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# Solid-Phase Functionalization of Peptides by an α-Hydrazinoacetyl Group

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A novel procedure for the preparation of α-hydrazinoacetyl peptides is reported on the basis of the solid-phase coupling of partially or fully Boc-protected hydrazino acetic acid derivatives. The degree of unwanted polymerization of the activated ester during both activation and coupling was found to be significant for the monoprotected derivative $BocNHNHCH_2CO_2H$ but could be minimized with the diprotected derivative $BocNHNH(Boc)CH_2CO_2H$ and suppressed with the fully protected acid. Despite the instability of the imidocarbonate group toward acids and bases, a low-cost and effective route was sought for the preparation of the tris(Boc)-protected derivative. The *N*,*N*,*N*′-tris(Boc)hydrazinoacetic acid could be introduced on the solid phase after or before peptide elongation using Fmoc/*tert*-butyl chemistry. In this latter case, HR MAS NMR analysis of model solid supports demonstrated the partial loss of one Boc group during the repetitive piperidine treatments. Despite this slight instability, *N*,*N*,*N*′-tris(Boc)hydrazinoacetic acid was found to be a highly convenient reagent for the robust and easily scalable preparation of hydrazinopeptides in good yield and high purity.

## Introduction

The modification of peptides by an α-hydrazinoacetyl moiety allows the site-specific derivatization of fully deprotected peptides owing to the easy discrimination between the hydrazino group and the other functionalities naturally present on peptides. For example, the large difference in p$K_a$ between an α-hydrazinoacetamide group and $\epsilon$ amino groups was exploited for the chemoselective acylation of the former by fatty acid succinimidyl esters, thus providing a valuable access to complex lipopeptides.[1] The α-hydrazinoacetyl moiety was also successfully engaged in hydrazone formation by reaction with α-oxo aldehydes, either for the synthesis of lipopeptides,[2] the site-specific anchoring of peptides or mannose-mimetic clusters onto the surface of vesicles,[3] the synthesis of oligonucleotide−peptide conjugates,[4] or the synthesis of clustered glycoside−antigen conjugates using two one-pot, orthogonal, chemoselective ligation reactions.[5]

Owing to the usefulness of these peptide derivatives, we have examined various methods for their solid-phase synthesis based upon the Fmoc/*tert*-butyl strategy, since the α-hydrazinoacetamide group was found to be stable in the concentrated TFA mixture used for side-chain deprotection.[6] In a first approach, the N−N bond was elaborated on the solid phase by *N*-electrophilic amination of a glycyl residue with *N*-Boc-3-(4-cyanophenyl)-oxaziridine (BCPO):[1a,b,7] Various hydrazinopeptides were successfully synthesized using this process, which however cannot be easily scaled up[8] and generalized.[5b] An interesting and practical alternative resides in the derivatization of the peptidyl resin by an α-bromoacetyl group followed by displacement of the bromine atom by *tert*-butylcarbazate.[9] In search of a robust and easily

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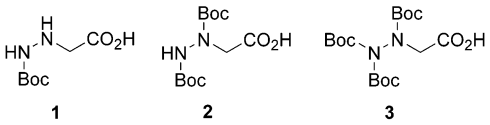


**FIGURE 1.** Mono, di and tri-protected α-hydrazinoacetic derivatives **1**−**3**.

scalable methodology for the synthesis of hydrazinopeptides using automated solid-phase techniques, we have envisioned the introduction of the hydrazino moiety through a classical acid activation using Boc-protected derivatives of α-hydrazinoacetic acid (Figure 1). We report in this paper the synthesis of mono-, di-, and triprotected derivatives **1**−**3** and the study of their usefulness for the synthesis of hydrazinopeptides. Some preliminary results were described before in a short report.[1a] Preparation of fully protected derivative **3** proved to be troublesome due to the sensitivity of one of the Boc groups to both aqueous acids and bases. Coupling of **1**−**3** to a model peptidyl resin was examined using various activation procedures. In particular, the impact of the activation method upon the extent of polymerization of diprotected derivative **2** was documented. The stability of supported triprotected α-hydrazinoacetyl moieties toward piperidine, diluted TFA, or acetic anhydride/diisopropylethylamine was also examined using high-resolution magic angle spinning (HR MAS) NMR spectroscopy to document its behavior during the Fmoc/*tert*-butyl solid-phase peptide synthesis.

## Results and Discussion

During the past decade, several methods allowing the preparation of either free,[10] $N_\beta$-protected or $N_\beta$,$N_\alpha$-orthogonally bisprotected[11] α-hydrazino acids have been reported. However, little attention has been devoted to the coupling of these derivatives to peptidyl resins for the solid-phase synthesis of hydrazinopeptides. Side reactions arising during the activation and coupling of $N_\beta$-protected α-hydrazino acids have been partially addressed only recently by Guy et al.[12] Coupling with mixed anhydrides prepared from $ClCO_2$-*i*-Bu failed, since once the anhydride formed, its acyl group was immediately transferred to the $N_\alpha$. The use of *N*-hydroxysuccinimidyl esters led to diketopiperazine and oligomer formation. This coupling worked reasonably well provided the activated ester was formed in situ with hindered hydrazino acids. Protection of the $N_\alpha$-position by a benzyl group solved these problems but required an additional hydrogenolysis step following peptide assembly.

To avoid this additional step following the separation of the α-hydrazinoacetyl peptide from the solid support, we have focused our attention on the synthesis and coupling of α-hydrazinoacetic acid derivatives **1**−**3**, which were synthesized as depicted in Scheme 1.

**Synthesis of Boc-Protected α-Hydrazinoacetic Acid Derivatives 1−3.** Two routes have been devised for the access to Boc-protected derivatives (Scheme 1). Path 1 was found suitable for the production of the three protected acids **1**−**3** on a laboratory scale and to document their ability to be coupled on solid phase. Path 2 was developed as an attractive, low-cost alternative for the preparation of triprotected derivative **3** on a larger scale.

