PREPARATION AND PROPERTIES OF A NEW DALARGINE ANALOGUE
L-Tyr-D-Ala-Gly-L-Phe-L-Tle-L-Arg

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Stepwise synthesis in solution provided tert-butyloxycarbonyl-O-benzyl-L-tyrosyl-D-alanyl-glycyl--L-phenylalanyl-L-tert-leucyl-L-arginine which was then catalytically reduced and treated with trifluoroacetic acid. The product was purified by ion exchange chromatography and free electrophoresis. The hexapeptide containing L-tert-leucine in position 5 exhibited 63% biologic activity (guinea pig ileum) of Dalargine (L-Tyr-D-Ala-Gly-L-Phe-L-Leu-L-Arg).

Thousands of enkephalin[1] analogues have been prepared up to the present. Frequently, syntheses were motivated by preparation of analogues having enhanced affinity to different receptor populations, increased selectivity of their effect and greater metabolic stability. Substitution of L-aminoacids in the chain by D-enantiomers[2,3], preparation of prohormone-type substances[4] or dimers[5,6] should be mentioned here. One of the approaches to synthesis is replacement of coded aminoacids by non-coded ones having sterically constrained side chains either of the L- or D-form, with the consequence that the conformational flexibility of the peptide studied is limited. Thus, for example, enkephalin analogues modified in position 5 were prepared, L-tert-leucine, which has a bulky butyl side chain, was introduced into position 5. Application of this non-coded amino acid led to higher stability with respect to enzymatic degradation and 2 to 3 times higher activity in investigations of opiate activity compared to [D-Ala^2, L-Leu^5]enkephalin[7].

The aim of the present study was substitution of the amino acid residue in position 5 in the known, efficient enkephalin analogue Dalargine (L-Tyr-D-Ala-Gly-L-Phe-L--Leu-L-Arg)[8], *i.e.* synthesis of L-Tyr-D-Ala-Gly-L-Phe-L-Tle-L-Arg* and comparison of its biologic properties (guinea pig ileum) with Dalargine and some related analogues[10].

* Nomenclature and symbols employed in accordance with IUPAC recommendations[9]. The abbreviation Tle is used for tert-leucine (2-amino-3,3-dimethylbutane acid).

The hexapeptide L-tyrosyl-D-alanyl-glycyl-L-phenylalanyl-L-tert-leucyl-L-arginine was synthesised from two fragments, prepared by step-wise condensation of active amino acid esters. Benzyloxycarbonyl-L-phenylalanyl-L-tert-leucyl-L-arginine was prepared from free arginine. In the course of preparing tert-butyloxycarbonyl-O--benzyl-L-tyrosyl-D-alanyl-glycine, the carboxyl group was protected in the form of its salt. Fragments were then linked by active esters and the protecting groups were then removed by hydrogenolytic and acidolytic cleavage.

Table I presents a review of the biologic activity (IC_{50}) in the standard GPI test[11]. Comparison of the activity of Dalargine with that of the newly prepared analogue shows, that the activity of the new analogue is 1/3 lower. Comparison with published data[7] of the effect of substituting L-leucine by L-tert-leucine in the case of pentapeptides shows, that the substitution can have different consequences of dependence on the nature of the peptide in which this substitution is performed. For comparison of the activity of Dalargine, data are included for shorter peptides, *i.e.* [D-Ala^2, L-Leu^5]enkephalin and its chloromethyl ketone[10], which latter appears to be one of the most efficient analogues available to us.

EXPERIMENTAL

Purity of the initial amino acids and intermediates was checked by silicage thin layer chromatography (Merck–Kieselgel 60) in the systems: Chloroform–methanol–32% acetic acid (60 : 45 : 20; A), butanol–acetic acid–water (3 : 1 : 1; B), ethylacetate–pyridine–acetic acid–water (45 : 20 : : 6 : 11; C), chloroform–methanol–acetic acid–water (3 : 2 : 2 : 1; D), dichloromethane–methanol– –50% acetic acid (85 : 15 : 2; E), chloroform–methanol–formic acid (9 : 1 : 0·5; F), butanol–formic acid–water (15 : 3 : 1; G). Melting points were measured using a Boetius block. Optical rotation was measured using a Perkin–Elmer Model 241 polarimeter in chloroform (*c* 0·2), unless stated otherwise. Amino acid analysis was carried out with a Labotron-Liquimat 111 automatic analyser. Substances were purified by high-pressure liquid chromatography using an Altex 334 instrument.

TABLE I

Opioid activity of Dalargine and its analogues on guinea pig ileum

Substance	IC_{50}[a] nmol l^{-1}	Relative activity
Dalargin	369 (8)	1
L-Tyr-D-Ala-Gly-L-Phe-L-Tle-L-Arg	599 (5)	0·62
[D-Ala^2, L-Leu^5]enkephalin chloromethyl ketone	82 (4)	4·50[b]
[D-Ala^2, L-Leu^5]enkephalin	285 (4)	1·29

[a] The number of experiments is given in brackets; [b] Ref.[12].

