PREPARATION AND CHARACTERIZATION OF NEW ANALOGUES OF DALARGIN

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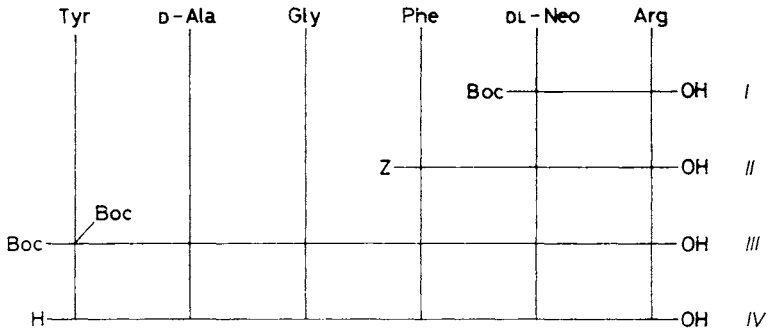
Tert-butyloxycarbonyl-O-tert-butyloxycarbonyl-L-tyrosyl-D-alanyl-glycyl-L-phenylalanyl-DL--neopentylglycyl-L-arginine was prepared by means of fragment condensation in solution. Protecting groups were removed with trifluoroacetic acid and the diastereoisomeric peptides were separated employing high-performance liquid chromatography (HPLC) and the absolute configuration of neopentylglycine in each diastereomer was determined by two different methods: analysis of enzymatic degradation with carboxypeptidase B and ^{1}H NMR spectroscopic data. The hexapeptide with L-neopentylglycine in position 5 exhibited a 20 times higher activity in a standard test on isolated guinea-pig ileum (GPI). The hexapeptide with D-neopentylglycine in position 5 exhibited a 4 times higher activity than Dalargin in the GPI test but its effect was prolonged in comparison to the analog with L-neopentylglycine and Dalargin.

From the time of the discovery of enkephalin[1], thousands of its synthetic analogues have been prepared up to the present time. Frequently, syntheses were motivated by attempts to obtain analogues with enhanced affinity to various populations of receptors, greater selectivity of action and metabolic stability. Substitutions of L-amino acids in the chain by D-enantiomers[2,3], preparation of compounds of the prohormone type[4] or of dimers[5,6] should be mentioned here. One of the possible approaches to the synthesis is a replacement of natural amino acids by amino acids with sterically constrained side chains either of the D- or L-configuration, which results in the restriction of the flexibility of the prepared peptide and, frequently increases its stability against enzyme action[7,8].

The aim of the present study was to replace the amino acid residue in position 5 in an active enkephalin analog Dalargin[9] by a noncoded amino acid, neopentylglycine of the L- and D-form and to compare biological activities of these analogues with that of Dalargin.

Neopentylglycine ([Neo^5]) analogues were synthesized by means of fragment condensation of active *p*-nitrophenylester of tert-butyloxycarbonyl-O-tert-butyloxy-

carbonyl-L-tyrosyl-D-alanyl-glycine with L-phenylalanyl-D,L-neopentylglycyl-L-arginine according to Scheme 1.



SCHEME 1

The obtained diastereoisomeric analogues were separated on a column with Silasorb C-18 by means of HPLC employing a gradient elution (0–100% methanol–water). The two compounds – [L-Neo^5]Dalargin and [D-Neo^5]Dalargin – isolated in this manner were differentiated enzymatically using carboxypeptidase B. This enzyme (E.C.3.4.17.2) catalyzing the splitting-off of the basic amino acid from the carboxyl end of the peptide chain is stereospecific. The terminal amino acid has to be of the L-configuration and the rate of hydrolysis depends also on the character of the preceding amino acid[10]. As the molecule of [D-Neo^5]Dalargin contains D-neopentylglycine in the position preceding L-arginine, the peptide was not hydrolyzed by the enzyme and, in contrast to [L-Neo^5]Dalargin, it retained its biological activity after incubation with the enzyme.

^{1}H NMR spectra of the diastereoisomers support the assignment of the compound resistant to enzymatic degradation with carboxypeptidase B as the D-neopentylglycyl

TABLE I

Biological activities of Dalargin, [L-Neo^5] and [D-Neo^5]Dalargin; results from eight independent experiments

Substance	IC_{50}, nmol l^{-1}	Relative activity
Dalargin	389·0 ± 20	1
[L-Neo^5]Dalargin	19·9 ± 7	19·5
[D-Neo^5]Dalargin	84·5 ± 8	4·6

diastereomer. In ^{1}H NMR spectra the alkyl side chains of the amino acids adjacent to aromatic side chains experience a ring current shift that is very pronounced when the two residues are of opposite configuration[11-13]. The spectrum of enzymatically resistant compound shows in water solution an 0·11 ppm upfield shift of the δ neopentylglycyl proton resonance (0·83 ppm) relative to the chemical shift of the same resonance in the other diastereomer (0·94 ppm).

