Synthesis of N-[2-^{3}H] acetyl-D-muramyl-L-alanyl-D-iso-glutaminyl-L-alanyl-2-(1',2'-dipalmitoyl-sn-glycero-3'-phosphoryl) ethylamide of high specific radioactivity

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SUMMARY

The title compound (MTP-cephalin) was synthesised with specific activity of 22 Ci mmol^{-1} and with radiochemical purity of 96-98% by a direct radiochemical synthesis using [^{3}H] muramyl dipeptide. Several unsuccessful approaches to the target compound are documented.

Key Words: MTP-cephalin, CGP 19835, immune modulator

INTRODUCTION

MTP-cephalin (CGP 19835) is an experimental compound of CIBA-GEIGY Ltd., Basle, Switzerland. It shows a variety of interesting biological response-modifying activities (1,2) and increases non-specific resistance in murine candidiasis (3). It also has prophylactic and therapeutic activity in several experimental virus infections (4) and in liposomes activates tumoricidal human blood monocytes (5). Material labelled to a high level with tritium was required for studies of metabolism and distribution. As highly-tritiated muramyl dipeptide was already available (6), it appeared reasonable to attempt synthesis directly from this by coupling to alanyl-cephalin.

RESULTS AND DISCUSSION

A cold synthesis, which was later repeated successfully with low specific activity (20 mCi mmol^{-1}) [^{3}H] muramyl dipeptide

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([^{3}H] MDP), was developed. This involved activation of the γ-carboxyl group of the isoglutamine residue of MDP as the N-hydroxysuccinimide (-OSu) ester and subsequent coupling to alanyl-cephalin (Scheme). The coupling to produce MTP-cephalin was complete in 15 min and yielded 10% of product. The solvent used to dissolve the alanyl-cephalin for reaction was chloroform: methanol (65:25, by vol.). The material has been used in studies of the incorporation of MTP-cephalin into liposomes (7).

When the synthesis of MTP-cephalin was repeated using [^{3}H] MDP of high specific activity (15-20 Ci mmol^{-1}) (6), a yield of 1% was obtained after a coupling time of 15 min. Using a coupling time of 30 min, no product was recoverable. These observations suggested that radiation-induced decomposition was taking place and it seemed reasonable to assume that chlorine radicals were giving rise to destruction of the product, possibly by oxidation of the hemi-acetal or even by more extensive oxidation of the sugar residue. If this hypothesis was correct, avoidance of chlorinated hydrocarbons in the reaction solvent should allow synthesis of MTP-cephalin without concomitant radiolytic decomposition. Alanyl-cephalin could only be solubilised sufficiently in warm alcohols. The final conditions chosen employed tert.-butanol at a temperature of 50°C. Model experiments suggested reaction was complete within a few minutes.

