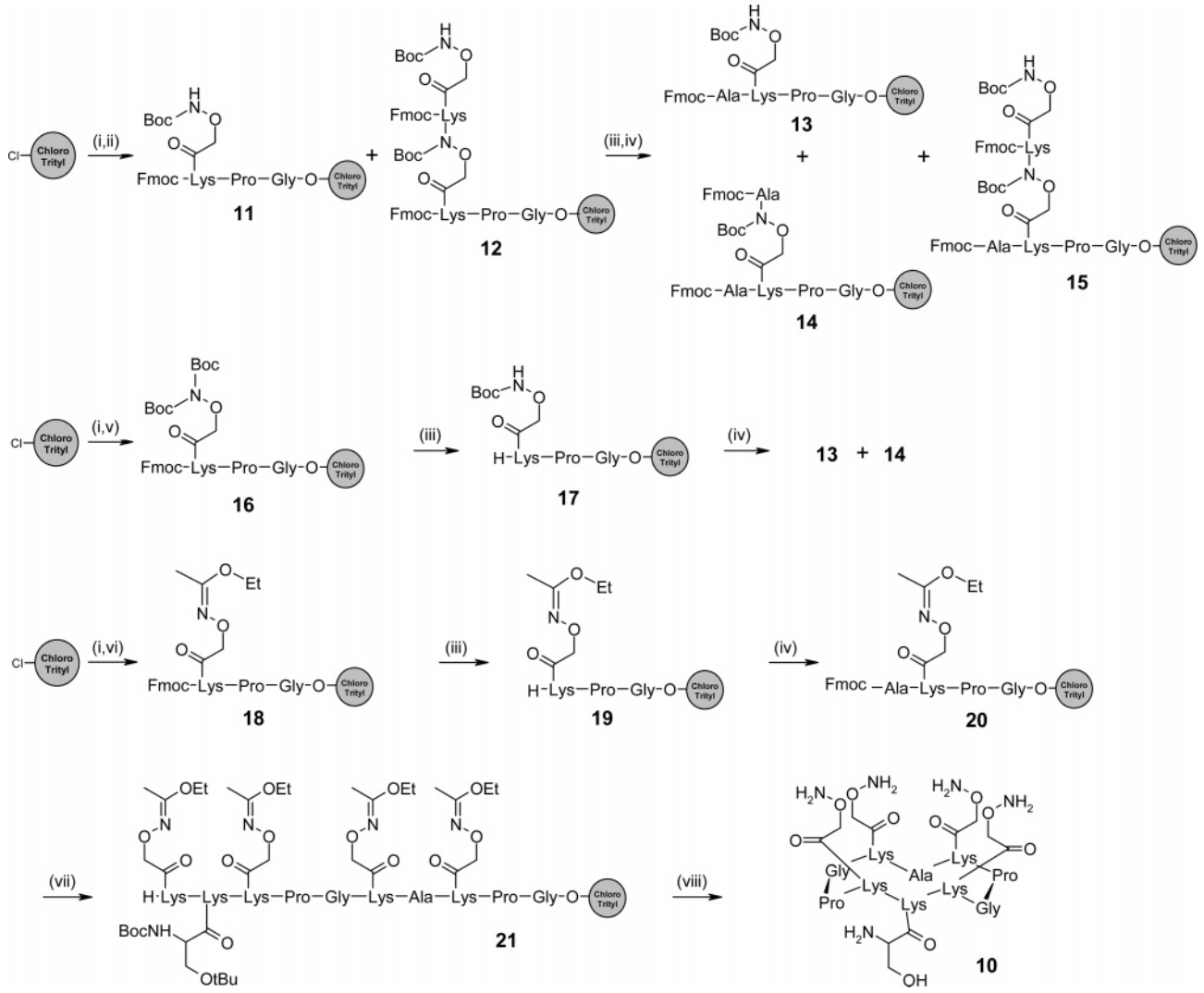
SCHEME 1. Synthetic Ways To Prepare Building Blocks 1−4



and analyzed by mass spectrometry. Compounds are easily isolated from a few resin beads. After incorporation of glycine and proline residues on the resin, building block **1** was coupled and the products were analyzed (Figure 2A). The expected peptide **11** was obtained with a byproduct **12** (∼10% estimated from HPLC). Compound **12** results without ambiguity from the *N*-overacylation of the Boc-protected aminooxy function by itself, namely Fmoc-Lys[Boc-(Boc-Aoa)Aoa]-Pro-Gly resin. Since **1** was devoid of *N*-acylation product, this demonstrates that the formation of **12** occurred during the coupling step to the growing peptide-chain. Fmoc group deprotection and subsequent PyBOP coupling to that mixture with Fmoc-Ala-OH gave the two expected *N*-Fmoc-Ala-tetrapeptides **13** and **15** as well as a new compound **14** (in a ratio 81:5:14, respectively). The latter corresponds to *N*-overacylation by Fmoc-Ala-OH of the Boc-protected NH−O function as inferred by mass spectroscopy. These results confirm that the use of Boc protection of Aoa is not suitable for the incorporation of **1** and demonstrates that it is not compatible with a subsequent peptide-chain elongation step.

Consequently, we investigated compound **2** in which *N*,*N*′-Boc-protection of the Aoa nitrogen moiety appears to be the best promising candidate to avoid *N*-overacylation during the peptide coupling reaction (Scheme 2). We were pleased to find that the coupling of the building block **2** provided exclusively the expected pure tripeptide **16** without indeed any *N*-overacylation compound in agreement with result from the literature.[10] However under typical Fmoc removal conditions (Scheme 2), the unexpected free-amino peptide **17** was obtained in which concomitant loss of one Boc group on the aminooxy function occurred. Further evidence of this unexpected release of the Boc group under basic conditions was obtained when commercial

SCHEME 2. Synthesis of Chemoselectively Addressable Template 10[a]