Comparison of biological activities of [L-Neo^5]Dalargin, [D-Neo^5]Dalargin and Dalargin is presented in Table I. [D-Neo^5]Dalargin exhibited a markedly prolonged effect. After the administration of the same dose ($2 . 10^{-3}$ mg), the duration of its effect was doubled in comparison to the effect of the analogue containing L-neopentylglycine.

EXPERIMENTAL

^{1}H NMR spectra were recorded at 500 MHz using a Bruker — M 500 spectrometer operating in the pulsed Fourier transform mode. Resonances assignment in spectra was performed using difference spin decoupling experiments. Solutions of 5mM samples were prepared in $(CD_3)_2SO$ and D_2O. To get cationic form of the peptides, small amount of trifluoroacetic acid was added into DMSO. Chemical shifts were measured relative to sodium 2,2-dimethyl-2-silapentane-5--sulfonate in D_2O and relative to DMSO resonance (δ 2·5 ppm) in dimethyl sulfoxide solution. Melting points were measured on Boetius block and are uncorrected. Systems for thin-layer chromatography on silica gel: chloroform–methanol–32% acetic acid (60 : 45 : 20, A); butanol–-acetic acid–water (3 : 1 : 1, B); ethyl acetate–pyridine–acetic acid–water (45 : 20 : 6 : 11, C).

The opioid activity of the peptides was determined on the basis of their ability to inhibit electrically stimulated contractions of the guinea-pig ileum mucosa layer (1 pulse per sec, amplitude 8 V cm^{-1} of the distance between the electrodes[14] and expressed in IC_{50} values (i.e. concentration of peptides causing 50% inhibition).

Tert-butyloxycarbonyl-D,L-neopentylglycyl-L-arginine (*I*)

To the solution of L-arginine (0·28 g, 1·62 mmol) 0·59 g (1·62 mmol) of *p*-nitrophenyl ester of tert-butyloxycarbonyl-D,L-neopentylglycine in 8 ml of dimethylformamide was added. The reaction mixture was agitated for 12 h at laboratory temperature and then, dimethylformamide was evaporated in vacuum. The residue was pulverized under ether. The precipitated substance was filtered, washed with ether, dissolved in 2-propyl alcohol and precipitated again with ether. The yield of compound *I* was 0·51 g (88%); m.p. 175—177°C; R_F 0·66 (A), 0·59 (B), 0·41 (C). Amino acid analysis: Neo 1; Arg 0·96. For $C_{18}H_{35}N_5O_5.H_2O$ (419·5) calculated: 51·53% C, 8·89% H, 16·69% N; found: 51·14% C, 8·76% H, 16·48% N.

Benzyloxycarbonyl-L-phenylalanyl-D,L-neopentylglycyl-L-arginine (*II*)

Trifluoroacetic acid (5 ml) was added to compound *I* (0·54 g, 1·35 mmol) and the mixture was agitated for 1 h. The solution was dried in vacuum and the residue was washed with ether, filtered and rinsed with ether again. The product was dissolved in a minimum volume of water and passed through a column of Dowex 1 in OH^- cycle. The filtrate was evaporated to dryness and the residue was dissolved in dimethylformamide. Benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (0·56 g, 1·35 mmol) was added to the solution and the reaction mixture was agitated

for 12 h. Dimethylformamide was evaporated in vacuum and the residue was agitated with ether and filtered. The solid substance was dissolved in ethyl alcohol and precipitated with ether. Yield: 0·67 g (85%) of compound *II*; m.p. 155—157°C; R_F 0·87 (A), 0·66 (B), 0·48 (C). Amino acid analysis: Phe 0·98; Neo 1; Arg 0·97. For $C_{30}H_{42}N_6O_6.2\ H_2O$ (618·7) calculated: 58·23% C, 7·49% H, 13·58% N; found: 58·75% C, 7·06% H, 13·43% N.

Tert-butyloxycarbonyl-O-tert-butyloxycarbonyl-L-tyrosyl-D-alanyl--glycyl-L-phenylalanyl-D,L-neopentylglycyl-L-arginine (*III*)

Compound *II* (0·62 g, 1·13 mmol) was hydrogenated in methanol in the presence of Pd/C (0·60 g). After the termination of the reaction, the catalyst was filtered-off and the filtrate was evaporated in vacuum and dried in the desiccator over KOH. The residue was dissolved in 10 ml of dimethylformamide and 0·72 g (1·13 mmol) of tert-butyloxycarbonyl-O-tert-butyoxycarbonyl-L-tyrosyl--D-alanyl-glycine *p*-nitrophenyl ester was added to the solution. The reaction mixture was agitated for 24 h at laboratory temperature. Dimethylformamide was removed by evaporation in vacuum and the residue was dissolved in chloroform and chromatographed on a silica gel column using chloroform–methanol (7 : 3). Fractions corresponding to the main product were collected and evaporated to dryness. After dissolving in ethyl alcohol, the product was precipitated with ether. Yield: 0·86 g (73%) of compound *III*; m.p. 180—182°C; R_F 0·71 (A), 0·63 (B), 0·52 (C). Amino acid analysis: Tyr 1·06; Ala 1·07; Gly 1·06; Phe 1·01; Neo 1; Arg 0·98. For $C_{46}H_{69}O_9O_{12}$ .2 H_2O (976·1) calculated: 56·60% C, 7·54% H, 12·91% N; found: 56·25% C, 7·44% H, 12·43% N.

