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Structure of Actinotetraose Hexatiglate, a Unique Glucotetraose from an Actinomycete Bacterium

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An Actinomycete strain A499 belonging to the genera *Amycolatopsis* or *Amycolata* isolated from a Western Australian soil sample produced the cyclic decapeptide antibiotic quinaldopeptin (**1**), together with the actinotetraose hexatiglate (**2**), the hexa-ester of a novel non-reducing glucotetraose.

During the course of a screening program directed towards the discovery of nematocidal metabolites from Australian microorganisms, an Actinomycete bacterium strain A499 isolated from a Western Australian soil sample was found to produce the cyclic decapeptide quinaldopeptin (**1**),[1] a known antimicrobial and cytotoxic antibiotic which was responsible for the anthelmintic activity, together with abundant quantities of the unique tetrasaccharide derivative (**2**) (Fig. 1). This latter metabolite lacks significant antimicrobial activity, and its biological role will be addressed elsewhere. It is distinguished structurally by the presence of a novel glucotetraose skeleton to which are attached six tiglate ester units, the whole having a two-fold axis of symmetry. Since the parent tetrasaccharide probably has wider occurrence than as the present tiglate derivative, we have named it actinotetraose in recognition of its origin. We describe here the taxonomy of the producing strain, and the production, isolation and structure determination of this actinotetraose hexatiglate (**2**).

Results and Discussion

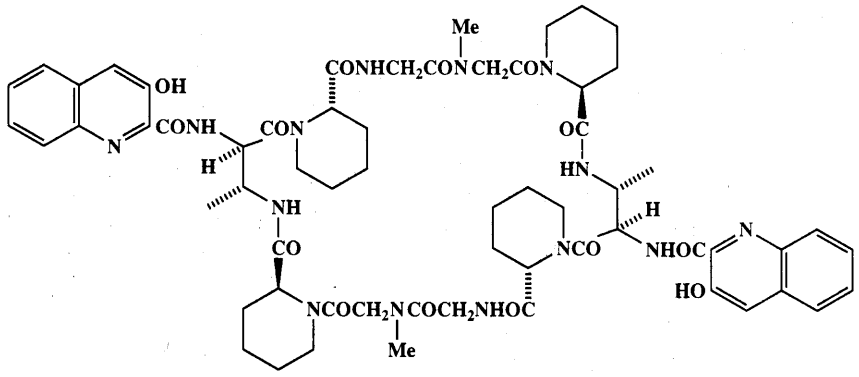
Taxonomy of the Producing Strain

The taxonomy of the Actinomycete strain A499 was examined by the American Type Culture Collection, and their report is summarised here. Strain A499 produced an extensively branched yellow-brown vegetative mycelium exhibiting some fragmentation, while the white aerial mycelium was very well developed and fragmented into ellipsoidal spore-like elements in long straight, flexuous or zig-zag chains. No endospores, sheaths, synnema, sporangia, sclerotia or motility of spore-like fragments were observed. Cell wall analysis indicated the presence of *meso*-diaminopimelic acid in hydrolysates, whole cell sugar analysis indicated arabinose and galactose as the major constituent sugars, and whole cell methanolysis showed the absence of mycolic acids. Glucose, arabinose, fructose, mannitol, inositol, xylose and galactose were utilised for growth, which was poor with raffinose, rhamnose and salicin and doubtful with sucrose. No soluble pigments or melanin were produced. Degradation of tyrosine and hydrolysis of hippurate and urea were positive, but degradation of xanthine and hydrolysis of aesculin were negative. While the strain was morphologically similar to several genera of the order Actinomycetales, including *Nocardiopsis*, *Nocardioides*, *Nocardia*, *Saccharothrix*, *Amycolata*, and *Amycolatopsis*, the chemotaxonomic profile indicates that it belongs to the genera *Amycolatopsis* or *Amycolata*.

Production and Isolation of the Metabolites

The Actinomycete strain A499 produced extractable anthelmintic activity when grown on ISP-2 agar, but not in shaken liquid culture using the same nutrient. The agar cultures were homogenised with acetone after 10～14 days of growth, the aqueous acetone separated and removed under vacuum, and the aqueous residue extracted with ethyl acetate. Chromatography of the

Fig. 1. Structures of quinaldopeptin (**1**) and the actinotetraose hexatiglate (**2**).



