SYNTHESIS OF THE 1-^{14}C-GLYCINE-LABELLED C-TERMINAL PENTAPEPTIDE OF GASTRIN

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The pentachloro- and pentafluorophenyl esters of *t*-butyloxycarbonyl (BOC)-1-^{14}C-glycine*, were prepared, and hence the 1-^{14}C-glycine-labelled C-terminal pentapeptide of gastrin. Three pentapeptides of different specific radioactivities were prepared for a study of *in vivo* metabolism and transport phenomena.

The C-terminal tetrapeptide of gastrin (H-Trp-Met-Asp-PheNH$_2$) is known to exhibit the full range of biological activity of the original heptadecapeptide [1]. Numerous analogues of this tetrapeptide have been synthesized to study the connections between chemical structure and biological activity [2]. We wished to study the mechanism of action, transport and metabolism of gastrin and its C-terminal pentapeptide. Therefore, the pentapeptide BOC-1-^{14}C-Gly-Trp-Met-Asp-PheNH$_2$ was synthesized, first with two different low specific radioactivities (168 μCi/mmole and 450 μCi/mmole), and used them in a study of the metabolism [3]. Later, a pentapeptide of higher specific activity (7.5 mCi/mmole), necessary for autoradiographic investigations with light- and electronmicroscopy, was synthesized.

1-^{14}C-glycine was prepared from 1-^{14}C-bromoacetic acid of three different specific radioactivities by the method described by MURRAY and WILLIAM [4]. We tried to elaborate a method which permits application of radioactive glycine with the highest efficiency possible. To this end the pentapeptide was synthesized by two different methods.

The tetrapeptide used in the synthesis of both pentapeptides of lower specific radioactivity was prepared by applying pentachlorophenyl esters of BOC-amino-acids, and by protecting the β-carboxyl group of the aspartic acid with a *t*-butyl group (Fig. 1). The yield of the coupling of active esters was 80—85% in each case. Coupling of BOC-1-^{14}C-glycine was also achieved *via* the pentachlorophenyl ester,

* Abbreviations accepted by the IUPAC-IUB (1967) for peptide chemistry are used in this paper: Z = carbobenzyloxy = benzyloxycarbonyl; BOC = *t*-butyloxycarbonyl; FOR = formyl; OBut = *t*-butylester.

with an 85% yield in this case, too. The pentapeptides proved not quite homogeneous; further purification was necessary, presumably because of partial oxidation of the tryptophan or methionine. After purification, the yield was about 70% of the starting BOC-1-^{14}C-glycine.

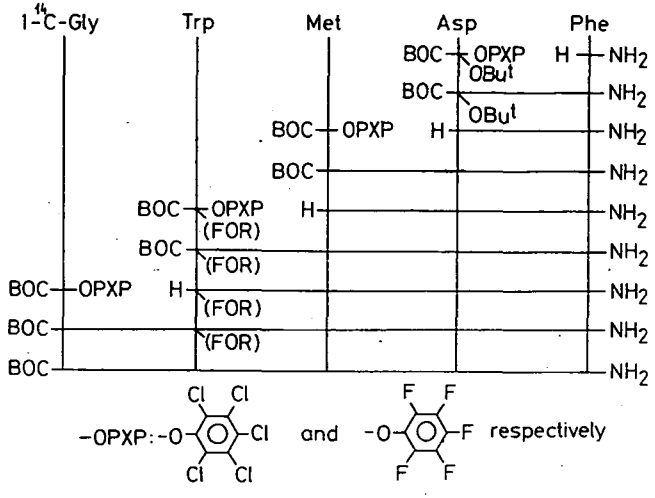

Fig. 1

In the synthesis of the radioactive pentapeptide of higher specific activity, use was made of the experience obtained in the above syntheses. The tetrapeptide was prepared by the method of KISFALUDY *et al.* [5]. Application of the pentafluorophenyl esters of BOC amino acids in a twofold excess resulted in very rapid synthesis. The β-carboxyl group of the aspartic acid was protected with a *t*-butyl group in this case, too; the indole ring of the tryptophan was protected with a formyl group [6] to avoid side-reactions (Fig. 1). The yields of the coupling steps were about 90%, and we succeeded in obtaining the pure tetrapeptide H-Trp-Met-Asp-PheNH$_2$. The pentafluorophenyl ester of BOC-1-^{14}C-glycine was prepared in 97% yield, and coupled with the equivalent quantity of the above tetrapeptide. Splitting-off of the N^{im}-formyl group from the pentapeptide gave an 85% yield of radiochromatographically-pure pentapeptide (checked in 3 different solvent systems). The purity was also checked by amino acid analysis.

Comparison of the two methods of synthesis indicated the use of pentafluorophenyl esters to be the more favourable.

