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ISOLATION AND CHARACTERIZATION OF CYTOTOXIC AND ANTIBACTERIAL TETRASACCHARIDE GLYCOSIDES FROM *IPOMOEA STANS*

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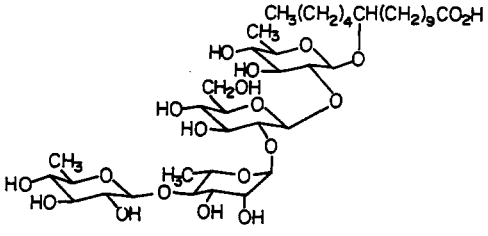
ABSTRACT.—Three new tetrasaccharide glycosides, differing from one reported previously in the type of short-chain fatty acids ester-linked to the tetrasaccharide core, have been isolated and identified from an oligosaccharide fraction of *Ipomea stans*. Preliminary screening tests showed that the fraction containing these compounds had pronounced cytotoxicity towards three human tumor cell lines as well as specific antibiotic activity against two bacterial strains.

Extracts from *Ipomoea stans* Cav. (Convulvulaceae) have been used in Mexican traditional medicine for treating epileptic seizures (1). We have recently undertaken a detailed phytochemical investigation of this species in the hope of isolating and identifying pharmacologically useful compounds (2). Initially, two polysaccharide fractions of different polarity were isolated from the roots of *Ipomoea stans*, with the less polar one being isolated in much higher yield. The ^{13}C-nmr spectral data suggested that the fractions were actually a mixture of closely related compounds. Mild acid hydrolysis of portions of the two fractions yielded a single glycosidic acid derivative which proved to be a tetrasaccharide with 11-hydroxypalmitic acid ether-linked to a terminal anomeric carbon. Detailed characterization using 2D nmr techniques allowed identification of the hydrolysis product as (*S*)-11-hydroxypalmitic acid 11-*O*-β-D-quinovopyranosyl-(1→4)-α-L-rhamnosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-quinovopyranoside [**1**]. Subsequent chromatographic purification of the less polar crude fraction yielded a



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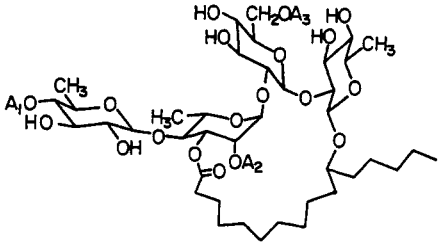

2 $A_1=A_2=$2-Methylbutanoic acid
$A_3=$3-Hydroxy-2-methylbutanoic acid

3 $A_1=A_2=$2-Methylpropanoic acid
$A_3=$3-Hydroxy-2-methylbutanoic acid

4 $A_1=$2-Methylpropanoic acid
$A_2=$2-Methylbutanoic acid
$A_3=$3-Hydroxy-2-methylbutanoic acid

5 $A_1=$2-Methylbutanoic acid
$A_2=$3-Methylbutanoic acid
$A_3=$3-Hydroxy-2-methylbutanoic acid

TABLE 1. Assigned ^{13}C- and ^{1}H-Nmr Chemical Shifts for Tetrasaccharide Components of Compounds **3–5** in $(CD_3)_2CO$.

Sugar	Carbon	Compound					
		3		**4**		**5**	
		δ_C[b]	δ_H[b]	δ_C	δ_H	δ_C	δ_H
glc	1	100.16	5.08	100.25	5.07	100.24	5.06
	2	79.08	3.45	79.10	3.44	79.18	3.44
	3	77.18	3.58	77.26	3.58	77.25	3.58
	4	72.08	3.34	72.13	3.34	72.06	3.34
	5	74.50	3.48	74.53	3.47	74.42	3.49
	6		4.18		4.16		4.17
		64.45	4.45	64.48	4.44	64.49	4.45
rha	1	96.69	5.37	96.86	5.37	96.89	5.34
	2	69.83	5.52	69.86	5.53	69.96	5.52
	3	71.73	5.47	71.79	5.46	71.76	5.47
	4	78.50	3.74	78.34	3.74	78.38	3.74
	5	68.45	4.26	68.57	4.24	68.59	4.24
	6	18.20	1.28	18.29	1.27	18.27	1.27
qui_1	1	100.28	4.52	100.40	4.51	100.42	4.52
	2	79.18	3.53	79.25	3.53	79.29	3.54
	3	77.96	3.52	78.06	3.52	78.01	3.53
	4	76.95	3.00	76.99	2.99	77.02	2.99
	5	72.10	3.24	72.16	3.22	72.13	3.23
	6	18.02	1.20	18.09	1.20	18.09	1.20
qui_2	1	104.42	4.46	104.43	4.44	104.40	4.44
	2	75.18	3.24	75.31	3.20	75.29	3.22
	3	74.88	3.50	75.00	3.49	75.06	3.50
	4	76.15	4.57	76.19	4.56	76.08	4.58
	5	70.48	3.49	70.58	3.46	70.55	3.48
	6	17.72	1.12	17.90	1.11	17.93	1.12

