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Bacterial resistance modifying tetrasaccharide agents from *Ipomoea murucoides*

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ABSTRACT

As part of an ongoing project to identify oligosaccharides which modulate bacterial multidrug resistance, the $CHCl_3$-soluble extract from flowers of a Mexican arborescent morning glory, *Ipomoea murucoides*, through preparative-scale recycling HPLC, yielded five lipophilic tetrasaccharide inhibitors of *Staphylococcus aureus* multidrug efflux pumps, murucoidins XII–XVI (**1**–**5**). The macrocyclic lactone-type structures for these linear hetero-tetraglycoside derivatives of jalapinolic acid were established by spectroscopic methods. These compounds were tested for in vitro antibacterial and resistance modifying activity against strains of *Staphylococcus aureus* possessing multidrug resistance efflux mechanisms. Only murucoidin XIV (**3**) displayed antimicrobial activity against SA-1199B (MIC 32 μg/ml), a norfloxacin-resistant strain that over-expresses the NorA MDR efflux pump. The four microbiologically inactive (MIC > 512 μg/ml) tetrasaccharides increased norfloxacin susceptibility of this strain by 4-fold (8 μg/ml from 32 μg/ml) at concentrations of 25 μg/ml, while murucoidin XIV (**3**) exerted the same potentiation effect at a concentration of 5 μg/ml.

1. Introduction

All Mexican medicinal arborescent members of the genus *Ipomoea* (Convolvulaceae) share two therapeutic properties: the raw flowers, used antiseptically, are rubbed directly on skin infections, itching and rashes, and as decoctions, plasters and poultices, and in some instances the leaves, stem and bark are used for rheumatism, inflammation, and muscular pain (Chérigo and Pereda-Miranda, 2006). "Cazahuate", Nahuatl (Aztec language) for "tree to cure mange", is the vernacular name in contemporary Mexican Spanish for this group of arborescent species belonging to the genus *Ipomoea* series Arborescentes, e.g., *Ipomoea murucoides* Roem. et Schult. In the 16th century account of Mexican pre-Hispanic herbolaria "De la Cruz-Badiano Codex" (Emmart, 1940), the antiseptic properties for this medicinal plant complex are confirmed in a description of how Aztec healers used this herbal drug to prevent hair loss.

Murucoidins, a series of related lipophilic pentasaccharides of jalapinolic acid, were first reported in the chemical analysis of resin glycosides from this crude drug (Chérigo and Pereda-Miranda, 2006). A second investigation followed for the identification of new resin glycoside inhibitors of bacterial growth and in some instances inhibitors of multidrug efflux to treat infections resulting from multidrug-resistant *S. aureus* strains. All tested murucoidins exerted a potentiation effect of norfloxacin against the NorA over-expressing strain SA-1199B by increasing the activity 4-fold (8 μg/ml from 32 μg/ml) at concentrations of 5–25 μg/ml (Chérigo et al., 2008). This work was undertaken to increase the recognition of chemical diversity of the target oligosaccharides which modulate bacterial multidrug resistance, basically by isolating compounds from a new plant collection of *I. murucoides* that displayed variations in its resin glycoside composition.

2. Results and discussions

$CHCl_3$-soluble extracts of a new collection of the crude drug "Cazahuate" were compared by C_{18} reversed-phase HPLC with reference solutions of the previously reported resin glycosides from this species (Chérigo et al., 2008). This analysis confirmed a higher complexity in their composition and allowed the detection of the known pentasaccharides stoloniferin I, pescaprein III, intrapilosin I, and murucoidins I–V, as well as five new constituents, murucoidins XII–XVI (**1**–**5**) which were separated and purified by using a recycling HPLC technique (Pereda-Miranda and Hernández-Carlos, 2002). Several NMR techniques and FABMS were used to characterize their structures which were found to be macrolactones of the known operculinic acids C and E, linear hetero-tetraglycosides of jalapinolic acid, with *n*-dodecanoic or (2*S*)-methylbutyric acids esterifying the C-2 or C-3 positions on the second rhamnose unit of the oligosaccharide core and (2*S*)-methylbutyric acid at C-4 on the third rhamnose moiety.

A small portion of the glycosidic mixture was saponified to liberate an H_2O-soluble mixture of five oligosaccharides of jalapinolic acid: the major products were identified as operculinic acid A and

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simonic acids A and B by HPLC retention time comparison with those of authentic samples (Chérigo et al., 2008). Two additional glycosidic acids represented minor constituents and were characterized as operculinic acid C: (11*S*)-hydroxyhexadecanoate 11-*O*-α-L-rhamnopyranosyl-(1 → 4)-*O*-α-L-rhamnopyranosyl-(1 → 4)-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranoside, and operculinic acid E: (11*S*)-hydroxyhexadecanoate 11-*O*-α-L-rhamnopyranosyl-(1 → 4)-*O*-α-L-rhamnopyranosyl-(1 → 4)-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside, both previously isolated from *I. operculata* (Ono et al., 1989). Evidence for the absolute stereochemistry of the sugars, the configuration of the anomeric linkages as well as the sequence of glycosidation was published when their oligosaccharide cores were first elucidated (Ono et al., 1989, 1990). Sugar analysis confirmed that all monosaccharides were in ther naturally occurring form as previously described (Chérigo et al., 2008).

