Synthesis and Biological Evaluation of Dehydrophenylalanine Containing Substance P Fragments

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Peptide fragments of Substance P corresponding to the C-terminal segments (6-11) and (4-11), which contain a putative β-turn, were synthesised using solution phase methodology. Analogs of Substance P were synthesised where the phenylalanine residue at position 8 was replaced by dehydrophenylalanine (ΔPhe) and the glycine residue in position 9 was replaced by alanine, valine, sarcosine (N-Me-Gly) and α-aminoisobutyric acid. In two of the analogs [8-ΔPhe, 9-Sar]-SP (4-11) (**5**) and [5,8-ΔPhe, 9-Aib]-SP (4-11) (**7**) glutamine residue at position 5 was also substituted by ΔPhe. These analogs were evaluated for their ability to cause smooth muscle contraction in guinea pig ileum preparation (*in vitro*) and for hypotensive activity (*in vivo*) in female rats anesthetized with urethane. In the hexapeptide series [8-ΔPhe]-SP (6-11) (**1**) and [8-ΔPhe, 9-Sar]-SP (6-11) (**4**) showed significant activity in both the assays. Octapeptide [5,8-ΔPhe, 9-Sar]-SP (4-11) (**5**) with ΔPhe residue in positions 5 and 8 and sarcosine in position 9 was found to be the most potent analog both *in vitro* and *in vivo* systems.

Substance P (SP) is widely distributed in central and peripheral nervous system of vertebrates.[1] SP is recognized for its hypotensive, vasodilatory, smooth muscle contracting properties and also as a neurotransmitter.[2,3] Moreover, it is involved in the transmission of pain, and related to enkephalin secretion.[4] Studies with amino-terminus deletion analogs have established[5] SP (6-11) to be the shortest fragment with full intrinsic activity. [6-p-Glu]-SP (6-11) has been shown to be more potent than SP in some of the pharmacological preparation,[6,7] while in others it is at least as potent[8] as SP. Methionine residue at the C-terminal is the most important element[9] for activity of SP, while Leu and Phe at positions 10 and 7 respectively are almost irreplaceable.[10] Structure–activity correlations on SP have led to the conclusion that Phe at position 7 is likely to be involved in binding to the receptor.[11] In contrast to the importance of Phe at position 7, the requirements of Phe at position 8 are not strict.[12] It has also been shown that the side chain amide groups of Gln residues at positions 5 and 6 do not participate in an important way in the hormone-receptor interaction.[13] Glycine at position 9 of SP has been of special interest[11] because of its location between the relatively rigid N-terminal part 1-8 and the hydrophobic part 10-11. Conformational studies,[14] in solution, have suggested a U-turn in the molecule that allows Gly–Leu–Met–NH_2 segment to establish hydrogen bonds between the terminal amide N-atom (donor) and each of the side chain carbamoyl groups (acceptors) of Gln-5 and Gln-6 residues.

Working with model peptides we have shown that introduction of α,β-dehydroamino acids confers some conformational rigidity and that dehydrophenylalanine (ΔPhe) and dehydroleucine (ΔLeu) help to nucleate a β-turn structure in acyclic peptides.[15,16] In addition to providing conformational constraint, introduction of

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (SP)
1 2 3 4 5 6 7 8 9 10 11

(1) [8-ΔPhe]-SP (6-11)

(2) [8-ΔPhe, 9-Ala]-SP (6-11)

(3) [8-ΔPhe, 9-Val]-SP (6-11)

(4) [8-ΔPhe, 9-Sar]-SP (6-11)

(5) [5,8-ΔPhe, 9-Sar]-Sp (4-11)

(6) [8-ΔPhe, 9-Aib]-SP (6-11)

(7) [5,9-ΔPhe, 9-Aib]-SP (4-11)

Fig. 1. Primary sequences of Substance P and seven partial Substance P analogs containing ΔPhe residues.

ΔPhe results in peptides that are poor substrates for peptidases[17] and therefore of potential interest in the development of SP analogs with prolonged biological activity.

In this paper we present the synthesis and biological activity of ΔPhe containing analogs of SP (Fig. 1) and discuss the structure–activity relationship of these analogs.