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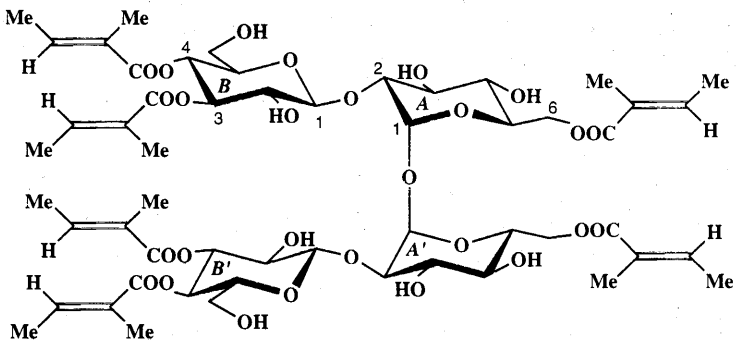

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extract gave major white and pale yellow fractions, all nematocidal activity being concentrated in the latter. Further purification by absorption and gel permeation chromatography, respectively, provided the actinotetraose hexatiglate (**2**), and quinaldopeptin (**1**).

Physico-chemical Properties and Structure of Actinotetraose Hexatiglate

FAB-MS in negative ion mode of the actinotetraose hexatiglate afforded intense deprotonated and chloride-carrying molecular ions at *m/z* 1157 and 1193, and in positive mode protonated and sodiated molecular ions at *m/z* 1159 and 1181. HR-MS of the sodiated species indicated the molecular formula $C_{54}H_{78}O_{27}$. EI-MS gave no molecular ion, but several series of ions separated by 82 or 100 amu, which HR-MS established as corresponding to successive losses of neutral C_5H_6O or $C_5H_8O_2$ units from ionised molecular fragments. Such losses suggested the EI-induced cleavage of dimethylacrylate ester units with loss of the corresponding neutral ketene or carboxylic acid fragments. In agreement, the base peak in EI-MS was *m/z* 83, the corresponding acylium ion $C_5H_7O^+$. We comment further on the EI spectrum below.

Despite its optical activity, the ^{13}C NMR spectrum of the actinotetraose hexatiglate in both $CDCl_3$ and D_5-pyridine showed only 27 carbon resonances, half the number expected, implying the presence of a two-fold axis of symmetry and greatly simplifying the subsequent structure determination. The carbonyl, two olefinic, and two methyl signals of three dimethylacrylate moieties were visible near δ 167, 137, 128, 14, and 12 ppm, respectively, confirming the inference from EI-MS fragmentations. Of the remaining 12 resonances, 10 were located between δ 82 and 61 ppm and bonded to single oxygen atoms, and the 2 at δ 106.4 and 94.6 ppm were bonded to two oxygen atoms, in agreement with the molecular formula and defining the asymmetric half molecule as a disaccharide. The ^{13}C NMR spectrum also necessitates that this disaccharide moiety exists as a single anomer and entirely in the cyclic form. ^1H NMR spectra of the tetraose in both $CDCl_3$ and D_5-pyridine also showed only half the expected resonances, confirming the presence of the symmetry element in the structure.

Complete assignments of the ^1H and ^{13}C NMR resonances of the asymmetric half molecule of the

Table 1. ^{13}C and ^{1}H NMR data for the actinotetraose hexatiglate (**2**) in D_5-pyridine, and α-methyl sophoroside (**4**) and α,α-trehalose (**5**) in H_2O.