[a] Reagents and conditions: (i) (a) Fmoc-Gly-OH (0.8 equiv), DIPEA (4 equiv), CH_2Cl_2, 30 min; (b) methanol/DIPEA/CH_2Cl_2 (2:1:17), 2 × 10 min; (c) piperidine/DMF (1:4), 3 × 10 min; (d) Fmoc-Pro-OH (2 equiv), PyBOP (2 equiv), DIPEA (5 equiv), DMF, 30 min; (e) piperidine/DMF (1:4), 3 × 10 min; (ii) building block **1** (2 equiv), PyBOP (2 equiv), DIPEA (5 equiv), DMF, 30 min; (iii) piperidine/DMF (1:4), 3 × 10 min; (iv) Fmoc-Ala-OH (2 equiv), PyBOP (2 equiv), DIPEA (5 equiv), DMF, 30 min; (v) building block **2** (2 equiv), PyBOP (2 equiv), DIPEA (5 equiv), DMF, 30 min; (vi) building block **3** (2 equiv), PyBOP (2 equiv), DIPEA (5 equiv), DMF, 30 min; (vii) (a) piperidine/DMF (1:4), 3 × 10 min; 6×; (b) Fmoc-aa-OH (2 equiv), PyBOP (2 equiv), DIPEA (5 equiv), DMF, 30 min then piperidine/DMF (1/4), 3 × 10 min; (viii) (a) TFE/acetic acid/CH_2Cl_2, (1:1:8), 2 × 30 min; (b) PyBOP (2 equiv), DIPEA (5 equiv), DMF, 1 h; (c) TFA/TIS/H_2O (95:2.5:2.5), 2 h.

bis-Boc-aminooxyacetic acid was treated with a solution containing piperidine or DBU (data not shown). We hypothesize that one Boc group can be released through a base catalyzed β-elimination. Consequently, the subsequent coupling of Fmoc-Ala-OH to pure **17** afforded peptide **13** and about 13% of *N*-overacylated compound **14** (Figure 2B). Accordingly, the *N*,*N*′-Boc deprotection provides a complete *N*-overacylation free incorporation of Aoa-containing building block but, it is not compatible with peptide-chain elongation under Fmoc/*t*-Bu strategy. Therefore, its use is limited to the introduction of Aoa residue at the last stage of peptide synthesis.

In the light of these results, we decided to devise a completely new masking of aminooxy reactivity through functional transformation rather than direct protection. We reasoned that this can be achieved through the use of *N*-hydroxyacetimidate function. Indeed as for *N*,*N*′-Boc, it makes the aminooxy function devoid of hydrogen thus preventing further substitution on the nitrogen atom to occur upon acylation conditions (Scheme 1). In addition, it can be cleaved under mild acid condition as demonstrated recently for the synthesis of an oxime library.[21] We thus decided to prepare a building block **3** in which Aoa moiety is protected by 1-ethoxyethylidene group (Eei). Ethyl *N*-hydroxyacetimidate was reacted to iodoacetic acid in aqueous NaOH to provide compound **7** in 73% yield (Scheme 1). After DCC/HOSu activation of **7**, subsequent acylation of Fmoc-lysine side-chain provided the Eei-protected building block **3** in good 63% overall yield. The purity of **3** was estimated from ^{1}H and ^{13}C NMR analyses. The stability of building block **3** was subsequently checked under peptide synthesis conditions. All reactions were monitored by analytical RP-HPLC (214 and 299 nm). As expected, the Eei protective group is stable under amino acid coupling conditions, under Fmoc removal conditions (20%

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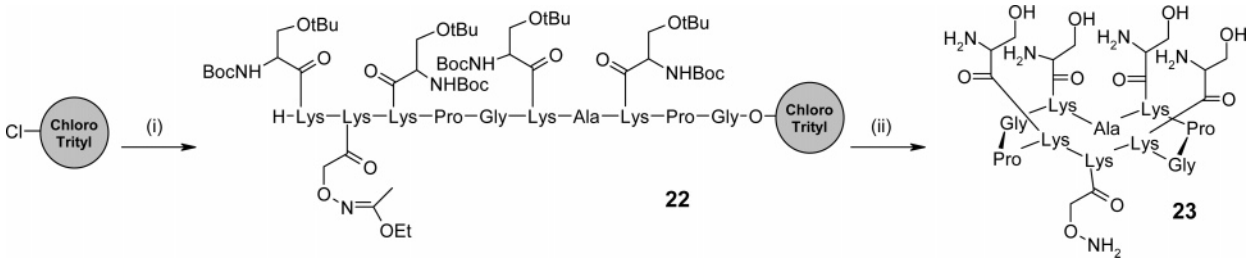
SCHEME 3. Synthesis of Chemoselectively Addressable Template 23[a]



[a] Reagents and conditions: (i) (a) Fmoc-Gly-OH (0.8 equiv), DIPEA (4 equiv), CH_2Cl_2, 30 min; (b) methanol/DIPEA/CH_2Cl_2 (2:1:17), 2 × 10 min; (c) piperidine/DMF (1:4), 3 × 10 min; 9×; (d) Fmoc-aa-OH (2 equiv), PyBOP (2 equiv), DIPEA (5 equiv), DMF, 30 min then piperidine/DMF (1:4), 3 × 10 min; (ii) (a) TFE/acetic acid/CH_2Cl_2, (1:1:8), 2 × 30 min; (b) PyBOP (2 equiv), DIPEA (5 equiv), DMF, 1 h; (c) TFA/TIS/H_2O (95:2.5:2.5), 2 h.

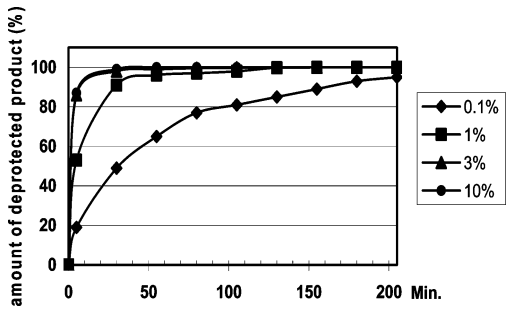


FIGURE 3. Kinetics of aminooxy deprotection using 2 mM of compound **3** in solutions containing TFA (0.1−10%) in water.

of piperidine in DMF), and under mild acid condition using acetic acid over 24 h. Besides, it was important to study the removal conditions of the Eei group. Deprotection of aminooxy function was examined using increasing concentrations of TFA (0.1% up to 10%) in water (Figure 3). Eei groups were completely removed with a solution containing 3% of TFA in 30 min whereas using a dilute solution of 0.1% of TFA half of protected compound **3** was found.

