Note

Determination of fatty acids of the bacteria *Streptomyces* R61 and *Actinomadura* R39 by capillary gas chromatography—mass spectrometry

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Actinomycetes have been used extensively by Ghuysen et al. [1] as sources of exocellular and membrane-bound DD-peptidases to study the mode of action of penicillin. The plasma membranes of various *Streptomyces* strains (R61, K15 and *rimosus*) have an atypical penicillin-binding protein (PBP) pattern characterized by the presence of a predominating low-molecular-weight (26 000-Mr) PBP which was characterized as a DD-transpeptidase [2]. The DD-transpeptidase of *Streptomyces* R61 exhibited unusual properties in that temperatures of —35°C (or below) were required to completely inhibit the enzyme when plasma membranes and the co-substrates Ac_2-L-Lys-D-Ala-D-Ala and Gly-Gly were incubated together in the frozen state. Under these conditions, the purified enzyme is devoid of activity [3]. Since the lipids could play an important role in providing the hydrophobic environment necessary for the membrane-bound enzyme to function, it was of interest to study the lipid composition of *Streptomyces* R61. The present paper reports the identification and quantitative analysis of the fatty acids of the phospholipids of *Streptomyces* R61 by gas chromatography—mass spectrometry (GC—MS) using high-resolution support-coated open tubular (SCOT) columns [4]. The same analysis was applied to another *Actinomycetes*, namely *Actinomadura* R39. The membrane-bound PBPs of this organism have not been investigated, but

both *Actinomadura* R39 and *Streptomyces* R61 excrete during growth exocellular serine DD-peptidases that are able to catalyse concomitant carboxypeptidation and transpeptidation reactions [1].

EXPERIMENTAL

Growth of bacteria

Streptomyces R61, and *Actinomadura* R39, kindly provided by Professor J.M. Ghuysen, Université de Liège, were grown in batch culture in peptone—oxoid medium at 25°C and with vigorous shaking. At the onset of the stationary phase (65 h), cells were harvested by centrifugation, washed twice with water, and then lyophilised.

Extraction of lipids and separation of phospholipids

Lipids were extracted from the cells by the method of Bligh and Dyer [5] and fractionated on a silicic acid column (200—325 mesh), with elution by chloroform, acetone and methanol, to yield the total phospholipids in the methanol eluate. The total phospholipids were subjected to two-dimensional chromatography over Whatman SG-81 silica-impregnated paper, and two-dimensional thin-layer chromatography (TLC) over silica gel G. For both methods the solvent mixtures of Wurthier [6] were used, viz. chloroform—methanol—diisobutyl ketone—acetic acid—water (45:15:30:20:4) (first dimension) and chloroform—methanol—diisobutyl ketone—pyridine—0.5 M aqueous ammonium chloride (60:35:50:70:12) (second dimension). Rhodamine 6G solution (0.0012%), Zinzarde's reagent [7] and ninhydrin were used for detection. Individual phospholipid fractions were compared with phospholipid standards, using both solvent mixtures.

Conversion to fatty acid methyl esters and hydrogenation of unsaturated esters

The phospholipids of *Streptomyces* R61 and *Actinomadura* R39 were separately cleaved and converted to fatty acid methyl esters by refluxing over 14% boron trifluoride in anhydrous methanol [8] for 30 min in an all-glass apparatus, followed by pentane extraction after addition of water. In the present work, this treatment did not cause detectable decomposition of the fatty acids. Thus using GC the compositions of methyl esters were substantially the same when the following esterification methods were applied as tests to the phospholipids of *Actinomadura* R39: reaction with 14% boron trifluoride in methanol [8], with or without prior alkaline hydrolysis, and (for comparison) reaction with 10% boron trichloride in methanol [9], with or without prior alkaline hydrolysis. Furthermore, MS analysis showed that, when subjected to the identical treatment with boron trifluoride in methanol, methyl 3-hydroxydecanoate (a β-hydroxy ester) was not dehydrated.

Hydrogenation of unsaturated fatty acid methyl ester mixtures was carried out by bubbling hydrogen for 1 h into a solution in methanol in the presence of 10% palladium-on-charcoal. This treatment did not cause ring cleavage of cyclopropane rings (see Results and discussion).

Gas chromatography

GC was carried out on ethylene glycol succinate cross-linked to silicone (EGSS-X) 3% on Gas-Chrom Q (100—120 mesh) and flame ionization detection was used.