HPLC Spherisorb ODS 5 μ, mobile phase A: 0·05M-KH_2PO_4 (pH = 3·0), B: ACN, gradient 5%—90% 20 min. Solutions of substances were concentrated in a vacuum evaporator at temperatures up to 40°C.

Tert-butyloxycarbonyl-L-tert-leucyl-L-arginine (*I*)

1·1 g (8 mmol) *p*-nitrophenol were added to a solution of 1·8 g (8 mmol) tert-butyloxycarbonyl-L--tert-leucine in 30 ml ethylacetate. The mixture was cooled to −15°C and a solution of 1·7 g (8 mmol) N,N'-dicyclohexylcarbodiimide in 10 ml ethylacetate was added. The mixture was agitated for 45 min and then allowed to stand at 0°C for 16 h. Precipitated N,N'-dicyclohexyl-urea was filtered under suction and the ethylacetate solution was evaporated. The residue was dissolved in 5 ml dimethylformamide and 1·4 g (8 mmol) L-arginine were added. The mixture was agitated at room temperature for 24 h, evaporated and the residue agitated with ether. The substance precipitated was filtered under suction, washed with eter and dried *in vacuo*: Yield 1·1 g (34%), m.p. 173—175°C, $[\alpha]_D^{20}$ −2·9°, R_F 0·75 (A); 0·48 (B); 0·55 (C).

Benzyloxycarbonyl-L-phenylalanyl-L-tert-leucyl-L-arginine (*II*)

0·50 g (1·3 mmol) tert-butyloxycarbonyl-L-tert-leucyl-L-arginine (*I*) were dissolved in a solution of 10 ml 4M-HCl in ethylacetate and agitated at room temperature for 45 min. Ethylacetate was evaporated and the residue dissolved in 30 ml water and filtered over Dowex-1 in the OH cycle. The aqueous solution was evaporated, the residue was dried in a dessicator *in vacuo*, dissolved in 5 ml dimethylformamide and added to a solution of 0·5 g (1·4 mmol) benzyloxycarbonyl-L--phenylalanine-p-nitrophenylester in 5 ml dimethylformamide. The mixture was agitated at room temperature for 24 h, evaporated and the residue was dissolved in 3 ml methanol and the solid substance was precipitated with 200 ml ether, filtered under suction and evaporated *in vacuo*: Yield 302 mg (41%), m.p. 156—158°C, $[\alpha]_D^{20}$ −4·3°. R_F 0·8 (A); 0·57 (C).

Benzyloxycarbonyl-D-alanyl-glycine (*III*)

0·5 g (7·7 mmol) glycine were dissolved in 8 ml 1M-NaOH and 2·7 g (7·8 mmol) benzyloxycarbonyl-D-alanine *p*-nitrophenylester in 20 ml dimethylformamide were added to this solution. The mixture was agitated for 24 h at room temperature. The solvent was then evaporated, the residue dissolved in water and extracted three times with 50 ml ether. The aqueous layer was acidified with 1M-H_2SO_4 and extracted with ethylacetate (50 ml, 3×). The ethylacetate solution was washed with water and the residue dried by azeotropic distillation with 2-propanol. The residue was agitated with 100 ml ether and the crystals obtained were filtered under suction, washed with ether and dried in a dessicator: Yield 1·4 g (65%), m.p. 126·5—127°C, $[\alpha]_D^{20}$ +20·7° (methanol), R_F 0·67 (A); 0·71 (C); 0·35 (E).

Tert-butyloxycarbonyl-O-benzyl-L-tyrosyl-D-alanyl-glycine (*IV*)

A solution of 1·7 g (5·9 mmol) substance *III* in 20 ml methanol was hydrogenated in presence of Pd black for 3 h. The catalyst was removed by filtering, washed with ethanol and the solution distilled *in vacuo*. The residue was dissolved in 5·9 ml 1M-NaOH. 2·9 g (5·9 mmol) N-tert-butyloxycarbonyl-O-benzyl-L-tyrosine *p*-nitrophenylester in 25 ml dimethylformamide were added to this solution. The mixture was agitated at room temperature for 24 h. The solvents were removed by distillation and the residue was dissolved in water and extracted with ether (50 ml, 2×). The aqueous layer was acidified with 1M-H_2SO_4 and extracted with ethylacetate (70 ml, 3×). The ethylacetate solution was washed with water (50 ml, 2×) and the residue was dried by azeotropic

distillation with 2-propanol. The residue was then agitated with 150 ml ether and the crystals obtained were filtered under suction, washed with ether and dried in a dessicator: Yield: 2·6 g (89%), m.p. 100—100·5°C, $[\alpha]_D^{20}$ −9·3, R_F 0·74 (C); 0·57 (E); 0·36 (F).

