Synthesis of Heptacosapeptide (AA_{111}–AA_{137}) of the γ-Chain of a Human Immunoglobulin (IgGl) Using *N-tert*-Butyloxycarbonyl-L-amino Acid Pentachlorophenyl Esters

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Abstract □ The synthesis of the heptacosapeptide (AA_{111}–AA_{137}) of the γ-chain of a human immunoglobulin (IgGl) is described. The solid phase method was used, and coupling of each amino acid residue was achieved by use of the corresponding *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl ester.

Keyphrases □ Heptacosapeptide (AA_{111}–AA_{137}) of γ-chain of a human immunoglobulin—synthesis using *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters □ *N-tert*-Butyloxycarbonyl-L-amino acid pentachlorophenyl esters—used in the synthesis of heptacosapeptide (AA_{111}–AA_{137}) of the γ-chain of a human immunoglobulin □ Immunoglobulins, human—synthesis of the heptacosapeptide (AA_{111}–AA_{137}) of the γ-chain using *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters □ Peptides—synthesis of the heptacosapeptide (AA_{111}–AA_{137}) of the γ-chain of a human immunoglobulin (IgGl) using *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters

Recently the total primary structure of the human immunoglobulin IgGl Eu[1] was elucidated (4–14). The domain hypothesis (15) for immunoglobulins suggests that each region of the molecule containing disulfide linkage forms a compact domain, and that these areas are connected by a more extended peptide chain. The switch peptide, sequence AA_{111}–AA_{137}, of the γ-chain of this immunoglobulin is most probably one of these extended peptide regions bridging between two respective domains.

Some *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters were previously described (16), and they were shown to be useful intermediates for rapid peptide synthesis by the solution method (17) and also for the solid phase method of peptide synthesis (18).

This article reports the characterization of some new *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters and their application for the synthesis of the fragment AA_{111}–AA_{137} of the γ-chain of a human IgGl.

DISCUSSION

The *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl esters (Table I) were prepared from the corresponding free acids using the general procedure outlined in the *Experimental* section. These derivatives were used to synthesize a fragment of the γ-chain of a human IgGl comprised of 27 amino acids (I and II).

The fully protected, resin-bound peptide I was synthesized by the stepwise addition of the appropriate *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl ester to an insoluble polystyrene resin (19). The cycle for the addition of each amino acid residue consisted of the following steps: removal of the *N-tert*-butyloxycarbonyl protecting groups by treatment with excess anhydrous trifluoroacetic acid in methylene chloride, neutralization of the resulting salt with triethylamine in methylene chloride, and then double coupling the resulting free amino residue with three equivalents of the appropriate *N-tert*-butyloxycarbonyl-L-amino acid pentachlorophenyl ester in dimethylformamide for 4 hr., after which a further three equivalents of the same amino acid derivative was added and coupled for a further 4 hr. Where sterically hindered amino acid residues such as valine and leucine were attached to the resin, coupling times were increased to 12-hr. periods. At the end of the chain-lengthening sequence, the protected peptide was cleaved from its polymer support by liquid hydrogen fluoride (20, 21); all of the side-chain protecting groups were removed under these conditions. The peptide was purified by passage through two columns[2].

EXPERIMENTAL

General Procedure for Preparation of *N-tert*-Butyloxycarbonyl-L-Amino Acid Pentachlorophenyl Esters—The general procedure of preparation of these esters is illustrated by the preparation of *N-tert*-butyloxycarbonyl-β-benzyl-L-aspartic acid pentachlorophenyl ester. To a solution of 9.7 g. (30 mmoles) of *N-tert*-butyloxycarbonyl-β-benzyl-L-aspartic acid (22, 23) and 8 g. (30 mmoles) of pentachlorophenol in 100 ml. of methylene chloride, cooled to 0°, was added 6.8 g. (33 mmoles) of *N,N'*-dicyclohexylcarbodiimide. The mixture was stirred overnight at room temperature. The solid *N,N'*-dicyclohexylurea was filtered off, and the filtrate was evaporated to dryness under reduced pressure. The solid was dissolved in ethyl acetate and cooled, and a further crop of *N,N'*-dicyclohexylurea was removed by filtration. The filtrate was evaporated to dryness and the solid material was crystallized from methanol to yield the pentachlorophenyl activated ester, 13.0 g, 75%, m.p. 129–130°; $[\alpha]_D$ − 28.9° (c 2.6, dimethylformamide).

