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Ca^{2+}-mediated thiolysis of peptide–resin linkage as an option for preparing protected peptide thioesters

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Abstract

A mild new procedure for preparing protected peptide thioesters, based on Ca^{2+}-assisted thiolysis of peptide–Kaiser oxime resin (KOR) linkage, is described. Ac-Ile-Ser(Bzl)-Asp(OcHx)-**SR** (Ac: acetyl; Bzl: benzyl; cHx: cyclohexyl), model peptide, was readily released from the resin by incubating the peptide–KOR at 60 °C in mixtures of DMF with *n*-butanethiol [$R = (CH_2)_3CH_3$] or ethyl 3-mercaptopropionate [$R = (CH_2)_2COOCH_2CH_3$] containing $Ca(CH_3COO)_2$. After serine and aspartic acid side-chain deprotection under acid conditions, Ac-Ile-Ser-Asp-$S(CH_2)_2COOCH_2CH_3$ was successfully obtained with good quality and high yield. This type of C-terminal modified peptide may act as an excellent acyl donor in peptide segment condensation by the thioester method, native chemical ligation and enzymatic methods.

Keywords: Solid-phase peptide synthesis; Kaiser oxime resin; Ethyl 3-mercaptopropionate; *n*-Butanethiol; Metal ion

Preliminary accounts of certain aspects of this work are described as a communication in Ref. 35.

Peptide thioesters—the key building blocks in convergent peptide synthesis through the thioester method,[1,2] native chemical ligation[3,4] or protease-mediated peptide condensation[5,6]—can be prepared by a stepwise solid-phase synthesis through *t*-butyloxycarbonyl (Boc) or fluorenylmethoxycarbonyl (Fmoc) strategy using highly efficient procedures.

Regarding the Boc strategy, peptide thioesters can be obtained by peptide elongation starting from Boc-amino acid-$S(CH_2)_2CO$-4-methylbenzhydrylamine (MBHA) resin or -hydroxymethylphenylacetamidomethyl resin (PAM resin) followed by HF treatment of the resulting peptide–resin.[1,2] The use of 3-mercaptopropionic acid as a highly versatile multidetachable linker, stable to the protocols used in the Boc strategy and HF treatment, but cleavable by thiol nucleophile, has also been explored.[7] Since KOR is an appropriate resin to prepare C-terminally modified peptides,[8,9] it has also been employed to furnish protected peptide thioesters through aminolysis of oxime ester bond with thioesterified amino acid.[6]

As to the Fmoc strategy, several attempts have overcome the lability of the thioester functionality to bases.[10] Such attempts include (i) using alternative Fmoc-deprotection cocktails, such as the one-to-one mixture of *N*-methylpyrrolidone–DMSO containing 1-methylpyrrolidine (25% v/v), hexamethyleneimine (2% v/v) and *N*-hydroxybenzotriazole (2% w/v);[11,12] (ii) employing the backbone amide linker (BAL) strategy in which the thioester moiety is introduced at the end of the solid-phase peptide synthesis via amino acid thioester coupled to the C-terminal residue;[13,14] (iii) performing alkylaluminium-mediated thiolysis of peptide–PAM or Wang resin linkage;[15,16] (iv) carrying out the solution-phase thioesterification of a fully protected peptide obtained from 2-chlorotrityl resin (2ClTrt resin) in the presence of coupling reagents.[17–19] Employing a potentially more general procedure, peptide thioesters have also been prepared by Fmoc-based SPPS using 'safety-catch' linkers, which form with the growing peptide a linkage

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stable to acidic and basic conditions. Among the examples are (i) the sulfonamide linker that can be activated by alkylation with trimethylsilyldiazomethane or iodoacetonitrile and cleaved by a thiol nucleophile to release the respective protected peptide thioester;[20–23] (ii) the arylhydrazine linker, which renders the resulting peptide hydrazide resin capable of undergoing mild oxidation and being converted into a reactive acyl diazene intermediate that, in turn, can be cleaved by an α-amino acid *S*-alkyl thioester providing the correspondent protected peptide thioester.[10,24]