**Path 1.** This strategy relies on the mono acylation of commercially available ethyl hydrazinoacetate hydrochloride **4** using $Boc_2O$ in a $H_2O$/EtOH mixture to give derivative **6a**. Saponification of ester **6a** in ethanolic sodium hydroxide solution yielded $N_\beta$-Boc-protected acid **1**, which could be purified by simple precipitation in *t*-BuOMe (49% overall yield). Attempts to obtain ester **7a** starting directly from ethyl hydrazinoacetate hydrochloride **4** failed and gave only mixtures of mono- and diprotected esters. Thus, synthesis of ester **7a** was performed by treatment of intermediate **6a** with an excess of $Boc_2O/Et_3N$ in the presence of a catalytic amount of DMAP. Triprotected ester **7a** was next treated in a NaOH 1 N/EtOH/$H_2O$ mixture for 3 h at rt. Unexpectedly, this experiment yielded exclusively diprotected acid **2** [13] (66% overall yield) and not the expected triprotected acid **3**. Additional experiments demonstrated the sensitivity of the Boc group to both aqueous NaOH and pH 3−5 aqueous media. The electron-withdrawing effects exerted by the hydrazine substituents in compound **7a** may render the imidodicarbonate more sensitive to nucleophiles. A method to selectively cleave off a Boc-group from a Boc tri-protected hydrazine in acidic conditions has been previously described by Mäeorg et al.[14]

These data in hand, access to triprotected acid **3** could be secured by the careful control of both saponification and acidification steps in term of time, temperature, and pH. Saponification of ethyl *N*,*N*,*N*′-tris(Boc)hydrazinoacetate **7a** was performed at 0 °C for only 25 min. The reaction mixture was acidified to pH 4 with citric acid and quickly extracted to limit the exposure of **3** to aqueous acid. Compound **3** could be purified by precipitation from an $Et_2O$/heptane mixture with a 63% overall yield on a 10 g scale (49% yield on a 50 g scale).

**Path 2.** An alternative route to intermediate ester **6a** starting from the cheap starting materials ethyl bro-

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(12) Guy, L.; Vidal, J.; Collet, A. *J. Med. Chem.* **1998**, *41*, 4833−4843.

(13) The diprotected derivative obtained following saponification was unambiguously identified as corresponding to chemical structure **2** using a heteronuclear multibond coupling (HMBC) NMR experiment. A clear coupling between the $N_\beta$-H and the carbonyl carbon and no correlation with the methylene were observed.

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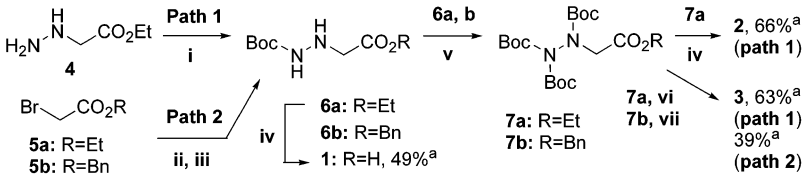
**SCHEME 1**[a]




[a] Key: (a) overall isolated yield; (i) $Boc_2O$ (1.1 equiv), *N*-methylmorpholine (1.1 equiv), $H_2O$/EtOH (1/1), rt, 2 h; (ii) *tert*-butyl carbazate (3 equiv), DMF, 8 h, rt; (iii) glyoxylic acid (4 equiv), $Na_2HPO_4$/NaOH, pH 5.2, rt, overnight; (iv) NaOH (2.2 equiv), $H_2O$/EtOH, 0 °C, 30 min, rt, 3 h; (v) $Boc_2O$ (3 equiv), DMAP, $Et_3N$, $CH_2Cl_2$, rt, 2 h; (vi) NaOH 1 N (1.1 equiv), $H_2O$/EtOH, 0 °C, 25 min; (vii) $H_2$/$Pd(OH)_2$/C, 4°C, 24 h.

moacetate and *tert*-butyl carbazate was examined as shown in Scheme 1 (path 2). In a first approach, the reaction was carried out using a slight excess of ethyl bromoacetate since preliminary experiments indicated that excess of *tert*-butyl carbazate was difficult to separate from ester **6a** during workup. The nucleophilic substitution was found to be faster in DMF than in $CH_3CN$. However, optimization of the reaction parameters could not preclude the formation of a mixture of the expected compound **6a** and a side product arising from dialkylation of *tert*-butyl carbazate.

To overcome this problem, we switched to the use of an excess of *tert*-butyl carbazate (3 equiv). To avoid activation of the hydrazino moiety of **6a** which could lead to overalkylation, the reaction was carried out without base, the generated HBr being trapped by the excess of *tert*-butyl carbazate. Using these experimental conditions, only traces of the byproduct were detected by TLC.

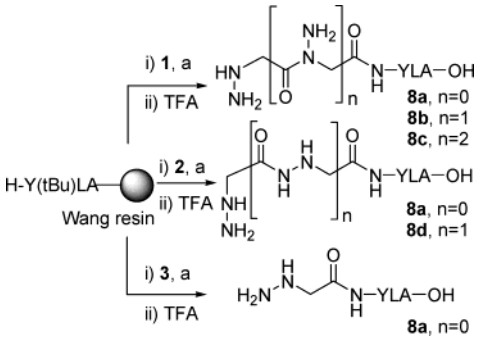
At this stage of the process, the unreacted *tert*-butyl carbazate had to be removed as it was anticipated that **3** and the tetra-Boc hydrazine, inevitably formed during the next step, would be difficult to separate. To eliminate the excess by a simple aqueous extraction, we sought to transform the carbazate into an acidic compound to facilitate its separation from the intermediate ethyl Boc-hydrazinoacetate **6a** during workup. Indeed, the addition of glyoxylic acid to the reaction mixture resulted in the formation of the corresponding hydrazone, which was readily soluble in water at basic pH.

The monoprotected ester **6a** synthesized using this alternative pathway was further converted into the triprotected acid **3** as described before with a 45% overall yield on a 9 g scale.

In a final optimization step, ethyl ester **6a** was replaced by the corresponding benzyl ester to avoid the saponification step which can lead to a partial loss of one $N_\beta$-Boc group. Indeed, removal of the benzyl ester of compound **7b** was easily performed by hydrogenolysis in the presence of palladium hydroxide on carbon (20% Pd). Starting from benzyl bromoacetate **5b**, compound **3** was obtained via the formation of intermediates **6b** and **7b** in a 39% overall yield on a 49 g scale.