All three radioactive pentapeptides prepared proved to possess the full biological activity of the product ICI 50, 123 ("Peptavlon"). The measuring of biological activities was performed by conductometric titration of chloride ion liberated in rat stomach on the *iv.* dosage of pentapeptides.

These preparations were mainly used to study the metabolism of the pentapeptide [3, 7]. We recently started an investigation of the transport and the mechanism of action of the gastrin pentapeptide of higher activity.

Experimental

Pentachlorophenyl ester of t-butyloxycarbonyl-1-^{14}C-glycine

1.17 g (4.4 mmole) pentachlorophenol, dissolved in 15 ml ethyl acetate, was cooled to 0 °C, and 0.824 g (4.0 mmole) dicyclohexylcarbodiimide (DCC), dissolved in 2 ml ethyl acetate, was added. After 2 min stirring, 0.700 g (4.0 mmole) BOC-1-^{14}C-glycine, dissolved in 4 ml ethyl acetate, was added to the solution, which was subsequently stirred at 0 °C for 1 hr and left to stand overnight. The dicyclohexylurea (DCU) formed was filtered off, the solution was evaporated to dryness, and the residue was dissolved in tetrahydrofuran which was next cooled to 0 °C. After the precipitated DCU had been filtered off, the solution was evaporated to dryness and the active ester crystallized from ethyl acetate—petroleum ether. Yield: 1.43 g (85%) chromatographically-pure product; m.p.: 138—139 °C (lit. 142 °C [8]). The specific radioactivities of two preparates worked up with these quantities were 168 μCi/mmole and 450 μCi/mmole, respectively; these data are equal to those for the original BOC-1-^{14}C-glycine materials.

Pentafluorophenyl ester of t-butyloxycarbonyl-1-^{14}C-glycine

17.5 mg (0.1 mmole) BOC-1-^{14}C-glycine and 22 mg (0.12 mmole) pentafluorophenol, dissolved in 0.2 ml ethyl acetate, was cooled to 0 °C, 25 mg (0.12 mmole) DCC dissolved in 0.1 ml ethyl acetate was added, and the mixture was stirred at 0 °C for 1 hr and then left to stand at −10 °C for 30 min. After the DCU had been filtered off, the solution was evaporated to dryness and the crude product was triturated with *n*-hexane. Yield: 32 mg (95%) chromatographically-pure product; m.p.: 75—76 °C (lit. 79—80 °C [9]). Specific radioactivity: 7.5 mCi/mmole; this is equal to the value for the original BOC-1-^{14}C-glycine.

Tryptophyl-methionyl-asparaginyl-phenylalanineamide

Method (a) (with pentachlorophenyl esters). Starting from 3.28 g (20 mmole) phenylalanineamide, equivalent quantities of pentachlorophenyl esters of carbobenzyloxy or BOC amino acids were used in each step of the synthesis. Coupling was performed in DMF; after 24 hr reaction time the mixture was poured into 0.2 M aqueous $KHSO_4$ and ether with vigorous stirring at 0 °C. The protected peptides, which solidified after a short standing, were filtered and purified by crystallization. The data obtained for the different protected peptides were as follows: Z-Asp(OBut)-PheNH_2 7.05 g (15 mmole, 75%), m.p.: 158 °C, recrystallized from isopropanol; BOC-Met-Asp-PheNH_2 6.3 g (12.5 mmole, 83%), m.p.: 208—209°C, crystallized from 2-ethoxy-ethanol; BOC-Trp-Met-Asp-PheNH_2 6.95 g (10 mmole, 80%), m.p.: 212—213 °C. The carbobenzyloxy protecting group was split off by hydrogenation, and the other protecting groups (BOC- and OBut) by acidolysis with 90% trifluoroacetic acid (TFA) for 30 min; in the case of the tripeptide and tetrapeptide, a N_2-blanket was used and 5% thioglycolic acid was added to the mixture

in order to avoid oxidation. The tetrapeptide trifluoracetate was crystallized from methanol—ether, m.p.: 192—194 °C; m.p. of the free base: 245—248 °C