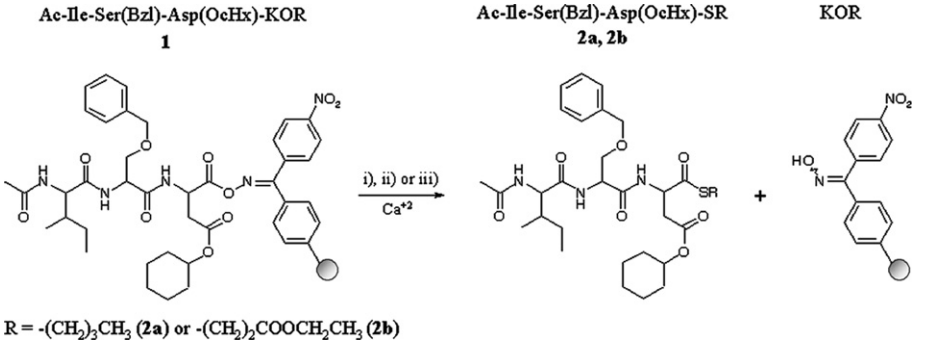
Evidently all the procedures cited above show advantages and disadvantages. Hence, new procedures for the synthesis of protected peptide thioesters are of interest in peptide and protein chemistry. While studying the preparation of protected peptide esters through Ca^{2+}-mediated alcoholysis of peptide–KOR linkage,[25] we preliminarily observed that the replacement of the alcohol by ethanethiol led to the desired peptide thioester selectively under very mild conditions. Nevertheless, the thiolysis was very slow and the procedure was compatible only with the Boc chemistry. In fact, the Boc chemistry has been supplanted by the Fmoc strategy. On the other hand, the Boc strategy is still used due to its lower cost, the option of replacing HF by other acids for peptide full deprotection/cleavage from the resin[26–28] and the fact that there are countries where HF is still commercially available and the laboratories specialized in peptide synthesis use it only on a very small scale mostly for scientific purposes.[29–31] Therefore, in this work we studied Ca^{2+}-mediated thiolysis of peptide–KOR linkage in an attempt to prepare an Ac-peptide-$S(CH_2)_2COOCH_2CH_3$. As in our previous work,[25] fragment 22–24 of the gastrointestinal hormone cholecystokinin-33 was chosen as a model peptide.

Boc-Asp(OcHx)-OH (1.74 equiv to the reactive groups in the resin) was first coupled to KOR (1 g; substitution level of 0.87 mmol/g) in methylene chloride (DCM; 10 mL) for 24 h, using *N*,*N*′-dicyclohexylcarbodiimide (DCC; 1.74 equiv),[32] to produce Boc-Asp(OcHx)–KOR (aminoacylation level of 0.53 mmol/g). Chain assembly was carried out manually by Boc chemistry using DMF as solvent and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/*N*,*N*-diisopropylethylamine (DIEA) as coupling reagents.[33] After completion, Ac-Ile-Ser(Bzl)-Asp(OcHx)–KOR (**1**; substitution level of 0.32 mmol/g) was characterized by total hydrolysis/amino acid analysis. Thiolysis reactions starting from **1** were performed in 20% *n*-butanethiol/DMF in the absence and presence of $Ca(CH_3COO)_2$ under the experimental conditions described in Scheme 1. Their monitoring by RP-HPLC and the analyses of 48 h-reaction aliquots by liquid chromatography coupled to electrospray ionization mass spectrometry (LC/ESI-MS) revealed that, as expected, the reaction in the presence of Ca^{2+} furnished more peptide thioester **2a** {ESI-MS $[MH]^+$: 620.6 (found); 621.2 (calcd)} than that conducted without Ca^{2+}.

Having confirmed our preliminary observation[25] and, hence, the ability of Ca^{2+} to mediate thiolysis of the oxime ester bond of **1**, attempts to prepare Ac-Ile-Ser(Bzl)-Asp(OcHx)-$S(CH_2)_2COOCH_2CH_3$ were done. In fact, the thioester corresponding to ethyl 3-mercaptopropionate is one of the most commonly employed in convergent peptide synthesis using either the thioester method[17,19] or the native chemical ligation.[20,22] In addition, it may also be used in enzyme-mediated peptide condensation due to its high susceptibility to nucleophiles and, consequently, high potential to form the acyl-enzyme complex.

