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Benzotriazole-Assisted Solid-Phase Assembly of Leu-Enkephalin, Amyloid β segment 34−42, and other "Difficult" Peptide Sequences

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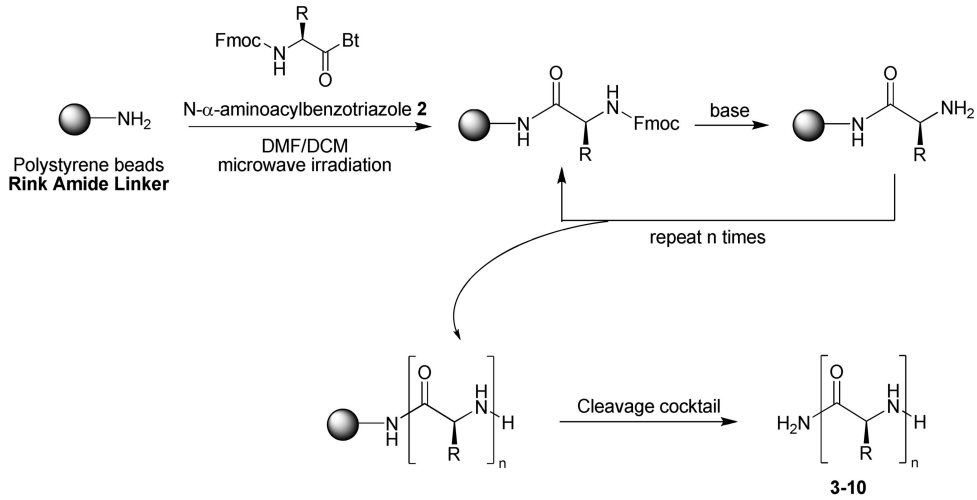

Microwave-assisted solid-phase syntheses of six "difficult" peptides, H-VVSVV-NH_2 (**3**), H-VVVSVV-NH_2 (**4**), H-VIVIG-OH (**5**), H-TVTVTV-NH_2 (**6**), H-VKDGYI-NH_2 (**7**), and H-VKDVYI-NH_2 (**8**), were achieved utilizing *N*-(Fmoc-α-aminoacyl)benzotriazoles. Extension to the syntheses of Leu-enkephalin (**9**) and amyloid-β (34−42) (**10**) demonstrates that this strategy comprises an efficient route to new and known "difficult" peptides.

Introduction

Solution-phase and especially the solid-phase peptide synthesis (SPPS) of potentially bioactive peptides is of great interest.[1a,b] While SPPS has enabled major advances in scope, yields, purities, and reaction times,[1a,c] SPPS[2a,b] has also encountered "difficult" peptide sequences when incomplete aminoacylation and/or deprotection reactions at various stages in the synthetic scheme [3a−d] have resulted in low yields and purities.[1a] Such difficulties can arise in attempting to form a peptide link (i) due to steric effects when both amino acid units possess β-branched side chains (e.g., valine, isoleucine and threonine)[3b] and (ii) as a result of the formation of secondary structures by intra- and interchain hydrogen-bonded associations.[3a−d] In such cases, the synthesized peptides can be partly

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racemized and/or adulterated with deletion sequences or form aspartimides and related side products.[4a,b]

Some of the problems associated with these difficult sequences in Fmoc-based SPPS have been alleviated by the use of (i) bases such as DBU and piperazine (less nucleophilic than the conventional piperidine) to suppress racemization and to reduce aspartimide formation;[4b,c] (ii) chemical ligation techniques, for example the "*O*-acyl isopeptide method" that can significantly reduce isomerization of the peptide backbone;[5a,b] and (iii) microwave acceleration of the deprotection and coupling steps,[4c,6] which decreases racemization as the growing peptide has less time available for α-carbon epimerization.

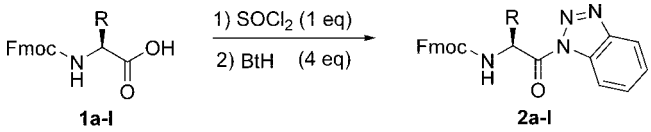
The formation of aspartimides and related side products remain a problem in SPPS. Backbone protection using the 2,4-dimethoxybenzyl (Dmb), 2-hydroxy-4-methoxybenzyl (Hmb), 2,4,6-trimethoxybenzyl (Tmb), or 2-nitrobenzyl (Nbzl) groups[7a−c] can help but requires additional steps.