Results and Discussion

Synthesis of SP Analogs Containing Phe Residue. Substance P analogs shown in Fig. 1 were synthesised by segment condensation approach in solution. Synthetic routes are shown in Figs. 2 and 3. *t*-Butoxycarbonyl (Boc) group was used for protecting the α-amino group. The Boc groups in the growing peptides were removed with HCl/acetic acid.[18] However, HCl/dioxane was used to remove the Boc group when glutamine was at the N-terminal.[19] Among many approaches utilized for introduction of a ΔPhe moiety, we selected the efficient synthetic route summarized in Figs. 2 and 3 (representative of SP (6-11) and (4-11) series respectively). Dehydro residue was incorporated as a dipeptide sequence which was obtained by β-elimination reaction of the C-terminal DL-phenylserine

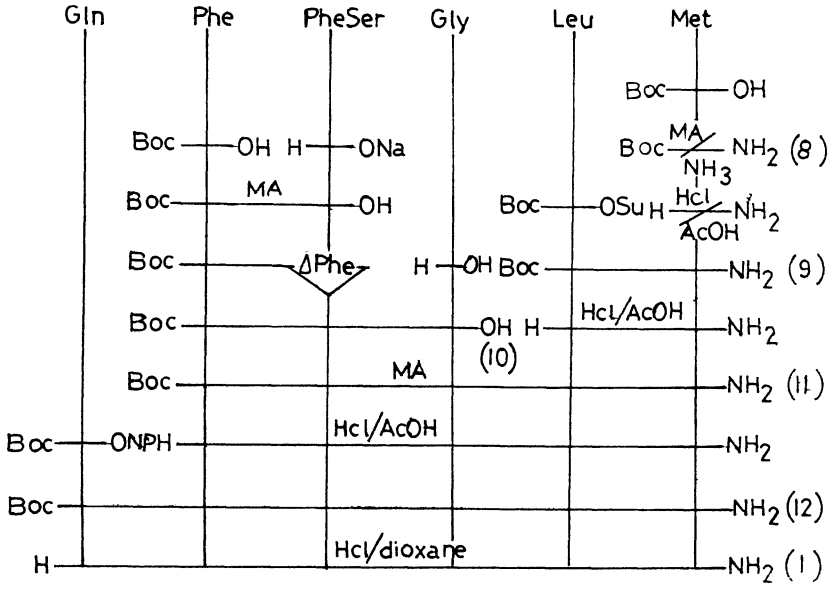

Fig. 2. Synthesis of [8-ΔPhe]-SP (6-11) (**1**).

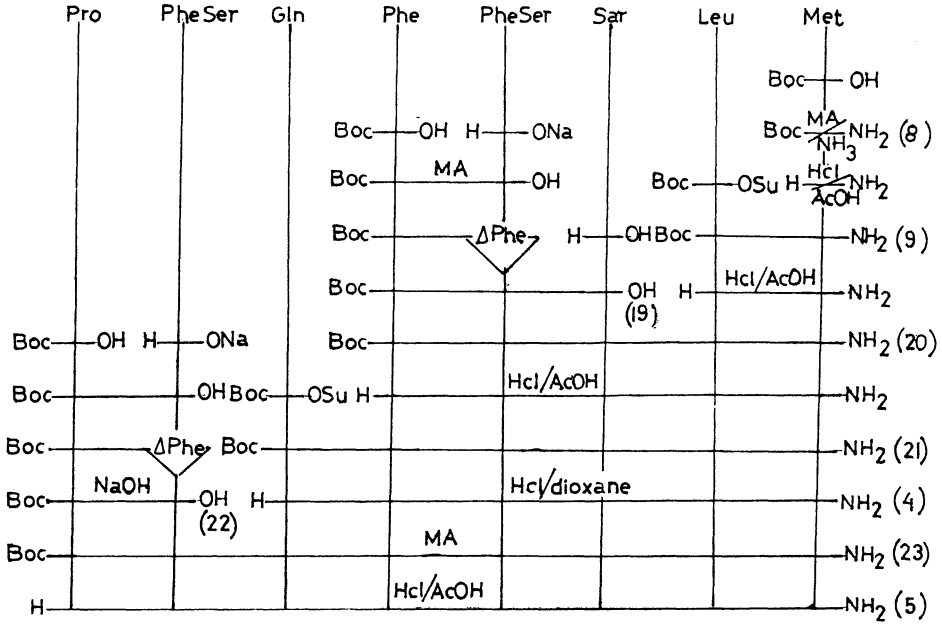

Fig. 3. Synthesis of [5,8-ΔPhe, 9-Sar]-SP (4-11) (**5**).

