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New tris-alkoxycarbonyl arginine derivatives for peptide synthesis

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Abstract: α-Alkoxycarbonyl protected ornithines were treated with N,N'-[Z(2Cl)]$_2$-*S*-methylisothiourea and N,N'-[Z(2Br)]$_2$-*S*-methylisothiourea, N,N'-Z_2-*S*-methylisothiourea and N,N'-Boc$_2$-*S*-methylisothiourea to form $N^{\alpha,\omega,\omega'}$-tris-alkoxycarbonyl arginines. Two of them, Boc-Arg-{ω,ω'-[Z(2Br)]$_2$}-OH and Boc-Arg-{ω,ω'-[Z(2Cl)]$_2$}-OH, were used for the synthesis of dermorphin fragments containing two or three arginine residues. Examination of the products by HPLC and ESI-MS revealed that the purity of the materials obtained with the use of the new derivatives was higher than that obtained in concurrent syntheses in which Boc-Arg(Tos) was used. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: arginine protection; dynorphin; guanidinylation; SPPS

INTRODUCTION

Despite the great variety of protection possibilities described for the arginine guanidine group, no ideal approach has been defined [1,2]. Nitro, arenesulfonyl and alkoxycarbonyl groups are used most frequently. Protection of the guanidine function with a single benzyloxycarbonyl or *tert*-butoxycarbonyl group does not prevent the formation of lactams during activation [3–5]. Derivatives of arginine bearing two alkoxycarbonyl groups for protection of the guanidine function have been developed as a solution of this serious problem: benzyloxycarbonyl [6], adamantyloxycarbonyl [7] and isobornyloxycarbonyl [8]. The required derivatives were obtained by reaction of arginine [6] or α-protected arginine [7,8] with the respective chloroformates. In the case of the bis-benzyloxycarbonyl derivative it has been unequivocally proved, using chemical methods, that the Z groups are located at the N^{δ} and N^{ε} positions [6]. Two Z groups were also introduced *via* N^{α}-Boc-N^{δ},N^{ω},O-[Me$_3$Si]$_3$-arginine [9]. Trialkylsilyl derivatives formed *in situ* from N^{α}-Z-arginine were also used for the preparation of several N^{α}-Z-N^{δ},N^{ω}-bis(alkoxycarbonyl)-arginines [10]. For the conversion of arginine to Z-Arg(Z_2)-OH using benzyl pentachlorophenyl carbonate as the alkoxycarbonyl donor has also been described [11]. Two Boc groups have been introduced by treatment of Z-Arg-OH with Boc_2O at room temperature [12]. The product was a mixture of two isomers in 2 : 1 proportions: Z-Arg(δ,ω-Boc$_2$)-OH and Z-Arg(ω,ω'-Boc$_2$)-OH. Syntheses of Fmoc-Arg(ω,ω'-Boc$_2$)-OH and Fmoc-Arg(ω,ω'-Z_2)-OH were accomplished by guanidinylation of appropriate ornithine derivatives with N,N'-Boc$_2$-*S*-methylisothiourea or N,N'-Z_2-*S*-methylisothiourea, respectively [13,14]. The Fmoc derivative of di-Boc protected arginine has been used successfully in the solid phase synthesis of dermorphin and deltorphin analogues [15] and α-MSH [16]. Transformation of N^{α}-Fmoc-ornithine to protected arginine has also been carried out using N,N'-Boc$_2$-N''-trifluoromethanesulphonylguanidine and N,N'-Z_2-N''-trifluoromethanesulphonylguanidine [17].

$N^{\omega}N^{\omega'}$-bis-Alkoxycarbonyl guanidines were obtained recently by guanidinylation of amines with N,N'-bis-alkoxycarbonyl-*S*-methylisothiourea [18]. Special attention was paid to two novel guanidinylation reagents: N,N'-[Z(2Cl)$_2$]-*S*-methylisothiourea and N,N'-[Z(2Br)$_2$]-*S*-methylisothiourea. These reagents were found to be more reactive than the corresponding Boc and Z derivatives. The present paper reports the preparation (Figure 1) and structure determination of several tris-alkoxycarbonyl arginines which are useful for peptide synthesis. The usefulness of two of them, with their guanidine groups bis-protected with Z(2Br) or Z(2Cl), is demonstrated in the syntheses of dynorphin analogues.