Thiolyses of **1** in 20% ethyl 3-mercaptopropionate/DMF were also performed at 60 °C for 6 h in the absence and presence of $Ca(CH_3COO)_2$ (Scheme 1). Their monitoring by RP-HPLC and the analyses of the 6 h-reaction aliquots by LC/ESI-MS demonstrated that our procedure is appropriate for the thiolysis studied and indicated that the equilibrium for the Ca^{2+}-mediated reaction was attained before 6 h. The yields of peptide detachment from KOR were determined through amino acid analyses of **1** before and after thiolysis.[33] Compound **2b** {ESI-MS $[MH]^+$: 664.6 (found); 664.8 (calcd)} was provided after 6 h in 49% (control reaction) and 94% (Ca^{2+}-mediated reaction) yields.

To determine the minimum reaction time for the Ca^{2+}-mediated thiolysis with ethyl 3-mercaptopropionate, two new reactions were performed starting from **1** with



Scheme 1. Ca^{2+}-mediated thiolysis of Ac-Ile-Ser(Bzl)-Asp(OcHx)–KOR oxime ester bond. Reagents and conditions: (i) 20% $CH_3(CH_2)_3SH$/DMF, 60 °C (**2a**); (ii) 20% $CH_3CH_2OOC(CH_2)_2SH$/DMF, 60 °C (**2b**); (iii) 50% $CH_3CH_2OOC(CH_2)_2SH$/DMF, 50 °C (**2b**). All the reactions were performed using 1 equiv of $Ca(CH_3COO)_2$/1 equiv of peptide under orbital shaking at 300 rpm.

$Ca(CH_3COO)_2$ in either 20% thiol/DMF at 60 °C or in higher thiol content and lower temperature (50% thiol/DMF at 50 °C; Scheme 1). Control reactions (with no additive) were also promoted. Their monitoring by RP-HPLC and the analyses of 2 h-reaction aliquots by LC/ESI-MS demonstrated that (i) the reaction mediated by Ca^{2+} attained its equilibrium in 2 h; (ii) the desired product **2b** {ESI-MS $[MH]^+$: 664.6 (found); 664.8 (calcd)} was obtained with good quality; (iii) the thiol dimer $CH_3CH_2OOC(CH_2)_2S–S(CH_2)_2COOCH_2CH_3$ (**3**) {ESI-MS $[MH]^+$: 266.9 (found); 266.4 (calcd)} was the main byproduct formed in either Ca^{2+}-mediated thiolyses or control reactions (**3** was also produced when the solution 20% $CH_3CH_2OOC(CH_2)_2$–SH/DMF was incubated with $Ca(CH_3COO)_2$ in the absence of the peptide–KOR). Figure 1 shows the RP-HPLC profiles for the reactions performed in 50% thiol/DMF at 50 °C. The yields of peptide thioester detachment from KOR described in Table 1 showed that both conditions used were suitable for Ca^{2+}-mediated thiolysis.

Ca^{2+}-containing reaction media were pooled and then placed in a 5 mL syringe connected to a Sep-Pak Plus C_{18} (Waters) to eliminate calcium salt, DMF and thiol from the desired protected peptide thioester. It was eluted from the cartridge with aqueous solutions of acetonitrile containing 0.1% trifluoroacetic acid (TFA). RP-HPLC and LC/ESI-MS analyses of the eluted fractions indicated that only byproduct **3** was not eliminated. On the other hand, its formation can be minimized using inert atmosphere for the reactions, which would not be possible, for example, in the aryl hydrazine linker procedure performed under an oxidant condition.[24]

Table 1
Yields of peptide **2b** detachment from KOR after oxime ester bond thiolysis with ethyl 3-mercaptopropionate for 2 h

Mediator	Solvent system (V/V)	*T* (°C)	Peptide detachment[a] (%)
—	20% thiol/DMF	60	28
Ca^{2+}	20% thiol/DMF	60	93
—	50% thiol/DMF	50	53
Ca^{2+}	50% thiol/DMF	50	92

[a] The yields of peptide detachment from KOR were determined through amino acid analysis of **1** before and after thiolysis.[33]

In order to be employed in convergent peptide synthesis through the thioester method, native chemical ligation or enzyme-mediated peptide condensation, our model peptide should have its serine and aspartic acid side-chains deprotected. Thus, after Sep-Pak C_{18} pre-treatment, compound **2b** contaminated with dimer **3** was submitted to HF treatment for 1.5 h in the presence of anisol. The RP-HPLC (Fig. 2) and LC/ESI-MS analyses of the resulting crude peptide revealed that (i) the desired product Ac-Ile-Ser-Asp-$S(CH_2)_2COOCH_2CH_3$ (**4**) {ESI-MS $[MH]^+$: 492.3 (found); 492.6 (calcd)} was successfully obtained; (ii)