Table 1. Yields, Physical Properties, and Analytical Data of Intermediate Peptides

Compound	Amino acid sequences	Yield/%	Mp/°C	$[\alpha]_D^{17}$/(*c* 1.0, MeOH)	Elemental analysis/% Found(F:), Calcd (C:)			R_f
					C	H	N	
13	Boc–Phe–ΔPhe–Ala–OH	74	154—155	−64.4	F: 64.96	6.45	8.68	R^1 0.59, R^3 0.87
					C: 64.85	6.49	8.73	
14	Boc–Phe–ΔPhe–Ala–Leu–Met–NH_2	63	115—116	−69.2	F: 61.43	7.19	11.55	R^1 0.8, R^{10} 0.9
					C: 61.30	7.23	11.59	
15	Boc–Gln–Phe–ΔPhe–Ala–Leu–Met–NH_2	56.4	125—126	−39.8	F: 59.15	7.04	13.14	R^2 0.81, R^3 0.83
					C: 59.62	7.14	13.20	
16	Boc–Phe–ΔPhe–Val–Leu–Met–NH_2	80	136—138	−8.05	F: 66.08	6.89	8.21	R^1 0.64, R^2 0.83
					C: 65.99	6.92	8.25	
17	Boc–Phe–ΔPhe–Val–Leu–Met–NH_2	71.2	174—175	+20.8	F: 62.02	7.4	11.10	R^1 0.64, R^2 0.83
					C: 62.21	7.49	11.16	
18	Boc–Gln–Phe–ΔPhe–Val–Leu–Met–NH_2	63	145—147	−5.8	F: 60.01	7.21	12.98	R^1 0.54, R^2 0.75, R^3 0.77
					C: 59.44	7.3	12.90	
19	Boc–Phe–ΔPhe–Sar–OH	63.5	145—147	−25.8	F: 64.72	6.52	8.75	R^1 0.35, R^2 0.82
					C: 64.85	6.49	8.73	
20	Boc–Phe–ΔPhe–Sar–Leu–Met–NH_2	71	171—172	−75.4	F: 60.96	7.02	11.34	R^1 0.6, R^2 0.8
					C: 61.30	7.23	11.59	
21	Boc–Gln–Phe–ΔPhe–Sar–Leu–Met–NH_2	45	125—126	−33.8 (*c* 0.65, MeOH)	F: 59.50	7.15	13.02	R^1 0.41, R^9 0.44, R^{10} 0.3
					C: 59.15	7.04	13.14	
24	Boc–Phe–ΔPhe–Aib–OH	80.5	110—112	−12.3	F: 65.55	6.78	8.40	R^1 0.56, R^3 0.94
					C: 65.44	6.71	8.48	
25	Boc–Phe–ΔPhe–Aib–Leu–Met–NH_2	49	127—128	+19.98 (*c* 1.3, MeOH)	F: 61.61	7.25	11.31	R^1 0.58, R^2 0.88, R^9 0.93
					C: 61.76	7.36	11.37	
26	Boc–Gln–Phe–ΔPhe–Aib–Leu–Met–NH_2	56	137—138	−65.9	—	—	—	R^1 0.57, R^2 0.87, R^9 0.87
27	Boc–Pro–ΔPhe–Gln–Phe–ΔPhe–Aib–Leu–Met–NH_2	56	127—129	−63.8	F: 61.51	7.02	12.50	R^1 0.47, R^2 0.71, R^9 0.81
					C: 61.62	6.93	12.61	