MATERIALS AND METHODS

N,N'-[Z(2Cl)$_2$]-*S*-methylisothiourea, N,N'-[Z(2Br)$_2$]-*S*-methylisothiourea, N,N'-Z_2-*S*-methylisothiourea and N,N'-Boc$_2$-*S*-methylisothiourea were obtained as described earlier [18]. Boc-Orn-OH was purchased from Senn Chemicals. Z-Arg(Z_2)-OH was purchased from Fluka. Boc-Lys[Z(2Cl)]-PAM resin was a Sigma product.

General Procedure for the Preparation of Tris-alkoxycarbonyl Arginines (1–6)

To a solution of $NaHCO_3$ (0.525 g, 6.25 mmol) in 30 ml of dioxane : water (1 : 1), N^{α}-alkoxycarbonylornithine (5 mmol)

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was added followed by *N*,*N*′-bis(alkoxycarbonyl)-S-methylisothiourea (3.75 mmol). The mixture was stirred for 48 h at 40 °C. The solvent was removed under reduced pressure and the crystalline residue was dissolved in ethyl acetate (400 ml). The solution was washed with aqueous HCl (26 ml of 0.3 M) and water (6 × 20 ml), dried with $MgSO_4$ and the solvent was evaporated to give an oil which solidified. After crystallization from methanol, a white crystalline material was obtained.

Boc-Arg{ω,ω′-(Z(2Cl))$_2$}-OH (1). Yield 60%; m.p. 133°–135 °C; $[\alpha]_D^{20} = +6.5°$ (c = 1, $CHCl_3$); Anal. calcd for $C_{27}H_{32}N_4O_8Cl_2$: C, 53.03; H, 5.27; N, 9.16. Found: C, 53.30; H, 5.45; N, 9.23; ^{1}H NMR ($CDCl_3$, 200 MHz): δ1.44 (s, 9H), 1.71–1.91 (m, 4H), 3.48 (m, 2H), 4.32 (m, 1H), 5.25 (s, 2H), 5.30 (s, 2H), 7.20–7.48 (m, 8H), 8.41 (br s, 1H), 11.79 (br s, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ25.20, 28.29, 29.51, 40.60, 53.06, 64.38, 65.45, 80.35, 126.83–134.40, 153.66, 155.75, 156.17, 163.40, 175.89.

Boc-Arg{ω,ω′-(Z(2Br))$_2$}-OH (2). Yield 74%; m.p. 123°–125 °C; $[\alpha]_D^{20} = +10.0°$ (c = 1, $CHCl_3$); Anal. calcd for $C_{27}H_{32}N_4O_8Br_2$: C, 46.30; H, 4.61; N, 8.00. Found: C, 46.25; H, 4.31; N, 7.92; ^{1}H NMR ($CDCl_3$, 200 MHz): δ1.44 (s, 9H) 1.71–1.92 (m, 4H), 3.48 (m, 2H), 4.33 (m, 1H), 5.22 (s, 2H), 5.28 (s, 2H), 7.10–7.61 (m, 8H), 8.41 (m, 1H), 11.60 (br s, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ25.18, 28.30, 29.51, 40.61, 53.05, 66.62, 67.64, 80.34, 122.52–136.03, 153.63, 155.75, 156.18, 163.37, 175.73.

Boc-Arg(ω,ω′-Z$_2$)-OH (3). Yield 74%; m.p. 114°–116 °C; $[\alpha]^{20}_D = +10.3°$ (c = 1, $CHCl_3$); Anal. calcd for $C_{27}H_{34}N_4O_8$: C, 59.77; H, 6.32; N, 10.33. Found: C, 59.76; H, 6.69; N, 10.59; ^{1}H NMR ($CDCl_3$, 500 MHz): δ1.43 (s, 9H), 1.67–1.90 (m, 4H), 3.43 (m, 2H), 4.32 (m, 1H), 5.11 (s, 2H), 5.16 (s, 2H), 7.32–7.37 (m, 10H), 8.37 (m, 1H), 11.72 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ25.17, 28.30, 29.53, 40.53, 53.03, 67.19, 68.24, 80.29, 127.92–136.64, 153.83, 155.72, 156.01, 163.56, 175.94.