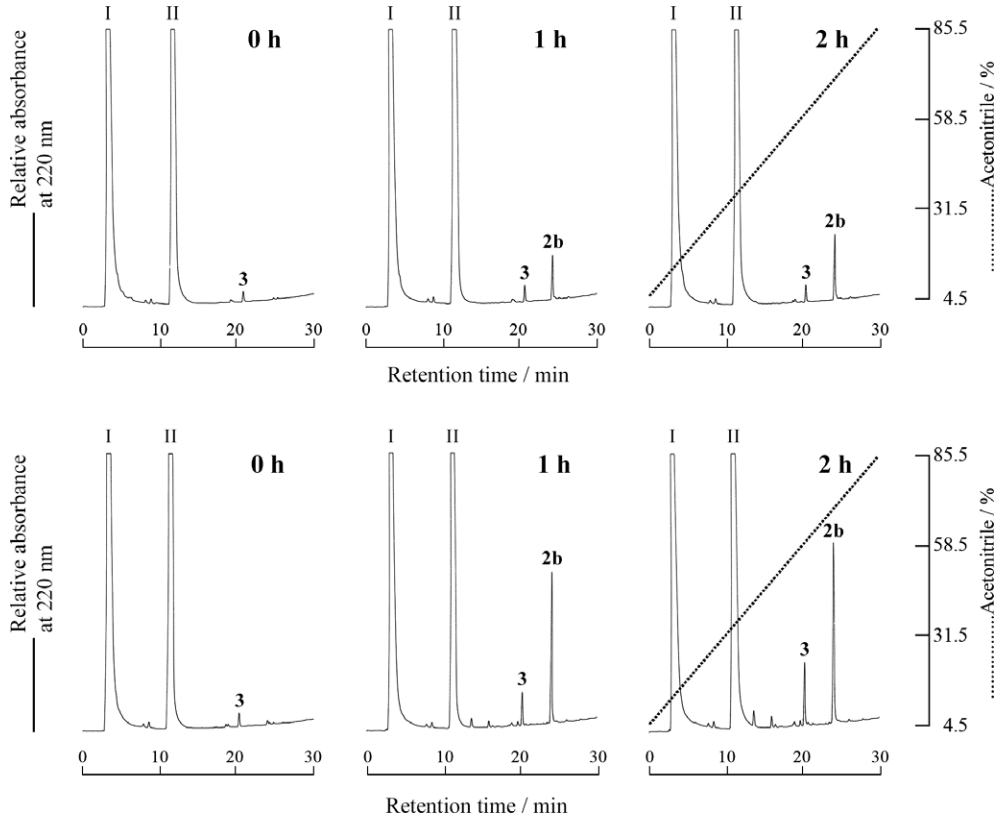


Fig. 1. RP-HPLC monitoring of oxime ester bond thiolysis of **1** in 50% $CH_3CH_2OOC(CH_2)_2SH$/DMF at 50 °C. (A) control reaction; (B) Ca^{2+}-mediated reaction. (I) DMF; (II) $CH_3CH_2OOC(CH_2)_2SH$. Elution conditions: column, Vydac C_{18} (4.5 × 250 mm) at a flow rate of 1 mL/min; eluent, aqueous acetonitrile containing 0.1% TFA.

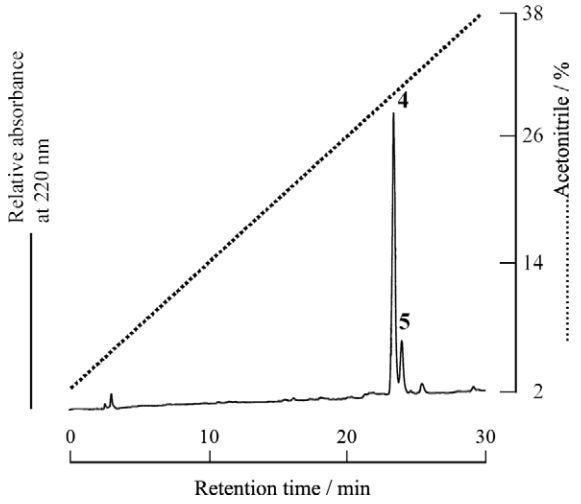


Fig. 2. RP-HPLC profile of the crude peptide thioester 4 remaining from HF treatment. Elution conditions: column, Vydac C18 (4.5 × 250 mm) at a flow rate of 1 mL/min; eluent, aqueous acetonitrile containing 0.1% TFA.

