SYNTHESIS AND BIOLOGICAL ACTIVITY IN GPI TEST OF SCYLIORHININ I AND ITS ANALOGUES MODIFIED IN POSITION 8 BY Leu, Sar AND Pro RESIDUES

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Three analogues of scyliorhinin I with Gly in position 8 substituted with Leu, Sar and Pro residues, were synthesized by the solid-phase method. The agonistic activity was determined on isolated guinea pig ileum. Analogues with Sar and Pro in position 8 appeared to be significantly more active than scyliorhinin I and the analogues with Leu in this position. For all analogues the Hill cooperativity coefficient is much lower than one suggesting that the interaction of these peptides with tachykinin receptor(s) in not a one-step reaction.

Scyliorhinin I**, a decapeptide belonging to the tachykinin family, was isolated in 1986 from extracts of the intestine of common dogfish caniculus[1]. The amino acid sequence of scyliorhinin I is presented below:

$$\overset{1}{\text{Ala}}\text{-Lys-Phe-Asp-Lys-Phe-Tyr-}\overset{8}{\text{Gly}}\text{-}\overset{10}{\text{Leu}}\text{-Met-NH}_2 .$$

In our previous publication we described the synthesis and GPI activity of scyliorhinin I (Scy I) and its two analogues modified in position 7 (ref.[2]). Introduction of Val and Ile in this position did not affect significantly the agonistic activity. In the present paper we focus our interest on the position 8. As it was shown by several research groups, this position seems to be very important for α-helix formation in substance P under conditions that mimic the membrane environment[3-5]. This was also observed in the case of scyliorhinin I (ref.[6]). Structure–activity relationships of substance P indicate that the substitution of Gly^9 with Sar and Pro increase the agonistic activity of such modified analogues towards NK1 (refs[7,8]) and NK2

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** 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)).

(ref.[8]) tachykin receptors. From the point of view of similar conformational behaviour of substance P and scyliorhinin I, we decided to synthesize following analogues of scyliorhinin I: [Pro^8]Scy I, [Sar^8]Scy I, and [Leu^8]Scy I. By introducing Leu in position 8 we intended to investigate whether the replacement of a strong α-helix breaker (Gly) with strong α-helix former (Leu) would have any significant effect on the pharmacological properties. All compounds were synthesized by the solid-phase method using *p*-methylbenzhydrylamine (MBHA) resin as a carrier. Pharmacological experiments were carried out using the GPI test. Synthetic details and physicochemical properties of [Sar^8]Scy I, [Pro^8]Scy I and [Leu^8]Scy I have been listed in Table I. The HPLC analysis of all peptides obtained was also carried out (see Fig. 1).

As shown by data given in Table II, all synthetic analogues display contraction-response activity on isolated guinea pig ileum (GPI). With regards to the effective dose (ED_{50}), the lowest activity was exerted by analogue [Leu^8]Scy I (80% of the native Scy I activity). The introduction of Sar and Pro in position 8 enhanced considerably the agonistic activity. In the GPI test, the activity of [Sar^8]Scy I and [Pro^8]Scy I amounted to 170% and 400% of that of Scy I, respectively, yet being still lower than that of substance P. The most surprising result was that the Hill cooperativity coefficient obtained for the analogues was distinctly lower than that obtained for native Scy I, which could be due to:

TABLE I

Physicochemical properties of Scyliorhinin I analogues modified in position 8

Compound	Yield, %	Formula (M.w.)	R_F A B	R_T, min[a]	$[\alpha]_D^{20}$ *c* 0·1; H_2O	Amino acid analysis
[Leu^8]Scy I	41	$C_{63}H_{96}O_{13}N_{13}S$. . 3 AcOH (1 455·7)	0·42 0·34	5·68	−60°	Lys 2·10; Asp 0·91 Ala 1·0; Met 0·81 Leu 2·11; Tyr 0·83 Phe 2·01
[Sar^8]Scy I	38	$C_{60}H_{90}O_{13}N_{13}S$. . 3 AcOH (1 413·6)	0·38 0·30	4·28	−25°	Lys 2·09; Asp 0·89 Ala 1·0; Met 0·85 Leu 1·06; Tyr 0·82 Phe 2·06
[Pro^8]Scy I	43	$C_{62}H_{92}O_{13}N_{13}S$. . 3 AcOH (1 439·7)	0·39 0·33	4·53	−40°	Lys 2·13; Asp 0·96 Pro 1·04; Ala 1·0 Met 0·76; Leu 1·10 Tyr 0·93; Phe 2·12

[a] RP C_{18} column (Ultrasphere-ODS, 4·6 × 150 mm); linear gradient from 25 to 70% of acetonitrile in 0·1% TFA; flow rate 2 ml/min.