Boc-Arg(ω,ω′-Boc$_2$)-OH (4). Yield 80%; m.p. 102°–103 °C; $[\alpha]^{20}_D = +18.8°$ (c = 1, $CHCl_3$); Anal. calcd for $C_{21}H_{38}N_4O_8$: C, 53.15; H, 8.07; N, 11.81. Found: C, 53.48; H, 8.42; N, 12.11; ^{1}H NMR ($CDCl_3$, 500 MHz): δ1.45 (s, 9H), 1.48 (s, 9H), 1.49 (s, 9H), 1.69–1.92 (m, 4H), 3.41 (m, 2H), 4.32 (m, 1H), 8.44 (br s, 1H), 11.49 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ25.29, 28.05, 28.20, 28.33, 29.70, 40.40, 53.19, 79.70, 80.11, 80.36, 153.21, 155.78, 156.33, 163.05, 175.18.

Z-Arg(ω,ω′-Boc$_2$)-OH (5). Yield 63%; m.p. 99°–101 °C; $[\alpha]^{20}_D = +17.9°$ (c = 1, $CHCl_3$); lit [13]: m.p. 102 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ1.46 (s, 9H), 1.49 (s, 9H), 1.61–1.73 (m, 4H), 3.38 (d, 2H), 4.38 (m, 1H), 5.11 (s, 2H), 7.29–7.35 (m, 5H), 8.45 (s, 1H), 11.46 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ22.70, 25.32, 27.91, 28.05, 28.15, 29.37, 29.54, 29.66, 31.17, 31.93, 40.42, 53.74, 66.99, 79.83, 83.45, 128.08–136.26, 153.18, 156.25, 156.33, 162.87, 174.99.

Z-Arg(ω,ω′-Z$_2$)-OH (6). Yield 72%; m.p. 105°–107 °C; $[\alpha]^{20}_D$ = +11.2 °C (c = 1, $CHCl_3$); Lit [14]: m.p. not reported, $[\alpha]^{25}$ = +16.41°, c = 1, in $CHCl_3$; Anal. calcd for $C_{30}H_{32}N_4O_8$: C, 62.49; H, 5.59; N, 9.72. Found: C, 62.51; H, 5.95; N, 9.38; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.66–1.89 (m, 4H), 3.39 (m, 2H), 4.38 (m, 1H), 5.08 (s, 2H), 5.09 (s, 2H), 5.15 (s, 2H), 7.24–7.38 (m, 15H), 8.38 (s, 1H), 11.72 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ25.13, 29.35, 40.48, 53.45, 67.14, 67.20, 68.27, 153.82, 156.12, 156.23, 163.46, 175.51.

Syntheses of Dynorphin A(1–13), Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys

Boc-Lys[Z(2Cl)]-PAM resin (286 mg, 0.1 meq; Sigma, 0.35 meq/g) was used for each synthesis. All amino acids were of L-configuration. α-Amino functions were Boc protected, and the side chains were blocked with the following groups: Tyr, Z(2Br); Lys, Z(2Cl); Arg, Tos in synthesis a, and Arg, {ω,ω′-[Z(2Cl)$_2$}] in synthesis b. The peptide chain assembly was performed according to standard procedures: (a) deprotection with 55% TFA in DCM (1 × 1 min, 1 × 20 min); (b) washing with DCM (3 × 1 min); (c) washing with 30% dioxane in DCM (2 × 1 min); (d) washing with DCM (3 × 1 min); (e) neutralization with 5% DIEA (1 × 1 min, 1 × 5 min) in DCM; (f) washing with DCM (6 × 1 min); (g) coupling of Boc-amino acid (0.3 mmole) in the presence of DIPC (0.3 mmole) in DCM for 2 h; (h) washing with DCM (6 × 1 min). The Boc group of the last amino acid was removed by running steps (a)–(f) of the schedule. The protected peptide-resin was treated with liquid HF (10 ml) in the presence of anisole (1 ml) for 1 h at 0 °C. The HF was removed under reduced pressure, and the residue was treated with cold Et_2O, extracted with 50% acetic acid and lyophilized. The only difference between synthesis a and b was that one of the Arg derivatives mentioned above was used for introducing Arg in positions 6, 7 and 9. The yield of crude product was 165 mg in synthesis a, and 170 mg in synthesis b. Analytical HPLC (conditions see below) of crude product a and b showed the yields to be about 41% and about 49.6%, respectively, of the main product, as judged from the area of the main peak. ESI-MS spectra were run to assess the quality of the products obtained, using a Finnigan MAT 95S spectrometer, Bremen, Germany (Figure 2). In the mass spectrum of a, peaks corresponding to des-Arg peptide were observed in addition to peaks for the target peptide. The crude peptides (20 mg samples) were purified using a Knauer HPLC system with a Vertex column Nucleosil-300 C_{18} (8 × 200 mm, 5 μm); solvent system: A, 0.1% TFA in water, B, 60% MeCN in A. Elution: 20%–35% B in 15 min, then 35%–40% B in 15 min and 40%–100% B in 10 min. Flow rate 2 ml/min. Fractions were analysed on a Vertex column Nucleosil-100 C_{18} (4 × 250 mm, 5 μm) using gradient of 20%–80% in 20 min; flow rate 1 ml/min; detection at 220 nm. The retention time of the main product was 14.1 min. Homogeneous fractions containing a single peak were combined and lyophilized. Fractions were pooled for maximum purity rather than yield. The yields of purified material in the two syntheses were comparable, but the mass spectrum of the purified product obtained after synthesis using Boc-Arg(Tos)-OH revealed persistent contamination with des-Arg-peptide.

Synthesis of Dynorphin A(1–8), Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile

Boc-Ile-Merrifield resin (498 mg, 0.6 meq/g; 0.3 meq) was used for synthesis. α-Amino functions were Boc-protected and side chains were blocked with the following groups: Tyr, [Z(2Br)] and Arg, {ω,ω′-[Z(2Br)]$_2$}. The synthesis was carried out as described above. The yield of crude product was 300 mg.

Analytical HPLC of the crude product showed that the yield of the main product was about 74% as judged from the area of the main peak. The mass spectrum of crude product (Figure 3) shows two major peaks corresponding to $[M + H]^+$ and $[M + 2H]^{2+}$. No deletion peptides were detected. The crude peptide was purified using the Knauer HPLC system described above. Elution: 20%–40%B in 20 min, then 40%–55% B in 40 min and 50%–100% in 10 min. Flow rate 2 ml/min. Fractions were pooled for maximum purity rather than yield. After lyophilization 7 mg of pure peptide was obtained.

RESULTS AND DISCUSSION

Several tris-alkoxycarbonyl arginines were obtained by the treatment of Boc-Orn-OH or Z-Orn-OH with *N*,*N*′-[Z(2Cl)]$_2$-*S*-methylisothiourea, *N*,*N*′-[Z(2Br)]$_2$-*S*-methylisothiourea, *N*,*N*′-Z_2-*S*-methylisothiourea or *N*,*N*′-Boc_2-*S*-methylisothiourea (Figure 1). In all cases the purified products were obtained in high yields (60–80%). The new compounds (entries *i*–*vi*) and compounds previously described, but obtained using different guanidinylation reagents (entry *v*), or other methods of synthesis (entries *vi*–*ix*) are listed in Table 1. Careful examination of the ^{1}H and ^{13}C NMR spectra of these compounds confirms that the products obtained with the use of our guanidinylation reagents are all N^{α},N^{ω},$N^{\omega'}$-tri-substituted Arg derivatives, as expected from the method of synthesis. The chemical shifts of $C^{\delta}\underline{H}_2$ are 3.4–3.5 ppm and the chemical shifts of $\underline{C}^{\delta}$ are 40.4–40.6 ppm, while for Z-Arg(δ,ω-Z_2)-OH (entry *ix*, a commercial product) they are 3.9 and 44.1, respectively. Grønwald *et al.* reported [12] that Z-Arg-OH when treated with Boc_2O gave two isomers (entries *vi* and *vii*): Z-Arg(δ,ω-Boc_2)-OH, $C^{\delta}\underline{H}_2$ 3.8 ppm and C^{δ} 44.3 ppm; Z-Arg(ω,ω'-Boc_2)-OH, $C^{\delta}\underline{H}_2$ 3.3 ppm and C^{δ} 40.3 ppm. It is not clear if this was the result of the formation of two products during the reaction in the first place or subsequent partial isomerization of one of them in harsh reaction conditions. Under the conditions described in this paper only one isolatable product was formed.