As for building blocks **1** and **2**, Fmoc-Lys(Eei-Aoa-)-OH **3** was stepwise incorporated under SPPS conditions (Scheme 2). PyBOP coupling of **3** afforded pure *N*-Fmoc-*N*′-Eei-protected peptide **18** devoid of *N*-overacylation. Subsequent Fmoc cleavage provided the pure desired tripeptide H-Lys(Eei-Aoa-)-Pro-Gly- **19** attached to the solid support. Following Fmoc-Ala-OH coupling led to the expected intermediate **20** in excellent purity compared to previous attempts using either **1** or **2**. Since **3** proved entirely compatible with SPPS conditions, we decided to complete the peptide-chain elongation by incorporating stepwise the following Fmoc-protected amino acids including the lysine building blocks **3** and **4**. Building block **4** was designed as masked glyoxylyl group since it is readily generated after sodium periodate oxidative cleavage of the 1,2-amino alcohol function of serinyl residue attached to the lysine side-chain.[22] Compound **4** was easily obtained from Boc-Ser(*t*-Bu)-OSu and Fmoc-lysine in 51% overall yield (Scheme 1). After completion of the SPPS cycle, homogeneous resin-bound decapeptide **21** exhibiting four Aoa moieties was obtained in very good purity. Subsequently, the protected decapeptide **21** was cleaved off the solid support under mild acid conditions (acetic acid, trifluoroethanol) without affecting the acetimidate function. The peptide was isolated after ether precipitation in sufficient purity to carry out the subsequent step. The corresponding head-to-tail cyclization and the total deprotection were performed according previously described procedures affording the CAT derivative **10**.[14] Using the same building blocks, we have also prepared the cyclodecapeptide **23** that presents four glyoxylaldehyde precursors and one aminooxy group (Scheme 3) tethered on lysine side chains. It is worth noting that incorporation of four Boc/*t*-Bu protected serine at the lysine side chains did not disrupt the cyclization step and peptide **23** was obtained in very good overall yield. To confirm these results and the usefulness of building block **3** all the peptide syntheses were carried out automatically on a peptide synthesizer. As found manually, only the building blocks **3** provided expected protected-decapeptide **21** whereas inextricable products mixture was obtained using the building block **1** (Figure 4) or **2**. By means of building block **3**, we commonly used this method for the synthesis of other peptides notably homing peptides such RGD or NGR-containing ligands (see the Supporting Information).

Finally, we validated the compatibility of our protecting group with classical reactions used for biomolecule conjugations. Therefore, a peptide that allows the assembling of biomolecules through oxime bond and disulfide bridge formation was designed. We prepared a CAT derivative **26** encompassing four Eei-protected aminooxy functions and NPys-protected cysteine (Scheme 4). The latter is routinely used to regioselectively form a disulfide bond. Nevertheless, this group is not stable under Fmoc removal conditions.[23] The coupling of NPys-containing cysteine was then carried out subsequent to the peptide elongation. Consequently, we introduced a regioselective group by inserting an Alloc-protected lysine within the CAT compound **24** (Scheme 4). As expected the SPPS and the following head-to-tail cyclizations provided the desired cyclodecapeptide. Alloc deprotection was performed using the well-established Pd^0/$PhSiH_3$ procedure[24] affording the desired cyclodecapeptide **25** in more than satisfactory yield (84%). These conditions are entirely compatible with the presence of Eei protecting groups and make more flexible the use of building block **3**. Compound **25** is an useful intermediate for development of new derivatives. In fact using activated ester, it is possible to link for example labels. Boc-Cys(NPys)-OH was then grafted to cyclodecapeptide **25** using PyBOP as coupling reagent affording CAT derivative **26** in high yield. To perform the subsequent disulfide bridge, we used the commercially available 2-mercaptoethanol. The reaction was carried out using 3 equiv. of thiol compound in

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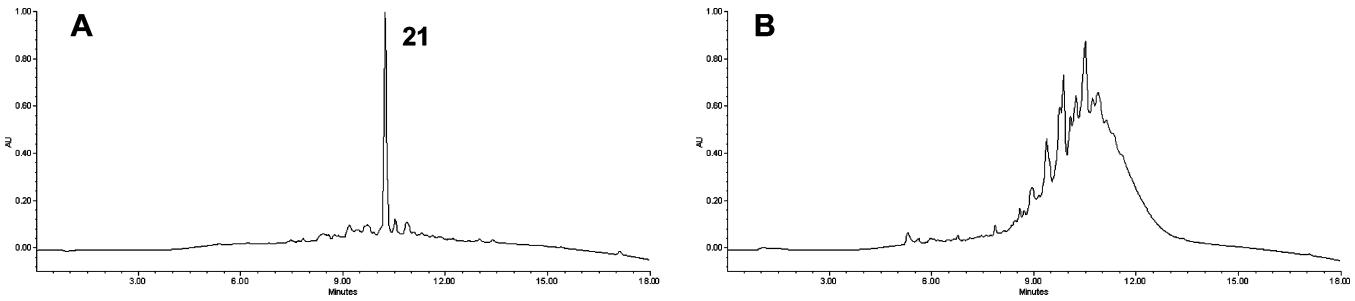
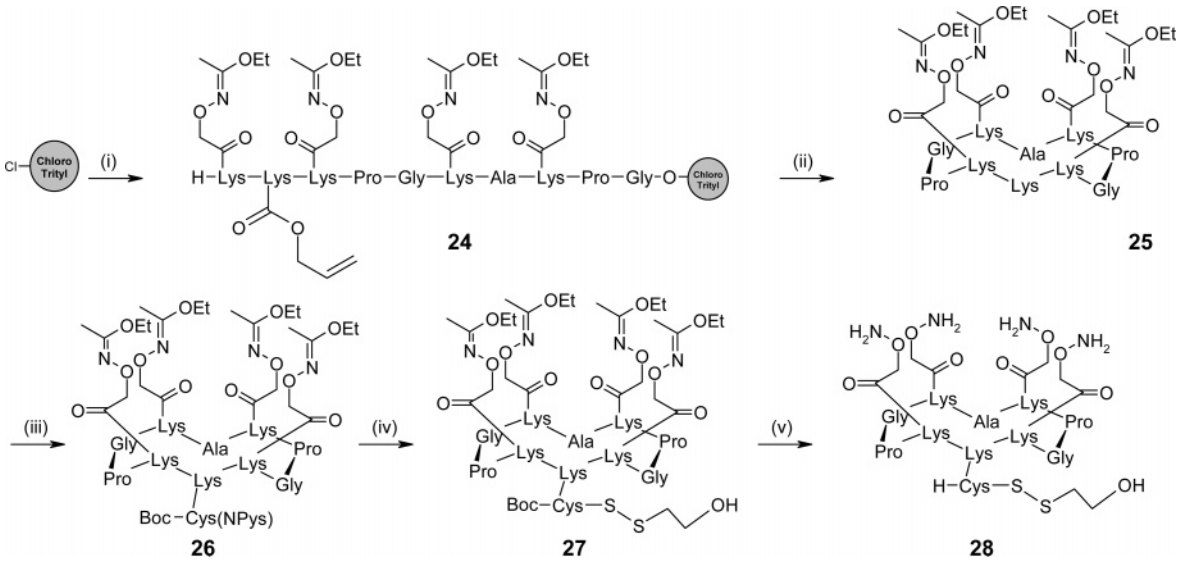