Gas chromatography—mass spectrometry

Fatty acid methyl esters were analysed by GC—MS using a 50-m SCOT SE-30 column and helium, methane and ammonia as chemical ionisation (CI) reactant gases. This system and its application to the analysis of fatty acid methyl esters have been described [4].

RESULTS AND DISCUSSION

Identification of fatty acid methyl esters derived from phospholipids

Streptomyces R61. GC—MS analysis under methane CI conditions of the fatty acid methyl esters from *Streptomyces* R61 before and after hydrogenation of unsaturated fatty acid methyl esters gave the chromatograms shown in Fig. 1. The following peaks were identified by comparison of their GC retention times and CI mass spectra (methane and helium gases) with those of authentic samples: peak 2 (14:0); peak 4 (anteiso-15:0); peak 5 (15:0); peak 7 (iso-16:0); peak 9 (16:0); peak 14 (17:0); peak 17 (iso-18:0); and peak 19 (18:0). Other peaks were identified by inspection of their helium CI mass spectra which yield information on the branching [4]. These are: peak 1 (iso-14:0); peak 3 (iso-15:0); peak 10 (10-Me-16:0); peak 12 (iso-17:0); peak 16 (10-Me-17:0); and peak 20 (10-Me-18:0). The remaining peaks in the chromatogram of the fatty acid methyl esters from *Streptomyces* R61 before hydrogenation (Fig. 1A) had mass spectra corresponding to mono-unsaturated or cyclopropane-containing esters. These peaks were not present on the chromatogram obtained after hydrogenation over palladium-on-charcoal (Fig. 1B) and must therefore correspond to unsaturated rather than cyclopropane esters. MS analysis showed that under the same hydrogenation conditions, the cyclopropane ring of methyl *cis*-9,10-methylenehexadecanoate was not opened. On the basis of the molecular ions observed in their mass spectra, peaks 6 and 8 corresponded to 16:1 isomers, peak 11 to 17:1, and peaks 15 and 18 to 18:1 isomers. Retention times indicated that peak 8 was probably 9-*cis*-16:1 and peak 18 was probably 9-*cis*-18:1.

The methane mass spectrum of peak 13 indicated that it corresponded to a co-chromatographing mixture of 17:0 and 17:1. This was confirmed by inspection of the ammonia CI mass spectrum of this peak. As described previously [4] the ammonia CI spectrum showed an enhanced peak area for the unsaturated fatty acid ester. After hydrogenation, which removed the 17:1 contribution, the helium CI mass spectrum of peak 13 showed enhanced cleavage ions at m/z 255 (M — C_2H_5) and 227 (M — C_4H_9) indicating the presence of anteiso-17:0.

Actinomadura R39. GC—MS analysis of the fatty acid methyl esters from *Actinomadura* R39 before and after hydrogenation gave chromatograms which were qualitatively identical to those described for *Streptomyces* R61.

