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Note

Determination of the absolute configuration of the glycerol component in poly(glycosylglycerolphosphates) by GLC–MS *

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The identification of an increasing number of teichoic acid-like capsular polysaccharide structures containing glycerol phosphate linkages (see, for example, refs 1–14), has necessitated the development of accurate and versatile methods for the determination of the absolute configuration of the chiral glycerol components. Only in a few instances has the assignment of the stereochemistry of *O*-glycosyl glycerol, phosphorylated at one of the primary hydroxymethyl groups, within this type of polymer been reported[12–15]. On the other hand, the stereochemistry of the teichoic acids in the cell walls and membranes of gram-positive bacteria appears to be well established[16,17].

Assignment of the stereochemistry of the glycerol residue in poly(glycosylglycerol phosphates) may be achieved by release of the chiral glycerol component, followed by its conversion to an appropriate derivative, and assignment of absolute stereochemistry by comparison with standards of known chirality by means of spectroscopic (ORD, CD, or NMR), chromatographic or enzymic methods[18,19]. In the case of polymers joined by $\rightarrow$1)-glycerol-(3$\rightarrow$ phosphodiester linkages, depolymerization can be accomplished with aqueous HF to afford a 1-*O*-glycosylated glycerol fragment. Configurational analysis is then possible by comparison of the chromatographic properties with standards of known absolute configuration[20] or by stereospecific enzymic assay following oxidation to the corresponding chiral glyceric acid[21]. More recently a method based on the measurement of the chiroptical properties of 2,3-di-*O*-benzyl-*sn*-glycerol obtained from 1-*O*-glycosyl-*sn*-glycerols has been described[22].

When the teichoic acid contains 2-*O*-substituted glycerol phosphate linkages,

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the glycerol component is rendered achiral following HF treatment. For these polymers an alternative depolymerization strategy may involve periodate oxidation. The application of this approach has been reported[15,23], and the configuration of the glycerol phosphate released was determined by enzymic oxidation with *sn*-glycerol 3-phosphate dehydrogenase[24].

Recently, we reported a method for determination of the absolute chirality of 1-*O*-(α-D-galactopyranosyl)glycerol in which the configuration of the glycerol methine carbon centre is related to that of the D-sugar by a combination of ^{1}H NMR ^3J-values and NOE measurements[12]. We now describe a more general method for determination of absolute configuration (and also enantiomeric purity) based on the chromatographic separation of diastereomeric glycerol derivatives.

The basis of this approach involves reaction of a free primary hydroxyl group of chiral di-*O*-acyl- or di-*O*-alkyl-glycerols with an enantiomerically pure chiral derivatizing reagent, (1*S*,4*R*)-(−)-camphanic acid chloride, followed by GLC–MS analysis of the derived diastereomeric esters. Camphanic acid chloride is readily available and has proved useful as a chiral derivatizing reagent for the estimation of the enantiomeric purity of stereoselectively deuterated primary alcohols[25] and for the resolution of the D- and L-enantiomers of substituted *myo*-inositols[26]. In this study, it was expected that this reagent would not only provide the enantiomeric composition of chirally di-*O*-substituted glycerols by GLC, but also give diastereomeric derivatives suitable for possible future NMR studies.