FIGURE 4. RP-HPLC profiles of automatic syntheses of linear protected aminooxy-containing decapeptides using (A) building block **3** and (B) building block **1**.

SCHEME 4. Synthesis of Chemoselectively Addressable Template 26[a]



[a] Reagents and conditions: (i) (a) Fmoc-Gly-OH (0.8 equiv), DIPEA (4 equiv), CH_2Cl_2, 30 min; (b) methanol/DIPEA/CH_2Cl_2 (2:1:17), 2 × 10 min; (c) piperidine/DMF (1:4), 3 × 10 min; 9×; (d) Fmoc-aa-OH (2 equiv), PyBOP (2 equiv), DIPEA (5 equiv), DMF, 30 min then piperidine/DMF (1:4), 3 × 10 min; (ii) (a) TFE/acetic acid/CH_2Cl_2, (1:1:8), 2 × 30 min; (b) PyBOP (2 equiv), DIPEA (5 equiv), DMF, 1 h; (c) $PhSiH_3$ (100 equiv), $Pd^0(PPh_3)_4$ (0.2 equiv), CH_2Cl_2, 30 min under argon; (iii) Boc-Cys(NPys)-OH (1 equiv), PyBOP (1 equiv), DIPEA (4 equiv), DMF, 30 min; (iv) $HO(CH_2)_2SH$ (3 equiv), PBS pH 4.8/DMF (1:3), 5 min; (v) TFA/TIS/H_2O (95:2.5:2.5), 2 h.

phosphate buffer for 5 min. The product **27** was obtained without any undesired byproduct. This result highlights the usefulness of our protective group since prior to oxime bond formation we can easily access to disulfide bridge.

Conclusions

In summary, we reported for the first time a complete study of the reactivity of aminooxy moiety upon peptide acylation conditions during the peptide-chain growth. For this purpose, Fmoc-protected lysine building blocks **1**−**3** exhibiting side-chain acylated by various protected aminooxy moiety were prepared. Compounds **2** and **3** devoid of acid hydrogen on the nitrogen atom were devised as *N*-overacylation-free building blocks for the incorporation of aminooxy into the peptide chain. Surprisingly, *N*,*N*′-Boc protected **2** was found to be unstable under Fmoc release conditions and therefore not compatible for the peptide-chain growth after its incorporation as the reference *N*-Boc protected compound **1**. We consequently developed 1-ethoxyethylidene (Eei) as a new protecting group of the aminooxy function for stepwise SPPS. Using standard SPPS procedures, we found that the *N*-Eei-protected building block **3** prevents the *N*-overacylation during its incorporation as well as the subsequent peptide-chain elongation. Fmoc-Lys(Eei-Aoa)-OH building block was successfully and up to four times incorporated in a stepwise manner in peptides under standard manual and automatic SPPS conditions. In comparison with the state of the art, this methodology considerably reduces the number of steps and the combination of protecting groups required so far for the construction of such aminooxy-peptides. It allows the production of our template in high yield (94% for **10**) while conventional protecting strategy gives moderate yield (42%[14] for **10**). This is emphasized with the straightforward preparation of chemoselectively addressable peptides with only one level of side-chain protection. Synthetic schemes like the one presented here will likely become the rule in the preparation of molecular conjugates and could be applied to a broad range of applications.

Experimental Section

General Procedure for Solid-Phase Peptide Synthesis. Assembly of all protected peptides was carried out using the Fmoc/*t*-Bu strategy manually in a glass reaction vessel fitted with a sintered glass frit or automatically on a peptide synthesizer using 2-chlorotrityl resin. Coupling reactions were performed manually by using 2 equiv of *N*-α-Fmoc-protected amino acid (relative to the resin loading) activated in situ with 2 equiv of PyBOP and

3−5 equiv of diisopropylethylamine (DIPEA) in DMF (10 mL/g resin) for 30 min except for the first coupling on chlorotrityl resin. Coupling reactions carried out on the synthesizer were performed twice. The coupling efficiency in manual synthesis was assessed by Kaiser and/or TNBS tests. *N*-α-Fmoc protecting groups were removed by treatment with a piperidine/DMF solution (1:4) for 10 min (10 mL/g resin). The process was repeated three times and the completeness of deprotection verified by UV absorption of the piperidine washings at 299 nm.

Synthetic linear peptides were recovered directly upon acid cleavage. Before cleavage, the resin was washed thoroughly with methylene chloride. The linear peptides were then released from the resin by treatments with a solution of acetic acid/trifluoroethanol/methylene chloride (1:1:8, 10 mL/mg resin, 2 × 30 min). Hexane (5−10 volumes) was added to the collected filtrates, and the crude peptides were isolated after evaporation as white solids. The residue was dissolved in the minimum of methylene chloride and diethyl ether was added to precipitate peptides. Then they were triturated and washed three times with diethyl ether to obtain crude materials that were used in the next step without further purification.

General Procedure for Cyclization Reactions. All linear peptides (0.5 mM) were dissolved in DMF and the pH values were adjusted to 8−9 by addition of DIPEA. PyBOP (1 equiv) was added and the solution stirred at room temperature for 1 h. as described.[15] Solvent was removed under reduced pressure and the residue dissolved in the minimum of methylene chloride. Diethyl ether was added to precipitate peptides. Then they were triturated and washed three times with diethyl ether to obtain crude materials that were used in the next step without further purification.

