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The Chemical Synthesis of Glucosaminylphosphatidylglycerol

COMPARISON WITH A NEW PHOSPHOLIPID ISOLATED FROM *BACILLUS MEGATERIUM*

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1. We describe the synthesis of a glucosamine derivative of phosphatidylglycerol having the same structure as that of the natural compound isolated from *Bacillus megaterium*. 2. 2-*O*-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-phthalimido-D-glucopyranosyl)-3-*O*-benzyl-1-iodo-*sn*-glycerol was prepared by a Königs–Knorr condensation between 3-*O*-benzyl-1-toluene-*p*-sulphonyl-*sn*-glycerol and 3,4,6-tri-*O*-acetyl-1-bromo-2-deoxy-2-phthalimido-D-glucopyranose followed by replacement of the toluene-*p*-sulphonyl group with iodine. The iodide was treated with the silver salt of 2-isolauroyl-1-oleoyl-*sn*-glycerol 3-(monobenzyl hydrogen phosphate) to form the fully protected phosphoglycolipid. 3. Removal of benzyl protecting groups by catalytic hydrogenolysis, phthaloyl group with hydrazine and acetyl groups with pH 10 buffer furnished 2-*O*-(2-amino-2-deoxy-D-glucopyranosyl)-1-(2-isolauroyl-1-stearoyl-*sn*-glycero-3-phosphoryl)-*sn*-glycerol. 4. The synthetic and natural compounds appeared identical when compared by chromatography and by identification of hydrolysis products from chemical and enzymic degradations.

The amino acid esters of phosphatidylglycerol are well substantiated compounds, and fairly detailed knowledge about their structure (Macfarlane, 1964*a*; Houtsmuller & van Deenen, 1963, 1965; Bonsen, de Haas & van Deenen, 1966), metabolism (Macfarlane, 1964*b*; Gale & Folkes, 1965; Houtsmuller & van Deenen, 1965; Op den Kamp, Houtsmuller & van Deenen, 1965), chemical synthesis (Baer & Jagannadha Rao, 1966; Bergelson & Molotkovsky, 1966; Bonsen, de Haas & van Deenen, 1965, 1967) and biosynthesis (Lennarz, Nesbitt & Reiss, 1966; Gould & Lennarz, 1967; Lennarz, Bonsen & van Deenen, 1967) has been accumulated over the past few years. As far as function is concerned, we are still very much at the speculative stage.

Recently, a new bacterial phospholipid, related to phosphatidylglycerol, was isolated from *Bacillus megaterium* (Op den Kamp *et al.* 1965) and its structure identified as 1-(1,2-diacyl-*sn*-glycero-3-phosphoryl)-2-*O*-(2-amino-2-deoxy-D-glucopyranosyl)-*sn*-glycerol (Op den Kamp & van Deenen, 1966; van Deenen, Op den Kamp & Bonsen, 1967) (see Fig. 1). A compound from *Pseudomonas ovalis*, which appeared to have a similar structure, was described at about the same time (Phizackerley, MacDougal & Francis, 1966). [The numbering system used in this paper is, for lipids, the suggested rules for the nomenclature of lipids of the IUPAC–IUB Commission on Biochemical Nomenclature (1967), and, for carbohydrates, the recommended rules of the Nomenclature Committee of the Division of Carbohydrate Chemistry of the American Chemical Society and the British Committee on Carbohydrate Nomenclature (1963)].

In order to confirm the structure of the phosphoglycolipid and to be able to study its properties in

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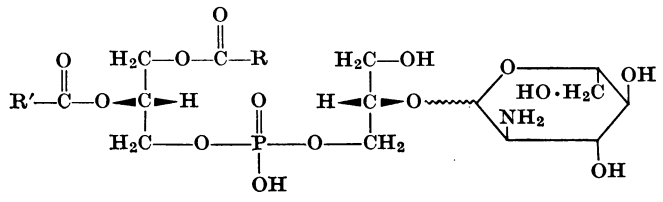
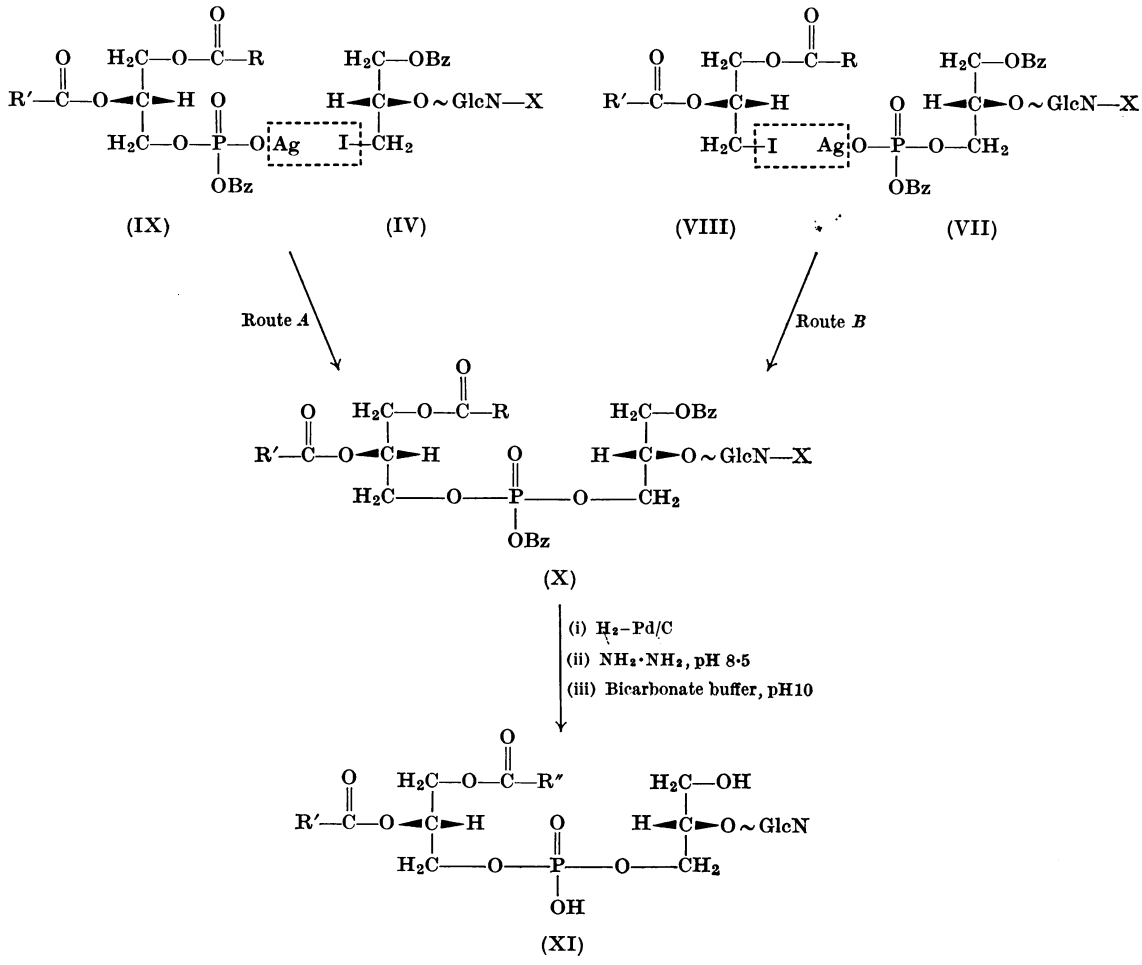