– presence of more than one type of tachykinin receptor in guinea pig ileum,
– negative receptor co-operativity,
– multiple-step reaction of analogues with the receptor.

In our experiments, the negative receptor cooperativity can be excluded as it was not observed in the case of Scy I. The most probable explanation of these results is that the analogues are bound with more than one receptor, as the both NK1 and NK2 receptors are present in the course of guinea pig ileum preparation[9]. GPI is a multireceptor system with NK1 and NK2 (in muscle) and NK3 (in the mesenteric plexus) receptors[9]. Since, in our experiments, mesenteric plexus was removed during preparation of GPI strips, NK3 receptors were eliminated.

TABLE II

Activity of scyliorhinin I, its analogues modified in position 8, and substance P on isolated guinea pig ileum

Agonist	Regression equation	Correlation coefficient	ED_{50} ± SEM nmol l^{-1}	Hill coefficient	*n*
Substance P	$Y = 0{\cdot}19x + 2{\cdot}03$	0·84	11·9 ± 3·2	0·38	12
Scyliorhinin I	$Y = 0{\cdot}49x + 3{\cdot}91$	0·91	123 ± 9·4	0·97	6
[Sar^8]Scy I	$Y = 0{\cdot}36x + 3{\cdot}1$	0·87	69·5 ± 5·2	0·70	6
[Pro^8]Scy I	$Y = 0{\cdot}24x + 2{\cdot}3$	0·75	30·1 ± 4·4	0·47	6
[Leu^8]Scy I	$Y = 0{\cdot}34x + 2{\cdot}8$	0·96	154 ± 12·3	0·73	6

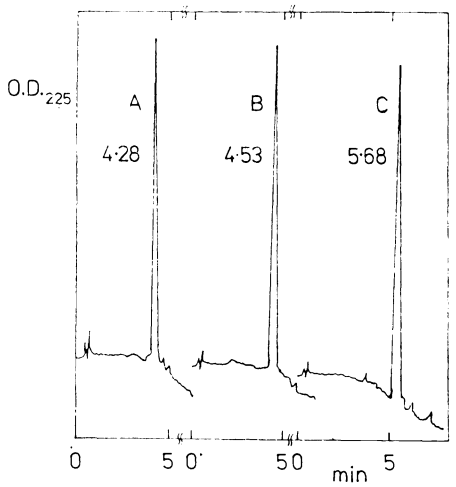

FIG. 1

RP HPLC traces of [Sar^8]Scy I (A), [Pro^8]-.Scy I (B) and [Leu^8]Scy I (C). Conditions see Table I.

It is also possible to interpret this phenomenon based on the receptor model, assuming that Scy I and its analogues exhibit both, different affinity to the NK1 receptor and different binding mechanism. Most probably the Scy I analogues, as well as substance P bind to NK1 receptor in multistep reactions whereas Scy I interacts in a one-step reaction which can be supported by the facts that:

- substance P and Scy I exhibit agonistic activity towards NK1 receptor present in guinea pig ileum,
- substance P is a selective agonist of NK1 receptor and has rather low affinity towards other tachykinin receptors,
- according to the previous investigations[10], Scy I interacts with both NK1 and and NK2 receptors, but in our test the Hill cooperativity coefficient amounts to about 1, which allows to assume that this peptide binds to one receptor type – NK1.