***N′*-Boc-aminooxyacetyl *N*-Hydroxysuccinimide Ester 5.** To a stirred solution of *N*-Boc-aminooxyacetic acid (0.500 g, 2.6 mmol) in ethyl acetate/dioxane (1:1, 10 mL) cooled on an ice bath were added *N*-hydroxysuccinimide (0.310 g, 2.7 mmol) and DCC (0.563 g, 2.7 mmol). The resulting mixture was stirred at room temperature for 5 h and was then filtered through a pad of Celite, and the filtrate was concentrated under vacuum. The obtained residue was redissolved in ethyl acetate (35 mL) and washed with 5% aqueous $NaHCO_3$ (3 × 5 mL), water (2 × 10 mL), and brine (10 mL). The organic phase was dried over Na_2SO_4 and evaporated in vacuo. The crude solid was re-crystallized from methylene chloride/diethyl ether/pentane, thereby providing pure compound **5** as a white solid (0.618 g, 2.14 mmol, 83%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.66 (1H, s), 4.78 (2H, s), 2.87 (4H, s), 1.49 (9H, s); DCI-MS calcd for $C_{11}H_{16}N_2O_7$ 288.1, found *m*/*z* 305.8 $(M + NH_4)^+$.

Fmoc-Lys[Boc-aminooxyacetyl]-OH 1. The ester **5** (0.301 g, 1.05 mmol) in methylene chloride (6 mL) was added dropwise to a stirred suspension of the TFA salt of Fmoc-lysine (0.409 g, 1.11 mmol) and DIPEA (0.190 mL, 1.09 mmol) in methylene chloride (6 mL). The resulting suspension was stirred for 7.5 h. The pH of the resulting reaction mixture was regularly adjusted to pH 8−9 by further additions of DIPEA. Unreacted Fmoc-lysine was then filtered off and the filtrate concentrated under vacuum. Ethyl acetate was added to the residue, and the organic solution was washed with water (2 × 10 mL) and brine (10 mL). The organic phase was dried over Na_2SO_4 and evaporated under vacuum. Fmoc-Lys-[Boc-aminooxyacetyl]-OH was precipitated from ethyl acetate/hexane. The solvents were removed and the solid rapidly washed with ice cold ether and dried under vacuum providing compound **1** as a white powder (0.472 g, 0.87 mmol, 84%): analytical HPLC t_R = 10.5 min; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.21 (1H, broad s), 7.75 (2H, d, *J*=7.5 Hz), 7.66 (1H, broad s), 7.60 (2H, t, *J*=7.5 Hz), 7.39 (2H, t, *J*=7.5 Hz), 7.30 (2H, t, *J*=7.5 Hz), 5.69 (1H, d, *J*=7.8 Hz), 4.38−4.31 (5H, m), 4.21 (1H, t, *J*=7.2 Hz), 3.34 (2H, m), 1.95−1.54 (6H, m), 1.45 (9H, s); ESI-MS calcd for $C_{28}H_{35}N_3O_8$ 541.2, found *m*/*z* 540.1 $(M - H)^-$.

***N′*,*N′*-Bis-Boc-aminooxyacetyl-*N*-hydroxysuccinimide Ester 6.** To a stirred solution of *N*,*N′*-bis-Boc-amino-oxyacetic acid (0.500 g, 1.72 mmol) in ethyl acetate/dioxaen (1:1, 5.5 mL) cooled on ice were added *N*-hydroxysuccinimide (0.198 g, 1.72 mmol) and DCC (0.354 g, 1.72 mmol). The resulting solution was stirred at room temperature for 3 h and was then filtered through a pad of Celite. The filtrate was concentrated under vacuum. The obtained residue was dissolved in ethyl acetate (35 mL) and washed with 5% aqueous $NaHCO_3$ (3 × 5 mL), water (2 × 5 mL), and brine (5 mL). The organic phase was dried over Na_2SO_4 and evaporated in vacuo affording compound **6** as a white solid (0.649 g, 1.67 mmol, 97%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.87 (2H, s), 2.85 (4H, s), 1.54 (18H, s); ESI-MS calcd for $C_{16}H_{24}N_2O_9$ 388.1, found *m*/*z* 411.1 $(M + Na)^+$, 427.1 $(M + K)^+$.

Fmoc-Lys[*N*,*N*-bis-Boc-aminooxyacetyl]-OH 2. Compound **6** (0.3 g, 0.77 mmol) in methylene chloride (4 mL) was added dropwise to a stirred suspension of TFA-salt of Fmoc-lysine (0.313 g, 0.85 mmol) and DIPEA (0.150 mL, 0.86 mmol) in methylene chloride (4 mL). The pH of the resulting reaction mixture was regularly adjusted to pH 8−9 by further additions of DIPEA. After 3 h, reaction of the excess Fmoc-lysine was filtered off and the filtrate washed with 5% aqueous $NaHCO_3$ (3 × 5 mL), water (2 × 5 mL), and brine (2 × 5 mL). The organic phase was dried over Na_2SO_4 and evaporated in vacuo. The crude product was purified by silica gel chromatography with methylene chloride/methanol/acetic acid (95:4.5:0.5), thereby providing building block **2** as a white powder (0.247 g, 0.385 mmol, 50%): analytical HPLC t_R = 12.1 min; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.75 (2H, d, *J* = 7.4 Hz), 7.60 (2H, broad d, *J* = 7.4 Hz), 7.39 (2H, t, *J* = 7.4 Hz), 7.30 (2H, td, *J* = 7.4, 1.2 Hz), 5.55 (1H, d, *J* = 7.7 Hz), 4.45 (2H, s), 4.39 (3H, m), 4.22 (1H, t, *J* = 6.8 Hz), 3.33 (2H, m), 1.94−1.13 (7H, m), 1.53 (18H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 175.0, 168.1, 156.1, 150.5, 143.8, 141.3, 127.7, 127.0, 125.1, 119.9, 85.3, 67.0, 53.6, 47.2, 38.6, 33.6, 31.7, 28.8, 28.0, 24.8, 22.2; ESI-MS calcd for $C_{33}H_{43}N_3O_{10}$ 641.3, found *m*/*z* 664.2 $(M + Na)^+$, 642.2 $(M + H)^+$.

