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Studies on Peptides. CXXXVIII.[1,2] Conventional Solution Synthesis of Bovine Hypothalamic Growth Hormone Releasing Factor (bGRF)

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A 44 residue peptide amide corresponding to the entire amino acid sequence of bovine hypothalamic growth hormone releasing factor (bGRF) was synthesized by assembling nine peptide fragments *via* the azide followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid. Met(O) was reduced by dimethylselenide during the above acid treatment. The synthetic peptide was as active as synthetic human GRF–44–NH_2 *in vitro* assay.

Keywords——bovine hypothalamic growth hormone releasing factor solution synthesis; dimethylselenide as Met(O) reducing reagent; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection; *in vitro* growth hormone releasing factor activity

After our solution syntheses of human growth hormone releasing factor (hGRF–44–NH_2)[3] and porcine GRF (pGRF–44–NH_2),[1] we wish to report the solution synthesis of bovine GRF (bGRF–44–NH_2), the structure of which was determined by Esch *et al.*[4] This factor possesses the same sequence as hGRF,[5] except for replacement of five residues at positions 28, 34, 38, 41 and 42, *i.e.*, Ser, Ser, Arg. Arg and Ala of hGRF with Asn, Arg, Gln, Lys and Val, respectively.

As described in the preceding paper,[1] amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA[6] were employed, *i.e.*, Arg(Mts),[7] Glu(OBzl), Asp(OBzl) and Lys(Z). The Met residue was reversibly protected as its sulfoxide.[8] Nine fragments were selected as building blocks to construct the peptide backbone of bGRF–44–NH_2 by the azide procedure[9] (Fig. 1). Of these, fragments [3] to [9] are those employed for our previous synthesis of pGRF–44–NH_2. In the present synthesis, two fragments, [1] and [2], which cover the area of species variation in the human, porcine and bovine factors, were newly synthesized.

Fragment [1] was prepared according to the scheme shown in Fig. 2. Starting with Z(OMe)–Val–Arg(Mts)–Leu–NH_2,[1] the C-terminal pentapeptide, Z(OMe)–Ala–Lys(Z)–Val–Arg(Mts)–Leu–NH_2, was prepared in a stepwise manner by successive addition of Z(OMe)–Lys(Z)–OH and Z(OMe)–Ala–OH *via* the mixed anhydride[10] and the Np active ester[11] methods, respectively, then Z(OMe)–Glu(OBzl)–Gln–Gly–$NHNH_2$[1] derived from the corresponding Tcboc-hydrazide derivative was condensed with a TFA-treated sample of the above pentapeptide amide *via* the azide procedure. The resulting octapeptide chain was elongated to [1] in a stepwise manner by the Np method or the mixed anhydride method. The purity of fragment [1] was ascertained by amino acid analysis, thin layer chromatography (TLC) and elemental analysis, as was done for other fragments.