Fig. 1. Proposed structure of the glucosamine-containing lipid from *B. megaterium*.

detail, we undertook the total chemical synthesis. The basic structure of the molecule is similar to that of the amino acid esters of phosphatidylglycerol except that in this case there is a glycosidic linkage between the glycerol moiety and the amino sugar, in contrast with an ester bond in the amino acid derivatives. Initially there was some doubt about whether the glucosamine was linked to the 1- or to the 2-hydroxyl group of the glycerol moiety. Another early difficulty in understanding the structure of the bacterial lipid was the apparent alkali-lability of the glycosidic bond (Op den Kamp & van Deenen, 1966), a phenomenon difficult to reconcile with the normal acid-lability and alkali-stability of such bonds, though not completely unknown (Ballou, 1954). Synthesis of the water-soluble fragments 2-glucosaminylglycerol and 2-glucosaminylglycerol 1-phosphate (J. A. F. Op den Kamp, P. P. M. Bonsen & L. L. M. van Deenen, unpublished work) and of the complete phospholipid were useful in clarifying these problems.

EXPERIMENTAL APPROACH

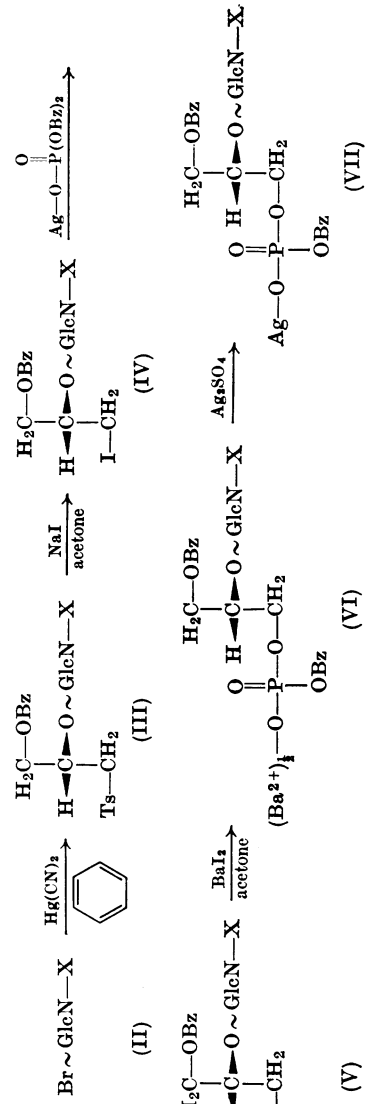
In view of the highly successful use made in this Laboratory of the condensation reaction between a silver salt and an iodo derivative for the synthesis of several classes of phospholipids, we have applied this principle in the present work. Once the choice of protecting groups had been decided, two possible



Scheme 1. Synthesis of 2-*O*-(2-amino-2-deoxy-D-glucopyranosyl)-1-(2-isolauroyl-1-stearoyl-*sn*-glycero-3-phosphoryl)-*sn*-glycerol (glucosaminylphosphatidylglycerol). Bz, benzyl; R, $[CH_2]_7 \cdot CH{:}CH \cdot [CH_2]_7 \cdot CH_3$ (oleoyl); R′, $[CH_2]_8 \cdot CH(CH_3)_2$ (isolauroyl); R″, $[CH_2]_{16} \cdot CH_3$ (stearoyl); ~GlcN, 2-amino-2-deoxy-D-glucopyranosyl (glucosaminyl); ~GlcN-X, 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-D-glucopyranosyl.