Recently, *N*-Fmoc-(α-aminoacyl)benzotriazoles of proteinogenic amino acids have been utilized in the synthesis of tri- to heptapeptides in crude yields of 65−77% on the Rink amide MBHA solid support.[6] We now describe the microwave-assisted syntheses of six short "difficult" α-peptide sequences in attempts to examine the extent of racemization, incomplete aminoacylation/deprotection reactions, and the formation of aspartimides when *N*-Fmoc-(α-aminoacyl)benzotriazoles are used as activating reagents for SPPS.

Results and Discussion

N-Fmoc-(α-aminoacyl)benzotriazoles **2a**−**l** (76−91%) were prepared as previously described[7d] by treatment of purchased Fmoc-L-protected amino acids **1a**−**l** with 4 equiv of benzotriazole and 1 equiv of $SOCl_2$ in THF at room temperature for 2 h (Scheme 1, Table 1).

SCHEME 1. Preparation of *N*-Fmoc-(α-Aminoacyl)benzotriazoles



BtH= benzotriazole

A standard SPPS approach was employed in the syntheses of the "difficult" peptides (**3**−**10**), in which the appropriate *N*-Fmoc-(α-aminoacyl)benzotriazole (**2**) was coupled in turn to the growing peptide (Scheme 2). Subsequent cleavage[7e] from the Rink amide MBHA resin and purification of the crude peptide provided the desired product.

TABLE 1. Preparation of *N*-Fmoc-(α-Aminoacyl)benzotriazoles (2) from the corresponding Fmoc-Protected α-Amino Acids (1)

		yield[a]		literature	
entry	compound	(%)	mp (°C)	mp (°C)	ref
1	Fmoc-L-Val-Bt (**2a**)	84	151.9−152.6	148.3−149.8	7d
2	Fmoc-L-Thr(*t*Bu)-Bt (**2b**)	80	64.6−66.8	62.2−65.0	7d
3	Fmoc-L-Ser(*t*Bu)-Bt (**2c**)	78	63.8−65.4	91.7−92.4[b]	
4	Fmoc-L-Tyr(*t*Bu)-Bt (**2d**)	84	99.0−100.5	138.4−139.3[b]	
5	Fmoc-L-Ile-Bt (**2e**)	78	165.4−167.2	168.8−170.0	7d
6	Fmoc-L-Lys(Boc)-Bt (**2f**)	78	138.2−141.7	138.4−140.6	7d
7	Fmoc-Gly-Bt (**2g**)	76	161.8−163.3	161.5−161.8	7d
8	Fmoc-L-Asp(O*t*Bu)-Bt (**2h**)	81	102.1−104.3	102.0−104.0	7d
9	Fmoc-L-Phe-Bt (**2i**)	78	157.0−158.3	159.1−160.2	7d
10	Fmoc-L-Leu-Bt (**2j**)	87	75.3−78.6	121.3−123.2[b]	
11	Fmoc-L-Met-Bt (**2k**)	91	129.1−131.8	122.7−123.3	7d
12	Fmoc-L-Ala-Bt (**2l**)	88	160.5−161.8	160.0−160.3	7d

[a] Isolated. [b] See Supporting Information for characterization of the polymorphs **2c**,**d**,**j**

β-Hydroxy-α-amino acids, such as serine, 3-hydroxyproline, threonine, and certain analogues (for example, β-hydroxyphenylalanine and β-hydroxytyrosine) are widely distributed as components of biologically active natural products.[8a−d] The syntheses of peptides containing β-hydroxy-α-amino acids can be challenging (i) due to the risk of racemization of such residues during both the stepwise and convergent approaches to Fmoc SPPS[5b,9] and (ii) because of aggregation, which may commence as early as the addition of the fifth amino acid residue in certain β-hydroxy-α-amino acid containing sequences.[5b,10a,b] It is also known hydrophobic and branched chain amino acids (BCAAs), such as valine, isoleucine, and leucine, promote aggregation during peptide synthesis and purification, particularly when a large percentage of such hydrophobic residues is present.