**NMR Characterization.** Unlike monoprotected compound **1**, hydrazino acids **2**, and **3** presented complex $^1$H NMR spectra in some deuterated solvents, suggesting a slow exchange on the NMR time scale between conformers. Indeed, $^1$H NMR spectra of diprotected acid **2** in DMSO-$d_6$ at 300 K showed four distinct sets of resonances for $N_\beta$-H proton (relative intensities 53/26/14/7). At 330 K, the same proton appeared as a broad singlet. This phenomenon could be the consequence of a cis/trans equilibrium for each carbamate. In DMF-$d_7$ at 300 K, the methylene group came out as a singlet for both compounds **2** and **3**, whereas in $CD_2Cl_2$ they appeared as a singlet for diprotected derivative **2** but as two singlets in a nearly 1/1 ratio for **3**.

**SCHEME 2**[a]




[a] Key: (a) BOP, DIEA, DMF.

**Solid-Phase Functionalization Studies.** With the three acid compounds **1**−**3** in hand, we next examined their coupling onto a solid phase for the functionalization of peptides by an α-hydrazinoacetyl moiety. A model tripeptide YLA was assembled on a Wang resin using the Fmoc/*tert*-butyl strategy (Scheme 2).

After peptide elongation, the resin was divided into three samples. The N-terminal Fmoc group was removed with piperidine, and the peptidyl resins were reacted with either derivative **1**−**3** (4 equiv) using an in situ BOP activation (chosen to minimize the potential risk of azalactone formation during the activation step). After 30 min at rt, a ninhydrin test[15] indicated the completion of the acylation. Following deprotection and cleavage in TFA, the peptides were precipitated in cold $Et_2O$ and freeze-dried. The purity of the target peptide **8a** was evaluated by RP-HPLC, CZE and ES-MS. We have found that only ES-MS allowed detection of the presence of oligomers ($n$ = 1−2, Table 1 and Scheme 2) in the mixture, which could be easily quantified as described elsewhere.[16] Their amount and distribution depended upon the number of Boc groups on the hydrazino moiety (Table 1). The use of the monoprotected acid **1** led to the formation of monomer **8a**, dimer **8b** and trimer **8c** in a 83/14/3 ratio (Table 1, entry 1). These results confirmed the observations of Guy et al., who reported the formation of polymeric products during activation of $N_\alpha$-unprotected

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**TABLE 1. ES-MS Analysis of Hydrazinopeptide 8 Synthesized Using Derivatives 1, 2, or 3**

| | | hydrazinopeptides[a,b] | | |
|---|---|---|---|---|
| entry | α-hydrazino acid | **8a** | **8b/8d** | **8c** |
| 1 | **1** | 83 (1.0) | 14 (0.8) | 3 (0.2) |
| 2 | **2** | 92 (1.2) | 7.8 (0.5) | 0 |
| 3 | **3** | 100 | 0 | 0 |

[a] % estimated by ES-MS as a percentage of the total area (orifice voltage of 30 V, sample dissolved in $H_2O$/MeCN (4/1) formic acid 0.1%, injection at 5 μL per min). [b] The standard deviation for three measurements is indicated in parentheses.

hydrazino acids. The authors solved this problem by using $N_\beta$-Boc-$N_\alpha$-Bn-bisprotected hydrazino acids. But in our hands, the use of diprotected derivative **2** did not preclude the formation of dimer **8d**, which was obtained at a rate of 8% (Table 1, entry 2). The fact that $N_\beta$ in acid **2** retained some nucleophilic character led us to examine the use of the fully protected derivative **3**. Indeed, acylation of the peptidyl resin with activated **3** led to the expected α-hydrazino acetyl peptide **8a** in a high purity. Oligomers could not be detected by ES-MS (Table 1, entry 3).

Nevertheless, regarding the low rate of dimer formed with the diprotected acid **2** and the better chemical stability of this compound compared to the fully protected one, we undertook the optimization of the coupling step. Various activating agents such as PyBOP, DIC, DIC/HOSu, HBTU/HOBt were also investigated as possible reagents for the solid-phase coupling of the diprotected acid onto the model peptidyl resin used before. The time of coupling was fixed at 40 min and the completion of the reaction was verified using either ninhydrin or TNBS tests.[17] Again, the rate of oligomer formation was quantified using ES-MS following deprotection and cleavage from the resin. Regardless of the coupling agent used, the trimer species **8c** were not detected. The amount of dimer **8d** ranged from 18.0% to 1.8% depending on the nature of the coupling agent (Table 2). Experiments performed with BOP or PyBOP gave a rate ranging from 4.9% to 7.8% (Table 2, entry 1 and 2). The best results were obtained using HBTU/HOBt/DIEA, DIC, or DIC/HOSu (Table 2, entries 3, 4, and 6), but dimer formation could not be wholly suppressed. As expected, increasing the time of preactivation from 15 min to overnight resulted in an increase in the amount of dimer formed (18%, Table 2, entry 5). Finally, the coupling carried out on an automated peptide synthesizer with 4 equiv of compound **2** using a standard HBTU/HOBt/DIEA activation procedure led to only 2% of dimer formation (Table 2, entry 6).

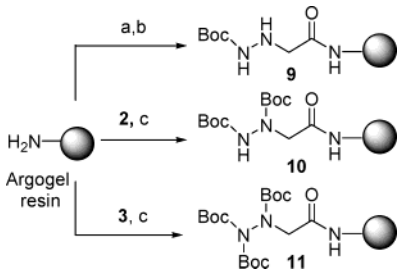
Such a low degree of dimer formation suggest that derivative **2** could be used for hydrazinopeptide synthesis. However, the problem is expected to be worse in the case of a difficult synthesis necessitating multiple couplings[19] or when a peptide has to be functionalized by multiple α-hydrazinoacetyl groups such as for the synthesis of lysinyl dendrimers. In this latter case, a side reaction,

**TABLE 2. Optimization of Diprotected α-Hydrazinoacetic 2 Coupling**

| | | | hydrazinopeptides[b] | |
|---|---|---|---|---|
| entry | coupling agents | activation | **8a** | **8d** |
| 1 | BOP (4 equiv)/ DIEA (12 equiv) | in situ | 92.2 | 7.8 |
| 2 | PyBOP (4 equiv)/ DIEA (12 equiv) | in situ | 95.1 | 4.9 |
| 3 | DIC(4 equiv) | 15 min | 97.7 | 2.3 |
| 4 | HOSu (8 equiv)/ DIC (4 equiv) | 15 min | 98.2 | 1.8 |
| 5 | HOSu (4 equiv)/ DIC (4 equiv) | overnight at 4 °C | 82.0 | 18.0 |
| 6 | HBTU (4 equiv)/ HOBt(4 equiv) DIEA (12 equiv)[a] | 1 min | 98.0 | 2.0 |

[a] Coupling performed on an automatic peptide syntheziser Applied Biosystems 431 A. [b] % estimated by ES-MS as a percentage of the total area (orifice voltage of 30 V, sample dissolved in $H_2O$/MeCN (4/1) formic acid 0.1%, injection at 5 μL per min).

