SYNTHESIS OF ^{14}C- AND ^{3}H-LABELLED β-CASOMORPHIN-5

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SUMMARY

β-casomorphin-5, tyrosyl-prolyl-phenylalanyl-prolyl-glicine, labelled with ^{14}C and ^{3}H in the N-terminal tyrosine residue, was synthesized by stepwise elongation, using [Dit][1]-β-casomorphin-5 as precursor for catalytic tritiation. The specific radioactivities of the two peptides were 1.6 mCi/mmol [59.2 MBq/mmol] and 44.1 Ci/mmol [1.63 /Bq/mmol], respectively. Both were biologically fully active as compared with a synthetic reference material.

Nomenclature is in accordance with the IUPAC-IUB rules: Biochem. J. 126, 773 1972 .
All optically active amino acids were of the L-configuration.
ABBREVIATIONS: BOC = tert.-butyloxycarbonyl, Z = benzyloxycarbonyl, OBzl = benzyl ester, Dit = 3,5-diiodotyrosine, OTcp = 2,3,5-trichlorophenyl ester, DMF = dimethylformamide, EtOAc = ethyl acetate, TLC = thin layer chromatography

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KEY WORDS

Peptide synthesis, β-casomorphin-5, [^{14}C] - and [^{3}H]-labelling, purification, biological activity.

INTRODUCTION

A novel type of natural opioid peptides derived from the β-chain of bovine casein, differing in structure from other endogenous opioid peptides e.g.(β-endorphin, enkephalins, dynorphin, kyotorphin), was recently described (1-4). In accordance with their biological origin and opiate-like activity, these were named β-casomorphins.

β-casomorphin-7 (H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH) and its analogues shortened at the C-terminal end β-casomorphin-6, /-5/ and /-4/ displayed opioid activity in an opiate receptor binding assay, in isolated mouse vas deferens, and in the guinea-pig ileum longitudinal muscle myenteric plexus preparation, and produced naloxone-reversible analgesia and inhibited the extinction and retrieval of a passive avoidance response in rats after intracerebroventricular injection (5-8). In all assays employed, β-casomorphin-5 was the most potent compound in this new group of naturally occurring opioid peptides.

In comparison with the much better investigated opioid peptides e.g. (β-endorphin, enkephalins), there is still very little knowledge about the physiological and/or pharmacological importance of the β-casomorphins.

RESULTS AND DISCUSSION

Radioactive labelled peptides allow further studies on their binding, mechanism of action, permeability into the nervous system and degradation. Since all tested bioassays show that the most potent peptide in the β-casomorphin group is the pentapeptide, we have prepared two radioactive derivatives, the ^{14}C - and ^{3}H - labelled pentapeptide (5) and (10), both labelled in the N-terminal tyrosine residue.

For preparation of both $[^{14}C(U)\text{-Tyr}]^{1}$-β-casomorphin-5 (5) and $[^{3}H\text{-Tyr}]^{1}$-β-casomorphin-5 (10) we applied stepwise elongation, which can be seen in Figures 1 and 2.

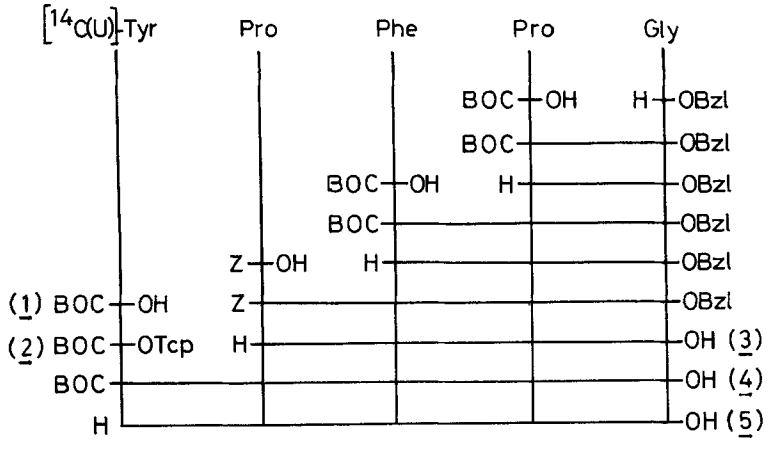

Fig. 1. Synthesis scheme of $[^{14}C(U)\text{-Tyr}]^{1}$-β-casomorphin-5

The synthesis of the C-terminal tetrapeptide H-Pro-Phe-Pro-Gly-OH (3) was accomplished by the mixed anhydride method corresponding to that of native β-casomorphin-5, the earlier

described (9,10) BOC/Bzl protecting group combination that is generally successful in peptide chemistry being used.