compound **3** did not hamper the side-chain deprotection reaction, nor was detected in the final solution, having probably been extracted in the ether phase during peptide precipitation after the HF treatment; (iii) byproduct **5** {ESI-MS $[MH]^+$: 492.3 (found); 492.6 (calcd)} seems to be an isomer of **4**, most likely a product from the *N–O* acyl shift[34] (although this secondary reaction can be reverted by the treatment of *O*-acyl-peptide with basic aqueous solution, it was not done due to the known thioester moiety lability to bases).[11] Compounds **2b** and **4** were purified by RP-HPLC and further characterized by amino acid analysis and ^{1}H NMR (see Supplementary data).

The significance of the data described here becomes noticeable when compared to the previous ones obtained by either Boc or Fmoc strategies. Indeed, Ca^{2+}-mediated thiolysis of the oxime ester bond from Ac-Ile-Ser(Bzl)-Asp(OcHx)–KOR in 20% ethanethiol/DCM required 75 h at 39 °C to yield only 37% of the corresponding protected tripeptide ester;[25] acid-catalyzed aminolysis of peptide–KOR with thioesterified amino acid required 50 h to provide the desired protected peptide thioester with a cleavage yield of 90%;[6] peptide detachment from 2ClTrt followed by the solution-phase peptide thioesterification with ethyl 3-mercaptopropionate required 12–50 h and large amounts of coupling reagents;[17–19] the synthesis of protected peptide ethylthioester using the 'safety-catch' aryl hydrazine linker requires prior preparation or acquisition of α-amino acid *S*-alkyl ethylthioester (Gly-SEt·HCl or Ala-SEt·HCl) followed by peptide–resin linkage activation through mild oxidation (10 min) and nucleophilic attack (30 min; yield of 60–70%);[24] peptide thioesters have been obtained using the sulfonamide linker, but the step for peptide–resin linkage activation by alkylation required 2–3 h (trimethylsilyldiazomethane) or 24 h (iodoacetonitrile) and the cleavage from resin with ethyl 3-mercaptopropionate required 24 h to provide cleavage yields varying from 48% to 85%.[20–22]

In conclusion, we have demonstrated that Ca^{2+}-mediated thiolysis of the oxime ester bond of a peptide–KOR using ethyl 3-mercaptopropionate may be an optional, simple and efficient one-step procedure for preparing precursors of acyl donors suitable for chemical convergent peptide syntheses. It employs mild conditions [$Ca(CH_3COO)_2$ as additive instead of acids or bases] avoiding significant side-reactions and the use of an ozone-depleting solvent (DMF as a solvent rather than methylene chloride).

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Supplementary data

This material contains the experimental procedures employed and the analysis data for the purified peptide thioesters. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.04.081.

References and notes

1. Aimoto, S. *Biopolymers* **1999**, *51*, 247–265.
2. Haginoya, E.; Hojo, H.; Nakahara, Y.; Nakahara, Y.; Nabeshima, K.; Toole, B. P.; Watanabe, Y. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 1338–1349.
3. Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776–779.
4. Nilsson, B. L.; Soellner, M. B.; Raines, R. T. *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 91–118.
5. Welker, E.; Scheraga, H. A. *Biochem. Biophys. Res. Commun.* **1999**, *254*, 147–151.
6. Cerovsky, V.; Bordusa, F. *J. Pept. Res.* **2000**, *55*, 325–329.
7. Camarero, J. A.; Adeva, A.; Muir, T. W. *Lett. Pept. Sci.* **2000**, *7*, 17–21.
8. DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1980**, *45*, 1295–1300.
9. Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins*; CRC Press: Florida, 1997, pp 144–145.
10. Camarero, J. A.; Mitchell, A. R. *Protein Pept. Lett.* **2005**, *12*, 723–728; Woo, Y.-H.; Mitchell, A. R.; Camarero, J. A. *Int. J. Pept. Res. Ther.* **2007**, *13*, 181–190.
11. Li, X.; Kawakami, T.; Aimoto, S. *Tetrahedron Lett.* **1998**, *39*, 8669–8672.
12. Clippingdale, A. B.; Barrow, C. J.; Wade, J. D. *J. Pept. Sci.* **2000**, *6*, 225–234.
13. Alsina, J.; Yokum, T. S.; Albericio, F.; Barany, G. *J. Org. Chem.* **1999**, *64*, 8761–8769.
14. Brask, J.; Albericio, F.; Jensen, K. J. *Org. Lett.* **2003**, *5*, 2951–2953.
15. Swinnen, D.; Hilvert, D. *Org. Lett.* **2000**, *2*, 2439–2442.
16. Sewing, A.; Hilvert, D. *Angew. Chem., Int. Ed.* **2001**, *40*, 3395–3396.