[a]Glc=D-glucose, rha=L-rhamnose, qui=quinovose, qui_1 is the sugar bonded to 11-hydroxypalmitic acid (2).

[b]Chemical shifts in δ (ppm) relative to internal $(CH_3)_4Si$.

pure compound which was shown to have structure **2** (2).

We now report the results of further study on *Ipomoea stans* involving the isolation and identification of three additional tetrasaccharides from the more abundant crude fraction, and the preliminary screening of the two crude fractions for antibacterial and cytotoxic activity.

Careful hplc separation of the less polar fraction of *L. stans* provided four further fractions. Area integration suggested that these fractions, listed in order of increasing retention time, were present in relative amounts of ca. 10%, 35%, 20%, and 35%, respectively. The fraction with the longest retention time was confirmed to be pure **2**. Each of the other three fractions contained ca. 80–90% of a single compound with traces of its nearest neighbors (by retention time). These were characterized without further purification. A combination of ^{1}H-, ^{13}C-, and DEPT nmr spectra in conjunction with the COSY, TOCSY, HMQC, HMBC, and ROESY nmr techniques was used to identify the major component of each fraction. TOCSY spectra were particularly useful for identifying and assigning ^{1}H-nmr spectra for individual monosaccharides and short-chain fatty acids since these allowed us to obtain "edited" ^{1}H-nmr sub-spectra for these individual molecular fragments (2). HMQC then allowed assignment of the corresponding carbons via one-bond ^{13}C-^{1}H correlations

TABLE 2. Assigned ^{13}C- and ^{1}H-Nmr Chemical Shifts for Short-Chain Fatty Acid Components of Compounds **3–5**.

Acid	Carbon	Compound					
		3		**4**		**5**	
		δ_C[b]	δ_H[b]	δ_C	δ_H	δ_C	δ_H
A-1	1	176.53	—	176.47	—	175.98	—
	2	34.56	2.56	34.57	2.55	41.92	2.37
	3	19.03	1.13[c]	19.15	1.12	27.30	1.42,1.65
	4	19.03	1.15[c]	19.15	1.12	11.85	0.90
	5	—	—	—	—	16.82	1.11
A-2	1	176.31	—	175.85	—	174.94	—
	2	34.73	2.61	41.63	2.44	43.77	2.25
	3	19.52	1.19	27.60	1.53,1.70	26.37	2.09
	4	19.52	1.19	11.82	0.97	22.57	0.99
	5	—	—	17.08	1.15	22.57	0.99
A-3	1	175.46	—	175.49	—	175.44	—
	2	48.40	2.49	48.48	2.47	48.41	2.49
	3	69.63	3.96	69.48	3.92	69.45	3.93
	4	20.52	1.15	20.60	1.14	20.58	1.14
	5	13.25	1.10	13.39	1.09	13.37	1.10

[a]No chemical shift data are listed for the 11-hydroxypalmitic acid fragment since the observed ^{13}C- and ^{1}H-nmr chemical shifts for the different compounds were identical within 0.05 ppm for ^{13}C and 0.02 ppm for ^{1}H with each other and with the previously reported values for **2** (2).

[b]Chemical shifts in δ (ppm) relative to internal $(CH_3)_4Si$.

[c]The CH_3protons showed a slight non-equivalence, reflecting the fact that the 2-methylpropanoic acid is bonded to a chiral carbon.

while HMBC spectra were used to assign the linkage sites within the tetrasaccharide core as well as the sites of esterification by short-chain fatty acids. The former were determined from observed three-bond ^{1}H-C-O-^{13}C-H correlations between different monosaccharide units while the latter were determined from three-bond ^{1}H-C-O-^{13}C=O correlations between sugar protons and ester carbonyl groups. The linkage sites within the tetrasaccharide core were confirmed by ROESY cross-peaks between the pairs of protons on the carbons forming the C-O-C linkages. ROESY spectra were more useful than NOESY spectra for these compounds since the molecular tumbling rates were such that nuclear Overhauser enhancements were near zero (2).

