SYNTHESIS AND STRUCTURE–ACTIVITY RELATIONSHIP OF SCYLIORHININ I ANALOGUES MODIFIED IN POSITIONS 3, 6, 7 AND 8

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Seven analogues of scyliorhinin I modified in positions 3, 6, 7 and 8 were synthesized by the solid-phase method. Their agonistic activity was determined on isolated guinea pig ileum (GPI). Except for two analogues modified in position 6, all exhibited dose-dependent activity. Analogues with Phe, D-Phe(F) in position 7 and Abu in position 8 appeared significantly more active than scyliorhinin I and substance P, whereas the analogue with NMeLeu in position 8 appeared to be twice as active as the native molecule, but displayed only 12% of the substance P activity. CD studies indicated that the analogues modified in position 8 behaved differently in the surroundings that mimics the biological membranes.

Scyliorhinin I (ScyI), the tachykinin family peptide, was isolated in 1986 from extracts of the intestine of common dogfish (*Scyliorhinus caniculus*)[1]. It consists of 10 amino acid* residues and shares a common C-terminal sequence with other tachykinins.

1 3 6 7 8 10
Ala-Lys-Phe-Asp-Lys-Phe-Tyr-Gly-Leu-Met-NH_2

In our previous papers we described the synthesis, biological activity and circular dichronism (CD) studies of five analogues modified in positions 7 and 8 (refs[2-4]).

Continuing these studies we synthesized the following analogues: [Phe^7]ScyI (*I*), [Phe(F)^7]ScyI (*II*), [D-Phe(F)^7]ScyI (*III*), [NMeLeu^8]ScyI (*IV*), [Abu^8]ScyI (*V*), [Ala^6]ScyI (*VI*), [Trp^3, Ala^6]ScyI (*VII*), where Phe(F) 4-fluorophenylalanine and Abu α-aminobuteric acid.

* Unless otherwise stated, all chiral amino acids belong to the L-series. The nomenclature and symbols of the amino acids, their derivatives and peptides obey the IUPAC recommendations (Eur. J. Biochem. *138*, 9 (1984)).

The introduction of hydrophobic Ile and Val residues in position 7 did not significantly change the GPI activity[2]. This time we decided to investigate the influence of aromatic residues in this position. We therefore substituted Tyr^7 with Phe which is present in the equivalent position in substance P (SP). The introduction of L- and D-Phe(F) was dictated by their positive effect on the biological activity of enkephalins[5,6] and LHRH (ref.[7]). As we have already demonstrated, the introduction in position 8 of such amino acid residues as Pro and Sar significantly increased the GPI activity of the modified analogues[3]. Following this direction, we synthesized an analogue containing NMeLeu in this position. Since the analogue with Leu^8 was less active than the parent Scyl (ref.[3]) it could prove the statement that the imino group increases the biological acivity. We decided to achieve the same effect by introducing Abu in the position discussed. This amino acid is very often present in synthetic peptides with immunogenic activity[8], displaying the presence of β-turns.

In order to investigate the role of position 6 in the biological response in the GPI test, we replaced Phe^6 with Ala. The last compound (*VII*), containing an additional substitution of Phe^3 with Trp was designed to investigate (by spectrofluorimetric methods – the experiments in progress) the distance between the aromatic chromophores.

All peptides were synthesized by the solid-phase method, using BOC-chemistry.

EXPERIMENTAL

Apparatus and Methods

Specific rotations were determined at 20 °C on a Perkin Elmer Model 141 polarimeter with an accurancy of 0.01°. HPLC analysis was performed by means of a Beckman Peptide System on reversed phase analytical column (RP C_{18}, Ultrasphere-ODS, 4.6 × 150 mm). The homogeneity of the substances was also checked by TLC using glass plates coated with a 0.25 mm layer of silica gel (Kieselgel G, Merck). Chromatograms were developed in 1-butanol–acetic acid–water–pyridine (51 : 12 : 12 : 25). Spots were rendered visible with iodine vapour, or 0.2% ninhydrin solution in ethanol. The final products were hydrolyzed for 20 h at 110 °C with 6 M HCl in ampoules sealed under vacuum. The amino acid analysis was performed on a Beckman 121 amino acid analyzer. CD spectra were measured at room temperature using a Jasco-J-20 spectropolarimeter. The results were plotted as the mean residue ellipticity Θ (deg cm^2 $dmol^{-1}$) against wavelength λ (nm). Trifluoroethanol (TFE) was of the spectroscopic quality, 10 mM phosphate buffer (pH 7.0) was prepared from the purest reagents. 1-Dodecylsulfate sodium salt (SDS) was of the analytical grade.