pathways immediately suggested themselves for the final condensation reaction and these are illustrated in Scheme 1. Route *A* involves the condensation of silver benzyl phosphatidate (IX) with 2-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-D-gluco-pyranosyl)-3-*O*-benzyl-1-iodo-*sn*-glycerol (IV). Route *B* involves the condensation of a diacyliodoglycerol (VIII) with the silver salt of 2-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-D-gluco-pyranosyl)-3-*O*-benzyl-1-monobenzylphosphoryl-*sn*-glycerol (VII). The synthesis of silver benzyl phosphatidate and of diacyliodoglycerols has previously been described (Bonsen *et al.* 1967; de Haas & van Deenen, 1961). The choice of the fatty acids was governed by two factors. First, the natural phospholipid contains a high proportion of branched-chain fatty acids, the exact proportion depending on the growth conditions. The remaining acids are mainly straight-chain saturated acids, with only about 5% or less of unsaturated acids. To imitate as closely as possible the structure of the natural compound, a branched-chain fatty acid was incorporated into the synthetic molecule. Though isolauric acid is not normally present in the natural lipid, it was the only branched-chain acid readily available in sufficient quantity. In synthetic phospholipids it is usually preferable to include one unsaturated fatty acid. Fully saturated phospholipids tend to have low solubilities in most organic solvents and are generally difficult to handle; introduction of an unsaturated acid eliminates this problem. However, unsaturated acids can only be employed if hydrogenation is not required for the removal of protecting groups. In the present synthesis, we had hoped to be able to use protecting groups that could be removed without the use of hydrogen, and accordingly oleic acid was employed. If, however, as turned out to be the case, it was eventually necessary to use hydrogen, the short chain length of the isolauric acid would help to offset the effect of stearic acid on the physical properties of the lipid. The choice of position at which these acids were esterified was governed solely by such practical considerations as the limited quantity of isolauric acid available.

The synthesis of the iodo compound (IV) and the silver salt (VII) are illustrated in Scheme 2. The preparation of the starting materials, compounds (I) (Howe & Malkin, 1951; Slotboom, de Haas & van Deenen, 1963) and (II) (Bergman & Zervas, 1931; Akiya & Osawa, 1957; Zervas & Konstas, 1960), are well documented in the literature. The method of synthesis of compound (I) was chosen so that the glycerol moiety carrying the glucosamine in the final product would, like that in the naturally occurring lipid, have the opposite configuration to that of the acylated glycerol. We used a Königs–Knorr type of reaction for the formation of the fully



Scheme 2. Synthesis of the silver salt of 2-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-D-glucopyranosyl)-3-*O*-benzyl-1-monobenzylphosphoryl-*sn*-glycerol.
Bz, benzyl; Ts, toluene-*p*-sulphonyl; ~GlcN-X, 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-D-glucopyranosyl.

protected glucosaminylglycerol (Hardy, 1965). The configuration of the glycosidic bond in products of the Königs–Knorr condensation seems to be in some doubt. Hardy (1965) claimed to have a mixture of α- and β-isomers in the ratio 0·39:1·00 when a secondary alcohol (1,3-di-*O*-benzylglycerol) was coupled with a glucosamine in which the amino group was protected by 4-methoxybenzylidene. When a diphenyloxyphosphinyl group was employed to protect the amino group, the product had 100% β-configuration. However, the condensing agent was silver carbonate in the former case and in the latter mercuric cyanide. No comment was made about the influence of the condensing agent on the configuration of the glycosidic bond. Akiya & Osawa (1957) claimed that, when the amino group was protected with a phthaloyl group and silver carbonate was used as a condensing agent, the product had exclusively the β-configuration. J. A. F. Op den Kamp, P. P. M. Bonsen & L. L. M. van Deenen (unpublished work) obtained synthetic 2-glucosaminylglycerol in the β-configuration by the method of Hardy (1965). In this case, the condensing agent was mercuric cyanide and the amino group was phthaloylated. The configuration of the glycosidic bond in the natural compound is still uncertain. In view of this uncertainty, we were not too concerned about the proportion of α- and β-components in our synthetic product, but assumed, in the light of the evidence of J. A. F. Op den Kamp, P. P. M. Bonsen & L. L. M. van Deenen (unpublished work), that the compound would most likely have predominantly the β-configuration. Because of this uncertainty, we have not specified the glycosidic configuration of any synthetic compounds mentioned in this paper.

Choice of protecting groups

Protecting groups that need strongly acidic conditions for their removal have to be avoided on account of the acid-labile glycosidic bond. Conversely, strongly or even moderately alkaline conditions would cause release of fatty acids in the final stages of the synthesis. Moreover, in an early examination of possible protecting groups, we rejected those that had to be removed by hydrogenolysis in order that oleic acid might be used.

Phosphate protection. Standard techniques used in this Laboratory employ the silver salt of a monobenzylphosphoryl compound. This benzyl group is easily removed from the resulting phosphotriester by the mild anionic debenzylation procedure if hydrogenolysis is not desirable.

Amino group protection. Several groups are available, but many suffer from lability or the need to employ strong acid or alkali for their removal. The phthaloyl group was finally chosen as being very stable but relatively easily removed by hydrazine under mildly alkaline or even neutral conditions.

Sugar hydroxyl group protection. One of the commonest and most convenient methods in carbohydrate chemistry is the use of the acetyl group. Initially we thought that the mildly alkaline reaction of hydrazine hydrate, employed for dephthaloylation, would be sufficient to deacetylate the sugar without release of fatty acids.

Glycerol hydroxyl group protection. The most convenient method available at the present time for fixing the second glycerol moiety in the correct configuration involves the use of a benzyl protecting group, which subsequently requires hydrogenolysis for its removal. It might be possible to avoid reducing the double bond of the oleic acid residue by removing the benzyl group directly after the Königs–Knorr condensation (see Scheme 2), leaving the 3-hydroxyl group free during the final condensation (Scheme 1). Though it is unlikely that the glycosidic bond would migrate from the 2- to the 1-position, as might be the case for an ester bond, this procedure was not adopted because it is probable that the proximity of the free hydroxyl group to the iodine atom would give rise to side reactions during the final condensation. Until more suitable groups for protecting primary hydroxyl groups can be found, which do not require hydrogenolysis for their removal, it seems that a lipid of this type can only be synthesized with saturated fatty acids.