**SCHEME 3**[a]




[a] Key: (a) $BrCH_2CO_2H$, DIC, NMP; (b) Boc-$NHNH_2$, DIEA, NMP; (c) HOBt, HBTU, DIEA, NMP.

which can be neglected during a linear synthesis, can be deleterious during the synthesis of branched dendrimers. Typically, 2% of dimer formation is expected to generate about 16% of impurities during the functionalization of a tetravalent lysinyl tree. We have experienced that oligomers formed during activation are very difficult to separate from the target hydrazinopeptide by RP-HPLC. Thus, we recommend the use of the fully protected derivative **3** which can be easily synthesized at low cost using the simple procedure described before.

**Solid-Phase HR-MAS NMR Reactivity Studies.** Regarding the partial instability of triprotected acid **3** in both basic or slightly acidic media, we have investigated the behavior of solid supports acylated with **3** during the standard procedures used for the Fmoc/*tert*-butyl peptide elongation. To carry out this study, *N*,*N*,*N*′-tris(Boc)hydrazinoacetyl group was anchored to an Argogel resin. The modified solid support was treated with 20% piperidine in DMF or 1% TFA in $CH_2Cl_2$, i.e., usual conditions used during peptide elongation for Fmoc or Mtt removal. The fate of the compound linked onto the resin was monitored by HR-MAS NMR spectroscopy.

Mono-, di-, and triprotected hydrazino derivatives were prepared onto the solid support as described in Scheme 3 and served as references for the chemical shifts. Around 5 mg of each resin were swollen in $CD_2Cl_2$ and studied by HR MAS NMR (Figure 2). The choice of the solvent for the analysis was found to be crucial to observe a difference between the three different resins. In DMF-$d_7$, the $CH_2$ protons appeared as a broad singlet for all

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(18) Warrass, R.; Wieruszeski, J. M.; Lippens, G. *J. Am. Chem. Soc.* **1999**, *121*, 3787−3788.

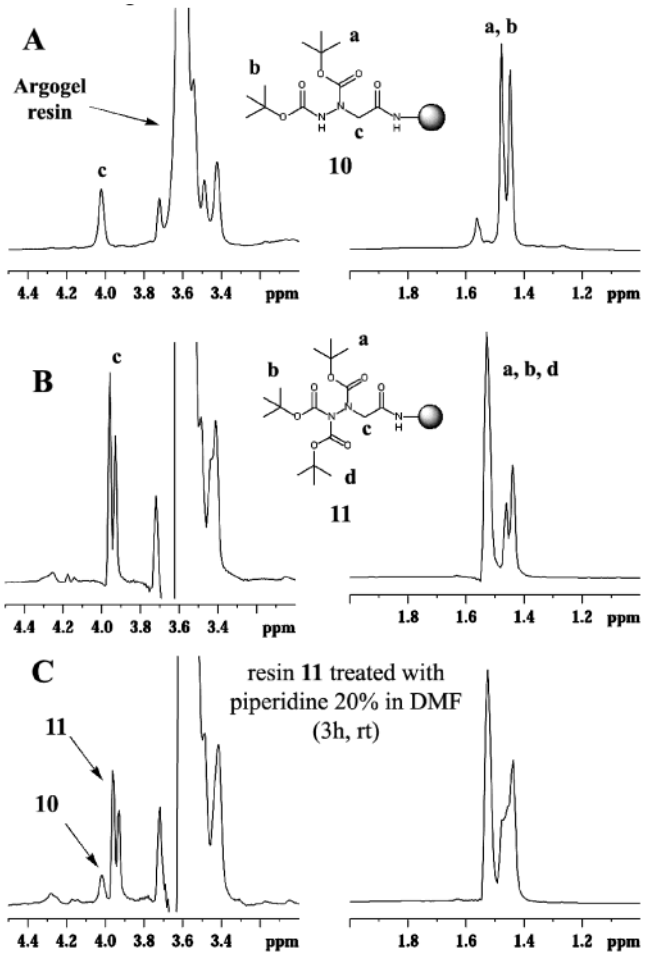
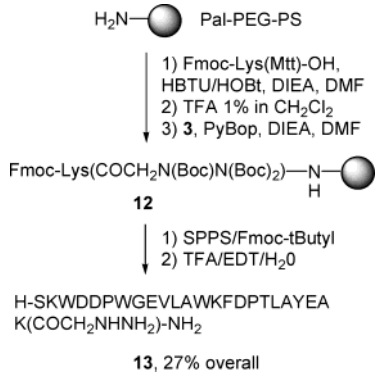



**FIGURE 2.** HR MAS NMR spectra in $CD_2Cl_2$ at 600 MHz recorded with the LED diffusion sequence: (A) resin **10**; (B) resin **11**; (C) resin **11** treated with piperidine 20% in DMF during 3 h at rt.

the three derivatives, whereas in $CD_2Cl_2$ the $CH_2$ protons were observed as a singlet for resin **10** but as two singlets for resin **11** (Figure 2A,B). $^1$H NMR spectra were recorded on a 600 MHz using the Longitudinal Eddy current Delay (LED) sequence to eliminate residual signals arising from protonated species diffusing freely in solution.[18] This sequence allowed us to perform the analyses in the presence of the reagents used in the stability study.

First, the potential removal of an Mtt group while keeping the chemical integrity of tri-protected hydrazinoacetyl group was examined by treating resin **11** with 1% TFA in $CH_2Cl_2$ for 30 min and 1, 3, or 5 h at rt. Within this time, the Boc group of the imidodicarbonate displayed excellent stability. The stability of the same Boc group toward 20% piperidine in DMF was then examined. After 30 min at rt, a sample of this resin was washed and loaded in a rotor. Based on the integration of the $CH_2$ signals, we found a conversion of 5% of the triprotected form into the diprotected one. An analysis after 3 h indicated about 17% of deprotection (Figure 2C). The resin was then washed and treated with $Ac_2O$ 10%/diisopropylethylamine 5% in $CH_2Cl_2$ for 10 min to determine the propensity of the $N_\beta$-deprotected group to undergo acetylation. No product of acetylation could be detected in the $^1$H HR MAS NMR spectrum.[19]

**SCHEME 4**




**Synthesis of a Model Peptide Using Triprotected Hydrazinoacetic Acid 3.** To illustrate the usefulness of triprotected acid **3** for the efficient preparation of α-hydrazinopeptides with a good yield and high purity, model peptide **13** derived from Nef protein encoded by the Simian Immunodeficiency Virus was synthezised using the Fmoc/*tert*-butyl strategy (Scheme 4).