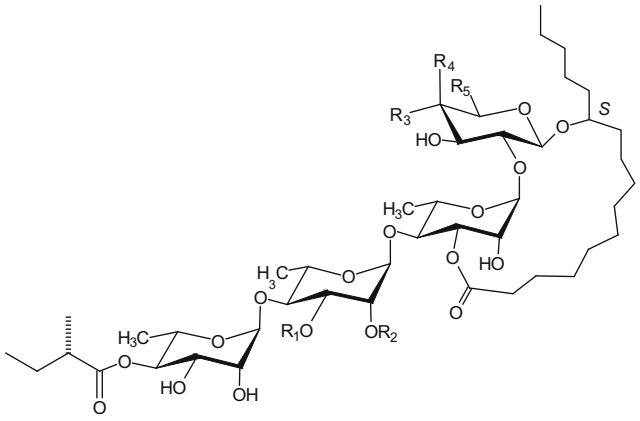
Negative-ion FAB mass spectra generated by murucoidins XII and XIII (**1–2**) were found to be very similar, with a pseudomolecular $[M–H]^-$ ion at *m/z* 1119, and therefore these constituents represented diastereoisomeric tetrasaccharides of molecular formula $C_{57}H_{100}O_{21}$. Compounds **3** afforded a FAB mass spectrum with the $[M–H]^-$ ion at *m/z* 1103 ($C_{57}H_{99}O_{20}$). Careful analysis of the negative FABMS generated by these tetrasaccharides **1–3** confirmed the nature of the oligosaccharide core for each glycolipid. The observed difference of 16 mass units in all diagnostic fragments for **3** in relation with **1** and **2** indicated the presence of a 6-deoxy-hexose (fucose) instead of the hexose unit (glucose). The initial loss of the dodecanoyl group afforded a peak at *m/z* 937 in **1** and **2** representing $[M–H–C_{12}H_{22}O]^-$, while the same elimination in **3** afforded the peak at *m/z* 921. The subsequent elimination of the methylbutyroyl unit (84 mass units) in **1** and **2** afforded a peak at *m/z* 853 $[937–C_5H_8O]^-$, and the peak *m/z* 837 in **3**. The ions produced by the rupture of each of the glycosidic linkages afforded the series of peaks at *m/z* 707, 561, 433 and 271 in **1** and **2** and at *m/z* 691 $[1067–2 \times 146\ (C_6H_{10}O_4)–C_5H_8O]^-$, 545 $[691–146\ (C_6H_{10}O_4)]^-$, which indicated that the lactonization was located at the first rhamnose unit (Rha), 417 $[545 + H_2O – 146\ (C_6H_{10}O_4)]^-$, and 271

Table 1
^{1}H (500 MHz) NMR spectroscopic data of compounds **1–5** (pyridine-d_5).[a]