RESULTS AND DISCUSSION

Synthesis

The yield of fully protected lipid from route *B* was grossly inferior to that from route *A*. This was largely due to the difficulties in handling and purifying the barium and silver salts, compounds (VI) and (VII). These compounds were poorly soluble in most solvents and it was difficult to free the silver salt from small traces of inorganic salts and water. In the coupling reaction of route *B* large quantities of diglyceride were therefore formed instead of the desired product. In most experiments involving the removal of protecting groups to form the final product the fully protected lipid synthesized via route *A* was therefore used.

In syntheses previously reported from this Laboratory, the condensation of a silver salt with an iodo derivative has usually required the temperature of boiling benzene, or in some cases of boiling toluene. In the present synthesis, owing to the bulky sugar moiety, only partial coupling occurred in boiling toluene after several hours, whereas in boiling xylene the reaction had proceeded to the extent of 90% in 30min.

The major difficulties in the entire synthesis arose during the removal of the protecting groups. The alkalinity of methanolic hydrazine (pH 8·5) was insufficient to remove all acetyl groups even when the compound had been completely dephthaloylated. In a control experiment, the minimum pH at which deacetylation of 1,3,4,6-tetra-*O*-acetylglucosamine was complete within reasonable time (24hr.) was 10·0. A control incubation of synthetic phosphatidylethanolamine demonstrated that no fatty acid was released under these conditions. The glucosamine-containing lipid, however, was rather more unstable and a small loss of fatty acids seemed to be inevitable under conditions for completely removing acetyl groups.

It proved to be more effective to use hydrogenolysis as the initial step in the deprotection sequence. If this step was employed after dephthaloylation, the reaction was often incomplete, even in an acidic medium, owing to poisoning of the catalyst by free amino groups. Hence the most suitable sequence was found to be: (i) hydrogenolysis to remove benzyl groups; (ii) hydrazine treatment to remove the phthaloyl group; (iii) treatment with a pH 10·0 buffer to complete the deacetylation.

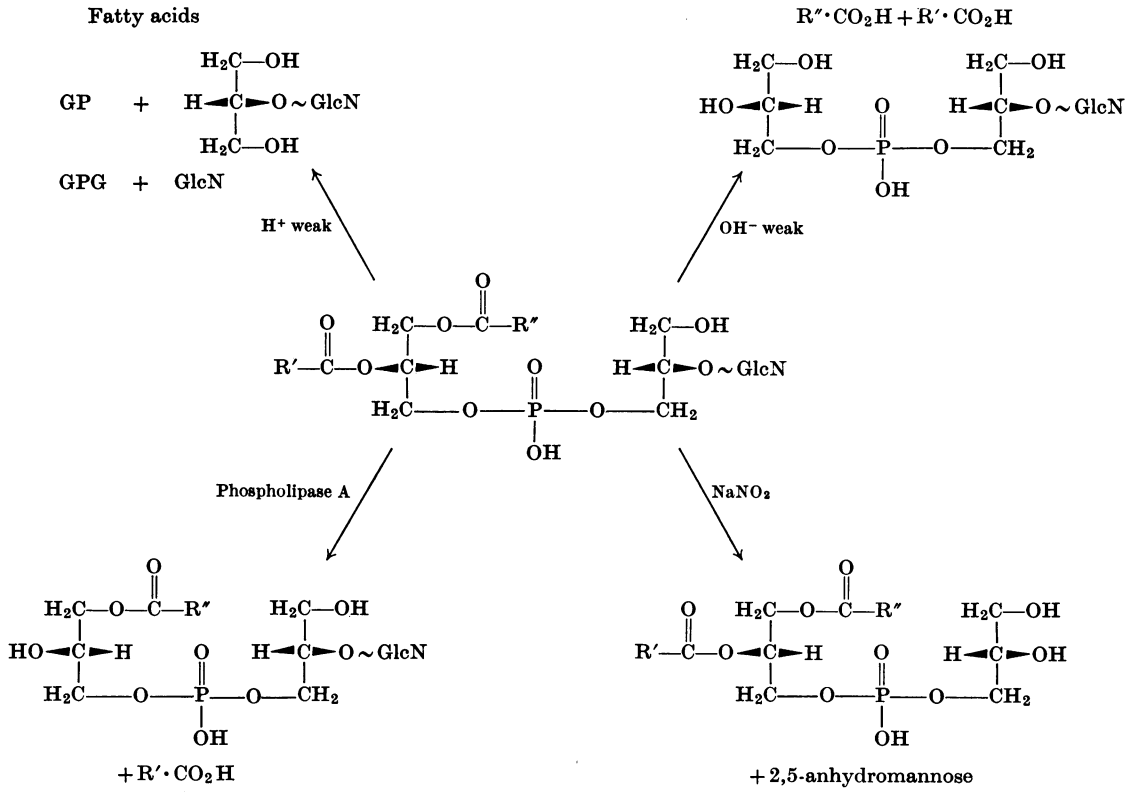
Comparison with the bacterial glucosaminylphosphatidylglycerol

Chromatography. The synthetic compound and the bacterial lipid both gave single spots with identical R_F values on silicic acid-impregnated paper in solvent 5, and on thin-layer chromatography in solvents 4, 5 and 6 (see the Materials and Methods section). Both compounds stained positively with the molybdate reagent (indicating a free phosphate ionization) and the ninhydrin reagent (indicating a free amino group) and yielded red spots with the tricomplex stain (see the Materials and Methods section). Neither compound produced a colour with the periodate–Schiff reagent, indicating the absence of vicinal hydroxyl groups (excluding the hydroxyl groups of the sugar ring, which react only very slowly with this reagent; there is a faint reaction after standing for 24hr.).