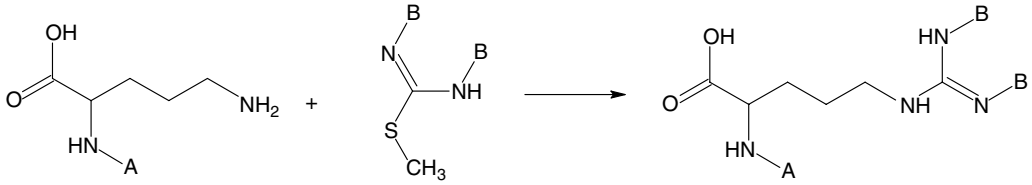
Table 1 Diagnostic ^{13}C and ^{1}H Chemical Shifts (in $CDCl_3$; values are given in ppm, relative to TMS) of Arginine Derivatives

Entry	Arginine derivative	^{13}C NMR $\underline{C}^{\delta}H_2$	^{1}H NMR $C^{\delta}\underline{H}_2$
i	Boc-Arg{ω,ω'-[Z(2Cl)]$_2$}-OH (**1**)	40.6	3.5
ii	Boc-Arg{ω,ω'-[Z(2Br)]$_2$}-OH (**2**)	40.6	3.5
iii	Boc-Arg(ω,ω'-Z_2)-OH (**3**)	40.5	3.4
iv	Boc-Arg(ω,ω'-Boc_2)-OH (**4**)	40.4	3.4
v	Z-Arg(ω,ω'-Boc_2)-OH (**5**)	40.4	3.4
vi	Z-Arg(ω,ω'-Boc_2)-OH[a]	40.3	3.3
vii	Z-Arg(δ,ω'-Boc_2)-OH[b]	44.3	3.8
viii	Z-Arg(ω,ω'-Z_2)-OH (**6**)	40.5	3.4
ix	Z-Arg(δ,ω'-Z_2)-OH[c]	44.1	3.9

[a] Lit [13] and [19].
[b] Obtained by reaction of Z-Arg-OH with Boc_2O [12].
[c] Purchased from Fluka.

Since Z(2Cl) and Z(2Br) groups are very resistant to treatment with trifluoroacetic acid, **1** and **2** were selected to demonstrate their usefulness for solid phase synthesis, with syntheses of dynorphin A(1–8) and dynorphin A(1–13), containing two and three Arg residues, respectively, using Boc methodology, as test cases. Dynorphin A(1–13) was synthesized using **1** and also in parallel for comparison using Boc-Arg(Tos)-OH. The yields of crude product in these two syntheses were comparable, but the purity of the material obtained in the synthesis using **1** was higher. The mass spectra of the products are presented in Figure 2. In both cases $[M + 2H]^{2+}$ and of $[M + 3H]^{3+}$ ions could be seen. But in the case of the product obtained using Boc-Arg(Tos)-OH, an additional ion at 724.4 was also observed, corresponding to the $[M + 2H]^{2+}$ ion from a peptide lacking one Arg residue.

Dynorphin A(1–8) was synthesized using **2** for introducing Arg residues. The yield and purity of crude product were good. The only significant ions observed



A = Boc, Z

B = Z(2Cl), Z(2Br), Z, Boc

1: A = Boc, B = Z(2Cl)
2: A = Boc, B = Z(2Br)
3: A = Boc, B = Z
4: A = Boc, B = Boc
5: A = Z, B = Boc
6: A = Z, B = Z

Figure 1 Preparation of tris-alkoxycarbonyl arginines.