Table 2. Amino Acid Analyses of the Final Peptides

Analog	HPLC retention time[a]	Amino acid composition Found (Calcd)					
	min	Pro	Glu	Phe	X	Leu	Met
[8-ΔPhe, 9-Ala]-SP (6-11) (**2**)	18.7		1.07 (1)	0.96 (1)	1.08 (1)	1.0 (1)	0.92 (1)
[8-ΔPhe, 9-Val]-SP (6-11) (**3**)	23.6		1.1 (1)	0.99 (1)	1.03 (1)	1.0 (1)	0.90 (1)
[8-ΔPhe, 9-Sar]-SP (6-11) (**4**)	27.5		1.05 (1)	0.97 (1)	—	1.0 (1)	0.89 (1)
[8-ΔPhe, 9-Aib]-SP (6-11) (**6**)	24.7		1.02 (1)	0.95 (1)	—	1.0 (1)	0.90 (1)
[5, 8-ΔPhe, 9-Aib]-SP (4-11) (**7**)	28.0	0.93 (1)	1.07 (1)	0.99 (1)	—	1.0 (1)	0.95 (1)

(**2**) X=Ala; (**3**) X=Val; (**4**) X=Sar; (**6**) X=Aib; (**7**) X=Aib.
a) See experimental section for details.

peptide, Boc-Phe-DL-Phe (β-OH)-OH. The compound thus obtained, Boc-Phe-ΔPhe azlactone, served as a key intermediate in the synthesis of all the seven analogs of SP. This method results in the formation of ΔPhe having only the *Z*-configuration.[20] Azlactone ring opening with H-Gly-Leu-Met-NH_2 (obtained by mixed anhydride coupling of Boc-Gly-OH with H-Leu-Met-NH_2) gave low yield (ca. 40%) of the protected pentapeptide. However, better yields of the pentapeptides were obtained if the dipeptide azlactone ring was first opened with the desired free amino acid, to give dehydrotripeptide acid, which was then coupled via a mixed anhydride reaction to H-Leu-Met-NH_2 (3+2 strategy). The coupling of Boc-Gln-ONP or Boc-Gln-OSu with deprotected pentapeptides was slow but gave the hexapeptides in ca. 55% yield. The octapeptides, [5,8-ΔPhe, 9-Sar]-SP (4-11) (**5**) and [5,8-ΔPhe, 9-Aib]-SP (4-11) (**7**), were obtained by subsequent segment condensation of Boc-Pro-ΔPhe-OH with the corresponding hexapeptide amino components using mixed anhydride coupling procedure. Final deprotection was achieved by treatment with HCl/acetic acid. Each intermediate product was checked for purity by TLC and characterized by melting point, optical rotation, chromatographic behavior and NMR measurements. After deprotection peptides (**1**—**7**) were purified by gel filteration followed by semipreparative reversed-phase high-performance liquid chromatography. Homogeneity of the peptides was ensured by analytical RP-HPLC. The peptides were characterized by amino acid analyses. Representative syntheses have been described in the experimental section. Tables 1 and 2 show physical data concerning all other compounds not included in the experimental section.

Biological Activities of SP Analogs Containing ΔPhe Residues. The biological activity of all the newly synthesised analogs of Substance P was examined both *in vitro* (smooth muscle contraction) and *in vivo* (hypotensive activity). It is clear from the data presented in Table 3 that replacement of Phe at position 8 by ΔPhe leads to an analog with reduced but significant[21] agonist activity. The double substitutions with Ala/Val at position 9 in place of Gly, and ΔPhe at position 8 resulted in almost complete loss of potency in this preparation. However, [8-ΔPhe, 9-Sar]-SP(6-11) (**4**) was a weak agonist with potency 0.66% that of SP. The corresponding analog with Aib at position 9 was found to be inactive in this assay system. It appears that requirements for the side chain of amino acids at position 9 are quite subtle and glycine or closely related amino acid like sarcosine may well be one of the main requirements in this position. Replacement of glycine with conformationally restricted amino acid Aib, or even with alanine or valine appears to be highly detrimental to the bioactivity of the analog. In the octapeptide series SP(4-11), an analog with substitutions at positions 5, 8 and 9 [5,8-ΔPhe, 9-Sar]-SP (4-11) (**5**) was found to be the most potent (66.6% of SP) in the present study. A similar analog with Aib at position 9 was found to be much less potent.