Chemical hydrolysis (*see Scheme* 3). The glycerol:phosphorus:glucosamine molar proportions, determined after very strong acid hydrolysis of the intact lipid, were 2·0:1·1:1·0 for the bacterial lipid (Op den Kamp & van Deenen, 1966) and 2·0:1·2:1·0 for the synthetic compound. The intact bacterial and synthetic lipids consumed 1·20 and 1·02 moles of periodate/g.atom of phosphorus respectively. (Under the same conditions bound glucosamine always consumed only 1 mole of periodate/mole of glucosamine; apparently the vicinal hydroxyl and amino groups are not attacked.) Insufficient material remained for a satisfactory elemental analysis to be done.

Hydrolysis with weak acid (N-hydrochloric acid at 120° for 4hr.) yielded glucosamine, 2-glucosaminylglycerol, 2-glucosaminylglycerol phosphate (trace amounts), glycerylphosphorylglycerol, glycerol phosphate and fatty acids. The proportions of the water-soluble products depended on the exact conditions; the milder the conditions, the higher the ratio of 2-glucosaminylglycerol to glucosamine. Stronger hydrolysis (4N-hydrochloric acid at 120° for 20hr.) yielded only glucosamine, glycerol phosphate, glycerol, fatty acids and some inorganic phosphate. Similar patterns were observed with the natural compound. The hydrolysis products, 2-glucosaminylglycerol and 2-glucosaminylglycerol phosphate, were identified by comparison with synthetic reference compounds (J. A. F. Op den Kamp, P. P. M. Bonsen & L. L. M. van Deenen, unpublished work). The synthetic isomers 1-glucosaminylglycerol and 2-glucosaminylglycerol could be distinguished from each other in a number of ways. On paper chromatograms they had different R_F values in butan-1-ol–ethanol–water–aq. ammonia (sp.gr. 0·88) (40:10:49:1, by vol.; upper phase). The 1-isomer reacted strongly with periodate–Schiff reagent on paper chromatograms and consumed 2 mol.prop. of periodate (i.e. 1 mol.prop. for the glycerol hydroxyl groups and 1 mol.prop. for the sugar ring hydroxyl groups), whereas the 2-isomer had no reaction with periodate–Schiff reagent and consumed only 1 mol.prop. of periodate (i.e. for the sugar ring hydroxyl groups only). Judged by these criteria, the hydrolysis product from both bacterial and synthetic lipids was 2-glucosaminylglycerol.

Mild alkaline hydrolysis by the procedure of Dawson (1960) yielded only one compound, identical from both bacterial and synthetic lipids, which stained positively with molybdate, ninhydrin and periodate–Schiff reagents. The R_F value in solvent 7 on paper chromatograms was similar to that of 2-glucosaminylglycerol phosphate, but it was distinguished from the latter compound by its strong reaction with periodate–Schiff reagent. The hydrolysis product from the synthetic lipid coincided exactly on paper chromatograms and electrophoresis with the single radioactive compound released by alkaline hydrolysis of a sample of the natural lipid isolated from bacteria grown in the presence of [^{32}P]orthophosphate. No synthetic reference compound is yet available for the final confirmation of this product, but the evidence strongly suggests that it is the expected deacylation product, glycerylphosphorylglycerylglucosamine. These observations necessitate a re-evaluation of the alkali-lability previously reported for the glucosamine–glycerol linkage in the natural product (Op den Kamp & van Deenen, 1966). If allowed to stand for several weeks in the refrigerator, even at



Scheme 3. Chemical and enzymic degradation of glucosaminylphosphatidylglycerol. GP, glycerol phosphate; GPG, glycerylphosphorylglycerol; GlcN, glucosamine.

neutral pH, both 2-glucosaminylglycerol phosphate and glycerylphosphorylglycerylglucosamine decomposed to give small amounts of glucosamine, glucosaminylglycerol, glycerol phosphate, inorganic phosphate and, in the latter case, glycerylphosphorylglycerol.

Deamination with sodium nitrite (*see Scheme* 3). Deamination of both synthetic and bacterial lipids with sodium nitrite cleaved the glycosidic bond and yielded phosphatidylglycerol, having identical R_F values with those of a synthetic reference compound on paper and thin-layer chromatography. The other deamination product appeared to be identical with 2,5-anhydromannose, prepared from D-glucosamine by deamination.

Enzymic hydrolysis (*see Scheme* 3). Incubation of the synthetic lipid with phospholipase A for 4–5 hr. gave complete degradation to lysoglucosaminylphosphatidylglycerol, and released almost exclusively (95–97%) isolauric acid. This confirmed the stereochemistry of the phosphatidyl moiety and the retention of isolauric acid at the 2-position throughout the synthesis. The lipid was a poor substrate for phospholipase C and the degree of hydrolysis was very variable from experiment to experiment. Degradation to diglyceride was confirmed, but the amount of water-soluble product was too small for a positive identification. Op den Kamp & van Deenen (1966) were able to demonstrate that the products obtained by phospholipase C degradation of the bacterial lipid were 1,2-diglyceride and 2-glucosaminylglycerol phosphate.