Proton[b]	1	2	3	4	5
glc-1	4.99 *d* (8.0)	5.00 *d* (8.0)			
2	4.30* *dd* (8.0, 8.0)	4.30* *dd* (8.0, 8.0)			
3	4.30* *dd* (8.0, 8.0)	4.30* *dd* (8.0, 8.0)			
4	4.17 *dd* (9.0, 9.0)	4.16 *dd* (9.0, 9.0)			
5	3.90*	3.90 *ddd* (2.0, 5.5, 9.0)			
6a	4.38 *dd* (5.6, 12.0)	4.40* *m*			
6b	4.52 *dd* (2.8, 12,0)	4.52 *dd* (3.0, 11.7)			
fuc-1			4.79 *d* (7.5)	4.69 *d* (7.5)	4.73 *d* (7.5)
2			4.54*dd* (7.5, 9.5)	4.14 *dd* (7.5, 9.5)	4.14 *dd* (7.5, 9.5)
3			4.19 *dd* (3.0, 9.5)	4.00 *dd* (3.0, 9.5)	4.07 *dd* (3.0, 9.5)
4			3.92*	3.97 *m*	3.97 *brs*
5			3.82 *dq* (1.0, 6.5)	3.72 *dq* (1.0, 6.5)	3.77 *dq* (1.0, 6.5)
6			1.52 *d* (6.5)	1.50 *d* (6.5)	1.50 *d* (6.5)
rha-1	6.49 *d* (1.0)	6.51 *d* (1.5)	6.39 *d* (2.0)	5.49 *d* (2.0)	5.47 *d* (2.0)
2	5.28 *dd* (1.0, 3.0)	5.25 *dd* (1.5, 3.0)	5.25 *dd* (2.0, 3.0)	5.92 *dd* (2.0, 3.5)	5.90 *dd* (2.0, 3.5)
3	5.59 *dd* (3.0, 9.5)	5.69 *dd* (3.0, 9.5)	5.66 *dd* (2.5, 9.5)	4.99 *dd* (3.5, 9.5)	5.00 *dd* (3.5, 9.5)
4	4.63 *t* (9.5)	4.76 *t* (9.5, 9.5)	4.71 *t* (9.5)	4.20 *t* (9.5)	4.22 *t* (9.5)
5	5.08 *dq* (6.5, 9.5)	5.16 *dq* (6.5, 9.5)	5.08 *dq* (6.0,9.5)	4.45 *dq* (6.5, 9.5)	4.45 *dq* (9.5, 6.5)
6	1.75 *d* (6.5)	1.75 *d* (6.5)	1.59 *d* (6.0)	1.64 *d* (6.5)	1.63 *d* (6.5)
rha′-1	5.57 *d* (1.5)	5.91 *d* (1.5)	5.89 *d* (1.5)	6.01 *d* (2.0)	6.16 *d* (2.0)
2	5.78 *dd* (1.5, 3.5)	4.71 *dd* (1.5, 3.0)	4.70 *dd* (1.5, 3.0)	5.95 *dd* (2.0, 3.5)	4.87 *dd* (2.0, 3.0)
3	4.59 *dd* (3.5, 9.5)	5.72 *dd* (3.0, 9.5)	5.71 *dd* (3.0, 9.5)	4.66 *dd* (3.5, 9.5)	5.74 *dd* (3.0, 9.5)
4	4.26 *t* (9.5, 9.5)	4.57 *t* (9.5, 9.5)	4.56 *t* (9.5, 9.5)	4.24 *t* (9.5, 9.5)	4.58 *t* (9.5, 9.5)
5	4.37 *dq* (6.0, 9.5)	4.43 *dq* (6.5, 9.5)	4.40 *dq* (6.0, 9.5)	4.38 *dq* (6.5, 9.5)	4.42 *dq* (6.5, 9.5)
6	1.69 *d* (6.0)	1.61 *d* (6.5)	1.58 *d* (6.0)	1.71 *d* (6.5)	1.65 *d* (6.5)
rha″-1	6.16 *d* (1.5)	5.68 *d* (1.5)	5.68 *d* (1.5)	6.13 *d* (1.5)	5.75 *d* (1.5)
2	4.77 *dd* (1.5, 3.0)	4.47 *dd* (1.5, 3.0)	4.46 *dd* (1.5, 3.5)	4.81 *brs*	4.53 *brs*
3	4.48 *dd* (3.0, 9.5)	4.40* *m*	4.38 *dd* (3.5, 9.5)	4.55 *dd* (3.0, 9.5)	4.47 *dd* (3.0, 9.5)
4	5.80 *t* (9.5)	5.76 *t* (9.5)	5.75 *t* (9.5, 9.5)	5.83 *t* (9.5, 9.5)	5.79 *t* (9.5, 9.5)
5	4.41 *dq* (6.0, 9.5)	4.32 *dq* (6.5, 9.5)	4.30 *dq* (6.0, 9.5)	4.43 dq (6.5, 9.5)	4.34 *dq* (6.5, 9.5)
6	1.44 *d* (6.0)	1.36 *d* (6.5)	1.35 *d* (6.0)	1.44 *d* (6.5)	1.35 *d* (6.5)
jla-2a	2.24 *ddd* (3.0, 8.0, 11.7)	2.13 *ddd* (3.7, 7.8, 11.5)	2.14 *ddd* (3.5, 7.0, 12.0)	2.22 *ddd* (3.5, 7.0, 12.0)	2.21 *ddd* (3.7, 8.0, 11.5)
2b	2.72 *ddd* (3.0, 8.0, 11.7)	2.25 *ddd* (3.7, 7.8, 11.5)	2.23 *ddd* (3.5, 7.0, 12.0)	2.44 *ddd* (3.5, 7.0, 12.0)	2.35 *ddd* (3.7, 8.0, 11.5)
11	3.90*	3.96 *m*	3.92*	3.84 *m*	3.85 *m*
16	1.00 *t* (7.0)	0.99 *t* (7.0)	0.99 *t* (7.0)	0.88 *t* (7.0)	0.88 *t* (7.5)
mba-2	2.52 *tq* (7.0, 7.0)	2.48 *tq* (7.0, 7.0)	2.47 *tq* (7.0,7.0)	2.40 *tq* (7.0, 7.0)	2.45* tq (7.0, 7.0)
2-Me	1.22 *d* (7.0)	1.20 *d* (7.0)	1.20 *d* (7.0)	1.21 *d* (7.0)	1.19 *d* (7.0)
3-Me	0.95 *t* (7.5)	0.92 *t* (7.5)	0.92 *t* (7.5)	0.94 *t* (7.5)	0.91 *t* (7.5)
mba′-2				2.51 *tq* (7.0, 7.0)	2.45* *tq* (7.0,7.0)
2-Me				1.08 *d* (7.0)	1.13 *d* (7.0)
3-Me				0.85 *t* (7.5)	0.86 *t* (7.5)
dodeca-2a	2.27 *ddd* (7.5, 7.5, 15.0)	2.30 *ddd* (7.5, 7.5, 15.0)	2.27 *ddd* (7.0, 7.0, 15.0)		
2b	2.35 *ddd* (7.5, 7.5, 15.0)	2.39 *ddd* (7.5, 7.5, 15.0)	2.38 *ddd* (7.0, 7.0, 15.0)		
12	0.87 *t* (7.5)	0.87 *t* (7.0)	0.88 *t* (7.5)		