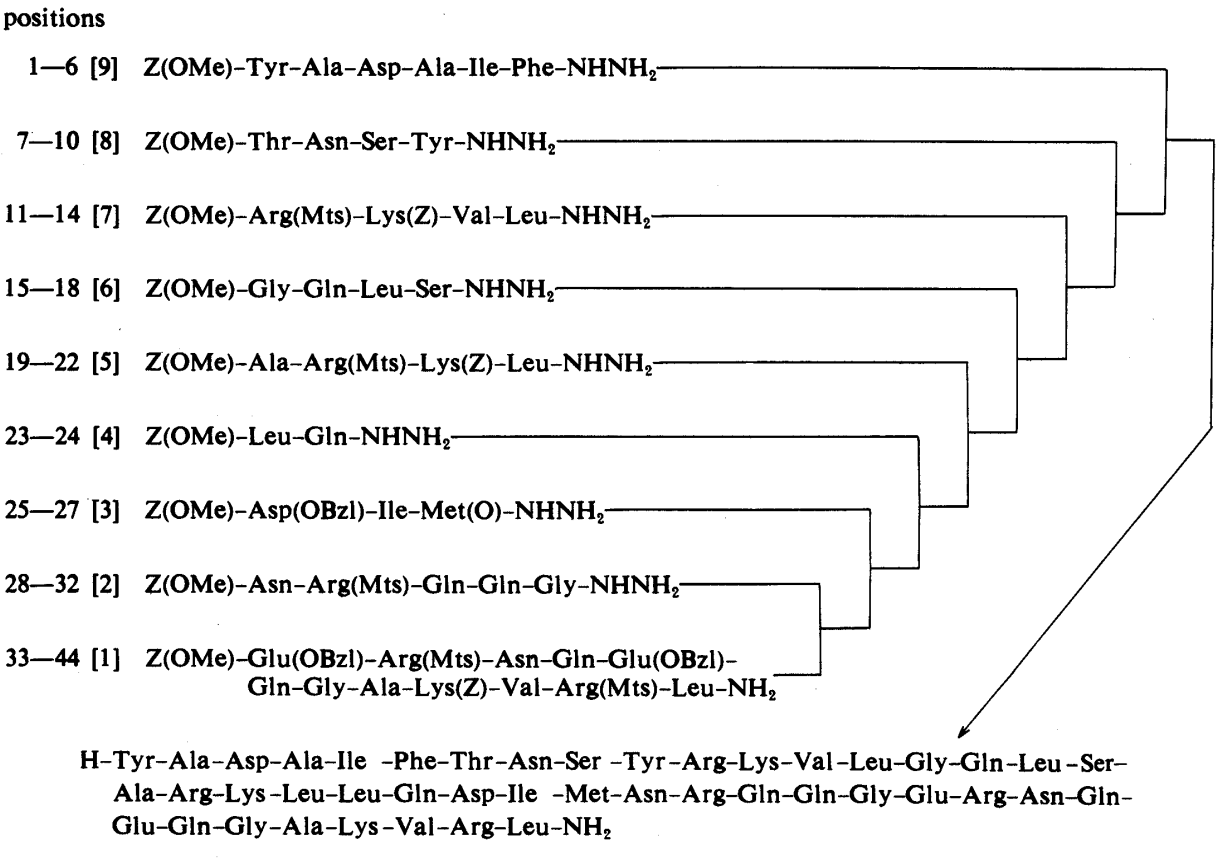

Fig. 1. Synthetic Route to Bovine GRF–44–NH_2

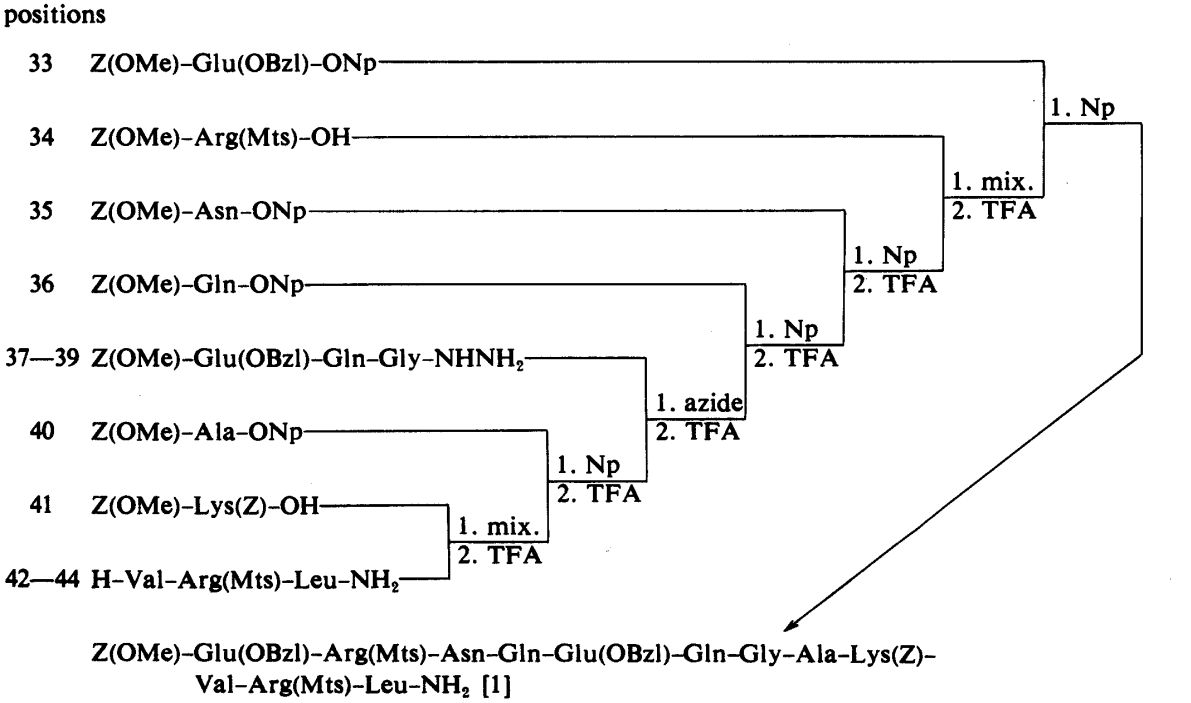

Fig. 2. Synthetic Scheme for the Protected Dodecapeptide Amide Z(OMe)-(bGRF 33—44)-NH_2 [1]

Fragment [2] (position 28—32) was prepared by condensation of Z(OMe)–Asn–ONp with a TFA-treated sample of Z(OMe)–Arg(Mts)–Gln–Gln–Gly–OMe, an intermediate of our previous synthesis of hGRF–44–NH_2,[3] followed by hydrazinolysis.

Table I. Amino Acid Ratios in 6N HCl Hydrolysates of Synthetic bGRF and Its Protected Intermediates

	Protected intermediates								Synthetic bGRF
	28—44	25—44	23—44	19—44	15—44	11—44	7—44	1—44	
Asp	1.91	2.87	3.02	3.14	3.12	3.10	4.13	4.80	5.00 (5)
Thr							0.95	0.82	0.98 (1)