Carbon	δC (ppm)	δH (ppm)	H Multiplicity, J (Hz)	δC (ppm)
Actinotetraose (**2**) units B, B'				α-Methyl sophoroside (**4**) unit B[5]
1	106.4	5.35	d, $J_{1,2}$ 8.0	105.0
2	73.6	4.13	m, overlapped	74.4
3	76.2	5.96	t, $J_{2,3}$ 9.6, $J_{3,4}$ 9.6	77.1
4	70.3	5.67	t, $J_{3,4}$ 9.7, $J_{4,5}$ 9.7	71.3
5	76.2	4.06	dt, $J_{4,5}$ 10.0, $J_{5,6}$ 4.0	77.1
6	61.8	4.15	m, overlapped	62.2
Actinotetraose (**2**) units A, A'				α,α-Trehalose (**5**) units A, A'[6]
1	94.6	5.83	d, $J_{1,2}$ 3.7	94.0
2	82.3	4.30	dd, $J_{1,2}$ 3.7, $J_{2,3}$ 9.7	72.0
3	74.0	4.72	t, $J_{2,3}$ 9.4, $J_{3,4}$ 9.4	73.5
4	71.0	4.22	t, $J_{3,4}$ 9.4, $J_{4,5}$ 9.4	70.6
5	71.2	4.96	dt, $J_{4,5}$ 10.0, $J_{5,6}$ 2.6	73.0
6	63.7	5.05	dd, $J_{5,6}$ 3.1, $J_{6,6}$ 12.0	61.5
		5.12	dd, $J_{5,6}$ 2.0, $J_{6,6}$ 12.0	
Tiglate esters				
1	167.9			
	167.5			
	167.0			
2	129.0			
	128.6			
	128.4			
3	138.3	6.87[a]	qq, $J_{3,4}$ 7.1, $J_{2',3}$ 1.4	
	137.8	6.82[a]	m, overlapped	
	137.2	6.87[a]	m, overlapped	
4	14.2	1.42[b]	dq, $J_{3,4}$ 7.0, $J_{2',4}$ 1.0	
	14.1	1.47[b]	dq, overlapped	
	14.0	1.49[b]	dq, overlapped	
2-Me	12.20	1.69[c]	bs	
	12.15	1.72[c]	bs	
	12.13	1.75[c]	bs	

Assignments marked [a,b,c] are arbitrary and may be interchanged within each group.

actinotetraose hexatiglate (**2**) are presented in Table 1. The close similarity of the ^{13}C and ^{1}H resonances of the three visible acrylate units indicated that all were identically substituted. The presence of $=CHCH_3$ proton spin systems necessitated 2,3- rather than 3,3-dimethyl substitution of these acrylates, the *E*-configuration of which followed from the ^{1}H and ^{13}C chemical shift correspondence of the H-3 and 2-CH_3 substituents with tiglic (2,3-*E*) but not angelic (2,3-*Z*) acid.[2,3] ^{1}H–^{1}H COSY traced two isolated 7-proton spin systems, defining the disaccharide components as two aldohexose units A and B. Both hexoses were present as pyranose forms with *gluco* relative stereochemistry, since with the exception of the anomeric proton of sugar A all the ring protons had at least one vicinal coupling constant exceeding 8.0 Hz, implying axially orientated protons on rings with chair conformations. The glycosidic linkages to rings A and B were thus axial and equatorial, respectively. With

Fig. 2. Important 3-bond HMBC relationships in the actinotetraose hexatiglate (**2**).

Relationships within glucose units and substituents in units A′ and B′ have been omitted for clarity.

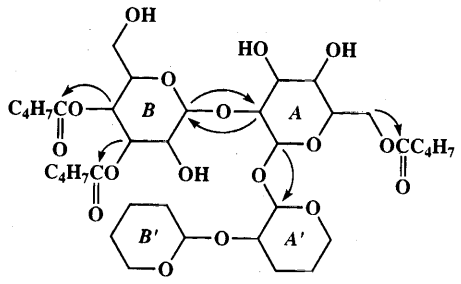
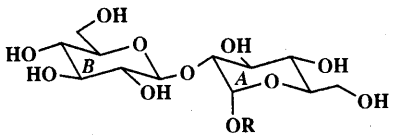
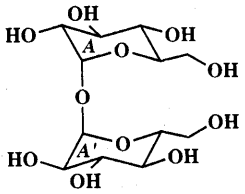


Fig. 3. Structures of the disaccharides α-sophorose (**3**), α-methyl sophoroside (**4**) and α,α-trehalose (**5**).



3 R = H
4 R = Me



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the proton assignments complete, HETCOR spectroscopy then assigned the resonances of the attached carbon atoms (Table 1).