Fmoc-Lys(Mtt)-OH was first coupled onto a Pal-PEG-PS resin using HBTU/HOBt activation. Following Mtt deprotection with 1% TFA in $CH_2Cl_2$,[20] *N*,*N*,*N*′-tris(Boc)-hydrazinoacetic acid **3** was incorporated on $\epsilon$-$NH_2$ group using a PyBOP in situ activation to furnish resin **12**. The peptide was elaborated on the solid phase using standard Fmoc/*tert*-butyl protocols, and then deprotected and cleaved from the resin with TFA/EDT/$H_2O$. Hydrazinopeptide **13** was isolated with a 27% overall yield following RP-HPLC purification. Peptide homogeneity was verified by ES-MS analysis and no byproducts arising from polymerization were detected.

It is worth noting that the organic solvent used for the RP-HPLC purification was found to be crucial. Indeed, we have observed a rapid decomposition of α-hydrazinopeptides when stored in the lyophilized form following RP-HPLC purification with acetonitrile as the mobile phase. This behavior was unexpected, since 6-hydrazinonorleucine-containing peptides were found to be stable for months when purified under the same conditions.[6] The decomposition occurred irrespective of the nature of the counterion (chloride, trifluoroacetate). Finally, α-hydrazinopeptides proved to be fully stable when acetonitrile was replaced by 2-propanol.[21]

These studies allow us to propose an efficient route for the solid-phase incorporation of α-hydrazinoacetyl moiety either on a C-terminal or N-terminal part of a peptide. The fully N,N,N′-triprotected acid **3** was found to be a valuable compound to suppress any oligomerization and gave access to highly pure hydrazinopeptides. As an alternative, the more stable N,N′-diprotected acid **2** can

(19) However, we have shown the propensity of the $N_\beta$ of the bis-Boc hydrazino acetic acid anchored onto a resin to be acylated using a BOP in situ activation. Indeed, following a first coupling of the diprotected acid, driven to completion as indicated by a negative Kaiser test, a second and a third coupling in the same conditions were performed on solid phase. Following the number of coupling, the proportion of dimer increased from 7.8% (1 coupling) to 13.4% (2 couplings) and 18.0% (3 couplings).

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(21) Bonnet, D.; Gras-Masse, H.; Melnyk, O. Manuscript in preparation.

be used using an HBTU/HOBt activation and as long as a 2% dimer contamination is acceptable.

## Conclusion

As part of our program on the solid-phase synthesis of hydrazinopeptides, we have developed a new strategy to prepare α-hydrazinoacetyl peptides in an efficient and readily scalable way. The approach reported in this work relies on the coupling of Boc-protected derivatives of α-hydrazinoacetic acid directly onto the peptide. A low cost route to a fully Boc-protected derivative is described. We have shown the need to fully protect the hydrazino moiety to avoid polymerization of the activated ester during the coupling. The process has been fully automated allowing the synthesis of various hydrazinopeptides in a good yield and high purity. In our laboratory, α-hydrazinoacetyl peptides have proved to be valuable partners in the hydrazone chemical ligation with α-oxo aldehyde derivatives giving a straightforward access to synthetic peptide conjugates and high molecular weight constructs.

## Experimental Section

**General Considerations.** $^1$H and $^{13}$C NMR spectra were recorded at 300 or 75.5 MHz. Chemical shifts are given in ppm and referenced to internal TMS. For the assignment of signals $^1$H, $^{13}$C, and $^1$H−$^{13}$C heteronuclear single quantum correlation (HSQC) spectroscopy experiments were used. Thin-layer chromatography was performed using E. Merck plates of silica gel 60 with fluorescent indicator. Visualization was effected by spraying plates with 5% phosphomolybdic acid in EtOH followed by heating at 120−140 °C. All reagents were used directly as obtained commercially.

*N*,*N*′,*N*′-Tris(*tert*-butyloxycarbonyl)hydrazino]acetic acid is now commercially available from NOVABIOCHEM.

Solid-phase peptide syntheses were performed using standard Fmoc/*t*-Bu chemistry on Perseptive Pioneer peptide synthesizers. The amino acids were activated using HBTU/HOBt/diisopropylethylamine (AA/HBTU/HOBt/DIEA: 4 equiv/4 equiv/4 equiv/8 equiv) in DMF. Side chain protections were as follows: Tyr(*t*-Bu), Lys(Boc) or Lys(Mtt), Ser(*t*-Bu), Trp(Boc), Thr(*t*-Bu), Asp(O-*t*-Bu), Glu(O-*t*-Bu). Fmoc amino acids were purchased from Senn Chemicals.

**Characterization of Purified Hydrazinopeptides.** The RP-HPLC analyses were performed on a C18 Vydac (4.6 × 250 mm) column using water/2-propanol linear gradient (0−100% B in 60 min, 1 mL/min, 50 °C, 215 nm). The following buffers were used: (eluent A) water containing 0.05% TFA by volume; (eluent B) 2-propanol/water 2/3 by volume containing 0.05% TFA by volume.

Capillary zone electrophoresis (CZE) was performed on an Applied Biosystems 270A-HT apparatus in a 75 $\mu$m × 50 cm fused silica capillary, with 40 mÅ current and a 30 kV field. The analyses were undertaken in a pH 3.0 sodium citrate buffer.

For electrospray mass spectrometry (ES-MS) studies, the *m*/*z* range 200−2100 was scanned using an ionspray voltage of 4500 V. The orifice plate voltage was ranged between 30 and 60 V. The temperature in the ionization chamber was 60 °C.

Amino acid compositions were determined using a Beckman amino acid analyzer model 7300 with ninhydrin detection, following hydrolysis in evacuated sealed tubes with 6 N HCl/phenol:10/1 (v/v) at 110 °C for 24 h.