Anal.—Calc. for $C_{15}H_{15}Cl_5N_2O_5$: C, 46.2; H, 3.5; N, 2.45. Found: C, 46.0; H, 3.4; N, 2.5.

BOC-Gly-Leu-Val-O-Bzl-Thr-Val-O-Bzl-Ser-O-Bzl-Ser-Ala-O-Bzl-Ser-O-Bzl-Thr-ε-N-Z-Lys-Gly-Pro-O-Bzl-Ser-Val-Phe-Pro-Leu-Ala-Pro-O-Bzl-Ser-O-Bzl-Ser-ε-N-Z-Lys-O-Bzl-Ser-O-Bzl-Thr-O-Bzl-Ser-Gly-Resin (I)—*N-tert*-Butyloxycarbonyl-Gly resin (2 g.), containing 0.56 mmole of Gly, was prepared from Bio-Beads S-X2, 200–400 mesh, by standard procedures (24). This substituted resin was treated for the incorporation of each amino acid by the following steps:

BOC-Gly-Leu-Val-Thr-Val-Ser-Ser-Ala-Ser-Thr-Lys-Gly-Pro-Ser-Val-Phe-Pro-Leu-Ala-Pro-Ser-Ser-Lys-Ser-Thr-Ser-Gly-Polymer

Bzl Bzl Bzl Bzl Bzl Z Bzl Bzl Bzl Z Bzl Bzl Bzl

I

H-Gly-Leu-Val-Thr-Val-Ser-Ser-Ala-Ser-Thr-Lys-Gly-Pro-Ser-Val-Phe-Pro-Leu-Ala-Pro-Ser-Ser-Lys-Ser-Thr-Ser-Gly-OH

II

[1] Nomenclature recommended by the International Union of Immunological Societies (1–3).

[2] First Dowex 1-X4 and then Sephadex G-25.

Table I—*N-tert*-Butyloxycarbonyl-L-amino Acid Pentachlorophenyl Esters

Compound	Yield, %	Melting Point	Molecular Formula	Analysis, % Calc.	Found	$[\alpha]_D^{25}$
BOC[a]-β-Bzl-Asp	70	130°	$C_{22}H_{20}Cl_5NO_6$	C 46.2 H 3.5 N 2.45	46.0 3.4 2.5	−28.9°(c 2.6, DMF[b])
BOC-Asn	63	176°	$C_{15}H_{15}Cl_5N_2O_5$	C 37.25 H 3.75 N 5.8	37.1 3.7 5.7	−41.7°(c 2.1, DMF)
BOC-S-Bzl-Cys	81	154°	$C_{21}H_{20}Cl_5NO_4S$	C 45.1 H 3.6 N 2.5	45.1 3.6 2.6	−38.2°(c 1.7, DMF)
BOC-γ-OBzl-Glu	80	138°	$C_{23}H_{22}Cl_5NO_6$	C 47.1 H 3.95 N 2.4	47.0 3.9 2.4	−27.1°(c 3.0, DMF)
BOC-Gln	57	186°	$C_{16}H_{17}Cl_5N_2O_5$	C 38.9 H 3.5 N 5.7	39.0 3.7 5.6	−24.4°(c 2.25, DMF)
BOC-N^{im}-DNP-His	51	170°	$C_{23}H_{18}Cl_5N_5O_8$	C 41.2 H 2.7 N 10.5	41.4 2.9 10.6	+3.9°(c 2.7, DMF)
BOC-Met	90	139°	$C_{16}H_{18}Cl_5NO_4S$	C 38.6 H 3.6 N 2.8	38.5 3.6 2.9	−37.0°(c 2.3, DMF)
BOC-Pro	93	117°	$C_{16}H_{16}Cl_5NO_4$	C 41.45 H 3.5 N 3.0	41.2 3.6 3.1	−32.5°(c 3.9, DMF)
BOC-Ser	39	157°	$C_{14}H_{14}Cl_5NO_5$	C 37.05 H 3.1 N 3.1	37.2 3.0 3.2	−31.0°(c 1.85, DMF)
BOC-O-Bzl-Ser	63	76°	$C_{21}H_{20}Cl_5NO_5$	C 46.4 H 3.7 N 2.6	46.5 3.6 2.8	−13.4°(c 2.25, DMF)
BOC-Thr	41	196°	$C_{15}H_{16}Cl_5NO_5$	C 38.5 H 3.45 N 3.0	38.3 3.6 3.0	−29.0°(c 2.35, DMF)
BOC-O-Bzl-Thr	65	126°	$C_{22}H_{22}Cl_5NO_5$	C 47.4 H 4.0 N 2.5	47.2 4.1 2.6	−16.0°(c 2.35, DMF)
BOC-Trp	70	195°	$C_{23}H_{19}Cl_5N_2O_4$	C 47.8 H 3.5 N 5.1	47.9 3.4 5.3	−49.2°(c 1.65, DMF)