Pulsed field gradient HMBC spectroscopy located the linkages between the monosaccharide units, and the positions of the tiglate ester units (Fig. 2). Strong 3-bond correlations between H-1 of pyranoside B (H^B-1) and C-2 of pyranoside A (C^A-2), and between H^A-2 and C^B-1, established the 1→2 linkage between units B and A. H^A-1 at δ 5.83 showed, in addition to the one-bond correlation to the carbon resonance at δ 94.6, a strong 3-bond correlation to the same resonance. This unusual situation arises from the fact that δ 94.6 is the chemical shift not only of C^A-1 itself but also of the symmetrically positioned $C^{A'}$-1, and the HMBC correlation established the 1↔1′ glycosidic linkage between units A and A′. The esterified hydroxyl groups were indicated by strong 3-bond correlations from H^B-3, H^B-4, and both protons at H^A-6 to the carbonyl resonances of the three tiglate residues at δ 167.5, 167.0 and 167.9, respectively. The ester positions were confirmed by the characteristic downfield acylation shifts[4] of the resonance frequencies of the secondary protons H^B-3 and H^B-4 in particular, and to a lesser extent of the primary protons H^A-6.

The anomeric proton coupling constants of 3.7 and 8.0 Hz for the monosaccharide units A and B defined the stereochemistry of the AA′ and AB glycosidic linkages as α and β, respectively, and complete the determination of the relative configuration of the actinotetraose hexatiglate (**2**). The absolute configuration of the metabolite as a D-*gluco*tetraose follows from the formation of β-methyl D-glucoside, identified by chiral HPLC, following hydrazinolysis of the peripheral ester groups and acid-catalysed methanolysis of the residual tetrasaccharide core. The AB and AA′ disaccharide segments thus correspond in structure and stereochemistry to the natural 1→2- and 1↔1-linked glucobioses sophorose, as its α-anomer (**3**), and α,α-trehalose (**5**) (Fig. 3). In agreement, the ^{13}C NMR resonances of the B,B′ and A,A′ monosaccharide units of the tetrasaccharide (**2**) match those of the unmethylated B unit of α-methyl sophoroside (**4**)[5] and of the A,A′ units of α,α-trehalose (**5**),[6] respectively, as seen from the comparison in Table 1. The sole exception to this correspondence is C^A-2, where the known substantial effect of ether formation[7] is reflected in the 10 ppm downfield shift from δ 72.0 to 82.3.

The EI-MS of the actinotetraose hexatiglate, however, was in apparent contradiction of the structure (**2**). The highest mass ion appeared at *m/z* 978, corresponding to the elimination of an unacylated glucose unit $C_6H_{12}O_6$ from a parent ion, *m/z* 1158. In addition to the expected intense oxonium ion from cleavage of the glycosidic bond of unit B, *m/z* 327, carrying two tiglate units, similar ions appeared at *m/z* 409 and 491, carrying three and four tiglate units. Cleavage of the glycosidic bond of unit A also occurred, leaving the AB fragment as the oxonium species *m/z* 571 and its dehydration product *m/z* 553, each carrying the expected three tiglate units. These ions, however, were accompanied by ions of similar origin at *m/z* 653 and 635, 735 and 717, and 817, carrying four, five and six tiglate moieties, respectively. These unexpected ions imply the presence of asymmetrically acylated tetrasaccharide species, in which the terminal

glucose B unit is quadruply acylated, the A unit diacylated, and the A′ and terminal B′ units unacylated. We believe that structures of this type arise by intramolecular migration of the tiglate units, promoted thermally on the direct insertion probe at the high temperatures of 280~350°C which were needed to obtain the EI spectrum. The resulting EI spectra are then dominated by ions arising from the more volatile hexatiglate esters of tri- and disaccharides, formed by thermal elimination of the unacylated B′ and A′ units.

The actinotetraose hexatiglate (**2**) is formally a derivative of *O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→2′)]-α-D-glucopyranosyl-(1↔1′)-α-D-glucopyranoside, *i.e.*, of 2,2′-di-(*O*-β-D-glucopyranosyl)-α,α-trehalose, which despite the structural simplification provided by its two-fold axis of symmetry appears to be a previously unreported non-reducing glucotetraose. This symmetry is maintained upon esterification with the six tiglate units, and the resulting unique metabolite is one of the 35 possible hexaesters of this tetrasaccharide which would retain such symmetry. It is also notable that quinaldopeptin (**1**), the major co-metabolite of the actinotetraose hexatiglate (**2**) produced by the strain A499, has a similar two-fold symmetry axis.