Peptide Synthesis

All peptides were synthesized by the solid-phase method using *p*-methylbenzhydrylamine (MBHA) resin (0.63 meq g^{-1}). The synthesis was carried out manually. Reversible protection of α-amino group was accomplished with the tert-butoxycarbonyl (Boc) group. tert-Butoxycarbonylamino acids were obtained using di-tert-butyldicarbonate[9]. L- and D-Phe(F) were obtained by the method described in the literature[10]. The phenolic hydroxyl group of Tyr was protected with benzyl moiety[11], β-carboxyl group of Asp was protected as benzyl ester[12], ε-amino group of Lys with benzyloxycarbonyl moiety[13], and indole group of Trp with formyl moiety[14].

Deblockings were performed with 40% trifluoroacetic acid (TFA) in CH_2Cl_2 in the presence of 2% thioanisole. Couplings were performed in DMF–CH_2Cl_2 (1 : 3, v/v) using symmetrical anhydride method.

For cleavage of the peptide from polymer support 1 g of peptide-MBHA resin was treated according to the low-high procedure[15]. In the first step, 1 g of peptide-resin reacted with 10 ml of the mixture DMS–*p*-thiocresol–*p*-cresol–HF (6.5 : 0.25 : 0.75 : 2.5), and 50 mg of Cys for 2 h at 0 °C, then HF and DMS were removed in vacuo. The reaction vessel was recharged with 14 ml of HF and the cleavage was completed at 0 °C for 1 h. After evaporation of HF, residue was washed with diethyl ether. The peptide was extracted with 30% AcOH and lyophilized to afford 30% to 45% yield of crude peptides.

Purification of the Peptides

Crude peptides were first purified on Sephadex G-15 column (2 × 100 cm), eluted with 1 M AcOH, then applied on CM 23-Cellulose column (1 × 15 cm) equilibrated with 0.01 M $AcONH_4$ (pH 4.5), 10% acetonitrile and eluted with a linear gradient of 250 ml 0.01 M $AcONH_4$–10% acetonitrile and 250 ml 0.4 M $AcONH_4$ (pH 6.5)–10% acetonitrile. Finally, the analogues were purified on semipreparative HPLC RP C_{18} column (Ultrasphere, 10 × 250 mm) using a linear gradient from 25 to 50% of B, t = 40 min (A: 0.1% TFA, B: 80% acetonitrile in 0.1% TFA), flow rate 1.5 ml min^{-1}, detector setting at 226 nm.

Pharmacological Methods

Experiments were carried out with male guinea-pigs (300 – 400 g) from Central Animal Farm of the Silesian Academy of Medicine, fed with water and food ad libitum. The animals were starved one day before the experiment. Guinea-pig isolated ileum was prepared according to Gyang and Kosterlitz[16]. Shortly, the longitudinal muscle of guinea-pig ileum (10 cm) divested of mesenteric plexus was placed in a glass container filled with 10 ml of Krebs solution oxygenated with a mixture of 95% oxygen and 5% carbon dioxide at 18 °C. The initial tension of ileum fragment was 500 mg. This specimen was incubated for 1 h. During this time, the incubation medium was replaced every 10 min. The tension of smooth muscles of ileum was registered using a tensometric transducer. After the incubation period, when acceptable baseline of ileum activity was obtained, the substances examined were added. Ten doses of the substances examined in a volume of 350 μm were added in the individual experiment. From obtained results dose-response curve was constructed[17]. Statistical analysis of results was based on occupational receptor theory of Clark with assumption of proportionality between the dose magnitude and biological response[18]. Calculations were performed using computer programs[19] IBM PC/XT. Experimental data were processed by plotting normalized effect (E/E_{max} × 100%) against logarithm of dose[20]. ED_{50} of examined substances was calculated using regression equation.

RESULTS AND DISCUSSION

Physicochemical properties of all seven ScyI analogues have been listed in Table I. CD spectra of analogues *IV* and *V* in aqueous and hydrophobic environment are presented in Fig. 1. Biological activity of compounds studied are summarized in Tables II and III.

Except for *VI* and *VII* the rest of the analogues displayed contraction-response activity on isolated guinea pig ileum. The lack of activity in the case of analogues in which Phe^6 was substituted by Ala indicates that this amino acid residue (Phe^6) is essential for biological activity, and seems to play the same role as does Phe^7 in SP. All compounds modified in position 7 and 8 appeared to be significantly more active than ScyI. The introduction of Phe and D-Phe(F) in position 7 increased the activity more than 27 and

48 times, respectively. These analogues were also much more active (1.9 and 3.3 times, respectively) than SP (Table II). Also analogue *II* with Phe(F) in position 7 exhibited a dose-contraction activity in this experiment. Unfortunately, its effect was "chimeric". This analogue behaves differently when applied in small and high doses. Its activity will be studied in detail and the results will be published separately. Our results indicated that the substitution of Tyr^7 with aliphatic amino acids Ile and Val did not significantly change the biological activity in the GPI test[2]. Very high activity achieved for analogues discussed herein suggests that the aromatic ring (especially substituted) is an important element for the biological activity in the GPI test.