2-(1-Ethoxyethylideneaminooxy)acetic Acid 7. To a stirred solution of iodoacetic acid (9.00 g, 48.4 mmol) in water (19 mL) at 0 °C was added aqueous NaOH (3.0 mL, 40% w/w). The resulting solution was allowed to heat to room temperature, whereafter ethyl *N*-hydroxyacetimidate (6.0 g, 58.3 mmol) was added followed by aqueous NaOH (4.5 mL, 40% w/w) and water (19 mL) (pH of solution >12). After 4.5 h of stirring at 80 °C and cooling to room temperature, water was added (70 mL) and the aqueous mixture was washed with methylene chloride (3 × 50 mL). The water phase was brought to pH 2−3 with a 1 M hydrochloride solution. The acidified water phase was then extracted with methylene chloride (4 × 50 mL), and the combined organic phases from this last extraction was washed with brine (50 mL), dried over Na_2SO_4, and concentrated in vacuum, providing compound **7** as a colorless oil (5.69 g, 35.3 mmol, 73%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.48 (2H, s), 4.00 (2H, q, *J* = 7.2 Hz), 2.01 (3H, s), 1.27 (3H, t, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 174.5, 165.0, 70.2, 62.8, 14.2, 14.0; DCI-MS calcd for $C_6H_{11}NO_4$ 161.2, found *m*/*z* 162.0 $(M + H)^+$.

***N*-Hydroxysuccinimidyl 2-(1-Ethoxyethylideneaminooxy)acetate 8.** To a stirred solution of compound **7** (5.69 g, 35.3 mmol) and *N*-hydroxysuccinimide (4.06 g, 35.3 mmol) in ethyl acetate/dioxane (120 mL, 1:1) at 0 °C was added DCC (7.28 g, 35.3 mmol) in one portion. The resulting mixture was stirred at room temperature for 5 h. The formed DCU was filtered off and the filtrate concentrated under vacuum. The obtained residue was dissolved in ethyl acetate (300 mL), and the solution was washed with 5% aqueous $NaHCO_3$ (2 × 75 mL), water (75 mL), and brine (75 mL). The organic solution was dried over Na_2SO_4 and evaporated under vacuum to oil which was used without further purification (8.69 g, 33.7 mmol, 95%): analytical HPLC t_R = 7.6 min; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.78 (2H, s), 4.01 (2H, q, *J* = 7.2 Hz), 2.84 (4H, s), 1.98 (3H, s), 1.28 (3H, t, *J* = 7.2 Hz).

Fmoc-Lys[*N*-Eei-Aoa]-OH 3. To a stirred mixture of Fmoc-lysine (6.22 g, 16.9 mmol) and DIPEA (3.0 mL, 17.3 mmol) in methylene chloride (100 mL) at room temperature was added dropwise over 20 min a solution of the above-prepared NHS-ester

8 (4.35 g, 16.9 mmol) in methylene chloride (40 mL). The pH of the resulting mixture was regularly adjusted to pH 8−9 by further additions of DIPEA. After 1 h of reaction, the unreacted Fmoc-lysine was filtered off and the filtrate was concentrated under vacuum providing an oily residue. After addition of methylene chloride (100 mL), the organic phase was washed with a concentrated citric acid solution. The aqueous phase was then extracted with methylene chloride (3 × 80 mL). The combined organic phases were then washed with brine (100 mL), dried over Na_2SO_4, and evaporated under reduced pressure. To the residue were added 60 mL of acetonitrile and 60 mL of water. The product was lyophilized, thereby providing the compound **3** as a white powder (7.47 g, 14.6 mmol, 86%): analytical HPLC t_R = 10.8 min; ^{1}H NMR ($CDCl_3$,-300 MHz) δ 7.75 (2H, d, J = 7.4 Hz), 7.60 (2H, d, J = 7.4 Hz), 7.38 (2H, t, J = 7.4 Hz), 7.29 (2H, t, J = 7.4 Hz), 6.50 (1H, t, J = 5.6 Hz), 5.70 (1H, d, J = 7.8 Hz), 4.38−4.36 (5H, m), 4.20 (1H, t, J = 6.9 Hz), 3.96 (2H, q, J = 7.1 Hz), 3.34 (2H, m), 1.96 (3H, s), 1.81 (2H, m), 1.58 (2H, m), 1.45 (2H, m), 1.24 (3H, t, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 174.7, 171.3, 164.4, 156.2, 143.7, 141.3, 127.7, 127.1, 125.1, 119.9, 72.6, 67.1, 62.8, 53.7, 47.2, 38.5, 31.7, 29.1, 22.1, 14.2, 13.9; ESI-MS calcd for $C_{27}H_{33}N_3O_7$ 511.2, found m/z 512.1 $(M + H)^+$.

Boc-Ser(*t*-Bu)-*N*-hydroxysuccinimide Ester 9. To a stirred solution of Boc-Ser(*t*-Bu)-OH (2.8 g, 10 mmol) and *N*-hydroxysuccinimide (1.2 g, 10.7 mmol) in ethyl acetate/dioxane (1:1, 100 mL) cooled on an ice bath were added DCC (2.2 g, 10.7 mmol). After 5 h of stirring at room temperature, the reaction mixture was filtered through a pad of Celite with ethyl acetate and the collected filtrate was concentrated in vacuo. The obtained oily residue was taken into 150 mLof ethyl acetate and washed with 5% aqueous $NaHCO_3$ (2 × 70 mL), water (2 × 70 mL), and brine (2 × 70 mL). The organic fraction was then dried over Na_2SO_4. Boc-Ser-(*t*-Bu)-NHS was obtained as a white powder after evaporation of the solvent (3.5 g, 9.8 mmol, 98%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.41 (1H, d, J = 9.0 Hz), 4.78 (1H, d, J = 9.0 Hz), 3.92 (1H, m), 3.66 (1H, m), 2.82 (4H, s), 1.46 (9H, s), 1.20 (9H, s); ESI-MS calcd for $C_{16}H_{26}N_2O_7$ 358.2, found m/z 381.1 $(M + Na)^+$, 360.3 $(M + H)^+$.