MATERIALS AND METHODS

Oleic acid was a product of The Hormel Institute (Austin, Minn., U.S.A.) and had a purity greater than 99%. Isolauric acid was prepared by the method of Hünig, Lücke & Benzing (1958) and had a purity greater than 99%. Fatty acid analyses were done with an F & M model 720 gas chromatograph (F & M Scientific Corp., Avondale, Pa., U.S.A.). The column material was polyethylene glycol adipate and the operating temperature 175°.

Micro-analyses were done by the Analytical Department of the Laboratory of Organic Chemistry (University of Groningen, The Netherlands). Melting points were determined on a Leitz Mikroscopheiztisch 350 and are un-

corrected. Optical rotations were measured in a Lichtelectrisches Präzisions polarimeter, with a limit of accuracy 0·005° (Carl Zeiss, Jena, Germany).

All solvents were dried and freshly distilled before use. Benzene and xylene (thiophen-free) were distilled first over P_2O_5, and then over $LiAlH_4$. Chloroform and ether were distilled over P_2O_5, and acetone was dried and distilled over anhydrous K_2CO_3.

Chromatographic methods. All analytical methods used in this work, with the exception of those dealing with carbohydrate chemistry, are referred to by Bonsen *et al.* (1965, 1967) and by Op den Kamp & van Deenen (1966).

The purity of intermediates and the end product was checked by thin-layer chromatography on microscope slides coated with silica and on silicic acid-impregnated paper. The solvent systems used were as follows and are designated by their numbers in the text: for thin-layer chromatography: solvent 1, ether–hexane (4:1, v/v); solvent 2, ether–hexane (9:1, v/v); solvent 3, methanol–benzene (1:9, v/v); solvent 4, chloroform–methanol–water (65:25:4, by vol.); solvent 5, di-isobutyl ketone–acetic acid–water (8:5:1, by vol.); solvent 6, chloroform – methanol – acetic acid – water (125:37:10:1, by vol.); for silicic acid-impregnated paper: solvent 5; for paper chromatography of water-soluble compounds: solvent 7, butan-1-ol–acetic acid–water (4:1:5, by vol.; upper phase); solvent 8, butan-1-ol–pyridine–water (6:4:3, by vol.).

Column chromatography was performed on Mallinckrodt silicic acid sieved to 60–140 mesh and activated at 120° for several hours before use. Alumina was a product (neutral, activity grade I) of M. Woelm (Eschwege, Germany) and was washed in chloroform before use.

Paper chromatography of hydrolysis products was done on Whatman no. 1 paper in solvent 7.

Electrophoresis of hydrolysis products was done on MN Chromatographie Papier nr 214 (Macherey, Nagel und Co., Düren, Germany) at 50 v/cm. for 30 min. in pyridine–acetic acid–water (1:10:89, by vol.), pH 3·6.

The deamination reaction with $NaNO_2$ was performed by the method of Baddiley, Buchanan, RajBhandary & Sanderson (1962). The products were separated on Whatman no. 1 paper in solvent 8.

Enzymic hydrolyses (Bonsen *et al.* 1967) were performed with (i) pancreatic phospholipase A (EC 3.1.1.4), a gift from Dr G. H. de Haas, or (ii) phospholipase C (EC 3.1.4.3) from *Bacillus cereus*. Identification methods used were (i) the ninhydrin reagent for free amino groups, (ii) the molybdate reagent for phosphate groups, (iii) the periodate–Schiff reagent for vicinal hydroxyl groups, (iv) charring with 30% (v/v) H_2SO_4 on thin-layer plates, (v) the tricomplex staining method for phosphatides on paper chromatograms (yielding red spots with phospholipids containing free amino groups and green spots with all other lipids) and (vi) the $AgNO_3$ dip (Trevelyan, Procter & Harrison, 1950) for sugars.

EXPERIMENTAL

The synthesis of intermediates (I), (II), (VIII) and (IX) are fully described in the literature, intermediate (I) by Howe & Malkin (1951) and Slotboom *et al.* (1963), intermediate (II) by Bergman & Zervas (1931), Akiya & Osawa (1957) and Zervas & Konstas (1960), and intermediates (VIII) and (IX) by de Haas & van Deenen (1961).

Synthesis of 2-O-(3,4,6-*tri*-O-*acetyl*-2-*deoxy*-2-*phthalimido*-D-*glucopyranosyl*)-3-O-*benzyl*-1-*toluene*-p-*sulphonyl*-sn-*glycerol* (*compound III, Scheme* 2)

The bromide, compound (II) (4·12g.; 18·2m-moles), was refluxed with compound (I) (3·35g.; 10m-moles; 20% excess) and 4·0g. of $Hg(CN)_2$ (15·8m-moles) in 50ml. of dry benzene, and the mixture was stirred magnetically. Analysis by thin-layer chromatography in solvent 2 demonstrated that the reaction was complete in 10min. After cooling, the residue was removed on a glass filter and washed with fresh benzene. The yellow solution was washed twice with an equal volume of 1% (w/v) NaCl and three times with water. After drying over $MgSO_4$, the solvent was removed *in vacuo* and the material purified by silicic acid chromatography. Excess of compound (I) was removed with 10% (v/v) ether in benzene and pure compound (III) was eluted in 1·5l. of 30% (v/v) ether in benzene. After drying *in vacuo* over P_2O_5 for 24hr. the yield of compound (III) was 5·34g. (86%, based on compound II). The product was glassy and had no real melting point (it softened gradually up to 50°, when it became completely transparent). It had $[\alpha]_D^{20}$ +21·6° (*c* 31 in chloroform). Thin-layer chromatography in solvent 2 revealed only one compound [Found: C, 58·5; H, 5·3; N, 1·7; S, 3·8. Calc. for $C_{37}H_{38}NO_{14}S$ (mol.wt. 752·8): C, 59·0; H, 5·0; N, 1·9; S, 4·3%].