In the literature the effect of the amino acid hydrophobicity is evident in the DIPCDI-HOBt (1,3-diisopropylcarbodiimide-hydroxybenzotriazole) stepwise SPPS of H-Val-Val-Ser-Val-Val-NH_2 (**3**)[5a,b] where the undesired N-protected peptide amide Fmoc-Val-Val-Ser-Val-Val-NH_2 was produced as the major compound (1.1-fold higher than the desired peptide **3** as evidenced by HPLC[5a]). In our presently reported work, using *N*-Fmoc-(α-aminoacyl)benzotriazoles, peptide **3** was obtained as the major product (Table 2, Figure 1; Figure 9S, Supporting Information) with no evidence of the undesired Fmoc-Val-Val-Ser-Val-Val-NH_2 peptide or any racemized product. A possible explanation for the absence of microaggregates in the benzotriazole-assisted synthesis of **3** when compared to the DIPCDI-HOBt route[5a,b] could be the impact of microwave irradiation on the environment of the growing peptide. In our present work, the alteration of the microenvironment by microwave irradiation may hinder the formation of insoluble microaggregates and facilitate the removal of the Fmoc groups from the resin bound peptide **3**.[4c] Additionally, our microwave-assisted stepwise protocol reduced the total coupling time for the synthesis of **3** from 10[5a] to 2.5 h (open vessel; 1.5 h when closed vessel

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SCHEME 2. SPPS Approach Using the Rink Amide MBHA Resin and *N*-Fmoc-(α-Aminoacyl)benzotriazoles