[a] BOC = *N-tert*-butyloxycarbonyl. [b] DMF = dimethylformamide.

1. Three washings with 20-ml. portions of methylene chloride.
2. Cleavage of the *tert*-butyloxycarbonyl group by the addition of 20 ml. of 20% trifluoroacetic acid in methylene chloride and shaking of the mixture for 30 min.
3. Six washings with 20-ml. portions of methylene chloride.
4. Neutralization of the trifluoroacetate salt with 15 ml. of a 10% solution of triethylamine in methylene chloride for 10 min.
5. Three washings with 20-ml. portions of methylene chloride.
6. Addition of three equivalents of *N-tert*-butyloxycarbonyl-amino acid pentachlorophenyl ester in 15 ml. of dimethylformamide–methylene chloride (1:1) and shaking for 4 hr., and then a further three equivalents of the same *N-tert*-butyloxycarbonyl-amino acid pentachlorophenyl ester in 15 ml. of dimethylformamide–methylene chloride (1:1) and shaking for a further 4 hr. Wherever *N-tert*-butyloxycarbonyl-L-valine pentachlorophenyl ester and *N-tert*-butyloxycarbonyl-L-leucine pentachlorophenyl ester were used, the coupling time of 12 hr. for each stage was used.
7. Three washings with 20 ml. of dimethylformamide.
8. Three washings with 20 ml. methylene chloride.

After 26 cycles, the resin was dried under vacuum to yield 3.3 g.

Gly-Leu-Val-Thr-Val-Ser-Ser-Ala-Ser-Thr-Lys-Gly-Pro-Ser-Val-Phe-Pro-Leu-Ala-Pro-Ser-Ser-Lys-Ser-Thr-Ser-Gly (II)—The above protected peptide resin was suspended in 30 ml. of redistilled anhydrous hydrogen fluoride containing 5 ml. of anisole. The resin was stirred for 1 hr. at 0°, after which the anhydrous hydrogen fluoride and anisole were removed under reduced pressure. The resin mixture was washed with two washes of 10 ml. of ethyl acetate. The resin was dried and the peptide was extracted into 50 ml. of 0.1 *M* acetic acid and lyophilized. The lyophilized crude peptide (0.4 g., 31%) was dissolved in 0.1 *M* acetic acid, placed on a column[3] (6.5 × 121 cm.), and eluted with pyridine–acetic acid–water solution, pH 8.0.

The lyophilized crude peptide was purified by chromatography on a column[4] (2.5 × 100 cm.) using pyridine–acetic acid–water solution, pH 6.1, as eluent. The major peak was collected and lyophilized to yield 103 mg. (8%, based on the starting substituted resin) of the free peptide. Amino acid analysis of an acid hydrolysate showed Ser, 7.8; Thr, 2.8; Gly, 3.1; Pro, 2.9; Val, 2.8; Leu, 2.0; Ala, 2.0; Phe, 0.9; and Lys, 2.0. The peptide II was found to be homogeneous by paper chromatography: R_f 0.67 (butanol–acetic acid–water–pyridine, 15:3:12:10); R_f 0.54 (pyridine–acetic acid–water, 5:3:1.5).