For introduction of [^{14}C(U)]^{1}-tyrosine we prepared the activated derivative BOC-[^{14}C(U)]^{1}-tyrosine-2,3,5-trichlorphenyl ester (2). After deprotection with hydrogen chloride in dioxane the crude pentapeptide was purified by preparative TLC on silica gel plates. The specific radioactivity after purification proved to be 1.6 mCi/mmol [59.2 MBq/mmol], quite satisfactory for different biological studies.

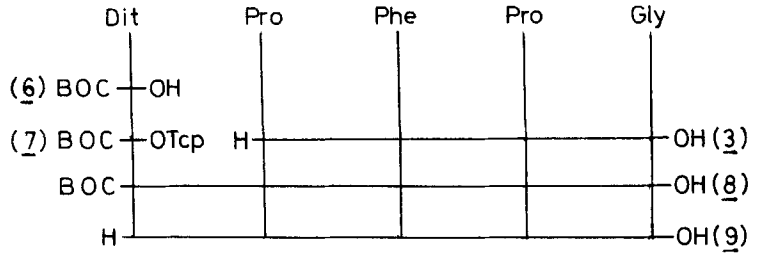

Fig. 2. Synthesis scheme of [Dit]^{1}-β-casomorphin-5

To prepare [^{3}H-Tyr]^{1}-β-casomorphin-5, we first synthesized the precursor pentapeptide [Dit]^{1}-β-casomorphin-5 (9). The coupling of BOC-Dit-OH (6) with the tetrapeptide (2) to give the protected pentapeptide (8) was achieved by using BOC-Dit-OTcp (7). After purification of the protected pentapeptide by column chromatography on silica gel and deprotection with hydrogen chloride in dioxane, the precursor peptide [Dit]^{1}-β-casomorphin-5 (9) was isolated as its hydrochloride.

[Dit]^{1}-β-casomorphin-5 (9) was catalytically dehalogenated using carrier-free tritium gas in the presence of palladium oxide (12). The crude product was purified by preparative TLC on silica gel plates. The specific radioactivity after purification proved to be 44.1 Ci/mmol [1.63 TBq/mmol].

The purities of the [^{14}C]- and [^{3}H]-labelled β-casomorphin-5 (5) and (10) were controlled by TLC in three different systems, by high-performance liquid chromatography on an analytical column, by comparison of the maxima in the ultraviolet absorption spectra (Table 1) and by radiochromatogram-scanning. Tritium was located only in the tyrosine residue. After enzymatic hydrolysis with dipeptidyl peptidase IV, among the identified fragments Tyr-Pro, Phe-Pro and Gly, only the dipeptide Tyr-Pro contained radioactivity.

Table 1. UV absorption spectra maximum values of β-casomorphin-5 derivatives

Substances	UV-maximum (nm)[x]
β-casomorphin-5	277.8
[Dit]^{1}-β-casomorphin-5	315.5
[^{14}C(U)]^{1}-β-casomorphin-5	277.8
[^{3}H]^{1}-β-casomorphin-5	275.0

[x] UV absorption spectra were normally measured in water, but in the case of [Dit]^{1}-β-casomorphin-5 a solution in 1% NH_4OH (pH = 9) was used.

Biological potencies of the labelled peptides were examined after intracerebroventricular administration to rats, using the pre-retention test of a passive avoidance task (7) as the test system. In addition, the influence of the [^{3}H]-labelled compound on the brain serotonin content of different brain areas was studied after intracerebroventricular administration to rats (13). Both labelled β-casomorphins showed the same activities as compared with the unlabelled synthetic peptide (Table 2 and 3).