Significant differences were observed in the GPI activity for analogues modified in position 8. Both were more active than ScyI. Analogue *IV* with NMeLeu in this position was almost twice as active as ScyI (Tables II and III). The comparison of these results with those previously reported[3] suggests that N-methylation of the peptide bond between the 7th and 8th amino acid residues enhances the biological acivity. The analogue with $NMeLeu^8$ was more than twice as active as that containing Leu in this position, and displayed an activity similar to that of the analogue with Sar (NMeGly) in the position considered (Table III). The activity of $[Pro^8]$ScyI did not dramatically differ either[3]. The similarity between these analogues was also observed in their CD spectra. Figure 1 shows that in 75% aqueous solution of trifluoroethanol (TFE) and in the presence of 1-dodecylsulfate Na salt (SDS) micelles, $[NMeLeu^8]$ScyI adopts a similar conformation. All the CD spectra display a broad negative ellipticity in the region 210 – 240 nm and a weak maximum around 208 nm. Although the negative ellipticity in the case of 75% TFE and 15 mM SDS was slightly higher, we conclude that the spectra may represent a folded structure of the peptide chain. It can also be noted, that the spectra are very similar to those recorded for $[Pro^8]$ScyI and especially for $[Sar^8]$ScyI in 75% TFE and in the presence 15 mM SDS (ref.[4]).

In the series of analogues modified in position 8 the highest activity was exerted by compound *V*, containing Abu in this position (Tables II and III), which rather promotes

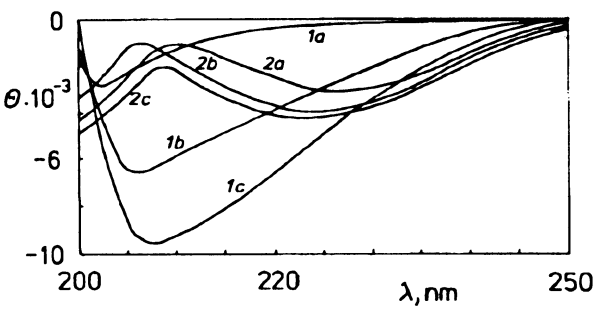

FIG. 1
CD spectra Θ, deg cm^2 $dmol^{-1}$ of $[Abu^8]$ScyI (*1*) and $[NMeLeu^8]$ScyI (*2*) in *a* phosphate buffer pH 7.0, *b* 75% TFE and *c* 15 mM SDS

TABLE I
Physico-chemical properties of scyliorhinin I analogues

Compound	Formula (M. w.)	R_F[a]	R_T[b] min	$[\alpha]_{589}$, ° c 0.1; H_2O	Amino acid analysis			
[Ala^6]Scyl	$C_{53}H_{83}N_{13}O_{13}S$ . . 3 CF_3COOH (1 484.4)	0.16	8.88	−31.5	Ala 1.98(2)	Lys 2.07(2)	Phe 1.01(1)	Asp 0.93(1)	Tyr 0.89(1)	Leu 1.00(1)	Gly 1.00(1)	Met 0.96(1)
[Trp^3, Ala^6]Scyl	$C_{55}H_{84}N_{14}O_{13}S$ . . 3 CF_3COOH (1 523.5)	0.24	5.91	−40.0	Ala 1.90(2)	Lys 1.74(2)	Asp 1.07(1)	Tyr 1.13(1)	Gly 0.89(1)	Leu 1.00(1)	Met 0.82(1)	Phe 1.01(1)
[Abu^8]Scyl	$C_{61}H_{93}N_{13}O_{14}S$ . . 3 CF_3COOH (1 606.6)	0.21	14.36	−10.0	Ala 1.17(1)	Lys 1.94(2)	Phe 1.76(2)	Asp 0.86(1)	Tyr 0.82(1)	Leu 1.00(1)	Met 1.01(1)	
[NMeLeu^8]Scyl	$C_{64}H_{97}N_{13}O_{13}S$ . . 3 CF_3COOH (1 640.8)	0.20	5.90	−22.0	Ala 1.03(1)	Lys 1.79(2)	Phe 1.72(2)	Asp 1.02(1)	Leu 1.00(1)	Tyr 0.95(1)	Met 1.05(1)	
[Phe(F)^7]Scyl	$C_{59}H_{86}N_{12}O_{13}SF$ . . 3 CF_3COOH (1 562.5)	0.34	17.79	−20.0	Ala 0.96(1)	Lys 1.90(2)	Phe 2.03(2)	Asp 0.89(1)	Leu 1.00(1)	Gly 1.01(1)	Met 1.12(1)	
[D-Phe(F)^7]Scyl	$C_{59}H_{86}N_{12}O_{13}SF$ . . 3 CF_3COOH (1 562.5)	0.36	17.97	−27.5	Ala 1.03(1)	Lys 2.05(2)	Phe 2.08(2)	Asp 0.89(1)	Gly 1.03(1)	Leu 1.00(1)	Met 1.20(1)	
[Phe^7]Scyl	$C_{59}H_{87}N_{12}O_{13}S$ . . 3 CF_3COOH (1 544.5)	0.30	13.79	−23.0	Ala 0.99(1)	Lys 1.82(2)	Phe 2.90(3)	Asp 0.76(1)	Gly 0.79(1)	Leu 1.00(1)	Met 0.96(1)	