In vivo, the hypotensive actions of SP analogs were tested on the rat blood pressure. The assay was performed in female rats anesthetized with urethane, because they maintain stable blood pressure values for several hours.[22] It was found that the intensity of pressure decrease is not dose related. In fact at higher dose the lowering in blood pressure was uniformly lower. The data presented in Table 4 shows a direct correlation between the *in vitro* and *in vivo* activities of the compounds suggesting that the same type of SP-receptor interaction is involved in these two test systems. In both the systems octapeptide **5** shows the highest activity; other dehydrophenylalanine peptides are mostly inactive.

The main objective in introducing dehydrophenylala-

Table 3. Relative Potencies of SP Analogs on Guinea Pig Ileum

Analog	GPI assay equi. active concn μg ml^{-1}	Relative potency (%SP)
(1)	0.16	1.25
(2)	a)	—
(3)	a)	—
(4)	0.3	0.66
(5)	0.003	66.6
(6)	a)	
(7)	6.0	0.03
Substance P	0.002	100

a) No contraction was produced upto 50 μg ml^{-1}.

Table 4. Biological Activities of SP Analogs

Analog	Concn of peptide M	Amount injected μl	Fall in i.v. blood pressure %
SP	1.0×10^{-3}	50	100
(1)	1.33×10^{-3}	50	39.5
		100	31.3
(2)	1.36×10^{-3}	50	10.0
(3)	1.20×10^{-3}	50	20.0
		100	15.8
(4)	1.29×10^{-3}	50	28.6
		100	22.9
(5)	1.01×10^{-3}	50	42.6
		100	34.8
(6)	1.26×10^{-3}	50	20.8
(7)	1.02×10^{-3}	100	16.6

nine residues in Substance P related sequences described above was to obtain linear, conformationally restricted peptide analogs. A single dehydrophenylalanine substitution induced a β turn in model peptide sequences. More recently, we and others have shown that two ΔPhe residue, separated by one or more amino acid residues stabilize helical conformations, in model peptides.[15,31,34] The results described above allow one to conclude that conformational constraints introduced by ΔPhe in position 8 are not favorable for maintaining high biological active. Even though turn structures have been described for Substance P as the active conformations, local rigidity at this position vis-a-vis backbone or the side chain orientation is deterimental for bioactivity in these peptides.

However, a substantial retention of bioactivity in [5, 8-ΔPhe, 9-Sar]-SP(4-11), may suggest that helical type of backbone could allow meaningful ligand-receptor interactions. Inactivity of the corresponding Aib position 9 analog may suggest that the C-terminal peptide needs to be somewhat flexible and the retention of glycine for this purpose may be crucial.

In conclusion, ΔPhe analogs of Substance P are not more active than the parent sequences unlike in the cases of bradykinin[25] or enkephalin were highly active analogs were obtained by such changes.[35]

As part of the screening process for the design and synthesis of potential inhibitors of SP degrading activities, the ΔPhe analogs represented a relatively simply achieved modification of the peptide backbone with the advantageous features of less susceptibility to proteolytic digestion[17] combined with its well-established tendency towards β-turn formation in acyclic[15,16] peptides.

Experimental

Melting points were determined on a Tropical capillary melting point apparatus and are uncorrected. NMR spectra were obtained with a JEOL FX-200 (200 MHz) spectrometer at the University Science Instrumentation Centre (USIC), University of Delhi. Chemical shifts are reported in ppm downfield from tetramethylsilane. Elemental analyses were obtained on Heraeus Rapid CHN analyser at USIC, Delhi University. The amino acid composition was determined with a LKB-4400 amino acid analyzer. The theoretical values of the amino acid ratios are shown in parentheses after each result. Optical rotations were measured on a JASCO DIP360 polarimeter (1 dm cell). UV and visible spectra were recorded on a Shimadzu UV-260 spectrophotometer. HCl in dioxane and HCl in glacial acetic acid were made by the method of Stewart and Young.[27]