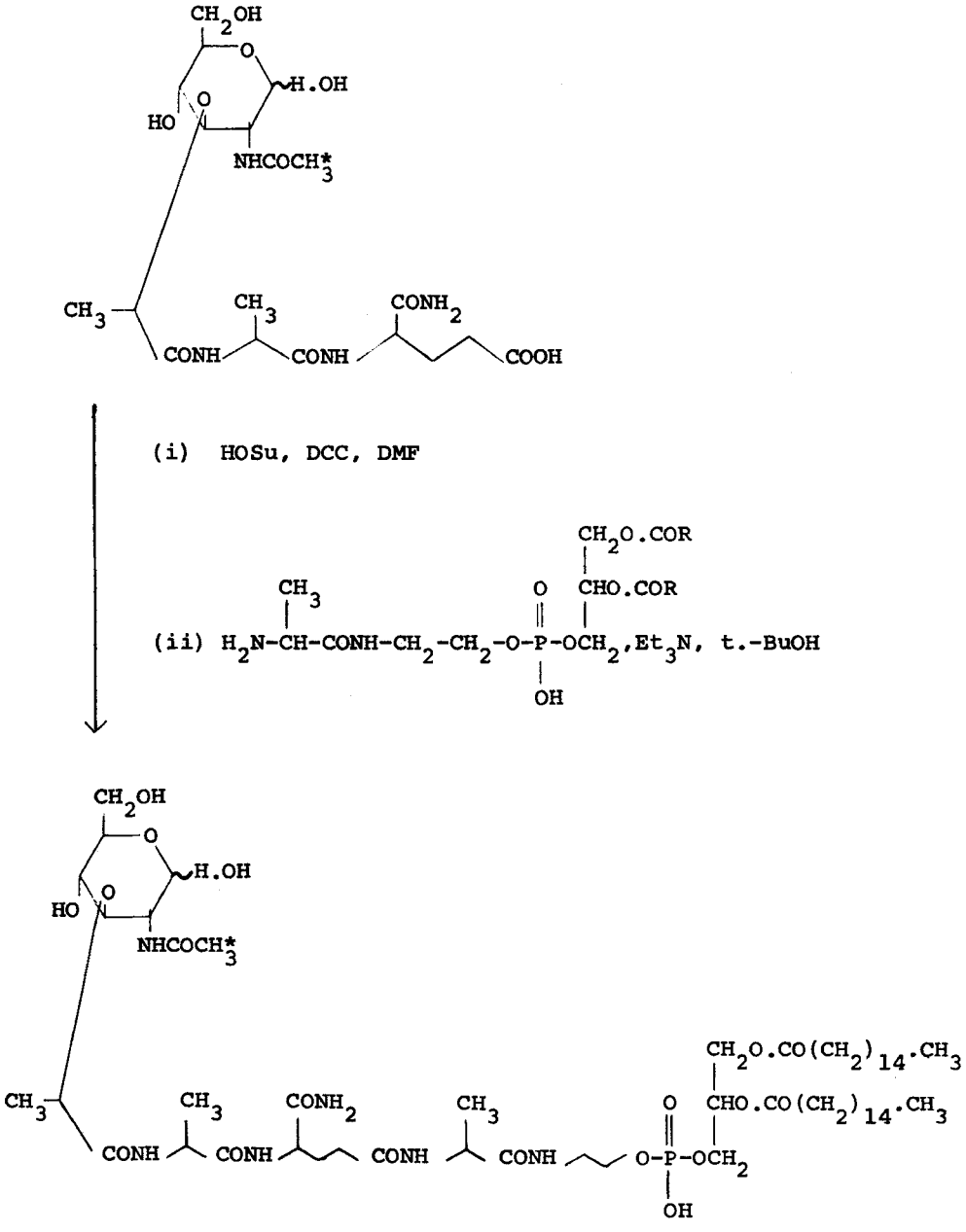
Purification was attempted by preparative high pressure liquid chromatography (h.p.l.c.) but was found to be irreproducible and to give poor recoveries. A two-stage chromatographic procedure was developed. The first step was open column silica chromatography which separated unreacted alanyl-cephalin from the product. As chloroform was a component of the elution solvent, eluted fractions were immediately diluted with ethanol to inhibit radical-induced oxidation. Final purification was by ion-exchange chromatography and the product was obtained in sodium phosphate buffer pH 7.0.

Some other routes to the product were also attempted but were not successful and these are documented here without experimental detail for the purposes of information.

1. The N-iodoacetyl analogue of MTP-cephalin was synthesised. It could not be catalytically reduced to MTP-cephalin probably due to catalyst poisoning by phosphorus. In any case, the great majority of catalysts investigated adsorbed MTP-cephalin irreversibly. This also precluded attempts to exchange-label the molecule.

SCHEME

Direct radiochemical synthesis of [^{3}H] MTP-cephalin



2. The N-tert.-butoxycarbonyl analogue of MTP-cephalin was synthesised. From it, by acid treatment, was obtained 'desacetyl MTP-cephalin'. This afforded product with a specific activity of 2-3 Ci $mmol^{-1}$ on acetylation with commercially available [^{3}H]acetic anhydride (from Amersham International Plc). This material was not sufficiently active for the uses envisaged.

EXPERIMENTAL

Materials

Phosphatidyl ethanolamine (synthetic L-dipalmitate) (cephalin) was purchased from Fluka AG, Switzerland.

Spray Reagents

Reagent A: Ninhydrin spray

Reagent B: Spray a) with H_2SO_4:2.5% ammonium molybdate: 0.85% NaCl = 1:6:5 (by vol.). Heat for 90 sec at 100°.

Reagent C: Chlorine-Starch/KI Spray

Solvent System

A: $CHCl_3$:MeOH:H_2O (65:25:4, by vol.).

N-Benzyloxycarbonyl-L-alanyl-2-(1',2'-dipalmitoyl-sn-glycero-3'-phosphoryl)ethylamide (1)

Benzyloxycarbonyl-alanine (186 mg, 0.98 mmol) and N-hydroxysuccinimide (HOSu) (109 mg, 0.95 mmol) were dissolved in N,N-dimethylformamide (DMF) (2.8 ml) and the solution was packed in ice-salt. Dicyclohexylcarbodiimide (DCC) (0.196 g, 0.95 mmol) was added and the solution was stirred for 5 h during which time the mixture was allowed to come to room temperature. The mixture was added during 5 min to a solution at room temperature of cephalin (0.465 g, 0.67 mmol) and Et_3N (0.28 ml, 2.0 mmol) in $CHCl_3$:MeOH = 10:4 (by vol.) (14 ml). The mixture was stirred for 16 h and examined by thin layer chromatography (t.l.c.) in the solvent system A using reagents B and C. No free cephalin could be detected in the mixture.