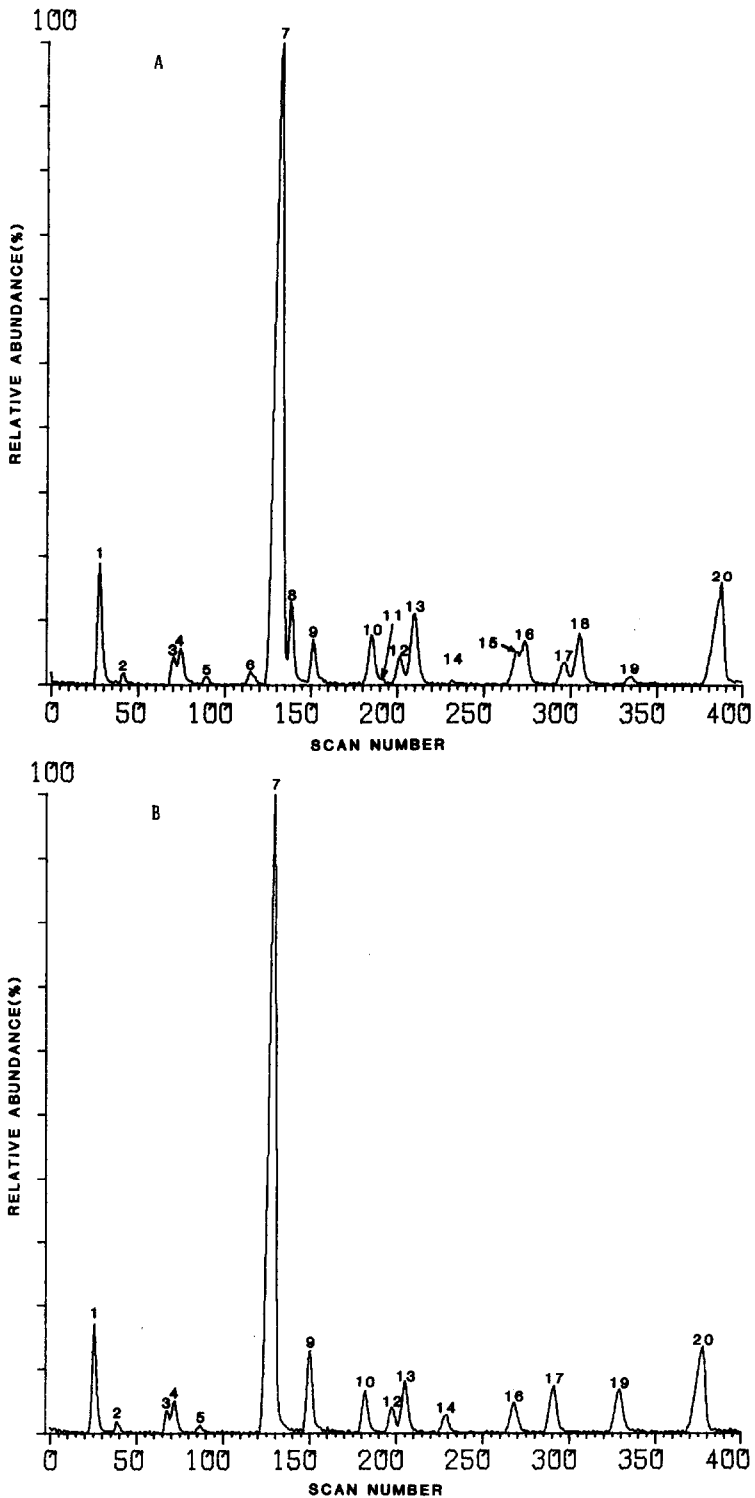A summary of the fatty acid methyl esters identified and the relative

Fig. 1. Gas chromatograms obtained by methane CI GC—MS analysis of fatty acid methyl esters from *Streptomyces* R61 before hydrogenation over palladium-on-charcoal (A) and after hydrogenation (B). Peak numbers refer to methyl esters listed in Table I.

amounts of each present in extracts of *Actinomadura* R39 and *Streptomyces* R61 is given in Table I. The most striking feature of the results shown in Table I is that branched saturated esters accounted for approximately 72% and 84% of the total fatty acid methyl esters from *Actinomadura* R39 and *Streptomyces* R61 respectively, whereas straight-chain saturated esters contributed only 3% of the total in each case. In both strains, the most abundant fatty acid methyl ester was iso-16:0 which constituted almost half of the total.

Unsaturated esters accounted for approximately 25% and 13% of the total fatty acid methyl esters from *Actinomadura* R39 and *Streptomyces* R61, respectively. After hydrogenation, the proportion of branched-chain esters

TABLE I

FATTY ACID METHYL ESTERS DERIVED FROM PHOSPHOLIPIDS OF *ACTINOMADURA* R39 AND OF *STREPTOMYCES* R61

GC—MS peak no.[*] (Fig. 1)	Relative retention time[**]	Identity of fatty acid[***]	Composition[§] (%)			
			Actinomadura R39		*Streptomyces* R61	
			Before hydro- genation	After hydro- genation	Before hydro- genation	After hydro- genation
1	0.407	iso-14:0	1.9	1.9	2.6	2.7
2	0.436	14:0	0.3	0.3	0.2	0.3
3	0.473	iso-15:0	0.6	0.7	1.0	0.9
4	0.479	anteiso-15:0	0.9	0.9	1.3	1.3
5	0.502	15:0	0.1	0.2	0.3	0.3
6	0.539	16:1	0.6	—	0.6	—
7	0.562	iso-16:0	41	40	48	49
8	0.574	16:1	4.0	—	3.5	—
9	0.596	16:0	1.5	5.7	2.0	5.2
10	0.636	10-Me-16:0	1.3	1.1	3.7	3.2
11	0.644	17:1	0.2	—	0.1	—
12	0.659	iso-17:0	1.8	1.8	2.2	2.1
13	0.672	{anteiso-17:0 / 17:1	} 5.6	3.8 / —	} 5.4	4.4 / —
14	0.708	17:0	<0.1	2.3	< 0.1	1.7
15	0.757	iso-18:1	3.1	—	2.9	—
16	0.763	10-Me-17:0	1.7	1.8	3.9	3.4
17	0.798	iso-18:0	2.8	6.0	2.2	5.6
18	0.812	18:1	14	—	5.0	—
19	0.853	18:0	1.6	17	0.7	6.1
20	0.930	10-Me-18:0	16	16	15	15
		Branched-chain	72	74	84	87
		Straight-chain	3	26	3	13
		Unsaturated	25	—	13	—