[a] Chemical shifts (δ) are in ppm relative to TMS. The spin coupling (*J*) is given in parentheses (Hz). Chemical shifts marked with an asterisk (*) indicate overlapped signals. Spin-coupled patterns are designated as follows: *s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, *m* = multiplet, *brs* = broad singlet.
[b] Abbreviations: fuc = fucose; rha = rhamnose; glc = glucose, jal = 11-hydroxyhexadecanoyl; mba = methylbutanoyl, dodeca = dodecanoyl.

[417–146 ($C_6H_{10}O_4$)]$^-$ in **3**, as previously reported for related tetrasaccharides of the tricolorin series (Bah and Pereda-Miranda, 1996). Therefore, operculinic acid E represented the oligosaccharide core for compounds **1** and **2** and operculinic acid C for **3**. A similar diastereoisomerism was observed for murucoidins XV (**4**) and XVI (**5**). Both compounds were also macrolactone derivatives of operculinic acid C and displayed in the negative-ion FAB mass spectrum a pseudomolecular [M–H]$^-$ ion at *m/z* 1005, which allowed for the calculation of a molecular formula of $C_{50}H_{86}O_{20}$. The initial elimination of two methylbutyroyl units produced the peaks at *m/z* 921 [M–H–C_5H_8O]$^-$ and 837 [921–C_5H_8O]$^-$. The lactonization was also located at the first rhamnose unit (Rha) by the observed peak at *m/z* 545, as previously reported (Pereda-Miranda et al., 2005) for related pentasaccharides of the murucoidin (Chérigo and Pereda-Miranda, 2006) and pescaprein series (Pereda-Miranda et al., 2005).



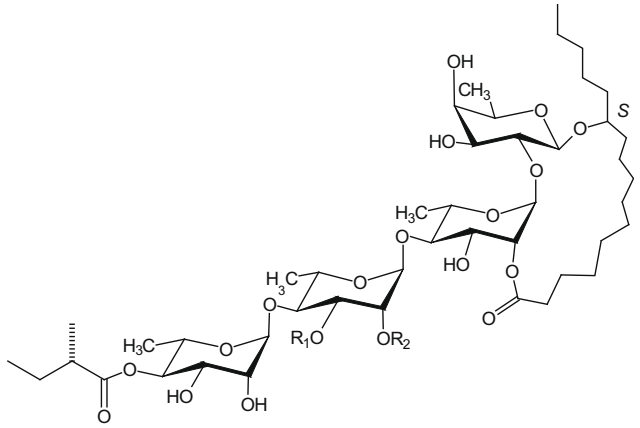
	R_1	R_2	R_3	R_4	R_5
1	H	*n*-dodecanoyl	OH	H	CH_2OH
2	*n*-dodecanoyl	H	OH	H	CH_2OH
3	*n*-dodecanoyl	H	H	OH	CH_3

Table 2
^{13}C (125 MHz) NMR spectroscopic data of compounds **1–5** (pyridine-d_5).[a]