**MAS NMR Experiments.** Resin was washed twice with $CH_2Cl_2$ and filtered. Then, 5 mg of this resin were loaded in a 4 mm rotor and swollen with $CD_2Cl_2$. Tetramethylsilane (TMS) was added as a internal reference. All NMR experiments were performed at 298 K on a 600 MHz spectrometer equipped with a 4 mm HR MAS probe using a 6 kHz spinning rate. $^1$H NMR spectra were recorded with 16 scans and diffusion filtered experiment (LED) with 64 scans. Gradient parameters for the LED based sequence were as previously described.[18]

**Synthesis of *N*-*tert*-Butyloxycarbonyl Hydrazinoacetic Acid 1.** Commercially available ethyl hydrazinoacetate (20.1 g, 130 mmol) and di-*tert*-butyl dicarbonate (31.3 g, 144 mmol) were dissolved in 130 mL of a mixture $H_2O$/EtOH (1/1). *N*-Methylmorpholine (15.8 mL, 144 mmol) were added dropwise, and the resultant mixture was stirred for 2 h in a water bath. NaOH (11.5 g, 287 mmol) was then added in five aliquots, and the solution was stirred for further 2 h at rt. Following dilution with a saturated solution of NaCl, the mixture was first extracted with diethyl ether (1 100 mL). The aqueous layer was then acidified to pH 3 with a solution of citric acid and extracted with diethyl ether (2 × 100 mL). The combined extracts were washed with water (50 mL), dried over $Na_2SO_4$, filtered, concentrated under reduced pressure, and dried under vacuum. The resultant solid was precipitated after dilution in methyl *tert*-butyl ether to afford **1** (12.2 g, 49% overall yield) as a white solid: mp 124−125 °C; HRMS $[M + H]^+$ calcd for $C_7H_{15}O_4N_2$ 191.1032, found 191.1028 (−2.2 ppm); MALDI-TOF $[M + Na]^+$ calcd 213.085, found 213.087; $^1$H NMR (MeOH-$d_4$, 300 MHz) $\delta$ 4.07 (s, 2H), 1.45 (s, 9H); $^{13}$C NMR (MeOH-$d_4$, 75 MHz) $\delta$ 173.3 and 172.2, 81.4, 53.0, 52.3, 51.8, 27.6, 27.5. Anal. Calcd for $C_7H_{14}N_2O_4$: C, 44.20; H, 7.42; N, 14.73. Found: C, 44.16; H, 7.59; N, 14.32.

**Synthesis of Ethyl [*N*-(*tert*-Butyloxycarbonyl)hydrazino]acetate 6a. Path 1.** Commercially available ethyl hydrazinoacetate (14.9 g, 96.4 mmol) and di-*tert*-butyl dicarbonate (26.1 g, 120 mmol) were dissolved in 70 mL of a mixture $H_2O$/EtOH (1/1). Following dissolution, the mixture was cooled to 0 °C, and 13.2 mL (120 mmol) of *N*-methylmorpholine was added dropwise. The resultant mixture was stirred for 15 min at 0 °C and 2 h at rt. The solution was then diluted with water saturated with $KH_2PO_4$ and extracted with diethyl ether (2 × 70 mL) and petroleum ether (2 × 70 mL). The combined organic layers were dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure to give crude **6a** (19.8 g) as an orange oil which was used without any further purification for the next step: $^1$H NMR ($CDCl_3$, 300 MHz, 323 K) $\delta$ 4.19 (q, 2H, $J$ = 7.1 Hz), 4.11 (s, 2H), 1.45 (m, 9H), 1.26 (t, 3H, $J$ = 7.1 Hz); $^{13}$C NMR ($CDCl_3$, 75 MHz, 323 K) $\delta$ 175.5, 174.3, 161.7, 160.8, 85.8, 85.2, 65.5, 65.4, 57.41, 32.8, 32.7, 32.6, 18.7. Anal. Calcd for $C_9H_{18}N_2O_4$: C, 49.53; H, 8.31; N, 12.84; O, 29.32. Found: C, 49.78; H, 8.36; N, 12.33; O, 29.27.

**Path 2.** Ethyl bromoacetate **5a** (5.6 mL, 50.4 mmol, 1 equiv) diluted in DMF (10 mL) was added by means of a dropping funnel to a stirred solution of *tert*-butylcarbazate (19.8 g, 151 mmol) in DMF (40 mL) at rt. The mixture was further stirred for 8 h. A solution of glyoxylic acid was added to the reaction vessel. The solution was prepared as follows: solid glyoxylic acid (18.6 g, 200 mmol) was dissolved in 0.4 M aqueous $Na_2HPO_4$ (50 mL), and the pH adjusted to 5.2 by addition of 5 N aqueous NaOH. Following overnight stirring, the crude mixture was poured into saturated aqueous $NaHCO_3$ (300 mL) and extracted by diethyl ether (5 × 100 mL). The combined extracts were washed with brine (2 × 50 mL), dried over $Na_2SO_4$, filtered, concentrated under reduced pressure, and dried under vacuum to give crude ethyl [*N*-(*tert*-butyloxycarbonyl)hydrazino]acetate **6a** (7.56 g) as a yellow oil which was directly engaged in the next step: $R_f$ = 0.48 (heptane/AcOEt 1:1).

**Synthesis of Benzyl [*N*-(*tert*-Butyloxycarbonyl)hydrazino]acetate 6b via Path 2.** Benzyl bromoacetate (51.1 mL, 321 mmol) diluted in DMF (64 mL) was added over 10 min by means of a dropping funnel to a stirred solution of *tert*-butylcarbazate (128 g, 968 mmol) in DMF (256 mL) at rt. The mixture was further stirred for 18 h. To the reaction vessel was then added a solution of sodium glyoxylate prepared as follows: solid glyoxylic acid (119.0 g, 1285 mmol) was dissolved

in $H_2O$ (192 mL) and then carefully added to a 10 N solution of NaOH (51.20 g, 1280 mmol) in $H_2O$ (128 mL) at 0 °C to give a yellow solution (pH ~4−5, paper). The reaction mixture, from which a precipitate appeared, was stirred for further 24 h at rt and then poured into a saturated aqueous $NaHCO_3$ solution (1.5 L) and extracted by diethyl ether (4 × 300 mL). The combined extracts were washed with brine (2 × 50 mL), dried over $Na_2SO_4$, filtered, concentrated under reduced pressure, and dried under vacuum to give crude benzyl [*N*-(*tert*-butyloxycarbonyl)hydrazino]acetate **6b** (83.0 g) as a yellow syrup which was directly used in the next step: $R_f$ = 0.53 (heptane/AcOEt 1:1); $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 1.44 (s, 3 × 3H), 3.74 (s, 2H), 5.17 (s, 2H), 7.35 (brs, 5H); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$ 28.2, 52.6, 67.1, 81.5, 125.2, 128.4, 128.6, 128.7, 135.1, 156.3, 170.5; TOF-PDMS MW calcd 280.1, *m/z* found 224.7 $[M + H - t\text{Bu}]^+$, 180.1 $[M + H - \text{Boc}]^+$.