[*]See Fig. 1 for chromatogram of *Streptomyces* R61 and Experimental for methane CI GC—MS conditions.
[**]Relative to docosane.
[***]First number, number of carbon atoms in the chain; second number, number of double bonds.
[§]Total does not come to exactly 100% due to rounding off.

increased only slightly whereas the proportion of straight-chain esters increased to 26% and 13%, respectively, which indicated that the unsaturated esters were predominantly straight-chain.

By comparison of the relative amounts of particular fatty acid methyl ester before and after hydrogenation it was possible to draw some inferences about the structure of some of the unsaturated esters. Peak 15 (3%, 18:1) decreased to zero after hydrogenation whereas peak 17 (iso-18:0) increased from 3% to 6% which implied that peak 15 represents iso-18:1. The same kind of argument may be used to show that peak 8 (16:1), peak 13 (17:1), and peak 18 (18:1) were straight-chain unsaturated fatty acid methyl esters.

The variation of fatty acid composition with phospholipid structure was assessed for the phospholipids of *Actinomadura* R39. Four individual phospholipids separated by preparative TLC were analysed by GC. The estimated fatty acid methyl ester compositions (expressed for groups of esters of similar retention time) as shown in Table II indicated that there were considerable variations. Phosphatidyl ethanolamine (a minor component) was different from the other phospholipids in not having iso-16:0 as the major fatty acid, but had instead an elevated 16:0 content. The level of 16:0 was also raised in cardiolipin. The results may reflect the dynamic nature of lipid turnover or the asymmetric nature of the bacterial membrane [10].

The fatty acids of most microorganisms including *Actinomycetes* species [11, 12] are known to contain branched and unsaturated fatty acids, but seldom do both types exist in appreciable quantities. The theory of homoviscous adaptation suggests that generally the degree of unsaturation and of

TABLE II

APPROXIMATE PERCENTAGE COMPOSITION OF GROUPS OF FATTY ACIDS FROM INDIVIDUAL PHOSPHOLIPIDS OF *ACTINOMADURA* R39

The approximate percentage composition is as analysed by GC under conditions given in Experimental.

Fatty acid	Phosphatidyl glycerol	Cardiolipin	Unidentified* phospholipid	Phosphatidyl ethanolamine
iso-14:0	1	7	1	1
14:0	0.2	2	1	3
iso-15:0 ⎫ anteiso-15:0 ⎬ 15:0 ⎭	1	5	3	2
iso-16:0	42	38	45	17
16:0	3	13	6	18
17:0 isomers ⎫ 16:1** ⎭	11	2	6	3
18:0 isomers ⎫ 17:1** ⎭	5	3	5	15
10-Me-18:0 ⎫ 18:1 isomers ⎭	37	29	33	41

*Representing 25% of the total phospholipids; stained negative to ninhydrin, ammoniacal silver nitrate and Dragendorff's reagents.
**Probably one of two isomers found in *Actinomadura* R39.

branching may be adjusted to maintain membrane fluidity [13, 14]. The membrane lipids of *Escherichia coli* K12 grown at 13°C was shown to be considerably more unsaturated than those of cells grown at 37°C [15]. Incorporation of branched fatty acids into *Acholeplasma laidlawii* membrane lipids resulted in non-appearance of particle aggregation on cooling of the modified cells [16]. It had been suggested that the branched chains could be more loosely packed than straight chains, and consequently had a lower crystalline state transition temperature [13, 16]. A number of studies support the idea [17] that a liquid—crystalline state for cell membrane lipids is necessary to support growth [18, 19], membrane transport [19, 20], and the activity of membrane-associated enzymes [21]. The fatty acid composition of both *Streptomyces* R61 and *Actinomadura* R39 is unusual in that the branched and unsaturated members together contribute as much as 97% of the total fatty acids. The 26000-Mr membrane-bound DD-transpeptidase of *Streptomyces* R61 remains active at temperatures below 0°C [2, 3]. It might be speculated that this is a consequence of the maintenance of membrane lipid fluidity by the reinforcing effects of the branched and unsaturated fatty acids.

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