Thin-layer chromatography (TLC) was performed on precoated silica (with fluorescent indicator) 60 F 254 plates (E. Merck) in the following solvent systems (volume ratios): R_f^1, chloroform–methanol (9 : 1); R_f^2, 1-butanol–acetic acid–water (4 : 1 : 1); R_f^3, 1-butanol–pyridine–acetic acid–wter (4 : 1 : 1 : 2); R_f^4, chloroform–methanol–acetic acid (85 : 10 : 5); R_f^5, chloroform–acetic acid (95 : 5); R_f^6, chloroform–methanol (4 : 1); R_f^7, chloroform–acetic acid–methanol (10 : 1 : 2); R_f^8, chloform–acetic acid–methanol–water (6 : 1 : 4 : 1); R_f^9, 1-butanol–pyridine–acetic acid–water (5 : 5 : 1 : 3). The deprotected peptides were extracted in 5—10% acetic acid, lyophilised, and purified by reverse phase high performance liquid chromatography (RP-HPLC) on Aquapore RP-300 Column (4.6×30 mm), using linear gradients of acetonitrile in 0.05% aqueous trifluoroacetic acid.

For *in vitro* tests, longitudinal muscle preparation of guinea pig ileum (GPI) was prepared from adult guinea pigs of either sex and mounted as described by Rang.[26] Graded concentrations of the test compound were added to the bath and the contraction produced at each concentration was noticed. The concentration of each compound producing a contraction height equal to 1 g tension was determined. Stimulatory effects of peptides were measured in the pesence of atropine in order to avoid the possible contribution by acetylchololine to the biological activities of Substance P related peptides. A summary of the biological results obtained with these analogs is recorded in Table 3.

***N*-*t*-Butoxycarbonyl-L-methionine Amide (8).** Compound **8** was prepared from *N*-butoxycarbonyl-L-methionine and aqueous ammonia in 78% yield. Mp 118—119 °C (lit,[28] 115—116 °C); R_f^7 0.67, R_f^4 0.8.

***N*-*t*-Butoxycarbonyl-L-leucyl-L-methioninamide (9).** Removal of *t*-Butoxycarbonyl group from **8** was achieved by treatment with 1.5 M HCl/AcOH (1 M=1 mol dm^{-3}) at room temperature for 1 h. By insolubilization and grinding with diethyl ether, the free methioninamide hydrochloride was

obtained.

Compound **9** was prepared from *N*-butoxycarbonyl-L-leucine succinimide ester and methionamide as usual. Yield 94%; mp 158—159 °C (lit,[29] 160—161 °C); R_f^1 0.88, R_f^2 0.83, R_f^3 0.85.

***N-t*-Butoxycarbonyl-L-phenylalanyl-α,β-dehydrophenylalanylglycine (10).** To a suspension of glycine (0.28 g, 3.83 mmol) in acetone (3.8 ml), NaOH (1M, 3.8 ml) was added dropwise over a period of 20 min. After 30 min, a solution of *N-t*-butoxycarbonyl-L-phenylalanyl-dehydrophenylalanine azlactone[30] (1.5 g, 3.83 mmol) in acetone (15 ml) was added and the mixture stirred at room temperature for overnight. The reaction mixture was concentrated in vacuo and the aqueous solution washed with ethyl acetate, acidified with solid citric acid to pH 3 and extracted with ethyl acetate. The organic layer was washed once with water and dried over anhydrous Na_2SO_4. Evaporation of ethyl acetate yielded the tripeptide free acid **10** (1.25 g, 70%); mp 170—171 °C; R_f^1 0.62, R_f^3 0.83. ^{1}H NMR (200 MHz, $CDCl_3$) δ=8.65 (1H, s, ΔPhe NH), 7.62—7.2 (11H, m, Phe and ΔPhe aromatic protons and ΔPhe C$^\beta$H), 4.4 (1H, m, Phe C$^\alpha$H), 4.0 (2H, m, Gly, CH_2), 3.35—3.2 (2H, m, Phe C$^\beta H_2$), 1.4 (9H, s, Boc CH_3). Found: C, 64.34; H, 6.2; N, 8.9%. Calcd for $C_{25}H_{29}N_3O_6$: C, 64.22; H, 6.25; N, 8.99%.