Table 2. Effects of [^{14}C]- and [^{3}H]-labelled-β-casomorphin-5 in comparison with unlabelled peptide on the avoidance latency in the pre-retention test /passive avoidance task/ in rats

Substances	Number of animals	24 h latency (s)	48 h latency (s)
Controls	8	78.5 ± 23.6	57.3 ± 16.1
β-casomorphin-5	8	16.4 ± 10.3	54.7 + 23.4
[^{14}C(U)]^{1}-β-casomorphin-5	10	20.3 ± 7.4	56.5 ± 25.1
[^{3}H]^{1}-β-casomorphin-5	10	20.1 ± 11.6	58.4 ± 27.3

Table 3. Changes in the brain serotonin content of different brain areas after intracerebroventricular administration of 200 ng [^{3}H]-labelled or unlabelled β-casomorphin-5.

Substances	Amygdala	Septum	Striatum
Controls	1.41±0.13[x](17)[xx]	1.39±0.17 (17)	0.67±0.05(17)
β-casomorphin-5	1.02±0.04 (7)	0.88±0.06(6)	0.51±0.03 (7)
[^{3}H]-β-casomorphin-5	1.00±0.03 (9)	0.95±0.05(10)	0.50±0.03 (10)

EXPERIMENTAL

1. Material and Methods

All amino acids were commercial products /Reanal/, except for 3,5-diiodotyrosine /Aldrich/ and [^{14}C(U)]-tyrosine /Isotope Institute, Prague/. Tritium gas was purchased from Techabexport /USSR/ and stored in the form of uranium tritide. The catalyst PdO was a product of Merck.

Melting points are uncorrected, as measured with a Boetius apparatus. Optical rotations were determined with a Polamat-A /VEB Carl Zeiss, Jena/ polarimeter. For analytical control by high-performance liquid chromatography a Hewlett-Packard apparatus /P-124 column and 0.01 M ammonium acetate /pH 4.2/-methanol/ were applied. TLC was carried out on precoated Silufol plates /Rf^{s}/ /Kavalier/ and Kieselgel-Fertigplatten /Rf^{k}/ /Merck/, using the following solvent systems:

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[x] Values are given in μg/g tissue, 60 minutes after the injection

[xx] Number of animals in parentheses

A	chloroform/methanol	9:1
B	ethyl acetate/pyridine/acetic acid/water	90:15:4.5:8.3
C	n-butanol/acetic acid/water	4:1:1
D	n-butanol/acetic acid/water/ethyl acetate	1:1:1:1
E	n-butanol/pyridine/acetic acid/water	30:20:6:24

TLC plates were developed by using ninhydrin and chlorine KI/amylase reagents. The purities of the labelled compounds on TLC plates were controlled with a Packard Radiochromatogram Scanner /model 7201/. The radioactivity values were measured with a Packard Tri-carb liquid scintillation system /model 3375/.

Ultraviolet absorption spectra were taken in a Specord-UV-VIS /VEB Carl Zeiss Jena/ spectrophotometer. For amino acid analyses the samples were hydrolyzed in 6 N HCl and analyzed by a Mikrotechna Amino Acid Analyzer /model AAA 881/.

2. Peptide synthesis

tert.-Butyloxycarbonyl-[^{14}C(U)]-tyrosine, (1)

0.65 mg [^{14}C(U)]-L-tyrosine /1 mCi, 270 mCi/mmol/ was diluted to a specific radioactivity of 2.7 mCi/mmol with 65 mg L-tyrosine. Amino group protection was carried out with di-tert.-butyl-dicarbonate according to the general method of Moroder et al. (14).

Yield: 76 mg, oil /75%/;

homogeneous on TLC: Rf^{s}_{A}: 0.31, Rf^{s}_{B}: 0.60.

tert.-Butyloxycarbonyl-[^{14}C(U)]-tyrosine-2,4,5-trichlorophenyl ester, (2)

76 mg /0.27 mmol/ (1) and 53.3 mg /0.27 mmol/ 2,3,5-trichlorophenol were dissolved in 1 ml EtOAc. The mixture was cooled to 0 °C, followed by addition of 55.7 mg/0.27 mmol/ dicyclohexylcarbodiimide. After stirring for 1 h at 0 °C and 3 h at room temperature, the dicyclohexylurea was filtered off. Addition of petroleum ether to the EtOAc solution resulted in a precipitate, which was collected and recrystallized from EtOAc.

Yield: 76 mg /61%/; m.p.: 172-174 °C; $[\alpha]_D^{22} = -36.0°$ /c=1, acetic acid/;

homogeneous on TLC: Rf_A^S: 0.64, Rf_B^S: 0.88.