17. Futaki, S.; Sogawa, K.; Maruyama, J.; Asahara, T.; Niwa, M. *Tetrahedron Lett.* **1997**, *38*, 6237–6240.
18. von Eggelkraut-Gottanka, R.; Klose, A.; Beck-Sickinger, A. G.; Beyermann, M. *Tetrahedron Lett.* **2003**, *44*, 3551–3554.
19. Kitagawa, K.; Adachi, H.; Sekigawa, Y.; Yagami, T.; Futaki, S.; Gu, Y. J.; Inoue, K. *Tetrahedron* **2004**, *60*, 907–918.
20. Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369–11374.
21. Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684–11689.
22. Quaderer, R.; Hilvert, D. *Org. Lett.* **2001**, *3*, 3181–3184.
23. Harris, P. W. R.; Brimble, M. A.; Dunbar, R.; Kent, S. B. H. *Synlett* **2007**, *5*, 713–716.
24. Camarero, J. A.; Hackel, B. J.; Yoreo, J. J.; Mitchell, A. R. *J. Org. Chem.* **2004**, *69*, 4145–4151.
25. Moraes, C. M.; Bemquerer, M. P.; Miranda, M. T. M. *J. Pept. Res.* **2000**, *55*, 279–288.
26. Tam, J. P.; Heath, W. F.; Merrifield, R. B. *J. Am. Chem. Soc.* **1986**, *108*, 5242–5251.
27. Fujii, N.; Otaka, A.; Ikemura, O.; Akaji, K.; Funakoshi, S.; Hayashi, Y.; Kuroda, Y.; Yajima, H. *J. Chem. Soc., Chem. Commun.* **1987**, 274–275.
28. Stewart, J. M. *Methods Enzymol.* **1997**, *289*, 29–44.
29. Chiva, C.; Barthe, P.; Codina, A.; Gairı, M.; Molina, F.; Granier, C.; Pugniere, M.; Inui, T.; Nishio, H.; Nishiuchi, Y.; Kimura, T.; Sakakibara, S.; Albericio, F.; Giralt, E. *J. Am. Chem. Soc.* **2003**, *125*, 1508–1517.
30. Machado, A.; Sforça, M. L.; Miranda, A.; Daffre, S.; Pertinhez, T. A.; Spisni, A.; Miranda, M. T. M. *Biopolymers* **2007**, *88*, 413–426.
31. Moraes, L. G. M.; Fázio, M. A.; Vieira, R. F. F.; Nakaie, C. R.; Miranda, M. T. M.; Schreier, S.; Daffre, S.; Miranda, A. *Biochim. Biophys. Acta* **2007**, *1768*, 52–58.
32. Nakagawa, S. H.; Kaiser, E. T. *J. Org. Chem.* **1983**, *48*, 678–685.
33. Proti, P. B.; Remuzgo, C.; Miranda, M. T. M. *J. Pept. Sci.* **2007**, *13*, 386–392.
34. Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical Company: Rockford, 1984; pp 42–46.
35. Proti, P. B.; Miranda, M. T. M. In *Peptides for Youth*, Proceedings of the 20th American Peptide Symposium; Escher, E.; Lubell, W. D., Eds.; Preparation of C-terminal Modified Peptides Through Alcoholysis and Thiolysis Mediated by Metal ions; American Peptide Society, in press.