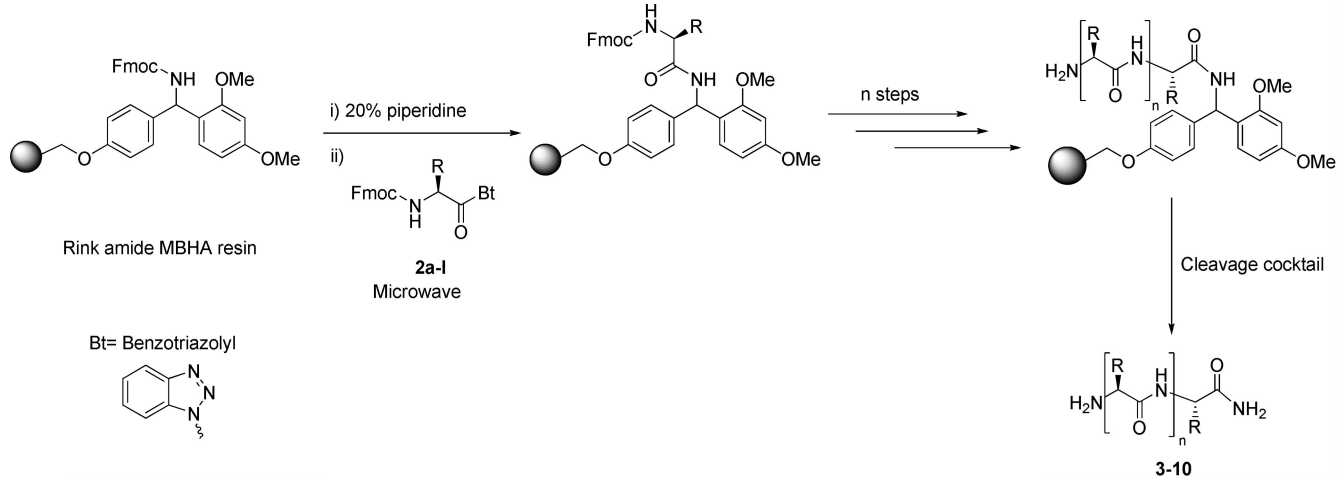

TABLE 2. Analytical Data of Peptides 3−10

sequence N−C terminus	retention time, t_R (min)	crude purity (%)	crude yield[b] (%)	after preparative HPLC purity (%)	after preparative HPLC yield (%)	$[M + H]^+$ found[d]
H-Val-Val-Ser-Val-Val-NH_2 (**3**)	7.57	60	63	>99	7	501.3385
H-Val-Val-Val-Ser-Val-Val-NH_2 (**4**)	9.79	60	34	nd[c]	nd[c]	600.4058
H-Val-Ile-Val-Ile-Gly-OH[a] (**5**)	10.82	70	40	>99	24	500.3643
H-Thr-Val-Thr-Val-Thr-Val-NH_2 (**6**)	8.70	50	35	89	16	618.3864
H-Val-Lys-Asp-Gly-Tyr-Ile-NH_2 (**7**)	8.31	30	68	>99	24	693.3916
H-Val-Lys-Asp-Val-Tyr-Ile-NH_2 (**8**)	9.10	37	64	>99	29	735.4419
H-Tyr-Gly-Gly-Phe-Leu-NH_2 (**9**)	11.54	80	86	>99	37	555.2948
H-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala-NH_2 (**10**)	13.82	28	42	89	22	857.5277

[a] On purification the peptide amide hydrolyzed to the corresponding acid. [b] Yields quoted are calculated from the amount of product obtained multiplied by % purity from the HPLC. [c] Not determined. [d] For calculated $[M + H]^+$ values see Supporting Information.

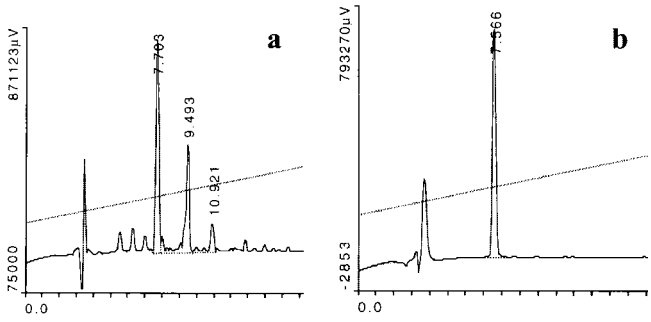

FIGURE 1. HPLC profiles of (a) crude and (b) pure peptide **3** obtained after SPPS using 20% piperidine−DMF for Fmoc cleavage (open vessel condition).

conditions are applied, Figure 11S, Supporting Information) and improved the overall yield from 1.4%[5a] to 7%. Although using the *O*-acyl isopeptide chemical ligation technique to synthesize **3** may provide a peak overall yield of 28% (extrapolated from the yield of the *O*-acyl isopeptide of **3**), this requires a coupling time of 40 h.[5b]

A synthesis analogous to that of **3** gave hydrophobic peptide **4** as the major product (20 mg of 60% purity, comparing to a yield of pure **4** of 34%), which is further evidence of the utility of *N*-Fmoc-(α-aminoacyl)benzotriazoles in the synthesis of difficult peptides containing β-hydroxy-α-amino acid residues. The HPLC and HRMS spectra of crude **4** showed no peaks for the analogous Fmoc-protected peptide amide or for any racemized product (Figures 12S and 13S, Supporting Information). A literature search for **4** gave no details of the synthesis or yield.

Peptide amides, such as **3** and **4**, contain valine, a naturally occurring branched chain amino acid (BCAA). BCAA compounds occur frequently in the cores of proteins. Such BCAAs are important in determining the three-dimensional structure of globular proteins and play a pivotal role in the interactions of membrane proteins with phospholipid bilayers.[11a] The absence of any peaks for the Fmoc protected peptides in both the HPLC and HRMS spectra of **3** and **4** suggests that interference by aggregation was negligible in these two examples of our present work.

We next synthesized peptide **5** (Table 2; Figures 14S and 15S, Supporting Information) possessing 80% BCAAs and with a hydrophobicity similar to that of **3**, in 24% isolated yield, with no evidence of interference by aggregation (Figure 15S, Tables 4S and 5S, Supporting Information; during purification of the peptide the amide was hydrolyzed to the corresponding acid). Previously, the Boc-based SPPS strategy along with the DCC/HOBt active ester coupling method was applied in the synthesis of **5**, while our present work utilized the milder Fmoc-based strategy along with *N*-(Fmoc-protected-α-aminoacyl)benzotriazoles. A comparison of the yield of **5** via the literature Boc-based SPPS with our yield by the Fmoc-based benzotriazole-assisted strategy could not be calculated from the information provided by the previous authors.[12]

Hexapeptide **6** contains only β-branched side chains and is less hydrophobic than **3** and **4**. We synthesized compound **6** (Table 2; Figures 16S and 17S, Table 6S, Supporting Information) by six successive couplings with (i) Fmoc-Val-Bt, (ii) Fmoc-Thr(*t*Bu)-Bt, (iii) Fmoc-Val-Bt, (iv) Fmoc-Thr(*t*Bu)-Bt,

