Acceleration of the N^{α}-Deprotection Rate by the Addition of *m*-Cresol to Diluted Methanesulfonic Acid and Its Application to the Z(OMe)-Based Solid-Phase Syntheses of Human Pancreastatin-29 and Magainin 1[1)]

Hirokazu TAMAMURA, Junko NAKAMURA, Katsuhiro NOGUCHI, Susumu FUNAKOSHI and Nobutaka FUJII*

Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan. Received August 10, 1992

In solid-phase peptide synthesis, the addition of *m*-cresol to diluted methanesulfonic acid (MSA) in dichloromethane accelerated the deprotection rate of the acid-labile α-amino protecting group, the *p*-methoxybenzyloxycarbonyl (Z(OMe)) group. Further, 0.1 M MSA, 20% *m*-cresol/CH_2Cl_2 was found to be a practically useful N^{α}-deprotecting reagent system, since the deprotection of the Z(OMe) group occurred selectively within 30 min at room temperature, leaving intact the other side chain protecting groups, such as benzyloxycarbonyl, benzyl ester, *S*-*p*-methoxybenzyl and N^{G}-mesitylene-2-sulfonyl groups. This reagent system was applied to the Z(OMe)-based solid phase syntheses of human pancreastatin-29 and magainin 1.

Keywords diluted methanesulfonic acid; *m*-cresol; *p*-methoxybenzyloxycarbonyl group; solid-phase peptide synthesis; human pancreastatin synthesis; magainin 1 synthesis

In the Boc-based solid-phase peptide synthesis, 40—60% trifluoroacetic acid (TFA)/CH_2Cl_2 is widely used for deprotection of the α-amino protecting groups. Wang *et al.*[2)] found that the deprotection of the Z(OMe) group[3)] proceeds rapidly and completely, requiring much less TFA than the deprotection of the Boc group. The greater lability of the Z(OMe) group to acid deprotection provides increased selectivity of removal between the N^{α}-protecting group and the benzyl-based protecting groups on the side chains or anchoring bonds to the resin. However, TFA is highly toxic, volatile and expensive, and there is the possibility of a trace amount of TFA forming termination side products[4)] and undesired pyroglutamyl formation[5)] during the coupling steps. It was previously demonstrated that diluted sulfonic acids, such as methanesulfonic acid (MSA), are preferable deprotecting reagents for the acid-labile α-amino protecting groups, such as Boc and Z(OMe).[6)] Kiso *et al.*[5,7)] reported the usefulness of 0.3 or 0.5 M MSA/CH_2Cl_2–dioxane (9:1) as an N^{α}-Z(OMe) or N^{α}-Boc deprotecting reagent in solid-phase peptide synthesis. It was demonstrated that with a diluted MSA reagent the side reaction of pyroglutamyl formation was suppressed to a much lower level than with the usual TFA reagent. The Boc-MSA strategy was successfully applied to the solid-phase syntheses of relatively complex peptides, such as porcine brain natriuretic peptide-26.[8)] We also tried to find an alternative diluted mechanesulfonic acid reagent system suitable for the Z(OMe)-based solid phase syntheses of relatively long and complex peptides.

In solid phase synthesis, *m*-cresol is an effective swelling reagent for peptidyl resins and the addition of *m*-cresol to a deprotecting reagent would provide a slightly acidic condition. The N^{α}-Z(OMe)-protected insulin A chain (residues 13—21) resin[9)] was selected as a model peptide resin and the deprotection rate of the Z(OMe) group was examined with the addition of 5% or 20% *m*-cresol to 0.1 M MSA (this concentration was preliminarily determined) in CH_2Cl_2 (Fig. 1). Increasing the *m*-cresol concentration from 5% to 20% remarkably accelerated the removal rate of the Z(OMe) group. The complete deprotection of the Z(OMe) group required only 5 min in the case of 20% *m*-cresol but 30 min in the case of 5% *m*-cresol. We have also examined the selectivity of various concentrations of MSA in 20% *m*-cresol/CH_2Cl_2 in order to find a suitable deprotecting condition under which the partial cleavage of