Carbon[b]	1	2	3	4	5
glc-1	101.4	101.5			
2	75.1	75.0			
3	79.8	79.8			
4	72.0	72.0			
5	78.2	78.3			
6	62.8	62.8			
fuc-1			101.7	104.4	104.2
2			73.1	79.9	80.1
3			76.9	73.5	73.4
4			73.6	72.9	72.9
5			71.2	70.9	70.8
6			17.2	17.4	17.3
rha-1	100.2	100.3	100.3	98.6	98.5
2	69.5	69.6	69.7	73.7	73.9
3	77.9	78.9	78.9	69.9	70.2
4	79.3	76.2	76.1	80.8	80.9
5	67.9	67.6	67.4	68.7	68.7
6	19.2	19.5	19.3	19.3	19.3
rha′-1	100.7	102.6	102.5	100.2	103.3
2	74.3	70.4	70.4	73.9	69.8
3	70.8	75.6	75.5	70.7	75.8
4	80.8	78.4	78.3	80.8	78.1
5	68.5	69.1	69.0	68.6	68.7
6	18.7	18.5	18.5	18.9	18.8
rha″-1	103.7	103.7	103.7	103.8	103.2
2	72.3	72.7	72.6	72.4	72.7
3	70.2	70.1	70.0	70.3	70.1
4	75.1	74.8	74.7	75.2	74.7
5	68.0	68.2	68.1	68.1	68.1
6	17.9	17.9	17.8	18.0	17.8
jal-1	174.9	174.4	174.4	173.1	173.0
2	34.2	34.6	34.6	34.3	34.2
11	79.3	79.8	79.8	82.4	82.4
16	14.6	14.6	14.6	14.3	14.3
mba-1	176.3	176.3	176.3	175.9	176.0
2	41.6	41.5	41.5	41.6	41.5
2-Me	17.0	17.0	17.0	16.7	16.9
3-Me	11.8	11.8	11.7	11.6	11.7
mba'-1				176.4	176.3
2				41.3	41.5
2-Me				17.1	16.8
3-Me				11.8	11.6
dodeca-1	173.2	172.6	172.6		
2	34.4	34.5	34.4		
12	14.3	14.3	14.3		

[a] Chemical shifts (δ) are in ppm relative to TMS.
[b] Abbreviations: fuc = fucose; rha = rhamnose; glc = glucose, jal = 11-hydroxyhexadecanoyl; mba = methylbutanoyl; dodeca = dodecanoyl.

The ^{1}H NMR spectrum of these compounds showed signals of four anomeric protons, and when compared with the values previously reported for operculinic acids C and E (Ono et al., 1989), exhibited the acylation shifts for H-3 of Rha, H-2 or H-3 of Rha′, and H-4 of Rha″. HMBC studies using long-range heteronuclear coupling correlation ($^{2,3}J_{CH}$) provided evidence for the location of each ester substituent at the oligosaccharide core (Duus et al., 2000; Pereda-Miranda and Bah, 2003). Thus, the site of lactonization by the aglycone for compounds **1–3** ($\delta_{C\text{-}1}$ 174) could be placed at C-3 (δ_H 5.59–5.69) of the second saccharide (Rha), while for compounds **4** and **5**, the lactonization by the aglycone ($\delta_{C\text{-}1}$ 173) was placed at C-2 (δ_H 5.90) of the same sugar unit; a methylbutyroyl group ($\delta_{C\text{-}1}$ 176) was located at C-4 of Rha″ (δ_H 5.75–5.83) in all murucoidins; and the additional acyl substituent, dodecanoic acid ($\delta_{C\text{-}1}$ 173) in compounds **1–3**, or methylbutyric acid ($\delta_{C\text{-}1}$ 176) in compounds **4** and **5**, exhibited a $^3J_{CH}$ coupling with either H-2 (δ_H 5.72 in compound **1** and δ_H 5.95 in compound **4**) or H-3 (δ_H 5.71–5.74 in compounds **2**, **3**, and **5**) signal of Rha′.



	R_1	R_2
4	H	(2*S*)-methylbutanoyl
5	(2*S*)-methylbutanoyl	H

Table 3
Susceptibility of *Staphylococcus aureus* to murucoidins XII–XVI (**1**–**5**) and their cytotoxicity.[a]

Compound	ED_{50} (μg/ml)			MIC (μg/ml)				
	KB	Hep-2	HeLa	ATCC 25923	XU−212	EMRSA−15	SA−1199B[b]	
							Nor (−)	Nor (+)
1	17.1	>20	>20	>512	>512	>512	512	8
2	>20	>20	>20	>512	>512	>512	512	8
3	13.6	3.6	16.0	>512	>512	>512	32	8[c]
4	19.0	16.4	15.5	>512	>512	>512	512	8
5	14.1	>20	15.9	>512	>512	>512	512	8
Tetracycline	–	–	–	0.125	64	0.125	0.0.25	–
Norfloxacin	–	–	–	0.5	8	0.25	–	32
Reserpine	–	–	–	–	–	–	–	8[d]
Vinblastine	0.003	0.007	0.008	–	–	–	–	–

[a] Abbreviations: KB = nasopharyngeal carcinoma; Hep-2 = laryngeal carcinoma; HeLa = cervix carcinoma; ATCC 25923 = standard *S. aureus* strain; EMRSA-15 = epidemic methicillin-resistant *S. aureus* strain containing the *mecA* gene; XU-212 = a methicillin-resistant *S. aureus* strain possessing the TetK tetracycline efflux protein; SA-1199B = multidrug-resistant *S. aureus* strain over-expressing the NorA efflux pump.
[b] Nor (−) = minimum inhibitory concentration (MIC) value determined in the susceptibility testing; Nor (+) = MIC value determined for norfloxacin in the modulation assay at the concentration of 25 μg/ml of the tested oligosaccharide.
[c] MIC value for norfloxacin in the modulation assay at the concentration of 5 μg/ml of the tested oligosaccharide.
[d] MIC value for norfloxacin in the modulation assay at the concentration of 20 μg/ml of reserpine which was used as positive control for an efflux pump inhibitor.