***N-t*-Butoxycarbonyl-L-phenylalanyl-α,β-dehydrophenylalanylglycyl-L-leucyl-L-methioninamide (11).** The dipeptide **9** (1.45 g, 4.02 mmol) was treated with 1.5 M HCl/AcOH for 1 h at room temperature. The corresponding hydrochloride salt was obtained after insolubilization and grinding over diethyl ether.

The tripeptide **10** (1.5 g, 3.3 mmol) was reacted with isobutyl chloroformate (0.42 ml, 3.3 mmol) in the presence of *N*-methylmorpholine (0.36 ml, 3.3 mmol) in *N,N*-dimethylformamide (15 ml). The amino component leucylmethioninamide hydrochloride (1.07 g, 3.6 mmol) was dissolved in *N,N*-dimethylformamide (5 ml) in the presence of triethylamine (0.5 ml, 3.6 mmol). The reaction mixture was stirred at 0 °C for 2 h and at room temperature for overnight. After reaction completion the triethylamine hydrochloride salt was filtered off and the remaining solution evaporated to dryness. The oily residue was taken into ethyl acetate and worked up as described for **9**. The crude pentapeptide showed two spots (major spot R_f^1 0.5) on TLC. It was purified by repeated precipitation from methanol with ether to yield **11** (1.74 g, 74%); mp 173—175 °C; $[\alpha]_D^{17}$ −38.2° (*c* 1.0, MeOH); R_f^1 0.51, R_f^2 0.75, R_f^9 0.9. ^{1}H NMR [200 MHz, $(CD_3)_2SO$] δ=9.54 (1H, s, ΔPhe NH), 8.5—8.3 (3H, m, Gly NH, Leu NH and Met NH), 7.56—7.24 (11H, m, Phe and ΔPhe aromatic protons and ΔPhe C$^\beta$H), 7.16 (1H, d, Phe NH), 4.56—4.32 (3H, m, Phe C$^\alpha$H, Leu C$^\alpha$H and Met C$^\alpha$H), 4.0 (2H, m, Gly CH_2), 3.08—2.88 (2H, m, Phe C$^\beta H_2$), 2.56 (2H, t, Met C$^\alpha H_2$), 2.05 (3H, s, Met C$^\delta H_3$), 1.92 (2H, m, Met C$^\beta H_2$), 1.64 (3H, m, Leu C$^\beta H_2$ and C$^\gamma$H), 1.48 (9H, s, Boc CH_3), 0.96 (6H, dd, Leu C$^\delta H_3$). Found: C, 60.38; H, 6.98; N, 11.75%. Calcd for $C_{36}H_{50}N_6O_7S$: C, 60.82; H, 7.08; N, 11.82%.

***N-t*-Butoxycarbonyl-L-glutaminyl-L-phenylalanyl-α,β-dehydrophenylalanyl-glycyl-L-leucyl-L-methioninamide (12).** The above pentapeptide **11** (1.7 g, 2.4 mmol) was treated with 1.5 M HCl/AcOH for 1 h at room temperature. The corresponding hydrochloride salt was obtained by insolubilization and trituration with diethyl ether. L-Phenylalanyl-dehydrophenylalanyl-glycyl-L-leucyl-L-methioninamide hydrochloride (1.4 g, 2.16 mmol) and triethylamine (0.3 ml, 2.16 mmol) were dissolved in *N,N*-dimethylformamide (20 ml) at 0 °C. To this solution was added *N-t*-butoxycarbonyl-glutamine *p*-nitrophenyl ester (0.72 g, 1.96 mmol) and the mixture was stirred at room temperature for 3 d. The solvent was removed under reduced pressure and the residue stirred with 10% Na_2CO_3 solution for 30 min. The separated solid was filtered and washed successively with 10% Na_2CO_3 solution, water, 10% citric acid solution and water. The product was purified by repeated precipitation from *N,N*-dimethylformamide with ethyl acetate to yield **12** (1.0 g, 60.7%); mp 158—160 °C; $[\alpha]_D^{17}$ −79.4° (*c* 0.7, MeOH); R_f^1 0.55, R_f^2 0.8, R_f^3 0.87. Found: C, 58.50; H, 6.92; N, 13.06%. Calcd for $C_{41}H_{59}O_9N_8S$: C, 58.69; H, 6.96; N, 13.35%.