Reference samples of the enantiomers of di-*O*-acetylglycerol were prepared from *sn*-glycerol 3-phosphate and its racemate. Attempted acylation with acetic anhydride in pyridine or in the presence of sodium acetate at elevated temperature (100–120°C), conditions routinely used for the preparation of alditol acetates, gave mixtures of acetylated α-, β-, and cyclic 5-membered glycerol phosphates as evidenced by GLC–MS of the corresponding *O*-trimethylsilyl-phosphate derivatives. However, when acetylation was carried out using less vigorous conditions, e.g., acetic anhydride in the presence of a catalytic amount of 1-methylimidazole at ambient temperature[27], the chiral integrity of the glycerol methine carbon centre was maintained to afford only 1,2-di-*O*-acetyl-*sn*-glycerol 3-phosphate. Dephosphorylation of *rac*-1,2-di-*O*-acetylglycerol 3-phosphate with aqueous HF afforded the racemic mixture of di-*O*-acetylglycerols. The (−)-camphanate ester derived from this mixture gave two GLC peaks (T_{GA} 1.31 and 1.34) of equal detector response that showed similar characteristic EI fragment ions in their MS at *m/z* 283 [M − CH_2OAc], 241 and 159. The camphanate esters obtained similarly from 1,2-di-*O*-acetyl-*sn*-glycerol 3-phosphate gave an enantiomeric excess (~ 73%) of the 1,2-di-*O*-acetyl-3-*O*-camphanyl-*sn*-glycerol which had a GLC retention time (T_{GA}) of 1.31. This latter result indicated that a significant amount of acetyl migration had occurred (ca. 30%) under the conditions employed. Moreover, GLC analysis of the trimethylsilyl derivatives of the acetylated products obtained by dephosphorylation of 1,2-di-*O*-acetyl-*sn*-glycerol 3-phosphate indicated a mixture of the 1,2- and 1,3-di-*O*-acetyl-*sn*-glycerols. Acid- or base-catalyzed migration of

acyl groups in partially acylated polhydric alcohols is well known[28], the direction of migration predominantly occurring from a secondary to a primary hydroxyl group. In the present context, the occurrence of acetyl migration during HF-mediated dephosphorylation of the acetylated glycerol phosphates led to a significant decrease in sensitivity of the configurational assay, limiting its applicability for determination of enantiomeric purity. We therefore investigated the applicability of a similar approach for configurational analysis involving the use of di-*O*-alkylglycerol derivatives. In the resulting diethers, alkyl migration would not be expected to be a problem.

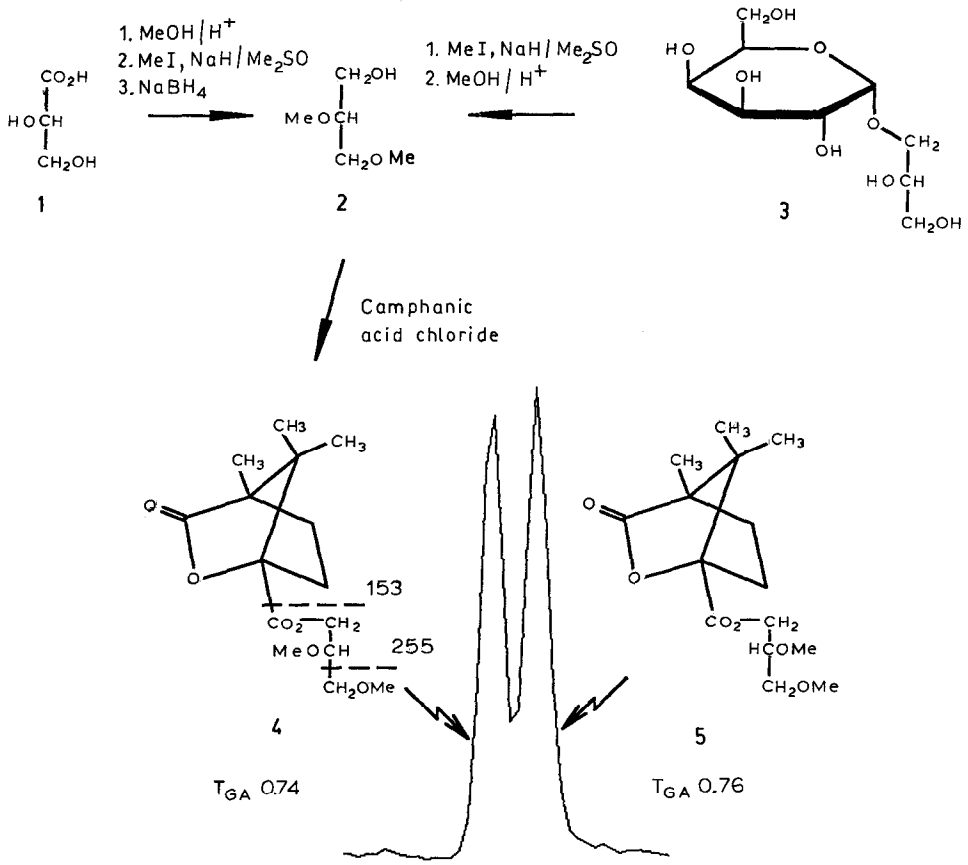
Samples of the two enantiomers of di-*O*-methyl glycerol were obtained from D-, L-, and DL-glyceric acid. Methyl glycerate was converted to the dimethyl ether by reaction with iodomethane in dimethyl sulfoxide containing sodium methylsulfinylmethanide, followed by reduction of the ester function with sodium borohydride to afford the corresponding glycerol derivative. Esterfication of the free hydroxy group of the di-*O*-methyl glycerol obtained from DL-glyceric acid with (−)-camphanic acid chloride afforded an equimolar mixture of diastereomeric camphanates that were separable by GLC (T_{GA} 0.74 and 0.76). The two diastereomers gave virtually identical mass spectra in which diagnostic EIMS fragment ions were observed at *m/z* 153 from the camphanoyl ring system and at *m/z* 255 arising from loss of the terminal methoxymethylene group (Scheme 1). The camphanate derivatives obtained from chirally pure L- and D-glyceric acid each gave rise to a single peak on GLC. In this way, the first eluting camphanate ester (**4**, T_{GA} 0.74) was identified as that derived from 2,3-di-*O*-methyl-*sn*-glycerol (**2**) by direct comparison with the sample obtained from L-glyceric acid (**1**). Analogously, by comparison with the sample from D-glyceric acid, the slower eluting ester (**5**, T_{GA} 0.76) was identified as that derived from 1,2-di-*O*-methyl-*sn*-glycerol.