The structures of the three additional compounds [**3–5**] (in order of increasing retention time) were also determined. They differ from **2** only in the identities of the short-chain fatty acids A_1 and A_2. ^{13}C- and ^{1}H-nmr spectral data for **3–5** are given in Tables 1 and 2. No data are reported for **2** since the chemical shifts for this compound were identical within experimental error to those previously reported (2). There is one earlier report of the isolation of two compounds with the same glycosidic acid as **1** from *Convolvulus scanononia* (3). However, neither of these compounds was identical to any of compounds **2–5**. Both had 2-methylbutanoic acid attached to C-2 of the rhamnose unit (as observed in **2** and **4**) but with no ester linkage at C-6 of glucose and with either no ester linkage or a tiglic acid ester at C-4 of the terminal quinovose unit.

Because compounds **2–5** were isolated in very limited (≤10 mg) amounts with **3–5** being no more than 90% pure, we decided to carry out preliminary screening tests for cytotoxic and antibacterial activity on the more abundant crude extract from which these compounds were

isolated, because the effort to obtain larger amounts of purified individual compounds for detailed testing could only be justified if the crude extract showed promising activity.

The less polar oligosaccharide extract (corresponding to a roughly 35:10:35:20 mixture of compounds **2**–**5**) was subjected to a cytotoxic bioscreening assay using cultured cancer cells representing nasopharyngeal carcinoma (KB), colon carcinoma (HCT-15), and squamous cell cervix carcinoma (SQC-1 UISO). Determined ED_{50} values using this extract were 1.5, 1.5, and 3.2 μg/ml for KB, HCT-15, and SQC-1 UISO, respectively. According to NCI guidelines, pure compounds with ED_{50} values ≤ 4 μg/ml are considered active (4). For purposes of comparison, the same tests were also carried out using the less abundant, more polar polysaccharide extract. In that case, ED_{50} values were 2.0, 25.1, and 16.2 μg/ml for KB, HCT-15, and SQC-1 USIO, respectively. It is known that the more polar extract is composed of two or more oligosaccharides with the same basic structures, **1**, but with two rather than three short-chain fatty acids (2). Thus, the two mixtures showed surprisingly large differences in cytotoxicity in spite of only minor structural differences. Because the less polar, more active fraction is known to contain four or more compounds, this sensitivity to structure raises the possibility that at least one of the compounds in the less polar fraction may have even more potent activity. Thus, detailed screening of individual pure compounds is desirable.

In order to establish selective or generalized toxicity towards various cells and microorganisms, the possible antimicrobial and fungicidal properties of the major polysaccharide mixture were also evaluated using different bacteria grown in vitro, and *Candida albicans*, and two dermatophytes in culture (see Experimental). The lowest concentration which elicited complete suppression of microorganism growth (MIC value) was determined in each case. The two polysaccharide mixtures exhibited strong antibiotic activity only towards *Staphylococcus aureus* and *Bacillus subtilis* (with respective MIC values of 25 and 12.5 μg/ml for the two bacteria, compared to MIC values of 8 and 16 μg/ml using nystatin with the same bacteria) and neither showed significant antifungal activity against the dermatophytes tested. Essentially identical results were obtained using the minor polysaccharide mixture, suggesting that, unlike cytotoxic activity, the antibacterial activity is insensitive to minor differences in structure.

A recent publication has described the isolation and characterization of a structurally similar tetrasaccharide, tricolorin A, from *Ipomoea tricolor* having different monosaccharide units and other minor structural differences (5). This compound was cytotoxic against P-388 and human breast cancer cells (ED_{50} 2.2 μg/ml) and antibacterial against *S. aureus*. The observation of both cytotoxic and antibacterial activity in different tetrasaccharide derivatives emphasizes the value of carrying out more detailed structure-activity investigations for such derivatives.