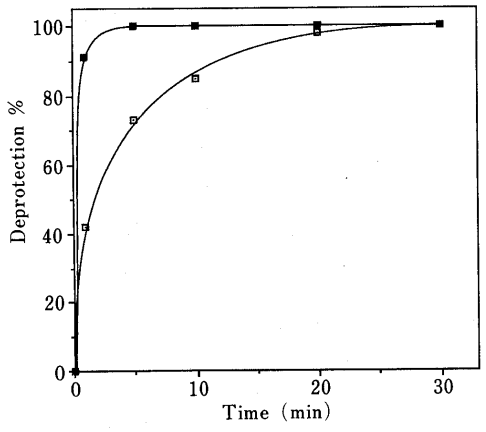

Fig. 1. The Deprotection of the Z(OMe) Group from the Protected Insulin A Chain (Residues 13—21) Resin by 0.1 M MSA in CH_2Cl_2 Containing 5% or 20% *m*-Cresol

⊡, 5% *m*-cresol; ■, 20% *m*-cresol.

TABLE I. Cleavage of Side-Chain Protecting Groups from Z(OMe)–Lys(Z)–OH, Z(OMe)–Glu(OBzl)–OH and Z(OMe)–Asp(OBzl)–OH

Reagent	Regeneration of parent amino acids (%)		
	Lys from Lys(Z)[a)] 24 h	Glu from Glu(OBzl)[b)] 24 h	Asp from Asp(OBzl)[c)] 24 h
0.5 M MSA[d)]	15		
0.2 M MSA[d)]	<1		
0.1 M MSA[d)]	0	3	<1
60% TFA[e)]	55		
25% TFA[e)]	3	8	2
0.3 M MSA[f)]	<1	4	3
0.5 M MSA[f)]	<1	14	3

a—*c*) Lys, Glu and Asp were measured by amino acid analysis. *d*) Containing 20% *m*-cresol in CH_2Cl_2. *e*) Containing 2% anisole in CH_2Cl_2. *f*) Containing 2% anisole in CH_2Cl_2–dioxane (9:1).

hPS-29 : H-Pro-Glu-Gly-Lys-Gly-Glu-Gln-Glu-His-Ser-Gln-Gln-Lys-Glu-Glu-Glu-Glu-Glu-Met-Ala-Val-Val-Pro-Gln-Gly-Leu-Phe-Arg-Gly-NH_2

magainin 1: H-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Gly-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Lys-Ser-OH

Fig. 2. Structures of Human Pancreastatin (HPS)-29 and Magainin 1

TABLE II. Stabilities of Boc–Gly–OCH_2–PAM–Resin and Boc–Gly–MBHA–Resin

Reagent	Cleavage of Gly (%) PAM-type[a]	Cleavage of Gly–NH_2 (%) MBHA-type[b]
0.1 M MSA, 20% *m*-cresol/CH_2Cl_2	0.6	1.1
25% TFA/CH_2Cl_2	1.3	1.5
60% TFA/CH_2Cl_2	1.3	4.5
0.3 M MSA/CH_2Cl_2–dioxane (9 : 1)	1.4	1.4
0.5 M MSA/CH_2Cl_2–dioxane (9 : 1)	3.3	2.6

a) Cleavage yield from Boc–Gly–OCH_2–PAM–resin (25 °C, 24 h). *b*) Cleavage yield from Boc–Gly–MBHA–resin (25 °C, 24 h).

the side chain protecting groups can be minimized.

Three amino acid derivatives, Z(OMe)–Lys(Z)–OH, Z(OMe)–Glu(OBzl)–OH and Z(OMe)–Asp(OBzl)–OH, were selected as model compounds, and the behavior of the protecting groups with the MSA and TFA reagents cited in Table I was examined. In each case, the complete deprotection of the Z(OMe) group was observed within 30 min by TLC. The generation of Lys, Glu or Asp after 24 hours' treatment is shown in Table I. From this experiment, the following tendencies were observed: 1) partial cleavage of the Z group occurred within 24 h upon standing with 0.2 to 0.5 M MSA in 20% *m*-cresol/CH_2Cl_2; 2) 0.1 M MSA in 20% *m*-cresol/CH_2Cl_2 is superior to TFA reagents and MSA reagents without *m*-cresol regarding selectivity in deprotection between the Z(OMe) and the Z or the Bzl group.

Furthermore, the behavior of other amino acid derivatives, Z(OMe)–Ser(Bzl)–OH, Z(OMe)–Thr(Bzl)–OH, Z(OMe)–Cys(MBzl)–OH, Z(OMe)–His(Bom)–OH, Z(OMe)–Arg(Mts)–OH, Z(OMe)–Trp(Mts)–OH and Z(OMe)–Tyr(Cl_2Bzl)–OH, was examined with the 0.1 M MSA reagent. The complete deprotection of the Z(OMe) group from each amino acid derivative within 30 min was observed and no partial cleavage of any side chain protecting group during 24 hours' treatment was detected by TLC.