H-Prolyl-phenylalanyl-prolyl-glycine HCOOH, (3)

As described earlier (9,10).

tert.-Butyloxycarbonyl-[^{14}C(U)]-tyrosyl-prolyl-phenylalanyl-prolyl-glycine, (4)

To a mixture of 55 mg /0.12 mmol/ (3), 30.8 l /0.22 mmol/ triethylamine and 27 mg /0.2 mmol/ 1-hydroxy-benzotriazol in 1.5 ml DMF, cooled to 0 °C, 46.1 mg /0.1 mmol/ (2) was added. After stirring for 24 h at 0 °C and 30 h at room temperature, the reaction mixture was evaporated to a small volume and added to 5 ml 5% $KHSO_4$ solution.

The precipitated product /oil/ was extracted with EtOAc and the combined extracts were washed several times with saturated NaCl, dried over Na_2SO_4 and evaporated. Crystallization from EtOAc/petroleum ether gave on oil, which was dissolved in a small amount of methanol and precipitated with EtOAc petroleum ether /1:1/. After standing overnight in a refrigerator, the

product was centrifuged off and dried in vacuo.

Yield: 45 mg /66%/.

The crude product was purified by preparative TLC /on Merck PSC-Fertigplatten, silica gel 60 F_{254}, 2 mm/ in solvent system C. The silica gel strip containing the product /detected with UV-light/ was removed from the plate, eluted with methanol, and the peptide was separated from the silica gel by centrifugation. The solvent was evaporated off, and the residue was crystallized from methanol/ether and dried in vacuo.

Yield: 36 mg /42%/; m.p.: 220-225 °C; $[\alpha]_D^{22} = -74.8°$ /c=1, methanol/;

homogeneous on TLC and radiochromatography:

Rf_A^k: 0.47, Rf_B^k: 0.19, Rf_C^k: 0.64, Rf_D^k: 0.77, Rf_E^k: 0.78.

H-[^{14}C(U)]-Tyrosyl-prolyl-phenylalanyl-prolyl-glycine HCl, (5)

To a solution of 30 mg /44 μmol/ (4) in 0.5 ml dioxane, 1 ml 4 N HCl in dioxane was added. After standing for 30 minutes at room temperature, the product was precipitated by addition of ether. The precipitate was collected, washed with ether and EtOAc and dried.

Yield: 22 mg /81%/; m.p.: 180-200 °C /dec./;

$[\alpha]_D^{25} = -67.8°$ /c=1, methanol/;

UV-maximum: 277.8 nm; specific radioactivity: 1.6 mCi/mmol [59.2 MBq/mmol]. The material proved to be chemically and radiochemically homogeneous on TLC: Rf_C^k: 0.33, Rf_D^k: 0.66, Rf_E^k: 0.60. It was also homogeneous on HPLC in comparison with β-casomorphin-5.

tert.-Butyloxycarbonyl-3,5-diiodotyrosine, (6)

Tert.-butylation was carried out by using di-tert.-butyl dicarbonate (14).
The product was recrystallized from EtOAc/petroleum ether or from diisopropyl ether.

Yield: 85%; m.p.: 182-184 °C; $[\alpha]_D^{22}$= +15.0° /c=2, methanol/;
homogeneous on TLC: Rf_A^S: 0.57, Rf_B^S: 0.89, Rf_D^S: 0.93.

tert.-Butyloxycarbonyl-3,5-diiodotyrosine-2,4,5-trichlorophenyl ester, (7)

2.67 g /5 mmol/ (6) was treated with 0.99 g /5 mmol/ 2,3,5-trichlorophenol and 1.03 g /5 mmol/ dicyclohexylcarbodiimide in 25 ml EtOAc and worked up exactly as described for compound (2).