Only murucoidins XIV (**3**) displayed antimicrobial activity at the concentration tested against SA-1199B (MIC 8 μg/ml), a *Staphylococcus aureus* strain that over-expresses the NorA MDR efflux pump. All of the murucoidins strongly potentiated the action of norfloxacin against this NorA over-expressing strain (Gibbons et al., 2003; Oluwatuyi et al., 2004) in experiments using a sub-inhibitory concentration of these oligosaccharides (Table 3). Compounds **1–5** exerted a potentiation effect which increased the activity of norfloxacin by 4-fold (8 μg/ml; from 32 μg/ml) at concentrations of 5–25 μg/ml. These increments in norfloxacin susceptibility were similar to those observed for the orizabins, tetrasaccharides from the resin glycoside mixture of Mexican scammony (*Ipomoea orizabensis*), and the lipophilic pentasaccharides previously isolated from *I. murucoides* (Chérigo et al., 2008; Pereda-Miranda et al., 2006). These compounds are amphiphilic with very similar logP values and they could cause non-specific membrane disruption, however if this was the case, all of these molecules would be active and the norfloxacin modulating activity could not be observed.

3. Concluding remarks

This is the first report of the presence of tetraglycosidic lactones of jalapinolic acid in a tree-like morning glory species since the section Arborescentes is characterized by the presence of pentasaccharides (Chérigo and Pereda-Miranda, 2006; Bah et al., 2007). The observed variation in the resin glycoside composition of *I. murucoides* flowers seems to depend on the geographical distribution of this species and deserves further studies.

The potential use as therapeutic agents of this class of bacterial resistance modifiers for new efflux pump inhibitors is under investigation in view of the fact that by combining these plant non-cytotoxic products with commercial antibiotics could facilitate the reintroduction of ineffective antibiotics into clinical use for the treatment of refractive infections caused by multidrug-resistant *S. aureus* strains.

4. Experimental

4.1. General experimental procedures

All melting points were determined on a Fisher–Johns apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer model 241 polarimeter. ^1H (500 MHz) and ^{13}C (125.7 MHz) NMR experiments were conducted on a Bruker DMX-500 instrument. The NMR techniques were performed in accordance with previously described methodology (Bah and Pereda-Miranda, 1996). Negative-ion low and high-resolution FAB–MS were recorded using a matrix of triethanolamine on a JEOL SX-102A spectrometer. The instrumentation used for HPLC analysis consisted of a Waters (Millipore Corp., Waters Chromatography Division, Milford, MA) 600 E multisolvent delivery system equipped with a refractive index detector (Waters 410).

4.2. Plant material

Flowers of *I. murucoides* were collected at Tepostlán, Morelos, Mexico, on April 15, 2001. The plant material was identified by Dr. Robert Bye and one of the authors (R.P.-M.) through comparison with an authentic plant sample (RP-05) archived at the Departamento de Farmacia, Facultad de Química, UNAM (Chérigo y Pereda-Miranda, 2006). A voucher specimen (Robert Bye 35906) was deposited in the Ethnobotanical Collection of the National Herbarium (MEXUE), Instituto de Biología, UNAM.

4.3. Extraction and isolation of compounds *1–5*

The whole plant material (500 g) was powdered and extracted exhaustively by maceration at room temperature with $CHCl_3$ to afford, after removal of the solvent, a dark brown syrup (35.8 g). The crude extract was subjected to column chromatography over silica gel (500 g) using gradients of CH_2Cl_2 in hexane (1:1 and 1:0), Me_2CO in CH_2Cl_2 (1:9 and 3:7), and MeOH in Me_2CO–CH_2Cl_2 (0.5:2.5:7, 1:2:7, 2:1:7). A total of 250 fractions (200 ml each) were collected, examined by TLC and combined in eight fractions. Fraction IV (eluates 122–126; MeOH–Me_2CO–CH_2Cl_2, 1:2:7) and fraction V (eluates 127–132; MeOH–Me_2CO–CH_2Cl_2, 2:1:7) were combined to give a pool containing a mixture of resin glycosides (18 g) which was subjected to fractionation by open column chromatography over reversed-phase C_{18} (150 g) eluted with MeOH. This process provided 40 secondary fractions (25 ml each). The subfraction 14–18 yielded a mixture of lipophilic resin glycosides (9.6 g) which were analyzed by reversed-phase HPLC using an isocratic elution with CH_3CN–MeOH (9:1). For their resolution, a Waters Symmetry C_{18} column (300 × 19 mm), a flow rate of 9 ml/min, and a refractive index detector were used. Peaks with t_R values of 7.0 (28 mg, peak I), 7.8 (18.6 mg, peak II), 12.6 min (26 mg, peak III), 13.4 (16.5 mg, peak IV), 14.0 min (25.8 mg, peak V), 16.5 (18 mg, peak VI), 19.5 min (20 mg, peak VII), 22.0