The stabilities of two peptide linkers, the PAM (*p*-oxymethylphenylacetamidomethyl) linker[10] of Boc–Gly–OCH_2–PAM resin and the MBHA (*p*-methylbenzhydrylamine) linker[11] of Boc–Gly–MBHA resin, were examined during each deprotecting treatment (24 h) (Table II). The Boc group was completely deprotected from both resins during each deprotecting treatment within 30 min (data not shown). Both of the peptide linkers were more stable to the 0.1 M MSA reagent than any other reagent used in this experiment. These results suggested that the solid-phase synthesis using the 0.1 M MSA reagent would provide a good overall yield.

Based on the above preliminary experiments, we conducted the Z(OMe)-based solid-phase syntheses of a Gln-rich 29-residue peptide amide, human pancreastatin (hPS)-29,[12] and a Lys-rich 23-residue peptide, magainin 1,[13] by employing 0.1 M MSA, 20% *m*-cresol/CH_2Cl_2 as the N^{α}-deprotecting reagent (Fig. 2). The results were compared with those of parallel syntheses of both peptides using the conventional Boc–TFA strategy.

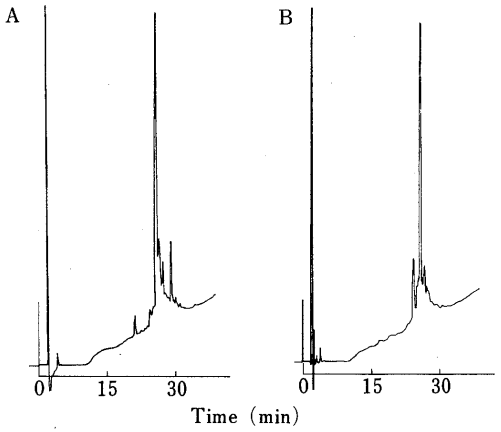

Fig. 3. Analytical HPLC of Crude Human Pancreastatin-29
A: Z(OMe)–MSA strategy. B: Boc–TFA strategy.

For the synthesis of hPS-29 in the Z(OMe)–MSA strategy, each Z(OMe)–amino acid derivative was condensed successively by means of DIPCDI–HOBt on the MBHA resin. After every condensation, the resulting resin was treated with 0.1 M MSA reagent to remove the Z(OMe) group and subsequently neutralized by treatment with 10% NMM/DMF. In the Boc–TFA strategy,[14] the chain elongation was carried out similarly using each Boc-amino acid derivative on the MBHA resin (60% TFA reagent was used as the N^{α}-deprotecting reagent). As a lysine derivative, Z(OMe)–Lys(Z)–OH was used for the Z(OMe)–MSA strategy, and Boc–Lys(Cl–Z)–OH, whose side chain protecting group (Cl–Z) is more stable under acidic conditions than the Z group,[15] was used for the Boc–TFA strategy. The yield of the chain elongation in the Z(OMe)–MSA strategy calculated from the starting MBHA resin was 81% based on the weight of the resin, while that in the Boc–TFA strategy was 64%. Each protected peptide resin thus obtained was treated with 1 M TMSOTf[16]–thioanisole/TFA in order to cleave the peptide amide from the resin and at the same time to remove all protecting groups employed except for O from Met(O). Next, the obtained peptide was treated with 1 M TMSBr[17]–thioanisole/TFA in order to reduce Met(O) to Met. Each crude peptide exhibited a sharp main peak (the same retention time) on HPLC (Fig. 3) and was then purified by preparative HPLC. The yields of the purified peptide in the Z(OMe)–MSA and Boc–TFA strategies calculated from the MBHA resins were 14% and 10%, respectively.