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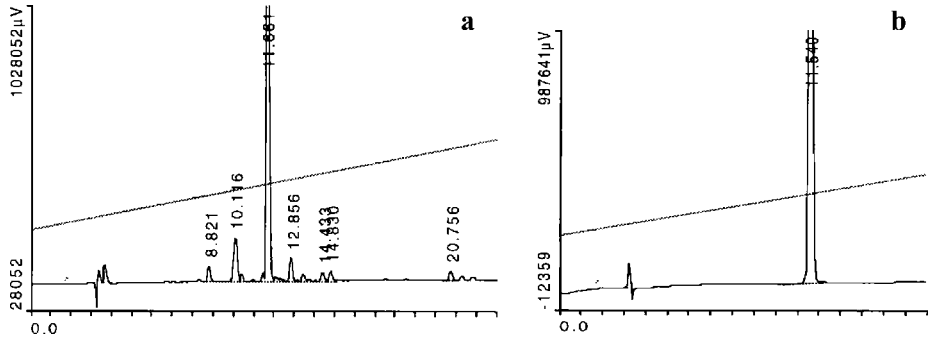

FIGURE 2. HPLC profiles of (a) crude and (b) pure peptide **9** obtained after SPPS using 20% piperidine−DMF for Fmoc cleavage.

(v) Fmoc-Val-Bt, and (vi) Fmoc-Thr(*t*Bu)-Bt. The linear geometry of **6** can be attributed to destabilizing steric effects and the restricted rotational freedom of peptides containing only β-branched amino acids.[11b] Peptides, such as **6** could be useful building blocks in tests for the stabilizing or destabilizing effect of BCAAs in peptide and protein α-helix formation.[11b,13] The nearest literature comparison appears to be the synthesis by Jolliffe et al.[13] of the TBS-protected linear peptide amide (Val-Thr)$_3$ as an intermediate in the formation of cyclo (Val-Thr)$_3$; again no direct comparison of yields is possible because of lack of data in the literature reference.[13]

In the SPPS of peptides containing asparagine or aspartic acid, aspartimides are frequently formed.[4a,b,14] The proportion of aspartimide side products depends on the base used for removal of the Fmoc group,[4b] the nature of the preceding amino acid residue,[4b,14] and to a lesser extent, the protecting group on the aspartyl residue.[4b,14] We chose to examine hexapeptide **7**;[4b,15] structure **7** is the peptide fragment 1−6 of toxin II from the scorpion *Androctonus australis* Hector[16] and contains the Asp-Gly fragment. Syntheses of compound **7** (Table 2; Figures 18S and 19S, Supporting Information) and analogue **8**[4b] (Table 2; Figures 20S and 21S, Supporting Information) containing Asp-Val serve to test the tendency for aspartimide formation using *N*-Fmoc-(α-aminoacyl)benzotriazoles. Our synthesis of peptide amide **7** (*m*/*z* 735.4419, t_R 8.31 min; Figures 18S and 19S, Supporting Information) did produce the corresponding aspartimide (*m*/*z* 675.3808, t_R 8.11 min; Figures 18S and 19S, Supporting Information) as a byproduct. However, our synthesis of peptide amide **8** (Table 2; Figures 20S and 21S, Supporting Information) proceeded without any of the analogous aspartimide byproduct, the formation of which was evidently suppressed by replacement of the glycine residue with valine. For both **7** and **8** we used 5% piperazine−DMF (and not 20% piperidine−DMF) for the removal of the Fmoc group, and this eliminated ring opening of the aspartimide to the corresponding piperidide in the case of **7**.

Although **7** was used as a test peptide by four sets of authors,[4b,14−16] no comparison of our yield with the literature can be made because each literature case reported only product purity and no yield was provided.[4b,14−16] Again, for **8**, only the product purity was provided in the literature,[4b] making a yield comparison impossible.