L-tyrosyl-D-alanyl-glycyl-L-phenylalanyl-L-neopentylglycyl-L-arginine (*IVa*) and L-tyrosyl-D-alanyl-glycyl-L-phenylalanyl-D-neopentylglycyl-L-arginine (*IVb*)

The compound *III* (0·80 g, 0·85 mmol) was dissolved in trifluoroacetic acid (5 ml) and mixed 1 h at laboratory temperature. Trifluoroacetic acid was removed by distillation under vacuum and the residue was agitated with ether, filtered, washed again with ether and dried in a desiccator over KOH. The yield was 0·60 g (96%); R_F 0·66 (A), 0·54 (B), 0·29 (C). The diastereoisomeric substances were separated by means of preparative HPLC on a column of Silasorb C-18 using a gradient of 0·05% aqueous trifluoroacetic acid–methanol (0—100%).

IVa: Amino acid analysis: Tyr 1·00; Ala 1·00; Gly 1·19; Phe 0·99; Neo 1·00; Arg 0·92. For $C_{36}H_{52}N_9O_8.2\ F_3CCOOH.2\ H_2O$ (1 002·93) calculated: 47·90% C, 5·83% H, 12·57% N; found: 47·50% C, 15·68% H, 12·50% N. ^{1}H NMR in DMSO (δ): Tyr: NH_3 8·08, $C^\alpha H$ 3·98, $C^\beta H_2$ 2·90, 2·84; D-Ala: NH 8·52, $C^\alpha H$ 4·33, $C^\beta H_3$ 1·04; Gly: NH 8·19, CH_2 3·71, 3·61; Phe: NH 7·97, $C^\alpha H$ 4·52, $C^\beta H_2$ 3·01, 2·73; Neo: NH 8·25, $C^\alpha H$ 4·37, $C^\beta H_2$ 1·63, 1·49, $C^\delta H_3$ 0·89; Arg: NH 8·00, $C^\alpha H$ 4·18, $C^\beta H_2$ 1·77, 1·60, $C^\gamma H_2$ 1·51, $C^\delta H_2$ 3·11, $N^\varepsilon H$ 7·58.

IVb: Amino acid analysis: Tyr 1·01; Ala 1·02; Gly 1·02; Phe 1·01; Neo 1·00; Arg 0·98. For $C_{36}H_{52}N_9O_8.2\ F_3CCOOH.2'H_2O$ (1 002·9) found: 47·56% C, 5·68% H, 12·52% N. ^{1}H NMR in DMSO (δ): Tyr: NH_3 8·07, $C^\alpha H$ 3·98, $C^\beta H_2$ 2·91, 2·85; D-Ala: NH 8·53, $C^\alpha H$ 4·31, $C^\beta H_3$ 1·05; Gly: NH 8·18, CH_2 3·70, 3·61; Phe: NH 8·07, $C^\alpha H$ 4·55, $C^\beta H_2$ 2·95, 2·73; D-Neo: NH 8·35, $C^\alpha H$ 4·35, $C^\beta H_2$ 1·57, 1·44, $C^\delta H_3$ 0·83; Arg: NH 8·12, $C^\alpha H$ 4·20, $C^\beta H_2$ 1·74, 1·60, $C^\gamma H_2$ 1·46, $C^\delta H_2$ 3·08, $N^\varepsilon H$ 7·52.

Hydrolysis of [L-Neo^5] and [D-Neo^5]Dalargin with carboxypeptidase B

Reaction mixture containing 10 µl of peptide solution (10 µg), 40 µl TES buffer (0·05 mol l^{-1}, pH 7·5) and 50 µl of enzyme (150 µg) was incubated at 37°C. The reaction was stopped after 0, 30, 60 or 120 min by 3 min boiling in a water bath. The samples were then tested for their bio-

logical activity in a standard GPI test (doses $2 . 10^{-3}$ and $5 . 10^{-3}$ mg). The results showed that 65–75% of Dalargin or [L-Neo^5]Dalargin is hydrolyzed after 30 min and 85% after 60 min of incubation with the enzyme, whilst the activity of [D-Neo^5]Dalargin remains unchanged veen after 120 min incubation.

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