(21.3 mg, peak VIII), 23.0 min (29 mg, peak IX), 24.9 (35.6 mg, peak X), and 30.5 (60 mg, peak XI) were collected by the technique of heart cutting and independently re-injected in the apparatus operating in the recycle mode to achieve total homogeneity after 15 consecutive cycles. An isocratic elution with CH_3CN–MeOH (7:3) was used for those peak with t_R values higher to 15 min. These techniques afforded pure murucoidin XIII (**2**, 15 mg) from peak III, murucoidin XII (**1**, 14 mg) from peak V, murucoidin XIV (**3**, 6 mg) from peak VII, and murucoidin XVI (**5**, 12 mg) from peak IX. Peak XI afforded murucoidin IV (28 mg) and murucoidin XV (**4**, 16 mg). Co-elution experiments with reference solutions of previously reported resin glycosides allowed the detection of the following compounds: murucoidin V from peak I (13.5 mg), stoloniferin I (10.2 mg) from peak II, murucoidin I (8.4 mg) from peak IV, pescaprein III (7.3 mg) from peak VI, murucoidin II (9.1 mg) from peak VIII, and murucoidin III (15 mg) from peak X. All known compounds were identified by comparison of NMR data with published values (Bah et al., 2007; Chérigo y Pereda-Miranda, 2006; Pereda-Miranda et al., 2005).

4.4. Compound characterization

4.4.1. Murucoidin XII (1)

White powder; mp 100–104 °C; $[\alpha]_D$–78° (*c* 0.2, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2; negative FABMS *m/z* 1119 $[M–H]^-$, 937 $[M–H–C_{12}H_{22}O]^-$, 853 $[937–C_5H_8O]^-$, 707 $[853–C_6H_{10}O_4]^-$, 561, 433, 271; HRFABMS *m/z* 1119.6676 $[M–H]^-$ (calcd for $C_{57}H_{99}O_{21}$ requires 1119.6679).

4.4.2. Murucoidin XIII (2)

White powder; mp 96–98 °C; $[\alpha]_D$–67° (*c* 0.1, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2; negative FABMS *m/z* 1119 $[M–H]^-$, 937 $[M–H–C_{12}H_{22}O]^-$, 853 $[937–C_5H_8O]^-$, 707 $[853–C_6H_{10}O_4]^-$, 561 $[707–C_6H_{10}O_4]^-$, 433 $[561–128]^-$, 271; HRFABMS *m/z* 1119.6674 $[M–H]^-$ (calcd for $C_{57}H_{99}O_{21}$ requires 1119.6679).

4.4.3. Murucoidin XIV (3)

White powder; mp 125–127 °C; $[\alpha]_D$–60° (*c* 0.47, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2; negative FABMS *m/z* 1103 $[M–H]^-$, 921 $[M–H–C_{12}H_{22}O]^-$, 837 $[921–C_5H_8O]^-$, 691 $[837–C_6H_{10}O_4]^-$, 545 $[691–C_6H_{10}O_4]^-$, 417, 271; HRFABMS *m/z* 1103.6724 $[M–H]^-$ (calcd for $C_{57}H_{99}O_{20}$ requires 1103.6729).

4.4.4. Murucoidin XV (4)

White powder; mp 108–111 °C; $[\alpha]_D$–66° (*c* 0.60, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2; negative FABMS *m/z* 1005 $[M–H]^-$, 921 $[M–H–C_5H_8O]^-$, 837 $[921–C_5H_8O]^-$, 775 [921–146 $C_6H_{10}O_4]^-$, 691 $[775–C_5H_8O]^-$, 545 $[691–C_6H_{10}O_4–C_5H_8O]^-$, 417, 271; HRFABMS *m/z* 1005.5630 $[M–H]^-$ (calcd for $C_{50}H_{85}O_{20}$ requires 1005.5634).

4.4.5. Murucoidin XVI (5)

White powder; mp 125–127 °C; $[\alpha]_D$–29° (*c* 0.35, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2; negative FABMS *m/z* 1005 $[M–H]^-$, 921 $[M–H–C_5H_8O]^-$, 691 $[921–C_5H_8O–C_6H_{10}O_4]^-$, 545 $[691–C_6H_{10}O_4]^-$, 417, 271; HRFABMS *m/z* 1005.5629 $[M–H]^-$ (calcd for $C_{50}H_{85}O_{20}$ requires 1005.5634).