Synthesis of 2-O-(3,4,6-*tri*-O-*acetyl*-2-*deoxy*-2-*phthalimido*-D-*glucopyranosyl*)-3-O-*benzyl*-1-*iodo*-sn-*glycerol* (*compound IV, Scheme* 2)

Compound (III) (5·02g.; 6·7m-moles) was refluxed in the dark for 48hr. with anhydrous NaI (4·05g., 27m-moles) in 100ml. of dry acetone. The course of the reaction was followed by thin-layer chromatography in solvent 2. After cooling, the precipitated sodium toluene-*p*-sulphonate was removed on a glass filter and the residue washed several times with dry acetone. The solvent was removed *in vacuo* and the residue dissolved in ether. The solution was washed once with an equal volume of 5% (w/v) $Na_2S_2O_3$ and once with water. After drying over anhydrous Na_2SO_4, the solvent was removed *in vacuo* and the material purified by silicic acid chromatography. The iodide, compound (IV), was eluted in 2l. of 10% (v/v) ether in benzene. After drying *in vacuo* over P_2O_5 for 36hr., the yield of compound (IV) was 3·63g. (77%, based on compound III). The iodide was kept in the dark throughout all these operations. It was glassy, with no proper melting point. It had $[\alpha]_D^{20}$ +11·6° (*c* 19·5 in chloroform). Thin-layer chromatography in solvent 2 revealed only one compound [Found: C, 50·9; H, 4·6; I, 17·7; N, 2·0. Calc. for $C_{30}H_{31}INO_{11}$ (mol.wt. 708·5): C, 50·9; H, 4·4; I, 17·9; N, 2·0%].

Synthesis of the fully protected lipid (*compound X, Scheme* 1) *by route* A

The iodide, compound (IV) (0·82g.; 11·6m-moles), was dissolved in 60ml. of dry xylene, and 1·04g. of silver monobenzyl phosphatidate, compound (IX) (12·8m-moles; 10% excess), was added. The mixture was refluxed (138°) in the dark with vigorous stirring for 1hr. AgI began to be precipitated after 10min., and after 30min. the reaction was

almost completed as judged by thin-layer chromatography in solvent 1. A trace of iodide remained after 1 hr., but also breakdown products began to appear. After cooling, the precipitated AgI was removed by centrifugation and the precipitate washed twice with fresh xylene. The pooled xylene extracts were evaporated to dryness *in vacuo*, the residue was dissolved in hexane and the solution was washed successively with equal volumes of (i) 5% (w/v) $NaHCO_3$, (ii) 1% (w/v) $Na_2S_2O_3$ and (iii) water until the pH was 6–7. The hexane solution was dried over Na_2SO_4, and the residue remaining after evaporation of the solvent was purified by chromatography on a column of silicic acid. The required product was eluted with 10% (v/v) ether in benzene. Small amounts of benzyl phosphatidic acid were present and could not be removed by silicic acid chromatography. This impurity was removed by passing the material, dissolved in chloroform, very rapidly through a very small column of alumina. Purified compound (X) was a colourless syrup, which consisted of one component when examined by thin-layer chromatography in solvents 1, 3, 4 and 5 and chromatography on silicic acid-impregnated paper in solvent 5. The yield was 1·30 g. (86%, based on compound IV). It had $[\alpha]_D^{20}$ +18·9° (*c* 12·6 in chloroform) [Found: C, 65·2; H, 7·8; N, 1·0; P, 2·1. Calc. for $C_{70}H_{99}NO_{19}P$ (mol.wt. 1289·7): C, 65·2; H, 7·7; N, 1·1; P, 2·4%].

Synthesis of the fully protected lipid (compound X, Scheme 1) by route B

2-O-(3,4,6-*Tri*-O-*acetyl*-2-*deoxy*-2-*phthalimido*-D-*glucopyranosyl*)-3-O-*benzyl*-1-*dibenzylphosphoryl*-sn-*glycerol* (*compound V, Scheme* 2). The iodo compound (IV) (3·38 g.; 4·8 m-moles) was dissolved in 75 ml. of dry xylene, and 2·5 g. of silver dibenzyl phosphate was added (6·5 m-moles; 35% excess). The suspension was stirred vigorously in the dark and brought to the boiling point of xylene (138°). About 10 ml. of xylene was distilled off to remove traces of water that might remain, and the mixture was refluxed for a further 1¼ hr. Thin-layer chromatography in solvent 2 demonstrated that maximum conversion had taken place in 20 min. and that beyond 1 hr. a breakdown product began to appear. Precipitated AgI was removed by centrifugation and washed again with fresh xylene. The solvent was removed to give 4·47 g. of an oil, which was purified by chromatography on a column of silicic acid. Pure compound (V) was eluted with 80% (v/v) ether in benzene and, after drying *in vacuo* over P_2O_5 for 24 hr., the yield was 3·19 g. (77% based on compound IV). The compound is glassy and has no proper melting point. Thin-layer chromatography in solvent 2 revealed only one compound. It had $[\alpha]_D^{20}$ +18·3° (*c* 14·7 in chloroform). [Found: C, 59·0; H, 5·2; N, 1·9; P, 3·0. Calc. for $C_{44}H_{45}NO_{15}P$ (mol.wt. 858·9): C, 61·5; H, 5·2; N, 1·6; P, 3·6%].