Application of this method to the determination of the absolute stereochemistry of 1-*O*-(α-D-galactopyranosyl) glycerol (**3**) obtained by aqueous HF treatment of the capsular antigen of *Pasteurella haemolytica* serotype T3 was in complete agreement with evidence from ^{1}H NMR analysis[12]. Permethylation of this glycerol-containing saccharide (**3**), followed by cleavage of the glycosidic linkage with methanolic HCl, afforded exclusively 2,3-di-*O*-methyl-*sn*-glycerol (**2**), as evidenced by comparison of the corresponding (−)-camphanate ester (**4**) with that obtained from L-glyceric acid (Scheme 1).

This approach has also been successfully applied to the determination of the absolute configurations of the bridging *sn*-glycerol 3-phosphate units present in the extracellular *O*-(glycosyl) glycerol containing teichoic acid from *Actinobacillus pleuropneumoniae* serotype 7 (ref 13).

EXPERIMENTAL

Analytical methods.—GLC employed a Hewlett–Packard 5710A gas chromatograph fitted with a hydrogen flame-ionization detector, a model 3380A electronic



Scheme 1. Conversion of L-glyceric acid (**1**) and 1-*O*-(α-D-galactopyranosyl)glycerol (**3**) into 1-*O*-camphanoyl-2,3-di-*O*-methyl-*sn*-glycerol (**4**). Characteristic EIMS fragments and GLC retention times (T_{GA}) of the two diasteriomeric glycerol camphanate derivatives (**4** and **5**, respectively) are indicated. The GLC trace obtained from *rac*-glycerol camphanate esters is shown in the inset.

integrator, and a fused-silica capillary column (0.3 mm × 25 m) containing 3% OV-17, with the temperature program 180°C (for 2 min) to 240°C at 2°C/min. Development was made with N_2 at 25 mL/min, and retention times are quoted relative to that of D-glucitol hexaacetate (T_{GA}). GLC–MS was performed with a Hewlett–Packard model 5958B system or Varian Saturn II ion trap, employing the above GLC conditions by EI (70 eV) or CI (methane).