**Synthesis of Ethyl [*N,N,N′*-Tris(*tert*-butyloxycarbonyl)hydrazino]acetate 7a via Path 1.** Crude ethyl [*N*-(*tert*-butyloxycarbonyl)hydrazino]acetate **6a** (19.8 g, 91.1 mmol) was dissolved in $CH_2Cl_2$ (16 mL) containing $Et_3N$ (38.5 mL, 276 mmol) at 0 °C under $N_2$. This mixture was then added by means of a dropping funnel to a solution of di-*tert*-butyl dicarbonate (60.2 g, 276 mmol) and DMAP (3.37 g, 27.6 mmol) in $CH_2Cl_2$ (20 mL) under $N_2$ at 0 °C. The reaction mixture was warmed to rt while stirring. After 2 h, the crude mixture was then diluted with $CH_2Cl_2$ (50 mL) and washed with saturated aqueous $KH_2PO_4$ (3 × 75 mL). The organic layer was dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure to give an orange oil which was further passed through a short column of silica gel (40−60 $\mu$m, 160 g, eluent: $CH_2Cl_2$/AcOEt 97/3 by volume). The filtrate was concentrated under reduced pressure and dried overnight over $P_2O_5$ to give the fully protected derivative **7a** which was directly used in the next step (yellow oil, 36.9 g): $R_f$ = 0.68 (heptane/AcOEt 1:1); MALDI-TOF MW calcd 418.2, *m/z* found 441.4 $[M + Na]^+$, 457.4 $[M + K]^+$; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 4.16 (s, 2H), 3.71 (q, 2H, $J$ = 7.2 Hz), 1.46 (m, 27H), 1.23 (t, 3H, $J$ = 7.2 Hz); $^{13}$C NMR ($CDCl_3$, 75 MHz) $\delta$ 167.74, 150.4, 150.2, 83.6, 82.4, 82.0, 60.9, 58.4, 53.3, 51.5, 28.0, 18.4, 14.2. Anal. Calcd for $C_{19}H_{34}N_2O_8$: C, 54.53; H, 8.19; N, 6.69. Found: C, 54.81; H, 8.25; N, 6.71.

**Synthesis of [*N,N′*-Bis(*tert*-butyloxycarbonyl)hydrazino]acetic Acid 2.** To a solution of crude ethyl [*N,N,N′*-tris(*tert*-butyloxycarbonyl)hydrazino]acetate **7a** (19.2 g, 88.3 mmol) in EtOH (135 mL) cooled at 0 °C was added cooled aqueous 1 N NaOH (135 mL, 135 mmol) by means of a dropping funnel. Following 30 min of stirring at 0 °C and a further 3 h at rt, the crude mixture was diluted with $H_2O$ (110 mL) and extracted with diethyl ether (2 × 80 mL). The aqueous layer was then acidified to pH 2 by means of a solution 1 N HCl and extracted with $CH_2Cl_2$ (2 × 80 mL) and $Et_2O$ (2 × 80 mL). The combined extracts were washed with brine (2 × 40 mL), dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure. The resultant oily residue was precipitated after dilution with a cooled mixture of diethyl ether/heptane to afford pure [*N,N′*-di-(*tert*-butyloxycarbonyl)hydrazino]acetic acid (17.9 g, 66% overall yield): $R_f$ = 0.24 (heptane/AcOEt 1:1); mp 113−114 °C; HRMS: calcd for $C_{12}H_{23}O_6N_2$ 291.1556, found 291.1551 (−1.7 ppm); MALDI-TOF $[M + Na]^+$ calcd 313.137, found 313.141; $^1$H NMR (DMSO-$d_6$, 300 MHz) $\delta$ 9.23 (s, 0.53H) 9,16 (s, 0.26H), 8.84 (s, 0.14H), 8.74 (s, 0.07H), 3.97 (s, 2H), 1.39 (m, 18H); $^{13}$C NMR (DMSO-$d_6$, 75 MHz, 300 K) 170.1, 155.0, 154.1, 80.1, 79.5, 54.6, 53.1, 27.9; HMBC $\delta_H$ NH 9.23 and 9.16, coupling to $\delta_C$ 155.0; $\delta_H$ $CH_2$ 3.97, coupling to $\delta_C$ 170.1 and 155.0. Anal. Calcd for $C_{12}H_{22}N_2O_6$: C, 49.65; H, 7.64; N, 9.65; O, 33.07. Found: C, 50.09; H, 7.84; N, 9.57; O, 32.64.

**Synthesis of [*N,N,N′*-Tris(*tert*-butyloxycarbonyl)hydrazino]acetic Acid 3 Following Saponification.** To a solution of crude ethyl [*N,N,N′*-tris(*tert*-butyloxycarbonyl)-hydrazino]acetate **7a** (15.05 g, 36.0 mmol) in EtOH (40 mL) cooled at 0 °C was added ice-cooled aqueous 1 N NaOH (39.6 mL, 39.6 mmol) by means of a dropping funnel. The reaction mixture was stirred for 25 min at 0 °C and then neutralized by slow addition of an aqueous citric acid solution (634 mg·mL$^{-1}$) to pH 4 (paper). The crude mixture was then diluted with $H_2O$ (50 mL) and extracted with diethyl ether (2 × 80 mL) and $CH_2Cl_2$ (2 × 80 mL). The combined extracts were washed with brine (2 × 40 mL), dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure. The oily residue was precipitated after dilution with a cooled mixture of diethyl ether/heptane to furnish pure [*N,N′*-tris(*tert*-butyloxycarbonyl)-hydrazino]acetic acid (9.6 g, 63% overall yield): $R_f$ = 0.24 (1:1 heptane/AcOEt); mp 119−120 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz) 4.04 (s, 2H), 1.42 (m, 27H); $^{13}$C NMR (DMSO-$d_6$, 75 MHz)169.3, 153.5, 149.8, 82.9, 81.7, 81.1, 53.1, 51.2, 27.8. Anal. Calcd for $C_{17}H_{30}N_2O_8$: C, 52.30; H, 7.74; N, 7.17; O, 32.78. Found: C, 52.63; H, 7.73; N, 7.21; O, 32.81.