Ser					0.82	0.87	1.87	1.51	1.84 (2)
Glu	6.34	6.22	6.65	7.76	8.80	8.73	8.83	8.70	8.33 (8)
Gly	1.96	2.01	2.15	2.18	3.16	3.24	3.34	3.09	3.12 (3)
Ala	1.04	1.00	1.07	2.05	2.06	2.15	2.21	3.66	3.84 (4)
Val	0.91	0.99	1.04	1.04	1.02	1.95	1.96	1.96	1.85 (2)
Met		0.81	0.77	0.82	0.85	0.84	0.86	0.87	0.79 (1)
Ile		0.89	0.91	0.95	1.03	0.98	1.00	1.68	1.83 (2)
Leu	1.00	1.00	2.00	3.00	4.00	5.00	5.00	5.00	5.00 (5)
Tyr							0.79	1.65	1.63 (2)
Phe								0.72	0.86 (1)
Lys	1.04	1.01	1.09	2.04	1.94	2.71	2.43	3.08	2.87 (3)
Arg	2.96	3.07	3.31	4.39	4.21	5.35	5.21	5.31	5.11 (5)
Rec.	94%	87%	77%	71%	75%	74%	62%	99%	65%

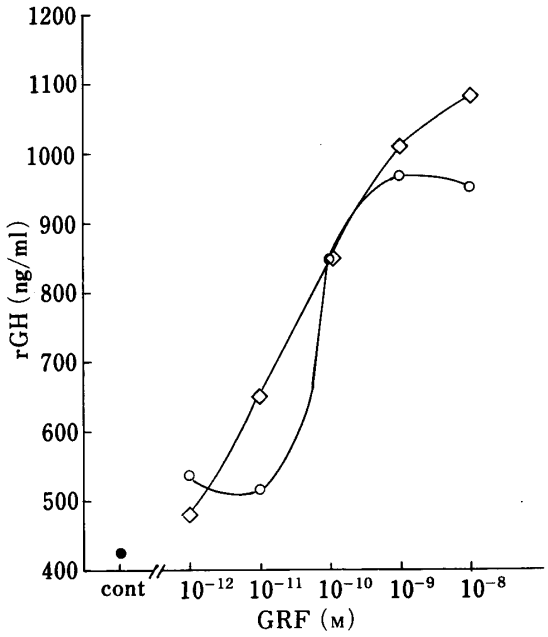

Fig. 3. *In Vitro* Assay of Synthetic bGRF–44–NH_2

○, hGRF–44–NH_2; ◇, bGRF–44–NH_2.