Leu-enkephalin (9)[17] and Amyloid-β segment (34−42) (10).[18] We also prepared **9** and **10**, two biologically important "difficult" peptides, by the above strategy. Leu-enkephalin (**9**) (Table 2, Figure 2) is a natural peptide neurotransmitter and a powerful painkiller. For **9** no direct yield comparison with the literature[17] can be made, as no literature yield is provided. Amyloid-β protein is the major plaque component in Alzheimer's disease (AD) and a commercially available product. The preparation of segment (34−42) (**10**) (Table 2, Table 10S, Figure 26S, Supporting Information) was previously described by Halverson[19] and co-workers, who used Kaiser oxime resin with the alanine residue already attached; they then coupled *N*-Boc-protected amino acids eight times to assemble **10**, stating that the solubilization was "extremely difficult" and recovery subsequent to HPLC analysis was "extremely sensitive" but claiming a 40% yield of material that was "quite susceptible" to oxidation.

Conclusions

As summarized in Table 2, our benzotriazole-assisted solid-phase assembly affords difficult peptides in crude yields of 34−86%. These benzotriazole-assisted syntheses, in tandem with microwave acceleration, have the following advantages over the previously used carbodiimide-based coupling methods: (i) our coupling reactions require no base, (ii) our conditions are comparatively mild, (iii) our reactions are more rapid, and (iv) importantly, our products are chirally homogeneous.

1-Hydroxybenzotriazole hydrate (HOBt) is a widely used coupling additive in peptide synthesis that suppresses racemization when used in combination with carbodiimides such as DCC. Other 1-hydroxybenzotriazole derivatives, for example, 1-hydroxy-7-azabenzotriazole (HOAt), 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt), phosphonium and aminium salts of hydroxybenzotriazoles, are also used as additives. Recently, the availability of HOBt has decreased because of the propensity for explosion during transportation.[20] Thus, this shows the efficacy of *N*-Fmoc-(α-aminoacyl)benzotriazoles in peptide synthesis.

There is a present and growing need for efficient synthetic methods for the assembly of proteins and peptides needed to study diverse systems in the body and/or develop a cure for neurodegenerative diseases such as AD. We have demonstrated

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that *N*-Fmoc-(α-aminoacyl)benzotriazoles are useful for the solid-phase assembly of "difficult" peptides and represent viable alternatives as SPPS reagents.

Experimental Section

Solid-Phase Protocol for the Preparation of Peptides 3−10. Standard stepwise solid-phase synthesis was performed manually in a 25-mL Discover SPS reaction vessel. Peptides **3−10** were each prepared on Rink amide MBHA resin. After swelling of the resin (0.1 mM) in DCM (4 mL, 0.5 h) and treatment with 20% piperidine−DMF (ca. 3 mL) for 20 min, the solvent was removed, the free-base amide resin was washed with DMF (5 mL × 3) and DCM (5 mL × 3), and dried, and a DMF−DCM (5:1 ca. 3 mL) solution of the appropriate *N*-Fmoc-(α-aminoacyl)benzotriazole (0.5 mM) was added. The coupling was induced using microwave irradiation (70−75 °C, 70−80 W, 10−30 min), and the completion of the coupling reaction was assessed by a negative ninhydrin (Kaiser) test. Successive *N*-Fmoc-(α-aminoacyl)benzotriazoles were similarly coupled to the growing peptide. Deprotection of the *N*-Fmoc-(α-aminoacyl)benzotriazole was achieved at each stage with 20% piperidine−DMF or 5% piperazine−DMF. Finally, the resulting peptidyl resin was cleaved with cleavage cocktail B[7e] (88% TFA/5% phenol/5% water/2% TIPS), K[7e] (82.5% TFA/5% phenol/5% water/5% thioanisole/5% EDT), or L[7e] (88% TFA/5% DTT/5% water/2% TIPS) for 1.5−2.0 h. Following cleavage, the peptide was precipitated with cold diethyl ether, and the ether-peptide mixture was incubated for 24 h at 4 °C and lyophilized to afford the crude peptides **3−10**.

Peptide Analysis. Analyses of the peptides **3−10** (Table 2) were carried out on 1100 series HPLC equipped with a Phenomenex Synergi 4u Hydro-RP 80A (2 mm × 150 mm, 4 μm) column plus a C18 guard column (2 mm × 4 mm). Mass analysis was performed using a LCQ ion trap mass spectrometer in electrospray ionization (ESI) mode.

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Supporting Information Available: General procedures and copies of HPLC and mass spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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