Method (b) (with pentafluorophenyl esters). In this synthesis we started from 2 mmole phenylalanineamide, used in a twofold excess, in this case with respect to the Z-Asp(OBut)-OPFP. In the further coupling steps, the corresponding BOC amino acid pentafluorophenyl esters were applied in twofold excess. The couplings were performed in DMF, the pH being adjusted to about 8—8.5 by addition of triethylamine. After 30—60 min reaction time the DMF was evaporated off and the residue was dissolved in ethyl acetate, washed with 0.5 M aqueous $KHSO_4$, then with water, dried and evaporated. The protected peptides were crystallized from mixtures of ethyl acetate—hexane or ether—hexane, in which the starting pentafluorophenyl esters remained dissolved. The protected tetrapeptide was isolated by pouring into a mixture of 0.2 M aqueous $KHSO_4$ and ether. The results obtained for the different protected peptides were as follows: Z-Asp(OBut)-$PheNH_2$ 0.42 g (0.9 mmole, 90%), m.p.: 156—158 °C; BOC-Met-Asp-(OBut)-$PheNH_2$ 0.40 g (0.76 mmole, 85%), m.p.: 172—173 °C; BOC-Trp-(FOR)-Met-Asp-$PheNH_2$ 0.47 g (0.66 mmole, 87%), m.p.: 209—210 °C. The carbobenzyloxy protecting group was split off by hydrogenation, and the BOC- and -OBut protecting groups with 4 N HCl in dioxan for 30 min; in the case of the tripeptide and tetrapeptide a N_2-blanket was used and 5% thioglycolic acid was added to the mixture in order to avoid oxidation. The free tetrapeptide hydrochloride was obtained in 97% yield; m.p.: 220—222 °C.

t-Butyloxycarbonyl-1-^{14}C-glycyl-tryptophyl-methionyl-asparaginyl-phenylalanineamide

Method (a) (with pentachlorophenyl ester). 2.04 g (3.4 mmole) tetrapeptide synthesized by method (*a*) and 1.43 g (3.4 mmole) BOC-1-^{14}C-glycine-pentachlorophenyl ester were dissolved in 15 ml DMF, and 0.77 ml (7 mmole) N-methylmorpholine was added. After 36 hr reaction time, the mixture was poured into a mixture of 100 ml 0.2 M aqueous $KHSO_4$ and 30 ml ether under vigorous stirring at 0 °C. After 30 min stirring the protected pentapeptide was filtered off, washed with water, then with ether, and dried in an exsiccator. The crude product (2.1 g, 85%) was purified by chromatography on a silica gel column and eluted with a 60:20:6:11 mixture of ethyl acetate—pyridine—acetic acid—water. Yield: 1.8 g (70%) radiochromatographically homogeneous product, R_f values: 0.42 (chloroform—methanol—acetic acid 70:30:1); 0.58 (*n*-butanol—water—acetic acid 4:1:1); 0.31 (ethyl acetate—pyridine—water—acetic acid 120:20::11:6). M.p.: 197—200 °C. $[\alpha]_D^{22} = -29 \pm 2°$ ($c = 1.0$, DMF). Amino acid analysis: Phe 1.00; Asp 0.95; Met 0.93; Gly 1.02; Trp 0.90.

Anal.: $C_{36}H_{47}O_9N_7S$ (753.8). Calc.: C 57.4, H 6.25, N 12.9%. Found: C 57.1, H 6.30, N 13.2%.

Method (b) (with pentafluorophenyl ester). 59 mg (0.095 mmole) H-Trp(FOR)-Met-Asp-$PheNH_2$ and 32 mg (0.095 mmole) BOC-1-^{14}C-Gly-OPFP were dissolved in 0.5 ml DMF, and 0.022 ml (0.2 mmole) N-methylmorpholine was added. During the reaction the pH was held constant at pH 8 by addition of N-methylmorpholine; the reaction was quantitatively complete in 6 hr. The reaction mixture was poured into a mixture of 5 ml 0.2 M aqueous $KHSO_4$ and 2 ml ether under vigorous stirring.

The protected pentapeptide was filtered off, washed with water, ether, and *n*-hexane and dried. In order to split off the N^{im}-formyl group, the protected pentapeptide was dissolved in 0.5 ml DMF, and 5 equivalents of piperidine were added [10]; after 0.5 hr the pentapeptide was poured into a mixture of 0.2 M aqueous $KHSO_4$ and ether, as described above. 60.3 mg (0.08 mmole, 85%) radiochromatographically-pure pentapeptide was obtained, R_f values: 0.40 (chloroform—methanol—acetic acid 70:30:1); 0.56 (*n*-butanol—water—acetic acid 120:20:11:6). M.p.: 204—205 °C, specific activity: 7.5 mCi/mmole. Amino acid analysis: Gly 1.00; Phe 1.04; Asp 0.95; Met 0.92; Trp 0.92.

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СИНТЕЗ С-КОНЦЕВОГО ПЕНТАПЕПТИДА ГАСТРИНА, СОДЕРЖАЩЕГО 1-^{14}С-ГЛИЦИНА

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Синтезированы пентафлор- и пентафторфениловый эфир трет-бутилоксикарбонил-1-^{14}С-глицина, и с применением этих эфиров С-концевого пентапептида гастрина, содержащего 1-^{14}С-глицина. Синтезированы 3 разных специфических радиоактивных пентапептида, для „*in vivo*" исследования метаболических и транспортных процессов.