**Synthesis of [*N,N,N′*-Tris(*tert*-butyloxycarbonyl)hydrazino]acetic Acid 3.** Crude benzyl [*N*-(*tert*-butyloxycarbonyl)hydrazino]acetate **6b** (80 g, 285 mmol) was dissolved in $CH_2Cl_2$ (51 mL) containing $Et_3N$ (125 mL, 891 mmol) and then cooled to 0 °C. This mixture was then added by means of a dropping funnel over 2 h to a solution of di-*tert*-butyl dicarbonate (194 g, 889 mmol) and DMAP (1.09 g, 0.89 mmol) in $CH_2Cl_2$ (64 mL) under $N_2$ at 0 °C. The reaction mixture was warmed to rt with stirring for 24 h. As the reaction was not completed (presence of a mixture of di- and tri-Boc-protected derivatives as monitored by TLC), additional di-*tert*-butyl dicarbonate (194 g, 889 mmol) was introduced to the reaction vessel. The mixture was stirred for 48 h and then diluted with $CH_2Cl_2$ (160 mL) and washed with saturated aqueous $KH_2PO_4$ (3 × 255 mL). The organic phase was dried over $Na_2SO_4$, filtered, and concentrated under reduced pressure to give an orange-brown oil which was further passed through a short column of silica gel (40−60 $\mu$m, 1.4 kg) (eluent: $CH_2Cl_2$/AcOEt 97:3). The filtrate was concentrated under reduced pressure and dried overnight over $P_2O_5$ to give the fully protected derivative **7b** as an orange oil which was used directly in the next step: $R_f$ = 0.53 (heptane/AcOEt 7:3). To a solution of crude benzyl [*N,N,N′*-tris(*tert*-butyloxycarbonyl)hydrazino]acetate **7b** (145 g, 302 mmol) in EtOH (1000 mL) cooled at 0 °C was added palladium hydroxide on carbon (20% Pd/C, 5.8 g, 4% w/w). The mixture was then hydrogenolyzed at atmospheric pressure of hydrogen for 24 h at 4 °C. The crude mixture was filtered through a pad of Celite which was washed with $CH_2Cl_2$. The solvent was concentrated under reduced pressure to give an orange oil. Following precipitation in a cooled mixture of diethyl ether/cyclohexane, pure [*N,N,N′*-tris(*tert*-butyloxycarbonyl)hydrazino]acetic acid (49 g, 39% overall yield) was obtained as a white solid: $R_f$ = 0.24 (heptane/AcOEt 1:1); HRMS calcd for $C_{17}H_{31}N_20_8$ 391.2080, found 391.2082; MALDI-TOF $[M + Na]^+$ calcd 413.190, found 413.1919. Anal. Calcd for $C_{17}H_{30}N_2O_8$: C, 52.30; H, 7.74; N, 7.17; O, 32.78. Found: C, 52.26; H, 7.77; N, 7.22; O, 32.63.

**Synthesis of H-SKWDDPWGEVLAWKFDPTLAYTY-EAK($COCH_2NHNH_2$)-$NH_2$.** Peptide **13** was elaborated starting from 3 mmol of Fmoc-Pal-PEG-PS resin (0.16 mmol/g, Applied Biosystems, Foster City, CA). Fmoc-L-Lys(Mtt)-OH (7.5 g, 12 mmol) was coupled using HBTU (4.55 g, 12 mmol)/HOBt (1.84 g, 12 mmol)/DIEA (3.38 mL, 48 mmol). The Mtt-protecting group was removed by 1% TFA in $CH_2Cl_2$, and the deprotection was monitored by RP-HPLC as previously reported.[20] Following washing with $CH_2Cl_2$ and DMF, $(Boc)_2$N-N(Boc)$CH_2$COOH (1.41 g, 3.6 mmol) was coupled using PyBop (1.87 g, 3.6 mmol) and DIEA (1.25 mL, 7.2 mmol) activation (30 min at rt). The completion of the reaction was monitored using a TNBS test. Resin was then successively rinsed with DMF, $CH_2Cl_2$, and diethyl ether and dried under vacuum, and 0.1 mmol of resin was loaded in a reactor vessel for SPPS in a Pioneer Perseptive Biosystems syntheziser. Peptide **13** was then elaborated using a Fmoc/*tert*-butyl strategy. Each coupling step was followed by treatment with $Ac_2O$/DIEA/DMF (3/0.3/96.7) by volume. Cleavage and deprotection steps were performed using 10 mL of TFA/EDT/$H_2O$ (95/2.5/2.5) by

volume mixture during 2 h at rt. The crude peptide was precipitated in cold diethyl ether, redissolved in water/acetic acid 5/1 by volume, and lyophilized. Purification was performed on a C3 Zorbax column (15 × 500 mm) using buffers A and B (20−50% B in 30 min, 50% for 25 min, 50−70% in 20 min, 3 $mL \cdot min^{-1}$, detection at 215 nm). Following a freeze-drying step, peptide **13** was obtained as a white powder (98.4 mg, 27%): ES-MS $[M + H]^+$ calcd 3188.8, found 3187.0; RP-HPLC purity (215 nm) one peak >95%.

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**Abbreviations:** BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; CZE, capillary zone electrophoresis; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; EDT, ethanedithiol; ES-MS, electrospray-mass spectroscopy; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOBt, *N*-hydroxybenzotriazole; HOSu, *N*-Hydroxysuccinimide; PyBOP, benzotriazole-1-yl-oxytrispyrrolidinophosphonium hexafluorophosphate.

**Supporting Information Available:** $^1$H NMR spectra for compounds **1**−**3**, **6a**,**b**, and **7a**,**b**, $^{13}$C NMR spectra for compounds **1**−**3** and **6a**,**b**, MS spectra for compounds **1**−**3** and **13**, and HPLC for peptide **13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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