Fmoc-Lys[Boc-Ser(*t*-Bu)]-OH 4. The above Boc-Ser(*t*-Bu)-NHS (8.00 g, 22.3 mmol) in methylene chloride (75 mL) was added dropwise over 30 min to a stirred suspension of finely powdered TFA-salt of Fmoc-lysine (10.90 g, 22.6 mmol) and DIPEA (15.6 mL, 90.0 mmol) in methylene chloride (130 mL). After 5 h of reaction, the solvent was evaporated in vacuo and the remaining residue was dissolved in ethyl acetate (400 mL), followed by washing with 10% aqueous citric acid solution (70 mL), water (2 × 100 mL), and finally brine (100 mL). The organic fraction was dried over Na_2SO_4, and evaporation under vacuum gave 11.85 g of a white solid. This solid was recrystallized from methylene chloride with a hexane−diethyl ether solution (95:5) giving Fmoc-Lys[Boc-Ser(*t*-Bu)]-OH **4** as a pure white powder (8.94 g, 14.6 mmol, 66%): analytical HPLC t_R = 11.7 min; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.68 (2H, d, J=7.5 Hz), 7.55 (2H, d, J=7.5 Hz), 7.33−7.18 (4H, m), 6.84 (1H, broad s), 6.25 (1H, broad d), 5.62 (1H, broad s), 4.34−4.06 (5H, m), 3.63 (1H, m), 3.36 (1H, m), 3.20−3.05 (2H, m), 1.84 (1H, m), 1.65 (1H, m), 1.47−1.32 (14H, m), 1.09 (9H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 178.0, 171.0, 156.6, 155.7, 144.0, 141.2, 127.5, 127.0, 125.2, 119.8, 73.8, 66.8, 62.0, 55.8, 52.7, 47.1, 38.9, 32.0, 29.1, 28.3, 27.4, 25.6, 22.5; ESI-MS calcd for $C_{33}H_{45}N_3O_8$ 611.3, found m/z 634.2 $(M + Na)^+$, 612.2 $(M + H)^+$.

c[-Lys(-CO-CH_2ONH$_2$)-Lys(Boc-Ser(*t*-Bu))-Lys(-CO-CH_2-ONH$_2$)-Pro-Gly-Lys(-CO-CH_2ONH$_2$)-Ala-Lys(-CO-CH_2-ONH$_2$)-Pro-Gly-] 10. The 2-chlorotrityl resin (100 mg) was preloaded for 30 min in dry CH_2Cl_2 with Fmoc-Gly-OH substituted at 0.57−0.74 mmol/g and capped with 6 mL of methanol/DIPEA/methylene chloride (2/1/17) for 5 min and then one more time for 10 min. Fmoc group removals and Fmoc-Pro-OH coupling reaction were performed as described in the general procedure providing H-Pro-Gly resin.

Procedure A. The building block **1** was introduced using the coupling reaction described in the general procedure. The reaction was repeated one time affording the tripeptide **11** and the branched tripeptide **12**. **11**: ESI-MS calcd for $C_{35}H_{45}N_5O_{10}$ 695.3, found m/z 696.2 $(M + H)^+$. **12**: ESI-MS calcd for $C_{63}H_{78}N_8O_{17}$ 1218.5, found m/z 1219.2 $(M + H)^+$.

Fmoc group removals and coupling of Fmoc-Ala-OH were performed as described in the general procedure yielding the tetrapeptide **13**, the branched tetrapeptides **14** and **15**. **13**: ESI-MS calcd for $C_{38}H_{50}N_6O_{11}$ 766.4, found m/z 767.3 $(M + H)^+$. **14**: ESI-MS calcd for $C_{56}H_{65}N_7O_{14}$ 1059.5, found m/z 1060.3 $(M + H)^+$. **15**: ESI-MS calcd for $C_{51}H_{73}N_9O_{16}$ 1067.5, found m/z 1068.1 $(M + H)^+$.

Procedure B. The building block **2** was introduced using the coupling reaction described in the general procedure. The reaction was repeated one time affording the tripeptide **16**: ESI-MS calcd for $C_{40}H_{53}N_5O_{12}$ 795.4, found m/z 796.2 $(M + H)^+$.

Fmoc group removals were performed as described in the general procedure providing the tripeptide **17**: ESI-MS calcd for $C_{20}H_{35}N_5O_8$ 473.2, found m/z 474.1 $(M + H)^+$. Coupling of Fmoc-Ala-OH was done as described in the general procedure yielding the tetrapeptide **13** and the branched tetrapeptide **14**.

Procedure C. The building block **3** was introduced using the coupling reaction described in the general procedure. The reaction was repeated one time affording the tripeptide **18**: ESI-MS calcd for $C_{34}H_{43}N_5O_9$ 665.3, found m/z 666.2 $(M + H)^+$.

Fmoc group removals were performed as described in the general procedure providing the tripeptide **19**: ESI-MS calcd for $C_{19}H_{33}N_5O_7$ 443.2, found m/z 444.0 $(M + H)^+$.

Coupling of Fmoc-Ala-OH was done as described in the general procedure yielding the tetrapeptide **20**: ESI-MS calcd for $C_{37}H_{48}N_6O_{10}$ 736.3, found m/z 737.2 $(M + H)^+$.

Fmoc group removals, coupling reactions of other amino acids, and cleavage of the peptide from the support were performed as described in the general procedure. The linear decapeptide **21** was then obtained as a white powder (117.0 mg, 0.059 mmol): ESI-MS calcd for $C_{83}H_{144}N_{20}O_{27}$ 1853.1, found m/z 1854.8 $(M + H)^+$.

The cyclization reaction was carried out using the crude linear peptide **21** (10 mg, 5.1 μmol) as described above affording the product as a white powder (10.3 mg, 5.1 μmol). Full deprotection was carried out on crude material (3.0 mg, 1.6 μmol) using 5 mL of a solution containing TFA/H_2O/TIS (95:2.5:2.5) at room temperature during 2 h. The product was isolated after removal of solvents under reduced pressure and precipitation from diethyl ether to yield compound **10** as a white powder (2.9 mg, 1.5 μmol, 94%): ESI-MS calcd for $C_{58}H_{102}N_{20}O_{20}$ 1398.8, found m/z 1399.6 $(M + H)^+$.

c[-Lys(Boc-Ser(*t*-Bu))-Lys(−CO−CH_2ONH$_2$)-Lys(Boc-Ser(*t*-Bu))-Pro-Gly-Lys(Boc-Ser(*t*-Bu))-Ala-Lys(Boc-Ser(*t*-Bu))-Pro-Gly-] 23. The linear peptide H-Lys(Boc-Ser(*t*-Bu))-Lys(-CO-CH_2O-N═C(OEt)Me)-Lys(Boc-Ser(*t*-Bu))-Pro-Gly-Lys(Boc-Ser(*t*-Bu))-Ala-Lys(Boc-Ser(*t*-Bu))-Pro-Gly-OH was assembled on 2-chlorotrityl resin (100 mg) using procedure C. The resin was preloaded for 30 min in dry CH_2Cl_2 with Fmoc-glycine substituted at 0.66 mmol/g and capped for 5 min with 6 mL of methanol/DIPEA/methylene chloride (2:1:17) and then one more time 10 min. Fmoc group removals, coupling reactions, and cleavage of the peptide from the support were performed as described in the general procedure. The linear decapeptide **22** was then obtained as a white powder (91.3 mg, 0.040 mmol, 61%): ESI-MS calcd for $C_{101}H_{180}N_{20}O_{30}$ 2153.3, found m/z 2154.4 $(M + H)^+$.