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[3] Dowex 1-X4.

[4] Sephadex G-25.

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Subcellular Binding of Halothane-1-^{14}C in Mouse Liver and Brain

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Abstract □ The extent of binding of halothane-1-^{14}C to mouse liver and brain 24 hr. after injection of a subanesthetic dose was measured utilizing several techniques: (*a*) exhaustive dialysis with phosphate buffer, (*b*) solvent extraction with 0.25 *M* sucrose and toluene, (*c*) protein precipitation with trichloroacetic acid, and (*d*) soxhlet extraction with methanol and benzene. A substantial portion of the radioactivity was not removed by these procedures and thus was bound both in liver and brain, the liver containing a greater amount of bound radioactivity on both an absolute basis and a protein content basis. The liver microsomal fraction, subjected to exhaustive dialysis, contained a higher specific activity (disintegrations per minute per milligram protein) than the whole homogenate, 9000×*g* pellet, or cytosol. The combination of trichloroacetic acid precipitation and soxhlet extraction with methanol and benzene (1:1) removed virtually all radioactivity from the brain, whereas 13% of the initial radioactivity remained in the liver as tightly bound material. Thus, halothane or a metabolite is tightly and probably covalently bound to a protein or other high molecular weight component in mouse liver but not in the brain.

Keyphrases □ Halothane, radiolabeled—*toxicity*, subcellular binding in mouse liver and brain □ Toxicity, halothane—subcellular binding in mouse liver and brain, radiolabeled, type of bonding □ Hepatitis—subcellular binding of halothane-1-^{14}C to mouse liver and brain, type of bonding, distribution of radioactivity

Halothane (1,1,1-trifluoro-2-bromochloroethane) possesses two properties of the ideal anesthetic: it is potent and nonexplosive. However, fatal hepatic necrosis has been reported in a small portion of exposed human subjects (1–4). While the incidence of hepatitis after halothane reportedly represents no greater a risk than surgical anesthesia itself (5), it is nevertheless a well-established untoward reaction that deserves investigation. The recurrence of hepatitis in sensitized anesthetists after exposure to only traces of halothane (6, 7) and the overall low incidence which increases with multiple exposures (5) suggest that halothane-induced hepatic damage is the result of an allergic reaction.

If halothane hepatitis is caused by a sensitization reaction, then halothane or a metabolite must form a hapten of an antigen by binding covalently to a macromolecule (8). Nonvolatile metabolites of halothane have been found to persist in animal liver (9) as well as human urine (10, 11). The presence of high molecular weight metabolites of halothane in liver and urine (12) indicates that a metabolite of halothane may be associated with a macromolecule; however, no direct evidence exists as to whether these metabolites are, in fact, covalently bound. In support of this suggestion, other halogenated hydrocarbons, such as carbon tetrachloride (13) and bromobenzene (14), do form covalent bonds with endogenous substances. Therefore, the binding character of halothane-1-^{14}C *in vivo* in mice was investigated.

EXPERIMENTAL

Dosage and Tissue Preparation—Female Swiss Webster albino mice[1] (28–30 g.) were fasted for 24 hr., and 0.5 mg. of halothane-1-^{14}C in saline[2], containing 4 μc. of radioactivity was administered by intraperitoneal injection. Twenty-four hours later, the mice were sacrificed, the livers were perfused with cold 0.25 *M* sucrose, and both the livers and brains were removed and placed in ice-cold 0.25 *M* sucrose. Ten percent homogenates of these organs were prepared in cold 0.25 *M* sucrose, using a Potter–Elvehjem tissue grinder[3]. An aliquot of these homogenates was removed for analysis of bound radioactivity and protein, and the remainder was centrifuged at 9000×*g* (average) in a refrigerated centrifuge[4] for 20 min. The pellet was removed for analysis, and the supernate was recentrifuged at

[1] Flow Research Laboratories, Dublin, Va.
[2] New England Nuclear, Boston, Mass.
[3] No. 62400, VWR Scientific Co., Baltimore, Md.
[4] Model HR-1, International Equipment Co., Boston, Mass.