Nine fragments thus obtained were assembled successively by the azide procedure according to the route illustrated in Fig. 1. The protected products obtained after condensation reactions of fragments [1] to [4] were purified by precipitation from DMSO–DMF with methanol and the rest of the products, including the protected bGRF, by gel-filtration on Sephadex LH-60 using DMF as an eluant. Throughout this synthesis the C-terminal Leu was selected as a diagnostic amino acid, as in our previous synthesis of pGRF. Amino acid ratios in 6N HCl hydrolysates are listed in Table I. The homogeneity of every product was further ascertained by elemental analysis and TLC.

In the final step, deprotection with 1M TFMSA–thioanisole in TFA and subsequent purification were carried out in essentially the same manner as described for pGRF synthesis. The Met(O) residue was reduced back to Met in two steps, firstly with thioanisole and

dimethylselenide[12] during the above acid treatment, and secondly with dithiothreitol during incubation of the deprotected peptide. The reduced product was purified by gel-filtration on Sephadex G-25, followed by reversed-phase high performance liquid chromatography (HPLC) on a Zorbax (Shimadzu) BP-ODS column using gradient elution with acetonitrile (30%—40%) in 0.3% TFA. The product thus purified exhibited a single peak in analytical HPLC and its homogeneity was further ascertained by amino acid analyses after acid hydrolysis and enzymatic digestion.

Synthetic bGRF–44–NH_2 was found to be as active as our synthetic hGRF in respect of increasing immunoreactive growth hormone in rat plasma (Fig. 3).

Experimental

General experimental procedures employed in this investigation were essentially the same as those used in our pGRF synthesis.[1] Protected peptides were purified by the precipitation procedure (A) or by gel-filtration (B). Solvents used for (A): A-1, DMSO–DMF(1 : 1)–MeOH; A-2, DMSO–DMF(1 : 3)–MeOH; A-3, DMSO–DMF(1 : 2)–AcOEt; A-4, DMSO–DMF (1 : 2)–MeOH; A-5, DMSO–DMF (2 : 1)–MeOH. Procedure employed for (B): Individual fractions (10 ml each) were examined by ultraviolet (UV) absorption measurement at 280 nm and the fractions corresponding to the front main peak were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder.

Analytical HPLC was conducted with a Shimadzu LC-4A instrument equipped with a Cosmosil (Nakarai Chem. Co.) $5C_{18}P$ column (4.6 × 150 mm) using linear gradient elution with acetonitrile (30% to 40%, 20 min) in 0.3% TFA at a flow rate of 1.0 ml/min.

TLC was performed on silica gel (Kieselgel 60 F_{254}, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8 : 3 : 1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2).

Synthesis of Fragments [1] and [2]——Fragment [1] was prepared according to the scheme shown in Fig. 2, and fragment [2] as described in the text. Purification procedure, physical constants and analytical data are summarized in Table II.

Synthesis of Protected bGRF——The nine fragments were condensed successively by the azide procedure according to the route shown in Fig. 1. The purification procedure, physical constants and analytical data of protected bGRF and its intermediates are listed in Table III. All protected peptides decomposed over 260 °C.

H–Tyr–Ala–Asp–Ala–Ile–Phe–Thr–Asn–Ser–Tyr–Arg–Lys–Val–Leu–Gly–Gln–Leu–Ser–Ala–Arg–Lys–Leu–

TABLE II. Physical Constants and Analytical Data of Fragments [1], [2] and Their Intermediates