The cyclization reaction was carried out using the crude peptide **22** (10 mg, 4.4 μmol) as described above affording the product as a white powder (8.8 mg, 4.1 μmol). Full deprotection was carried out on crude material (3.0 mg, 1.4 μmol) using 5 mL of a solution containing TFA/H_2O/TIS (95:2.5:2.5) at room temperature during

5 h. The product was isolated after removal of solvents under reduced pressure and precipitation from diethyl ether to yield compound **23** as a white powder (2.5 mg, 1.2 μmol, 90%): ESI-MS calcd for $C_{61}H_{108}N_{20}O_{20}$ 1440.8, found *m*/*z* 1441.5 $(M + H)^+$.

c[-Lys(-CO-CH_2O−N═C(OEt)Me)-Lys(Boc-Cys(NPys))-Lys(-CO-CH_2O−N═C(OEt)Me)-Pro-Gly-Lys(-CO-CH_2O-N═C(OEt)Me)-Ala-Lys(-CO-CH_2O−N═C(OEt)Me)-Pro-Gly-] 26. The linear peptide H-Lys(-CO-CH_2O-N═C(OEt)Me)-Lys(Alloc))-Lys(-CO-CH_2O−N═C(OEt)Me)-Pro-Gly-Lys(-CO-CH_2-O-N═C(OEt)Me)-Ala-Lys(-CO-CH_2O-N═C(OEt)Me)-Pro-Gly-OH was assembled on 2-chlorotrityl resin (750 mg) using procedure C. The resin was preloaded for 30 min in dry CH_2Cl_2 with Fmoc-glycine substituted at 0.3 mmol/g and capped for 5 min with 6 mL of methanol/DIPEA/methylene chloride (2/1/17) and one more time for 10 min. Fmoc group removals, coupling reactions, and cleavage of the peptide from the support were performed as described in the general procedure. The linear decapeptide **24** was then obtained as a white powder (480 mg, 2.7 mmol): ESI-MS calcd for $C_{75}H_{127}N_{19}O_{25}$ 1693.9, found *m*/*z* 1732.5 $(M + K)^+$.

The cyclization reaction was carried out using the crude peptide **24** (1.6 g, 956 μmol) as described above affording the product as a white powder (1.3 mg, 775 μmol, 81%): ESI-MS calcd for $C_{75}H_{125}N_{19}O_{24}$ 1675.9, found *m*/*z* 1698.4 $(M + Na)^+$.

Alloc group was removed using cyclic peptide (300 mg, 0.18 mmol) dissolved in a solution containing 18 mL of dry CH_2Cl_2 and DMF (3:1) under argon by adding phenylsilane (2.0 g, 18.5 mmol) for 3 min and then $Pd^0(PPh_3)_4$ (62.4 mg, 54 μmol) for 30 min at room temperature. The solvent was removed under reduced pressure. The oily residue was dissolved in the minimum of a solution containing a mixture of methylene chloride and methanol (1:1). Ether was added to precipitate the crude product **25**. Then it was triturated and washed 3 times with ether affording compound **25** as a white powder (240 mg, 0.151 mmol, 84%): ESI-MS calcd for $C_{71}H_{121}N_{19}O_{22}$ 1591.9, found *m*/*z* 1592.5 $(M + H)^+$.

Boc-Cys(NPys)-OH (60.1 mg, 0.16 mmol) and PyBOP (83.2 mg, 0.16 mmol) were added to a solution containing the peptide **25** (257 mg, 0.16 mmol) in 16 mL of DMF and DIPEA to adjust the pH at 8.0. The reaction was stirred for 30 min at room temperature and then concentrated under diminished pressure. The crude product was triturated and washed with ether to yield compound **26** as a white powder (300.2 mg, 0.154 mmol, 96%): ESI-MS calcd for $C_{84}H_{136}N_{22}O_{27}S_2$ 1948.9, found *m*/*z* 1988.2 $(M + K)^+$.

c[-Lys(-CO-CH_2ONH_2)-Lys(Boc-Cys(S-EtOH))-Lys(-CO-CH_2-ONH_2)-Pro-Gly-Lys(-CO-CH_2ONH_2)-Ala-Lys(-CO-CH_2ONH_2)-Pro-Gly-] 28. Compound **26** (10 mg, 5.13 μmol) and 2-mercaptoethanol (1 μL, 3 equiv) were added to a solution containing 500 μL of PBS (pH 5.0) and DMF (1:3). The reaction was stirred for 5 min at room temperature and then the product **27** purified using RP-HPLC (1.2 mg, 0.64 μmol, 13%): ESI-MS calcd for $C_{81}H_{138}N_{20}O_{26}S_2$ 1871.0, found *m*/*z* 1872.3 $(M + H)^+$.

Compound **27** (0.1 mg, 0.05 μmol) was added to a solution containing 100 μL of TFA and H_2O (1:1). The reaction was stirred for 1 h at room temperature, and then the product **28** analyzed by HPLC and ES-MS: ESI-MS calcd for $C_{60}H_{107}N_{20}O_{20}S_2$ 1490.7, found *m*/*z* 1491.5 $(M + H)^+$.

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Supporting Information Available: HPLC, MS, and NMR conditions; ^1H NMR spectra of **1**−**9**; ^{13}C NMR spectra of **2**−**4** and **7**; MS spectra of **1**−**7** and **9**−**23**; HPLC chromatograms of **1**−**4**, **8**, **10**, **16**−**28**, c[-RGDfK(ONH_2)], and c[-CNGRC-]-K(alloc)K(ONH_2) derivatives; experimental procedures for c[-RGDfK(ONH_2)] and c[-CNGRC-]-K(alloc)K(ONH_2) derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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