[a] TLC: 1-Butanol–acetic acid–water–pyridine (51 : 12 : 12 : 25); [b] HPLC: RP C_{18} column (Ultrasphere-ODS, 4.6 × 150 mm); (A: 0.1% TFA, B: 80% acetonitrile in 0.1% TFA); linear gradient from 25 to 50% of B; flow rate 1.5 ml min^{-1}.

the bending of the peptide chain. [Abu^8]ScyI was around 26 times more active than ScyI (Table III). In contradiction to the conformational behavior of [NMeLeu^8]ScyI, the CD spectra recorded for [Abu^8]ScyI in hydrophilic and hydrophobic environment are distinctly different. In water (not shown) and phosphate buffer the CD curves display a very weak ellipticity, which is probably due to the aggregation. Under the conditions that mimic biological membranes (75% TFE and SDS), significant changes are observed. The negative extremum appears at 205 – 206 nm and a strong negative ellipticity develops in the region 206 – 240 nm, indicating the presence of a significant degree of the ordered structure.

The examination of the CD spectra recorded for analogues containing amino acids with imino group (Pro, Sar) (ref.[4]) and NMeLeu in position 8 indicates the presence of a positive extremum at 208 nm, which significantly increases the ellipticity in the region 208 – 240 nm. Considering the above, the region 215 – 230 nm could be

TABLE II
Effect of substance P, scyliorhinin I and its analogues modified in positions 7 and 8 on the guinea-pig ileum

Agonist	Regression equation[a]	Correlation coefficient	ED_{50} ± SEM nmol l^{-1}	n
Substance P	$y = 202\,x + 18$	0.84	8.5 ± 2.1	6
Scyliorhinin I	$y = 392\,x + 49$	0.91	123.0 ± 9.4	6
[Phe^7]ScyI (*I*)	$y = 220\,x + 20$	0.87	4.46 ± 1.8	6
[D-Phe(F)^7]ScyI (*III*)	$y = 380\,x + 38$	0.94	2.54 ± 0.3	6
[NMeLeu^8]ScyI (*IV*)	$y = 399\,x + 49$	0.92	68.0 ± 4.2	6
[Abu^8]ScyI (*V*)	$y = 241\,x + 23$	0.81	4.7 ± 0.37	6

[a] All parameters of regression equation are significant ($p < 0.001$).

TABLE III
Comparison of relative potency of ScyI analogues modified in position 8 with ScyI

Compound	Relative activity	Compound	Relative activity
ScyI	1.00	[NMeLeu^8]ScyI	1.8
[Abu^8]ScyI	26.1	[Sar^8]ScyI	1.7[a]
[Pro^8]ScyI	4.1[a]	[Leu^8]ScyI	0.8[a]

[a] See ref.[3].

diagnostic for the prediction of biological activity. The lower ellipticity observed, the higher GPI activity can be expected.

CONCLUSIONS

The results presented above indicate, that position 6 in ScyI is essential for the biological activity in GPI test. The introduction of Ala in this position caused the loss of the biological response. On the other hand, the substitution of Tyr^7 with Phe and its derivatives significantly enhanced the activity of such modified analogues. Very promissing results were also obtained in the case of analogues containing in position 8 the amino acid which promotes the peptide chain bending. Our preliminary results showed that the biological activity of these analogues can be correlated with the CD spectra recorded under condition which mimics biological membranes.

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