Further work is planned to isolate as many pure compounds as possible from *I. stans*, and to test them individually for cytotoxic and antibacterial activity. In turn, this may suggest possible chemical modifications which would further enhance activity.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All measurements were carried out on a Varian Unity-500 spectrometer equipped with a 5-nm inverse detection probe. Portions (3–10 mg) of each sample were dissolved in ca. 0.75 ml of $(CD_3)_2CO$. COSY, HMQC, HMBC, and ROESY experiments were carried out using standard Varian software. COSY spectra were acquired in absolute value mode while TOCSY, ROESY, and HMQC spectra were acquired in phase-sensitive mode and HMBC was processed using mixed-mode processing (phase

sensitive along f_1, absolute value along f_2). Mixing times of 0.1 sec were used for TOCSY and ROESY experiments while HMBC experiments were optimized for 8 Hz coupling constants. A BIRD nulling delay of 0.25 sec was used for HMQC while J filters, BIRD pulses, and HMQC delays were all set corresponding to $J=155$ Hz.

PLANT MATERIAL.—Samples of *Ipomoea stans* were collected near km marker 93 on the Puebla-Orizaba highway, in the state of Puebla, Mexico. Botanical classification was carried out by Dr. M. Martinez, Instituto de Biologia, UNAM, and a voucher specimen (No. 2691) is on deposit at the IMSSM Herbarium in Mexico City.

EXTRACTION AND ISOLATION.—Details of the isolation and separation of the two oligosaccharide fractions are given in Ref. (2). Further separation of the less polar fraction was carried out with a Varian 9010 liquid chromatograph with a Varian variable uv detector model 9050 set at 220 nm. A semi-prep. (250×5 mm) MCH-10 column and a mixture of MeCN-H_2O (65:35) in isocratic mode were found to give a good separation at 30°. Elution of all components was complete within 55 min. Up to 1.3 mg of sample per injection could be used without losing resolution. Repeat injections were carried out until ca. 10 mg each of the two major components were collected, along with lesser amounts of the minor components.

BIOLOGICAL TESTING.—The KB, HCT-15, and SQC-1 UISO cell lines were maintained in RPMI culture medium with 10% fetal bovine serum (FBS). All cell lines were cultured at 37° in an atmosphere of 5% CO_2 in air (100% humidity). The cells at a log phase of their growth cycle were treated in triplicate at various concentrations of the compounds (0.5–100 μg/ml), and incubated for 72 h at 37° in a humidified atmosphere of 5% CO_2. The cell concentration was determined by protein analysis. Results were expressed as the dose that inhibits 50% control growth after the incubation period (ED_{50}). The values were estimated from a semi-log plot of the drug concentration (μg/ml) against the percent of viable cells. According to NCI guidelines described in the literature (4), pure compounds with ED_{50} values ≤4 μg/ml are considered active.

Antimicrobial screening studies were performed with cultures of *Escherichia coli* (ATCC 8937), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Shigella sonei* (ATCC 11060), and *Candida albicans* (ATCC 10231). The dermatophytes tested were *Trycophyton mentagrophytes* (NRRL 1942) and *Microsporum gypseum* (NRRL-A 2605). The bacteria were maintained in Trypticase soy agar (TSA), and the yeast and the dermatophytes on Sabourand's dextrose agar (SDA). The screening method was based on conventional disk assay procedures reported for the study of natural products with antibacterial activity and fungicidal action (6,7), using gentamicin and nystatin as reference standards. Observations were performed in duplicate and results expressed as the lowest concentration that elicited complete supression of microorganism growth (MIC values).

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LITERATURE CITED

1. J.L. Diaz, "Usos de las Plantas Medicinales de Mexico," Monografias Cientificas II, INEPLAM, Mexico, 1976, p. 329.
2. R.G. Enriquez, I. Leon, F. Perez, F. Walls, K.A. Carpenter, F.V. Puzzuoli, and W.F. Reynolds, *Can. J. Chem.*, **70**, (1992).
3. N. Noda, H. Kogitsu, T. Kawasake, and K. Mijahara, *Phytochemistry*, **29**, 3565 (1990).
4. R.I. Geran, N.H. Greenberg, M.M. McDonald, A.M. Schumacher, and B.J. Abbott, *Cancer Chemother. Rep.*, Part 3, 1 (1972).
5. R. Pereda-Miranda, R. Mata, A.L. Anaya, D.B.M. Wickramaratne, J.M. Pezzuto, and A.D. Kinghorn, *J. Nat. Prod.*, **56**, 571 (1993).
6. J.D. Paxton, in: "Methods in Plant Biochemistry, Vol. 6." Ed. by K. Hostettman, Academic Press, London, 1991, p. 33.
7. D.A. Vanden Berghe and A.J. Vlietinck, in: "Methods in Plant Biochemistry, Vol. 6." Ed. by K. Hostettman, Academic Press, London, 1991, p. 47.

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