EXPERIMENTAL

Methods

Specific rotation was determined on a Perkin–Elmer Model 141 polarimeter with an accurancy of 0·01°. The HPLC analysis was performed by means of a Beckman Peptide System by reversed phase analytical column (RP C_{18}, Ultrasphere-ODS, 4·6 × 150 mm). The homogeneity of the substance was also checked by thin layer chromatography (TLC) using glass plates coated with a 0·25 mm layer of silica gel (Kisselgel C, Merck). Chromatograms were developed in the systems: (A) 1-BuOH–AcOH–H_2O–pyridine (15 : 3 : 12 : 10), (B) 1-BuOH–AcOH–H_2O–pyridine (50 : : 12 : 12 : 25). Spots were detected with iodine vapour, or 0·2% ninhydrin solution in ethanol. Final products were hydrolyzed for 20 h at 110°C with 6M HCl in vacuo. Amino acid analysis was performed on a Mikrotechna type AAA 881 analyser. Fractions from chromatographic columns were analysed by means of a UV monitor (254 nm) and TLC. Tert-butoxycarbonylamino acids were obtained using di-tert-butyldicarbonate[11]. The hydroxy group of Tyr was protected with benzyl moiety[12]. β-Carboxyl group of Asp was protected as benzyl ester[13], and ε-amino group of Lys with benzyloxycarbonyl moiety[14].

Peptide Synthesis

All peptides were synthetized by the solid-phase method using *p*-methylbenzhydrylamine resin[15] (substitution 0·6 meq/g). The synthesis was carried out manually. For protection of the α-amino group the tert-butoxycarbonyl (Boc) group was used. 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 the symmetrical anhydride method.

Cleavage from the Resin

The mixture of 0·2 g of peptide–MBHA-resin, 200 μl of thioanisole, 200 μl of *m*-cresol and 4 ml of TFA was treated with 400 μl of trifluoromethanesulfonic acid at 0°C for 30 min. The resin was filtered off, and TFA was removed in vacuo. The residue was diluted with 50 ml of water, extracted twice with 10 ml of ethyl acetate, neutralized with 1M $NaHCO_3$ solution and lyophilized.

Purification of the Peptides

The crude peptides were first purified on a Sephadex G-25 column (2·5 × 38 cm) by elution with 0·2M AcOH containing 10% acetonitrile. Finally, the products were applied to CM-cellulose column (1 × 15 cm) equilibrated with 0·01M $AcONH_4$ (pH 4·5), 10% acetonitrile and eluted with a linear gradient (250 ml 0·01M $AcONH_4$, 10% acetonitrile; 250 ml 0·4M $AcONH_4$, pH 6·5, 10% acetonitrile). Fractions containing the products were collected and lyophilized. The purity of the final products was checked by HPLC (Fig. 1). Physicochemical properties of the products are presented in Table I.

Pharmacological Methods

Experiments were carried out on guinea-pigs (300—400 g) from Central Animal Farm of Silesian Academy of Medicine. Guinea-pig ileum was prepared according to Gyang and Kosterlitz[16]. The longitudinal muscle of GPI separated from the mesenteric plexus was placed for a short time in a glass container filled with 6 ml of Krebs solution oxygenated with a mixture of 95% oxygen and 5% of carbon dioxide at 37°C. The initial tension of ileum fragment was 100 to 150 mg. This specimen was incubated for 1 h, the incubation medium being replaced every 10 min. The tension of smooth muscles of the ileum was recorded with a tensiometric transductor. After the incubation period, when the base line of ileum activity was stabile the substances under examination were added. Three to seven doses (100 µl each) of the peptides were added in each individual experiment at intervales of at least 20 min. Based on the results obtained the dose--response curve was plotted[17]. Each substance examined was tested on 6 specimens of ileum.

Statistical analysis of the results was based on the occupational receptor theory of Clark, assuming proportionality between the magnitude of a dose of substance applied and the biological response[18]. The calculations were performed using computer programs[19] for an IBM PC/XT. Experimental data were elaborated by plotting the regression curve and calculating the correlation coefficient[20]. ED_{50} of the investigated substances was calculated using the regression equation. The Hill cooperativity coefficient was calculated as linear regression, $\log(C)$ being independent and $\log(E/(1-E))$ dependent variables (C is molar concentration of the compound examined and E is pharmacological effect calculated from 0 to 1). The slope of the regression analysis yields the Hill coefficent[21,22]. The results obtained are presented in Table II.

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