Compounds	Puri. proc.	Rf_1	Yield %	mp °C	$[\alpha]_D^{25}$ (in DMSO)	Formula	Analysis (%) Calcd (Found)		
							C	H	N
Z(OMe)–(41—44)–NH_2	A-1	0.62	73	155—158	−11.3°	$C_{49}H_{71}N_9O_{11}S$	59.19	7.20	12.68
							(58.64	7.24	12.58)
Z(OMe)–(40—44)–NH_2	A-1	0.46	71	211—216	−15.0°	$C_{52}H_{76}N_{10}O_{12}S$	58.63	7.19	13.15
							(58.35	7.21	12.98)
Z(OMe)–(37—44)–NH_2	A-2	0.43	71	237—240	−15.0°	$C_{71}H_{100}N_{14}O_{18}S\cdot H_2O$	57.32	6.91	13.18
							(57.10	6.72	13.44)
Z(OMe)–(36—44)–NH_2	A-4	0.38	89	260 (dec.)	−16.0°	$C_{76}H_{108}N_{16}O_{20}S\cdot 2H_2O$	55.87	6.91	13.72
							(55.91	6.72	13.44)
Z(OMe)–(35—44)–NH_2	A-4	0.35	90	260 (dec.)	−20.0°	$C_{80}H_{114}N_{18}O_{22}S\cdot H_2O$	55.54	6.76	14.58
							(55.48	6.72	14.63)
Z(OMe)–(34—44)–NH_2	A-5	0.38	96	260 (dec.)	−13.3°	$C_{95}H_{136}N_{22}O_{25}S_2\cdot 3H_2O$	54.22	6.80	14.64
							(54.15	6.62	14.50)
Z(OMe)–(33—44)–NH_2	A-5	0.35	88	260 (dec.)	−43.0°	$C_{107}H_{149}N_{23}O_{28}S_2\cdot 2H_2O$	55.74	6.69	13.97
							(55.56	6.57	13.72)
Z(OMe)–(28—32)–OMe	A-4	0.09	57	221—224	−39.9°	$C_{41}H_{59}N_{11}O_{14}S$	51.18	6.18	16.02
							(50.95	6.11	16.04)
Z(OMe)–(28—32)–$NHNH_2$	A-4	0.46	95	220—223	−32.7°	$C_{40}H_{59}N_{13}O_{13}S$	49.94	6.18	18.93
							(50.02	6.13	18.63)

TABLE III. Physical Constants and Analytical Data of Protected bGRF and Its Intermediates

Compounds	Puri. proc.	Rf_1	Yield %	$[\alpha]_D^{25}$ (in DMSO)	Formula	Analysis (%) Calcd (Found) C	H	N
Z(OMe)-(28—44)-NH_2	A-4	0.43	77	−17.1°	$C_{138}H_{196}N_{34}O_{38}S_3 \cdot 3H_2O$	53.65 (53.52)	6.59 6.58	15.42 15.51)
Z(OMe)-(25—44)-NH_2	A-3	0.40	72	−12.9°	$C_{160}H_{227}N_{37}O_{44}S_4 \cdot 7H_2O$	52.98 (52.97)	6.70 6.60	14.29 14.33)
Z(OMe)-(23—44)-NH_2	A-3	0.33	86	−15.6°	$C_{171}H_{249}N_{41}O_{47}S_5 \cdot 9H_2O$	51.95 (52.18)	6.81 6.52	14.53 14.08)
Z(OMe)-(19—44)-NH_2	B	0.45	83	−14.3°	$C_{209}H_{302}N_{48}O_{55}S_5 \cdot 10H_2O$	53.32 (53.32)	6.90 6.84	14.28 14.62)
Z(OMe)-(15—44)-NH_2	B	0.30	81	−25.7°	$C_{225}H_{329}N_{53}O_{61}S_5 \cdot 5H_2O$	53.49 (53.48)	6.82 6.52	14.97 14.84)
Z(OMe)-(11—44)-NH_2	B	0.41	75	−17.5°	$C_{265}H_{392}N_{62}O_{69}S_7 \cdot 8H_2O$	53.77 (53.69)	6.94 6.78	14.67 14.74)
Z(OMe)-(7—44)-NH_2	B	0.53	96	−30.0°	$C_{285}H_{419}N_{69}O_{77}S_7 \cdot 10H_2O$	53.08 (53.39)	6.86 6.56	14.99 14.54)
Z(OMe)-(1—44)-NH_2	B	0.43	91	−15.6°	$C_{319}H_{463}N_{73}O_{86}S_7 \cdot 10H_2O$	53.95 (54.14)	6.86 6.67	14.40 14.38)