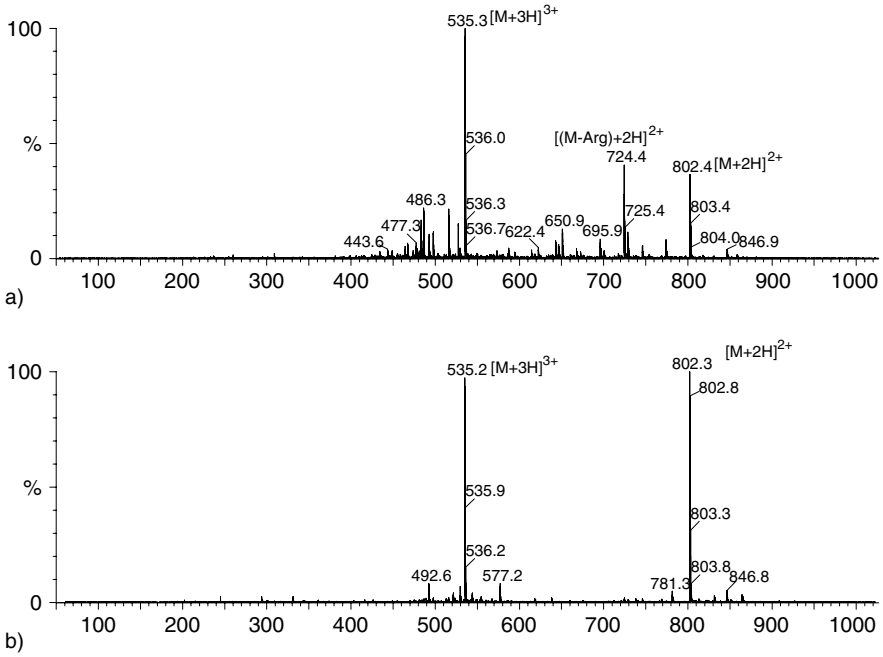


Figure 2 ESI-MS spectra of crude preparations of dynorphin A(1–13): (a) synthesis with the use of Boc-Arg(Tos)-OH; (b) synthesis with the use of Boc-Arg{ω,ω'-[Z(2Cl)]$_2$}-OH (**1**).

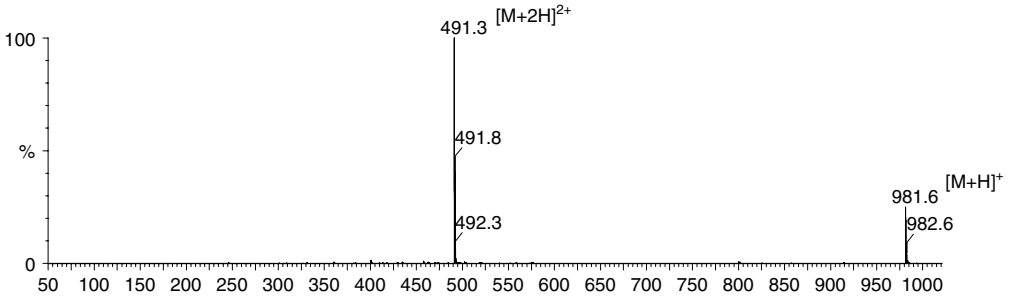


Figure 3 ESI-MS spectra of crude preparations of dynorphin A(1–8) obtained with use of Boc-Arg{ω,ω'-[Z(2Br)]$_2$}-OH (**2**).

in the mass spectrum were attributable to calculated mass (Figure 3).

CONCLUSION

An efficient method has been developed for the preparation of N^{α},N^{ω},$N^{\omega'}$-tris-alkoxycarbonyl arginines for use in peptide synthesis. The potential of two of them, Boc-Arg{ω,ω'-[Z(2Cl)]$_2$}-OH and Boc-Arg{ω,ω'-[Z(2Br)]$_2$}-OH in Boc-SPPS (especially for long sequences containing multiple Arg residues), has been demonstrated by their use in the assembly of dynorphin fragments.

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