Barium salt of 2-O-(3,4,6-*tri*-O-*acetyl*-2-*deoxy*-2-*phthalimido*-D-*glucopyranosyl*)-3-O-*benzyl*-1-*monobenzylphosphoryl*-sn-*glycerol* (*compound VI, Scheme* 2). Compound (V) (2·88 g.; 3·38 m-moles) was dissolved in 100 ml. of dry acetone. Anhydrous BaI_2 (1·5 g.; 3·7 m-moles; 10% excess) was added and the solution refluxed in the dark with vigorous stirring for 2 hr. Thin-layer chromatography in solvent 4 demonstrated that the reaction was almost complete at this time. The acetone was removed *in vacuo*, and ether and water were added to the brown residue. Most of the benzyl iodide and all of the unchanged starting material were extracted into the ether layer, and most of the product was extracted into the water layer. Free I_2 was removed by shaking with 1% (w/v) $Na_2S_2O_3$, and the compound was then converted into the free acid with 0·1 N-HCl. The free acid was readily extracted into ether, and after the solvent had been removed the colourless residue was dissolved in 50 ml. of acetone–water (1:1, v/v). Excess of saturated $Ba(OH)_2$ was added, and the slightly turbid solution was stirred for several minutes. Excess of $Ba(OH)_2$ was removed by passing CO_2 through the solution and then centrifuging off the precipitate of $BaCO_3$. The supernatant was evaporated to dryness, acetone was added and the remaining insoluble material was centrifuged off. Removal of solvent and drying over P_2O_5 *in vacuo* for several days yielded 1·5 g. of compound (VI) (54% yield based on compound V). Thin-layer chromatography in solvent 4 revealed only one compound, m.p. 139–143°.

Silver salt of 2-O-(3,4,6-*tri*-O-*acetyl*-2-*deoxy*-2-*phthalimido*-D-*glucopyranosyl*)-3-O-*benzyl*-1-*monobenzylphosphoryl*-sn-*glycerol* (*compound VII, Scheme* 2). The barium salt, compound (VI) (1·47 g.; 1·75 m-moles), was dissolved in 50 ml. of acetone–water (1:1, v/v) and 275 mg. of Ag_2SO_4 in 50 ml. of water was added. After shaking for 20 min., the suspension was centrifuged and the clear supernatant was evaporated to dryness in the dark. The solid white material was dried for 5 days in the dark *in vacuo* over P_2O_5. The yield was 0·96 g. (62%, based on compound VI). Thin-layer chromatography in solvent 4 revealed only one compound [Found: C, 49·1; H, 4·4; Ag, 13·5; N, 1·8; P, 3·3. Calc. for $C_{37}H_{38}AgNO_{15}P$ (mol.wt. 875·6): C, 50·7; H, 4·3; Ag, 12·4; N, 1·6; P, 3·5%].

Fully protected lipid (*compound X, Scheme* 1). The silver salt, compound (VII) (0·62 g.; 0·71 m-mole; 10% excess), was suspended in 25 ml. of dry xylene and refluxed in the dark with diacyliodohydrin, compound (VIII) (0·42 g.; 0·65 m-mole), under exactly the same conditions as described for the corresponding reaction of route *A*. The crude material was extracted and purified as described above. The yield was 80 mg. (9·1%, based on compound V). Thin-layer chromatography in solvents 1, 3, 4 and 5 and chromatography on silicic acid-impregnated paper in solvent 5 demonstrated that the compound was pure and identical with the product of route *A* [Found: C, 64·3; H, 7·5; P, 2·2. Calc. for $C_{70}H_{99}NO_{19}P$ (mol.wt. 1289·7): C, 65·2; H, 7·7; N, 1·1; P, 2·4%].

Removal of the protecting groups (*Scheme* 1)

Catalytic hydrogenation. The fully protected lipid, compound (X) (486 mg.; 0·38 m-mole), was dissolved in 15 ml. of methanol containing a few drops of acetic acid (pH 4–5) and hydrogenated in the presence of 5% (w/w) palladium on charcoal as catalyst. When the uptake of H_2 gas was complete the catalyst was removed by centrifugation and the product was extracted into chloroform and washed several times to remove acid. The chloroform solution was dried over Na_2SO_4 and examined by chromatography. One compound was present at this stage.

Removal of the phthaloyl group. The hydrogenated material was dissolved in 20 ml. of methanol containing 330 mg. of hydrazine hydrate (15-fold excess) and shaken at 20° for 65 hr. After cooling in ice, the reaction mixture was acidified to pH 4 with HCl and kept for 1 hr. at 0°. The product was extracted, washed and dried as before, and

examined by chromatography. Up to eight compounds were detectable at this stage, each having a free amino group.

Removal of acetyl groups. The dephthaloylated product was dissolved in 2ml. of methanol, and 4ml. of ether and 8ml. of bicarbonate buffer, pH10, were added. The emulsion was shaken at 20–24° for 24hr. and then cooled in ice and acidified with HCl to pH4, and the product was extracted into chloroform. After the solution had been washed and dried, the solvent was removed and the material applied to a small silicic acid column. Elution was done with methanol–chloroform mixtures and the desired product was eluted with 15% (v/v) methanol in chloroform. The product isolated in this way contained small amounts of the lyso compound and small quantities were purified when required by preparative thin-layer chromatography in solvent 6. Though only giving one spot on chromatography the material purified by thin-layer chromatography was slightly coloured and contained some insoluble material. After dissolving in the minimum quantity of chloroform with a few drops of methanol added (the purified phosphoglycolipid was only partially soluble in pure chloroform), acetone was added gradually until the solution became turbid. Crystals, which formed after the solution had stood overnight at −20°, were separated off by centrifugation and dried *in vacuo* over P_2O_5 for 24hr. Thin-layer chromatography in solvents 4, 5 and 6 and chromatography on silicic acid-impregnated paper in solvent 5 revealed only one compound. The yield was 68mg. (21%, based on compound X). Decomposition began at 245° but the material had not melted at 310°.

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