Experimental

General

NMR spectra were recorded on Varian Gemini-300, VXR-300S or VXR-500S spectrometers at 300 or 500 MHz for ^1H and 75.43 or 125.75 MHz for ^{13}C. The solvent ^1H and ^{13}C resonances, 7.27 and 77.0 ppm for $CHCl_3/CDCl_3$ and 7.19 and 123.5 ppm for D_4/D_5-pyridine, were used as internal references. Optical rotations were obtained on a Perkin Elmer 241 polarimeter, and UV spectra on a Hewlett Packard 8450A UV/VIS spectrophotometer. MS data were obtained on Fisons Instruments VG ZAB2-SEQ, AutoSpec and Quattro II spectrometers.

Fermentation of Actinomycete A499 and Isolation of Metabolites

Actinomyces species A499 was grown in glass petri dishes (14 cm diameter) on ISP2 agar (100 ml/dish) at 28°C for 10~14 days. The cultures were homogenised briefly in acetone (200 ml/dish) and then stirred for 2 hours. Filtration gave an extract from which the acetone was removed by rotary evaporation to leave an aqueous residue which was extracted with EtOAc. The dried (Na_2SO_4), evaporated extract (2.1 g/liter agar) was chromatographed on silica gel, elution with CH_2Cl_2: Me_2CO: MeOH (4:5:1) and then MeOH giving upon evaporation white and pale yellow (340 mg/liter agar) solids, respectively. The latter fractions were re-chromatographed on Sephadex LH-20 in MeOH to yield quinaldopeptin (**1**): FAB-MS positive *m/z* 1287 (MNa^+ of Na salt), 1265 (MNa^+), 1243 (MH^+); HRFAB-MS calcd for $C_{62}H_{78}N_{14}O_{14}Na$, 1265.572, found 1265.573; UV λ_{max} in neutral, acidic and alkaline EtOH identical with literature data[1]. In the absence of published ^{13}C and ^1H NMR data on quinaldopeptin itself, the identity was confirmed by preparation of the diacetate, ^{13}C NMR data of which matched that reported[1]: FAB-MS positive *m/z* 1327 (MH^+), 1349 (MNa^+). The quinaldopeptin carried the anthelmintic activity produced by the fermentation.

The white fractions were re-chromatographed on silica to afford the actinotetraose hexatiglate (**2**) (900 mg/liter agar): $[\alpha]_D^{23}$ +29.2° (*c* 1.0, CH_2Cl_2); FAB-MS positive *m/z* 1197 (MK^+), 1181 (MNa^+), 1159 (MH^+), negative *m/z* 1193 (MCl^-), 1157 ($M-H^-$), 1075 ($M-H^- - C_5H_6O$), 1057 ($M-H^- - C_5H_8O_2$); HRFAB-MS calcd for $C_{54}H_{78}O_{27}Na$, 1181.463, found 1181.471; EI-MS (probe 280~350°C, intensities variable and not reported) 978, 896, 878, 817, 814, 796, 778, 753, 735, 717, 714, 696, 653, 635, 612, 571, 553, 491, 471, 409, 389, 371, 327, 309, 245, 227, 209, 127, 83 (base peak); HREI-MS 327.1442, $C_{16}H_{23}O_7$ requires 327.1444, 245.1020, $C_{11}H_{17}O_6$ requires 245.1025; ^{13}C and ^1H NMR data as recorded in Table 1.

Hydrazinolysis and Methanolysis of Actinotetraose Hexatiglate (2)

Actinotetraose hexatiglate (**2**) (11 mg) in MeOH (3 ml) was treated with hydrazine hydrate (3 ml) for 3 days. Evaporation under vacuum and extraction with MeOH gave a soluble fraction (1.5 mg), shown by ^1H NMR spectroscopy (CD_3OD) and ESMS to contain free tetrasaccharide. Treatment of this fraction in MeOH (1 ml) with chlorotrimethylsilane (0.2 ml) for 18 hours, followed by evaporation to dryness, afforded a residue (2 mg) shown to contain methyl glucosides by ^1H NMR spectroscopy (CD_3OD) and ESMS, *m/z* 195 (MH^+), 217 (MNa^+). Chiral HPLC (Chiralpak AS column, Daicel Chemical Industries, Ltd) in isopropanol with methyl D- and L-glucosides (prepared as above from the corresponding glucoses) as reference compounds established the presence of β-methyl D-glucoside in this material.

Acknowledgments

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