L-Glutaminyl-L-phenylalanyl-α,β-dehydrophenylalanylglycyl-L-leucyl-L-methioninamide (1). The protected hexapeptide **12** (0.1 g) was treated with 4 M HCl–dioxane for 1 h at room temperature. The resulting hydrochloride salt was dissolved in 5% acetic acid and subjected to gel chromatography on a column (2.2×48 cm) of Sephadex G-15 using 15% acetic acid as eluent. The fractions containing the desired peptide were pooled and lyophilized. The peptide was purified by RP-HPLC. Amino acid ratios: Glu 1.05(1), Phe 1.03(1), Gly 1.03(1), Leu 1.0(1) and Met 0.95(1).

***N-t*-Butoxycarbonyl-L-prolyl-α,β-dehydrophenylalanine (22).** To a solution of Boc-Pro-ΔPhe azlactone[25] (6.83 g, 19.9 mmol) in methanol (100 ml) was added 4 M NaOH (7.6 ml). The reaction mixture was stirred for 10 h at room temperature and evaporated. The product was isolated in the same manner as described for **10**. Yield (6.3 g, 88%); mp 110—112 °C; R_f^1; 0.6, R_f^2 0.5. ^{1}H NMR (200 MHz, $CDCl_3$) δ=8.62 (1H, s, ΔPhe NH), 7.6—7.3 (5H, m, ΔPhe aromatic protons), 7.05 (1H, s, ΔPhe, C$^\beta$H), 4.44 (1H, br, ProC$^\alpha$H) 3.46 (2H, t, ProC$^\delta H_2$), 2.1 (2H, m, ProC$^\beta H_2$) 1.9 (2H, m, ProC$^\gamma H_2$), 1.49 (9H, s, Boc CH_3).

***N-t*-Butoxycarbonyl-L-prolyl-α,β-dehydrophenylalanyl-L-glutaminyl-L-phenylalanyl-α,β-dehydrophenylalanyl-sarcosinyl-L-leucyl-L-methioninamide (23).** It was prepared from Boc-Pro-ΔPhe-OH (**22**) (0.3 g, 0.83 mmol), *N*-methylmorpholine (0.09 ml, 0.83 mmol), isobutyl chloroformate (0.11 ml, 0.83 mmol) and Gln-Phe-ΔPhe-Sar-Leu-Met-NH_2·HCl (0.5 g, 0.64 mmol) and triethylamine (0.09 ml, 0.64 mmol) following the procedure described for **11**. The crude octapeptide was crystallized from *N,N*-dimethylformamide-ethyl acetate. Yield (0.4 g, 57%); R_f^1 0.51, R_f^2 0.72. Found: C, 61.09; H, 6.76; N, 12.62%. Calcd for $C_{56}H_{74}O_{11}N_{10}S$: C, 61.41; H, 6.81; N, 12.78%.

L-Prolyl-α,β-dehydrophenylalanyl-L-glutaminyl-L-phenylalanyl-α,β-dehydrophenylalanyl-sarcosinyl-L-leucyl-L-methioninamide (5). 0.4g of protected octapeptide **23** was treated with 1.5 M HCl/AcOH and the product isolated and purified in the same manner as described for **1**. It was further purified by HPLC. Mp 128—130 °C; $[\alpha]_D^{17}$ −61.3° (*c* 1.0, MeOH); R_f^8 0.76, R_f^9 0.84, R_f^{10} 0.35. Amino acid ratios: Pro 0.97 (1), Glu 1.1 (1), Phe 1.05 (1) Sar ND, Leu 1.0 (1), and Met 0.9 (1).

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