*Preparation of 1,2-di-*O*-acetyl-*sn*-glycerol and its racemate.*—Samples (5 mg) of *sn*- or *rac*-glycerol 3-phosphate (Sigma Chemical Co.) were suspended in Ac_2O (500 μL) containing 1-methylimidazole (50 μL)[27] and stirred for 30 min at 22°C, after which time the solution was concentrated to dryness under a stream of N_2. The sample was extracted with CH_2Cl_2–water, and the aqueous layer was concentrated in vacuo. The residue was dissolved in 48% HF (0.5 mL) in a Teflon

reaction vial, and the mixture was stored for 1 h at 2°C. Excess HF was neutralized with $CaCl_2$–solid CO_2–CH_2Cl_2, centrifuged and concentrated to dryness to give the di-*O*-acetylglycerol, which was converted directly to the camphanate ester. A portion of the di-*O*-acetylglycerols were converted to their *O*-trimethylsilyl derivatives for direct GLC analysis.

Preparation of rac *and 1,2(or 2,3)-di-*O*-methyl-*sn*-glycerol.*—In separate experiments D-, L-, and DL-glyceric acids (~ 40 mg), obtained as the calcium half-salts (Sigma Chemical Co.), were decationized by passing solutions through columns containing Rexyn 101 (H^+ form, 1 mL) cation-exchange resin, followed by concentration of the eluates. The glyceric acid was dissolved in MeOH containing 3% HCl (1 mL) and heated for 14 h at 90°C to afford the methyl ester, which was obtained as a clear oil following removal of excess methanolic HCl by flushing the solution with N_2 and drying in vacuo.

Permethylation of the methyl glycerates with sodium methylsulfinylmethanide and iodomethane in dimethyl sulfoxide as described[29] gave the methyl 2,3-di-*O*-methyl ethers which were recovered by partitioning the mixtures between CH_2Cl_2 and water, followed by concentration of the organic phase: EIMS, *m/z* 148 (M, 0.4%) 118 (M – CH_2O, 37), 116 (M – MeOH, 23), 103 (M – CH_2OMe, 19), 89 (M – CO_2Me, 100), 59 (70), 45 (99). The methyl ester was then reduced with $NaBH_4$ (50 mg) in 3:8 EtOH–THF (1.5 mL) over 14 h at 25°C[30]. Excess borohydride was destroyed by addition of glacial acetic acid, the solution was concentrated to dryness under a stream of N_2, and the borate was removed by repeated dissolution of the residue in MeOH containing 1% of acetic acid (1 mL), followed by evaporation under a stream of N_2 (3 times). The mixture was decationized by passing a 1:1 EtOH–water solution through a column containing Rexyn 101 (H^+ form, 1 mL) ion-exchange resin and evaporation of the eluate with N_2 to give 1,2- and/or 2,3-di-*O*-methyl-*sn*-glycerol. A portion of the di-*O*-methyl glycerol was converted to the 1-*O*-acetyl derivative: EIMS, *m/z* 117 (M – CH_2OMe, 100%), 89 (M – CH_2OAc, 36), 59 (46), 45 (40), 43 (92). Samples 2,3(and 1,2)-di-*O*-methyl-*sn*-glycerol and the racemate were then converted to their camphanate esters (see below).

*Conversion of 1-*O*-(α-*D*-galactopyranosyl)-*sn*-glycerol to 2,3-di-*O*-methyl-*sn*-glycerol.*—Depolymerization of the capsular antigen of *P. haemolytica* serotype T3 with aq HF afforded 1-*O*-α-D-galactopyranosyl-*sn*-glycerol (**3**), which was methylated and treated with methanolic HCl to give 2,3-di-*O*-methyl-*sn*-glycerol (**2**) as described[12].

Preparation of camphanate esters.—(1*S*,4*R*)-(–)-Camphanic acid chloride (Aldrich Chemical Co.) (10–20 mg), and > 10 mg of 4-dimethylaminopyridine (DMAP) were added to the di-*O*-methyl- or di-*O*-acetylglycerol (~ 5 mg) in 1:1 CH_2Cl_2–Et_3N (0.5 mL), and the solution was stirred or 1 h at 22°C. Aqueous 5% sodium carbonate (1 mL) was added, the product was extracted into CH_2Cl_2, and the extract was washed with water and dried over anhydrous sodium sulfate prior to GLC analysis.

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