Next, for the synthesis of magainin 1 in the Z(OMe)–MSA strategy, the chain elongation, deprotection and purification were carried out in essentially the same way as in the

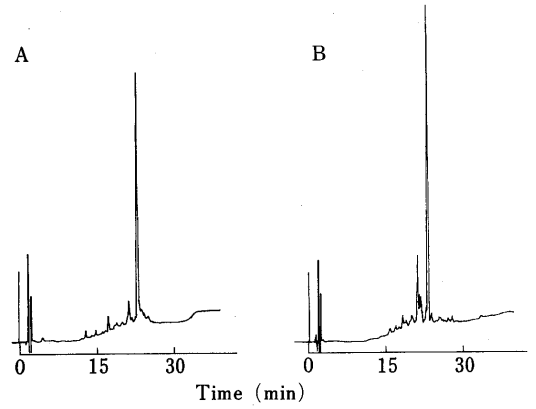

Fig. 4. Analytical HPLC of Crude Magainin-1

A: Z(OMe)–MSA strategy. B: Boc–TFA strategy (Applied Biosystems automated peptide synthesizer).

synthesis of hPS-29. The HPLC profile of the synthetic crude peptide was compared with that in the Boc–TFA strategy using an Applied Biosystems model 430 A automated peptide synthesizer. The crude peptides had similar purity and in each case the main peak had the same retention time on HPLC (Fig. 4).

This paper presents some advantageous features of the Z(OMe)-based solid phase synthesis using a diluted MSA deprotecting reagent system containing *m*-cresol. The addition of *m*-cresol (20%) to diluted MSA in CH_2Cl_2 accelerates the deprotection rate, and the concentration of MSA can be reduced to 0.1 M. As a result, for selectivity in deprotection between the N^{α}-protecting group and the side chain protecting groups or anchoring bonds to the resin, 0.1 M MSA, 20% *m*-cresol in CH_2Cl_2 is superior to the conventional TFA systems and diluted MSA systems without *m*-cresol. Relatively complex peptides, hPS-29 (Gln-rich) and magainin 1 (Lys-rich), could be successfully synthesized by this method without significant side reactions due to pyroglutamyl formation and/or partial cleavage of side chain protecting groups. We intend to evaluate further whether this deprotecting procedure with MSA and *m*-cresol can be applied to the syntheses of more complex peptides.

Experimental

Amino acid analysis and HPLC were conducted with a Hitachi 835 instrument and a Waters model 600M, respectively. The solvents for HPLC were H_2O and CH_3CN, both containing 0.1% (v/v) TFA. For analytical HPLC, Cosmosil C18 (5 μm particle size, 4.6 × 150 mm) was used at a flow rate of 1 ml/min. Preparative HPLC was performed on Cosmosil C18 (5 μm particle size, 10 × 250 mm). The eluate was monitored by UV absorption measurement at 220 nm. MBHA resin, Boc–Gly–OCH_2–PAM resin and Boc–Ser(Bzl)–OCH_2–PAM resin were purchased from Peptide Institute, Inc., Watanabe Chemical Industries, Ltd. and Applied Biosystems, Inc., respectively. In TLC on silica gel (Kieselgel G, Merck) the following solvent system was used: $CHCl_3$–MeOH–H_2O (8:3:1). FAB-mass spectra were recorded on a VG Analytical ZAB-SE instrument.

The Rate Measurement of Z(OMe) Group Deprotection by 0.1 M MSA in CH_2Cl_2 Containing 5% or 20% *m*-Cresol Treatment of 20 mg (5 μmol) of protected insulin A chain (13—21) resin with 0.1 M MSA in CH_2Cl_2 containing 5% or 20% *m*-cresol (2 ml each) was performed at 25 °C. At intervals (1, 5, 10, 20 and 30 min), part of the resin (3 mg each) was sampled and washed six times with CH_2Cl_2 (20 ml). The amount of free amino groups on the dry resin was then measured quantitatively by ninhydrin monitoring[18] (Fig. 1).

Treatment of Lysine, Glutamic Acid and Aspartic Acid Derivatives with Diluted MSA or TFA in CH_2Cl_2 Treatment of lysine, glutamic acid and aspartic acid derivatives (10 μmol each), Z(OMe)–Lys(Z)–OH, Z(OMe)–Glu(OBzl)–OH and Z(OMe)–Asp(OBzl)–OH, with each of the deprotection reagents cited in Table I (1 ml each) was performed at 25 °C. After 30 min, part of each solution was subjected to TLC, and then 12 N aqueous HCl and the ninhydrin reagent were sprayed on the plate, followed by heating in an oven (80 °C) for 15 min. In each case, the starting material was not detected and complete deprotection of the N^{α}-Z(OMe) group was confirmed. After 24 h, part of the solution (0.5 μmol each) was examined by amino acid analysis, and the amount of Lys, Glu or Asp was measured quantitatively. The results are listed in Table I.