p-Nitrophenylester Tert-butyloxycarbonyl-O-benzyl-L-tyrosyl-D-alanyl-glycine (*V*)

3·4 g (7·0 mmol) substance *IV* and 1 g *p*-nitrophenol were dissolved in 25 ml dimethylformamide and then 1·5 g (7·3 mmol) N,N'-dicyclohexylcarbodiimide in 10 ml dimethylformamide were added at −25°C under agitation. The mixture was agitated at −25°C for 45 min and allowed to stand at 0°C for 24 hours. Precipitated N,N'-dicyclohexylurea was filtered under suction and the filtrate was evaporated *in vacuo*. The residue was 3·6 g (83%), m.p. 154°C $[\alpha]_D^{20}$ −0·4°, R_F 0·87 (F); 0·69 (G); 0·41 (D).

Tert-butyloxycarbonyl-O-benzyl-L-tyrosyl-D-alanyl-glycyl-L-
-phenylalanyl-L-tert-leucyl-L-arginine (*VI*)

150 mg (0·3 mmol) substance *II* were dissolved in 15 ml methanol and hydrogenated for 3 h after addition of Pd black. The catalyst was removed by filtering and the filtrate evaporated. The residue was dissolved in 5 ml dimethylformamide and 163 mg (0·3 mmol) substance *V* were added. The mixture was agitated for 24 h at room temperature, evaporated *in vacuo*, the residue dissolved in 3 ml methanol and 250 ml ether were added. The precipitate was filtered under suction: Yield 190 mg (80%), m.p. 204—208°C, $[\alpha]_D^{20}$ −8·5° (dimethylformamide–acetic acid; 9 : 1), R_F 0·87 (A); 0·56 (B); 0·56 (C).

L-Tyrosyl-D-alanyl-glycyl-L-phenylalanyl-L-tert-leucyl-L-arginine (*VII*)

170 mg (0·2 mmol) substance *VI* were dissolved in 15 ml acetic acid and hydrogenated for 3 h after adding Pd black. The catalyst was then removed by filtering and the solution evaporated *in vacuo*. The residue was dissolved in 15 ml trifluoroacetic acid and allowed to stand for 15 min at room temperature. The solution was again evaporated *in vacuo* and agitated with 50 ml ether. The substance precipitated was filtered, washed with ether and then dissolved in 50 ml 10% acetic acid and filtered over Dowex-1 in the acetate cycle. The filtrate was evaporated *in vacuo*. The product obtained was purified by counter-current distribution in the system butanol–acetic acid–water (4 : 1 : 5): Yield 91 mg (68%), $[\alpha]_D^{20}$ +9·1° (water), R_F 0·63 (A); 0·27 (B); 0·40 (C).

Determination of Biological Activity

The opioid activity of the peptides was determined on the basis of their ability to inhibit contractions of guinea pig ileum caused by electric stimulation and expressed by the IC_{50} values, see Table I.

REFERENCES

1. Hughes J., Smith T. W., Kosterlitz H. W., Fothergill L. A., Morgan B. A., Morris H. R.: Nature *258*, 577 (1975).
2. Coy D. H., Kastin A. J., Scally A. V., Morin O., Caron N. G., Labrie F., Walker J. M., Fertel R., Berntson G. G., Sandman C. A.. Biochim. Biophys. Res. Commun. *83*, 977 (1978).
3. Pert C. B., Pert A., Chang J. K., Fong B. T. W.: Science *194*, 330 (1976).
4. Lewis R. W., Stern A. S.: Ann. Rev. Pharmacol. Toxicol. *23*, 353 (1983).
5. Lipkowski A. W., Konecka A. M., Srozcynska I.: Peptides *3*, 597 (1982).

6. Lipkowski A. W., Konopka M., Isipiak B., Gumulka W. S. in the book: *Peptides 1982, Proc. 17th Peptide Symposium, Prague 1982* (K. Bláha and P. Maloň, Eds) p. 481. Walter de Gruyter, Berlin 1983.
7. Fauchére J. L., Petermann C.: Chim. Acta *63*, 824 (1980).
8. Titov M. I., Vinogradov V. A., Bespalova Zh. D.: Bjull. Vsesoyuz. kardiolog. nauch. tsentr. AMN SSSR *2*, 72 (1985).
9. *IUPAC-IUB Commission on Biochemical Nomenclature. Symbols for Amino Acid Derivatives and Peptides. Recommendations 1971.* Biochemistry *11*, 717 (1972).
10. Medzihradszky K.: unpublished results.
11. Hutchinson M., Kosterlitz H. W., Leslie F. M., Waterfields A. A., Terenius L.: Brit. J. Pharmacol. *55*, 541 (1975).
12. Medzihradszky K., Szécsi J., Czanédy A., Hepp J. in the book: *Peptides 1982, Proc. 17th Peptide Symposium, Prague 1982* (K. Bláha and P. Maloň, Eds), p. 623. Walter de Gruyter, Berlin 1983.

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