The mixture was evaporated and the residue was pumped over conc. H_2SO_4 for 2 h. The residue was dissolved in 3% (by vol.) methanol in chloroform (50 ml) and applied to a column (30 x 3 cm) of silica which was eluted with portions (250 ml) of methanol (3%, 4%, 5%, 6% and 7%, by vol.) in chloroform. The pure product was

detected after t.l.c. in system A using reagent B in the 5% and 6% eluates while the 7% eluate contained impure product, but no cephalin. The combined 5% and 6% eluates afforded, by evaporation of solvent, 0.34 g of chromatographically-pure product. (Note: Cephalin eluted from a similar silica column on elution with 7% methanol in chloroform).

L-Alanyl-2-(1',2'-dipalmitoyl-sn-glycero-3'-phosphoryl) ethylamide (2)

Compound 1 (0.34 g) was dissolved in a mixture of $CHCl_3$ (10 ml) and EtOH (20 ml) and hydrogenated for 90 min at room temperature in the presence of a mixture of 10% palladium on charcoal (300 mg) and platinum oxide (300 mg). Examination of the mixture (t.l.c., reagent B) showed the reaction was complete. The catalysts were removed by filtration and the recovered product (0.29 g) after evaporation to dryness and trituration to a solid under petroleum ether (b.p. 60-80°) was stored at -20°. The ratio alanine:ethanolamine = 1.00:0.99 was obtained by amino acid analysis. Less than 0.02 molar proportion of ammonia was present.

[^{3}H] MTP-cephalin (3)

[^{3}H] MDP was synthesised on a scale that yielded ~8 µmoles (~140 mCi) of material (6). The compound was stored in water (20 ml) in liquid nitrogen while analysis (radiochemical purity by t.l.c., amino acid analysis) was carried out.

After receipt of a satisfactory analysis report, the solution was evaporated to dryness and DMF (2 ml) was distilled from the residue to remove traces of acetic acid. The residue was transferred to a small tube (5 ml) using several small portions (0.25 ml) of DMF, dried and redissolved in DMF (0.28 ml) containing HOSu (1.08 mg, 1.2 mol. equiv.) and DCC (2.56 mg, 1.5 mol. equiv.). The mixture was stirred at room temperature for 2 h. A solution of compound 2 (2.32 mg, 0.4 mol. equiv.) and Et_3N (1.6 µl, 1.2 mol. equiv.) in t-butanol (0.75 ml) at 50° was added and the mixture was stirred at 50° for 10 min.

The solution was dried, the residue dissolved in $CHCl_3$:CH_3OH:H_2O, 70:30:5 (v/v) (1 ml) and the solution applied to a column (10 x 1.1 cm) of silica gel 60 (70-230 mesh) which was eluted with the same solvent. Fractions (10 x 1 ml then 6 x 5 ml) were collected and assayed for radioactivity and compound 2 (reagent A). Fractions 3-7 contained compound 2, fractions 11-14 contained tritiated material. During the analysis, collected fractions were

diluted with 4 volumes of ethanol to inhibit radical-induced oxidation.

Fractions 11-14 were combined and evaporated, the residue was dissolved in water (1 ml) and applied to a column (3.5 x 0.8 cm) of diethylaminoethylcellulose (phosphate form) which was eluted with a linear gradient (40 ml) of 0-0.5 M pH 7.0 sodium phosphate buffer, collecting fractions (1 ml) automatically. The product was present in fractions 22-28. These fractions were combined and the solution was stored for use at the temperature of liquid nitrogen (-196°C) at a concentration of 2 mCi ml^{-1}.

The product was analysed after acidic hydrolysis by scintillation counting and amino acid analysis. It had the amino acid ratio alanine:ethanolamine = 2.00:1.02 and specific activity of 22.0 $\pm$ 3.0 Ci $mmol^{-1}$. The yield was 0.63 µmoles (8%), 13.9 mCi. A sample of solution was examined after chromatography on a thin layer of silica gel G which was developed with solvent system A using a Panax E.0111/XPD-05 radiochromatogram scanner. It had a radiochemical purity of 98.0 $\pm$ 0.15% (without carrier), 98.4 $\pm$ 0.15% (with carrier, 1 mg ml^{-1}). A sample (20 µl) of solution with the addition of carrier (20 µg) was examined by h.p.l.c. on a column (25 x 0.46 cm) of Nucleosil $10C_{18}$ ODS-silica which was eluted at a flow rate of 2.8 ml min^{-1} with acetonitrile:water:acetic acid (700:300:0.1, by vol.). The radiochemical purity, as estimated by counting the column effluent, was 96.3 $\pm$ 0.1%.

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