Treatment of Other Amino Acid Derivatives with 0.1 M MSA Reagent Treatment of other amino acid derivatives (10 μmol each), Z(OMe)–Ser(Bzl)–OH, Z(OMe)–Thr(Bzl)–OH, Z(OMe)–Cys(MBzl)–OH, Z(OMe)–His(Bom)–OH, Z(OMe)–Arg(Mts)–OH, Z(OMe)–Trp(Mts)–OH and Z(OMe)–Tyr(Cl_2Bzl)–OH, with 0.1 M MSA, 20% *m*-cresol/CH_2Cl_2 (1 ml) was performed at 25 °C. At 30 min and 24 h, part of each solution was examined by TLC. The complete deprotection of the N^{α}-Z(OMe) group (30 min) and no partial cleavage of each side chain protecting group (24 h) were confirmed.

Examination of the Stabilities of Two Peptide Linkers Treatment of Boc–Gly–OCH_2–PAM resin (0.35 mmol/g) (10 mg) with each of the deprotection reagents cited in Table II (1 ml each) was performed at 25 °C. After 24 h, part of the solution (400 μl each) was subjected to amino acid analysis (Table IIa).

Treatment of Boc–Gly–MBHA resin (0.36 mmol/g) 10 mg with each of the deprotection reagents (1 ml each) was similarly performed. After 24 h, part of the solution (400 μl each) was evaporated and the residue was subjected to amino acid analysis after 6 N HCl hydrolysis (Table IIb).

Solid-Phase Synthesis of Human Pancreastatin (HPS)-29 The synthesis based on the Z(OMe)–MSA or Boc–TFA strategy was carried out manually using the following Z(OMe) or Boc amino acid derivatives; *i.e.*, Gln, Gly, Ala, Val, Leu, Phe, Pro, Ser(Bzl), Glu(Bzl), Met(O), Lys(Z) [Z(OMe)–strategy], Lys(Cl–Z) [Boc–strategy], His(Bom) and Arg(Mts). Chain elongation was carried out according to the following cycle schedule. Each Z(OMe) or Boc amino acid derivative (2.5 eq) in DMF (2 ml) was condensed successively using DIPCDI (2.5 eq) in the presence of HOBt (2.5 eq) on the MBHA resin (263 mg, amine content 0.1 mmol). Every condensation reaction was continued until the resin became negative to the Kaiser test. After being washed with DMF (1 min × 5) and CH_2Cl_2 (1 min × 3), the resin was treated twice (5 and 30 min) with 0.1 M MSA, 20% *m*-cresol/CH_2Cl_2 or 60% TFA/CH_2Cl_2 and subsequently washed with the following solvents; CH_2Cl_2 (1 min × 5), MeOH (1 min × 2), CH_2Cl_2 (1 min × 5) and DMF (1 min × 5). Next, the resulting resin was treated with 10% NMM/DMF (2 min × 2) and washed with DMF (1 min × 5). Each protected hPS-29 resin was obtained in the following yield: Z(OMe)–MSA strategy, 598 mg (81%, calculated from the MBHA resin); Boc–TFA strategy, 475 mg (64%).

The Deprotection of the Protected HPS-29 Resins and the Preparation of HPS-29 Each protected hPS-29 resin (50 mg, 6.7 μmol) was treated with 1 M TMSOTf–thioanisole/TFA (5 ml) in the presence of *m*-cresol (0.25 ml, 500 eq) and EDT (0.1 ml, 300 eq) at 4 °C for 2 h. Each resin was removed by filtration and washed twice with TFA (1 ml). The filtrate and washings were combined and ice-chilled dry ether (50 ml) was added to precipitate each product. After centrifugation, the ether was removed by decantation. The residue was treated with 1 M TMSBr–thioanisole/TFA (5 ml) at 4 °C for 1 h; the resulting HBr gas was then discarded by evaporation and ice-chilled dry ether (50 ml) was added to precipitate each product. After centrifugation, the ether was removed by decantation and the residue was dissolved in 0.1 N AcOH (3 ml). The solution was applied to a Sephadex G-15 column (3.7 × 40 cm), which was eluted with 0.1 N AcOH. The eluate was monitored by measurement of UV absorption at 206 nm. The fractions corresponding to each front main peak were combined and the solvent was removed by lyophilization to give a powder: Z(OMe)–MSA strategy, 12 mg (52%, calculated from the protected hPS-29 resin): Boc–TFA strategy, 11 mg (50%). The gradient elution pattern on analytical HPLC with CH_3CN (10—40%, 30 min) is shown in Fig. 3. Each of the samples gave a sharp main peak (26 min retention time).