Yield: 2.92 g /82%/; m.p.: 188-192 °C; $[\alpha]_D^{22}$= -35.1° /c=1, DMF/;
homogeneous on TLC: Rf_A^S: 0.84, Rf_B^S: 0.98, Rf_D^S: 0.97.

tert.-Butyloxycarbonyl-3,5-diiodotyrosyl-prolyl-phenylalanyl-prolyl-glycine, (8)

740 mg /1.6 mmol/ (3), 0.45 ml /3.2 mmol/ triethylamine and 430 mg /3.2 mmol/ 1-hydroxy-benzotriazol were dissolved in 20 ml DMF and treated at 0 °C with 1.14 g /1.6 mmol/ (7). After stirring for 2 h at 0 °C and 30 h at room temperature the work-up was performed as described above for (4), and crystallization from EtOAc/cyclohexane gave 1.4 g /94%/ crude product, which was purified by column chromatography on silica gel /3 x 25 cm/; $CHCl_3$ /100 ml/, $CHCl_3$/methanol 9:1 /100 ml/

and $CHCl_3$/methanol 8:2 /elution of the product/ served as eluents. Fractions containing the product were pooled and evaporated in vacuo, and the residue was recrystallized from methanol/ether.

Yield: 1.0 g /63%/; m.p.: 208 °C /dec./;

$[\alpha]_D^{20} = -45.9°$ /c=1, acetic acid/;

homogeneous on TLC: Rf_A^s: 0.25, Rf_B^s: 0.45, Rf_D^s: 0.81.

H-3,5-Diiodotyrosyl-phenylalanyl-prolyl-glycine HCl, (9)

256 mg /275 µmol/ (8) was dissolved in 2 ml dioxane and treated with 1.3 ml 6 N HCl in dioxane. After 30 minutes at room temperature the product was precipitated by addition of ether. The precipitate was crystallized from methanol/ether, washed with ether and EtOAc and dried.

Yield: 210 mg /88%/; m.p.: 207-211 °C /dec./;

$[\alpha]_D^{22} = -47.2°$ /c=1, acetic acid/;

homogeneous on TLC: Rf_C^k: 0.55, Rf_D^k: 0.36, Rf_E^k: 0.51;

amino acid analysis: Tyr 0.98, Pro 2.03, Phe 1.01, Gly 1.00;

UV maximum: 315.5 nm.

H-^{3}H-Tyrosyl-prolyl-phenylalanyl-prolyl-glycine, (10)

3 mg /3.46 µmol/ (9) was dissolved in 500 µl water and 1.5 µl /10.38 µmol/ triethylamine and 12 mg PbO catalyst were added. The mixture was frozen using liquid nitrogen and, after connection of the reaction vessel to the tritiation manifold, melted and stirred magnetically at room temperature for 150 min. The reaction was followed by measuring the pressure decrease. The catalyst was then removed from the reaction solution by

filtration /Millipore filter, 0.45 μl/ and washed with 3 x 5 ml water. Exchangeable tritium was removed by repeated rotary evaporation /4 x 15 ml water/. The remaining crude product was dissolved in 5 ml water to obtain a stock solution.

Peptide content: 2.47 μmol /71%/ /by UV spectrum/;

radioactive concentration: 129 mCi /by LSC/;

specific radioactivity: 52 Ci/mmol; UV maximum: 275 nm.

The material proved chemically and radiochemically homogeneous by TLC: Rf_C^k: 0.33, Rf_D^k: 0.66, Rf_E^k: 0.60.

For biological experiments, about 1 μmol of the crude product was purified by preparative TLC in solvent system C /"Merck" Kieselgel-Fertigplatten DC-60, 20 x 20 cm/. The silica gel strip containing the product could be assayed via a β-casomorphin-5 standard. The autoradiochromatogram showed only one peak /Rf 0.33/, similar to the authentic β-casomorphin-5 spot detectable with ninhydrin. The labelled peptide was then eluted with methanol and separated from the silica gel by centrifugation. After evaporation of the solvent methanol, a stock solution in 2.5 ml water was prepared from the material.

Peptide content: 0.77 mol /77% with reference to the crude product, 55% with reference to [Dit]^1-β-casomorphin-5/;

radioactive concentration: 33.9 mCi;

specific radioactivity: 44.1 Ci/mmol [1.63 TBq/mmol]; UV maximum: 275 nm.

The product proved to be homogeneous both chemically and radiochemically on TLC: Rf_C^k: 0.33, Rf_D^k: 0.66, Rf_E^k: 0.60.
It was also homogeneous on HPLC in comparison with β-casomorphin-5. The product could be stored in ethanol /1 mCi/ml/ under liquid nitrogen or in a refrigerator at -20 °C, and it was stable over 6 months.

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