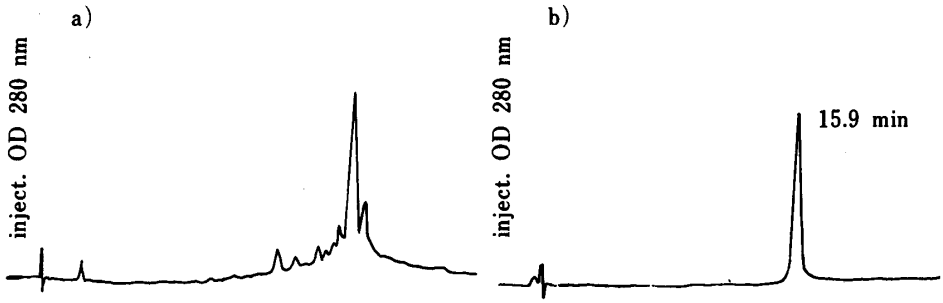

Fig. 4. HPLC of Synthetic bGRF

a) Sephadex-purified sample. b) Purified sample.

Leu-Gln-Asp-Ile-Met-Asn-Arg-Gln-Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln-Gly-Ala-Lys-Val-Arg-Leu-NH_2, bGRF-44-NH_2——The protected tetratetracontapeptide amide, Z(OMe)-(bGRF 1—44)-NH_2, (100 mg, 14.6 μmol) was treated with 1 M TFMSA–thioanisole in TFA (3.5 ml) in the presence of *m*-cresol (92 μl) and Me_2Se (67 μl) in an ice-bath for 90 min, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 30 min and dissolved in 50% AcOH (2 ml) containing dithiothreitol (112 mg). The solution was treated with Amberlite IRA-400 (acetate form, *ca.* 1 g) for 30 min and filtered. The pH of the filtrate was adjusted to 8.0 with 28% NH_4OH in an ice-bath and after 30 min, to 6.5 with 1 N AcOH. The solution was incubated at 40 °C for 8 h and lyophilized. The residue was purified by gel-filtration on Sephadex G-25 (1.8 × 90 cm) using 1 N AcOH as an eluant. The UV absorption at 280 nm was determined in each fraction (4 ml). The fractions corresponding to the front main peak (tube Nos. 21—34) were combined and the solvent was removed by lyophilization to give a powder; yield 58 mg (79%). Subsequent purification was performed by reversed-phase HPLC on a Zorbax (Shimadzu) BP-ODS column (7.9 × 250 mm). The sample (10 mg) was applied to the column, which was eluted with acetonitrile (gradient concentration from 30% to 40% within 20 min) in 0.3% TFA at a flow rate of 3 ml/min. The eluate corresponding to the main peak (retention time 22.8 min, Fig. 4a) was collected. The rest of the sample was similarly purified and the combined eluates were concentrated *in vacuo*. The residue was treated with Amberlite IRA-400 (acetate form, approximately 1 g) and lyophilized to give a fluffy white powder; yield 16 mg (26%), total yield from the protected bGRF was 21%. $[\alpha]_D^{16}$ −56.3° (c=0.8, 1 N AcOH), Rf_2 0.30. The synthetic peptide exhibited a single peak on analytical HPLC at a retention time of 15.9 min (Fig. 4b). Amino acid ratios after aminopeptidase M (Merck, Art. 24645 Lot. No. 2513445) digestion: Asp 2.03(2), Thr+Gln 6.71 (1+6, calcd as Thr), Ser 2.15 (2), Glu 2.40 (2), Gly 2.80 (3), Ala 3.84 (4), Val 2.10 (2), Met 0.90 (1), Ile 1.82(2), Leu 5.00 (5), Tyr 1.94 (2), Phe 0.96 (1), Lys 3.00 (3), Arg 4.77 (5); recovery of Leu 74%. Asn (3) was not determined. *Anal.* Calcd for

$C_{220}H_{366}N_{72}O_{66}S \cdot 9CH_3COOH \cdot 6H_2O$: C, 49.66; H, 7.25; N, 17.52. Found: C, 49.94; H, 7.18; N, 17.57.

References and Notes

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2) Amino acids and their derivatives used in this investigation were of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Mts = mesitylene-2-sulfonyl, Bzl = benzyl, Tcboc = 2,2,2-trichloro-*tert*-butoxycarbonyl, Np = *p*-nitrophenyl, DMF = *N*,*N*-dimethylformamide, DMSO = dimethylsulfoxide, TFMSA = trifluoromethanesulfonic acid, TFA = trifluoroacetic acid.
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