The crude peptide thus obtained was purified by preparative HPLC. The eluate corresponding to the main peak was collected and the solvent was removed by lyophilization to give a white fluffy powder: yield; Z(OMe)–MSA strategy, 3.8 mg (14%, calculated from the MBHA resin); Boc–TFA strategy, 3.5 mg (10%). Amino acid ratios in 6 N HCl hydrolysis (values in parentheses are theoretical): [Z(OMe)–MSA strategy] Ser 0.88 (1), Glu 12.00, (12), Gly 4.19 (4), Ala 1.04 (1), Val 1.72 (2), Met 0.11 (1), Leu 1.04 (1), Phe 1.03 (1), Lys 1.96 (2), His 0.99 (1), Arg 1.00 (1), Pro

1.87 (2) (recovery of Arg, 60%); [Boc–TFA strategy] Ser 0.89 (1), Glu 11.79 (12), Gly 4.08 (4), Ala 0.97 (1), Val 1.52 (2), Met 0.42 (1), Leu 0.99 (1), Phe 1.02 (1), Lys 1.84 (2), His 0.96 (1), Arg 1.00 (1), Pro 2.61 (2) (recovery of Arg, 60%). FAB-MS *m/z*: [Z(OMe)–MSA strategy] 3282.7 $(M+H)^+$; [Boc–TFA strategy] 3282.3 $(M+H)^+$ (Calcd 3281.5).

Solid-Phase Synthesis of Magainin 1 Boc–Ser(Bzl)–OCH_2–PAM resin (137 mg, content 0.1 mmol) was treated with 60% TFA/CH_2Cl_2 (5 and 30 min) and 10% NMM/DMF (2 min × 2). The chain elongation was carried out using the following Z(OMe) amino acid derivatives; *i.e.*, Gly, Ala, Val, Ile, Leu, Phe, Ser(Bzl), Glu(OBzl), Met(O), Lys(Z) and His(Bom) as in the synthesis of hPS-29 in the Z(OMe)–MSA strategy. The protected magainin 1 resin was obtained in a yield of 366 mg (80%, calculated from Boc–Ser(Bzl)–OCH_2–PAM resin).

The Deprotection of the Protected Magainin 1 Resin and the Preparation of Magainin 1 The protected magainin 1 resin thus obtained (50 mg, 11 μmol) was cleaved and deprotected in the same way as in the synthesis of hPS–29. The obtained crude material was purified by gel-filtration and preparative HPLC: yield, 8.6 mg (26%, calculated from Boc–Ser(Bzl)–OCH_2–PAM resin). Amino acid ratios in 6 N HCl hydrolysis (values in parentheses are theoretical): Ser 1.23 (2), Glu 1.18 (1), Gly 6.13 (5), Ala 2.14 (2), Val 1.00 (1), Met 0.05 (1), Ile 1.95 (2), Leu 1.16 (1), Phe 3.09 (3), Lys 3.91 (4), His 1.11 (1) (recovery of Val, 70%). FAB-MS *m/z*: 2410.4 $(M+H)^+$ (Calcd 2409.3). The gradient elution pattern of the synthetic crude peptide on analytical HPLC with CH_3CN (20—50%, 30 min) was compared with that in the Boc–TFA strategy using an Applied Biosystems model 430A automated peptide synthesizer (Fig. 4).

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References and Notes

1) The amino acids used here are of the L-configuration. The following abbreviations are used: Z(OMe) = *p*-methoxybenzyloxycarbonyl, MBzl = *p*-methoxybenzyl, Boc = *tert*-butoxycarbonyl, Z = benzyloxycarbonyl, Bzl = benzyl, Bom = benzyloxymethyl, Mts = mesitylene-2-sulfonyl, Cl_2Bzl = 2,6-dichlorobenzyl, Cl–Z = 2-chlorobenzyloxycarbonyl, PAM = phenylacetamidomethyl, MBHA = 4-methylbenzhydrylamine, DIPCDI = 1,3-diisopropylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, DMF = *N*,*N*-dimethylformamide, NMM = *N*-methylmorpholine, TMSOTf = trimethylsilyl trifluoromethanesulfonate, TMSBr = trimethylsilyl bromide, EDT = 1,2-ethanedithiol, DMBHA = 2,4′-dimethoxybenzhydrylamine.
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