4.5. Alkaline hydrolysis of resin glycoside mixture

A solution of the crude resin glycoside mixture (100 mg) in 5% KOH–H_2O (5 ml) was heated at 95 °C for 4 h. The reaction mixture was acidified to pH 4.0 and extracted with $CHCl_3$ (30 ml). The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The aqueous phase was extracted with *n*-BuOH (20 ml) and concentrated to dryness. The residue extracted (42 mg) from the aqueous phase was methylated with CH_2N_2 to further perform the separation by HPLC of the methyl ester derivatives, using a Waters μBondapak NH_2 column (7.8 × 300 mm), an isocratic elution with CH_3CN–H_2O (5:1), a flow rate of 3 ml/min, and a sample injection of 500 μl (20 mg/ml). This procedure yielded operculinic acid C methyl ester (6.4 mg, t_R = 5.8 min), operculinic acid E methyl ester (8.6 mg, t_R = 10.9 min), simonic acid A methyl ester (4.3 mg, t_R = 14.0 min), operculinic acid A methyl ester (8.6 mg, t_R = 20.9 min), and simonic acid B methyl ester (14.7 mg, t_R = 28.2 min), which were identified by comparison of their physical constants and NMR data with published value (Ono et al., 1989, 1990).

The residue from the organic phase was analyzed by GC–MS to allow the detection of the major liberated esterifying residues which were identified as 2-methyl propanoic, 2-methyl butanoic, and *n*-dodecanoic acids by comparison of their retention times and spectra with those of authentic samples as previously described (Bah et al., 2007; Chérigo et al., 2008). Previously described procedures were used for the preparation and identification of 4-bromophenacyl (2*S*)-2-methylbutyrate from the resin glycoside mixture: mp 40–42 °C; $[\alpha]_D$ + 18 (*c* 1.0, MeOH) (Pereda-Miranda and Hernández-Carlos, 2002).

4.6. Biological assays

4.6.1. Bacterial strains and media

Staphylococcus aureus EMRSA-15 containing the *mecA* gene was provided by Dr. Paul Stapleton, The School of Pharmacy, University of London. Strain XU-212, a methicillin-resistant strain possessing the TetK tetracycline efflux protein, was provided by Dr. E. Udo (Gibbons and Udo, 2000). SA-1199B, which over-expresses the NorA MDR efflux protein (Kaatz et al., 1993), and *S. aureus* ATCC 25923 were also used. All strains were cultured on nutrient agar (Oxoid, Basingstoke, UK) before determination of MIC values. Cation-adjusted Mueller–Hinton broth (MHB; Oxoid) containing 20 and 10 mg/l of Ca^{2+} and Mg^{2+}, respectively was used for susceptibility tests.

4.6.2. Susceptibility testing

Minimum inhibitory concentration values (MIC) were determined at least in duplicate by standard microdilution procedures. An inoculum density of 5×10^5 cfu of each of the test strains was prepared in 0.9% saline by comparison with a McFarland standard. MHB (125 μl) was dispensed into 10 wells of a 96-well microtiter plate (Nunc, 0.3 ml volume per well). All test compounds were dissolved in DMSO before dilution into MHB for use in MIC determinations. The highest concentration of DMSO remaining after dilution (3.125% v/v) caused no inhibition of bacterial growth. Then, glycolipids **1–5** or appropriate antibiotic (125 μl) were dispensed into well 1 and serially diluted across the plate (512–1 μg/ml), leaving well 11 empty for the growth control. The final volume was dispensed into well 12, which being free of inoculum served as the sterile control. The inoculum (125 μl) was added into wells 1–11 and the plate was incubated at 36 °C for 18 h. The MIC was defined as the lowest concentration which yielded no visible growth. Tetracycline and norfloxacin from Sigma (Poole, UK) were also tested as positive drug controls. A methanolic solution (5 mg/ml) of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliium bromide (MTT; Lancaster) was used to detect bacterial growth by a color change from yellow to dark blue. For the modulation assay, the murucoidins were tested at final concentrations of 25 or 5 μg/ml. Serial doubling dilutions of norfloxacin ranging from 1 to 512 μg/ml were added and the microtitre plates were then interpreted, after inoculum addition and incubation, in the same manner as MIC determinations. The activity of reserpine at a con-

centration of 20 μg/ml was also tested as an efflux pump inhibitor for comparison purposes. All samples were tested in duplicate.

4.6.3. Cytotoxicity assay

Nasopharyngeal (KB), cervix (HeLa), and laryngeal carcinoma (Hep-2) cell lines were maintained in RMPI 1640 (10×) medium supplemented with 10% fetal bovine serum. Cell lines were cultured at 37 °C in an atmosphere of 5% CO_2 in air (100% humidity). The cells at log phase of their growth cycle were treated in triplicate with various concentrations of the test samples (0.16–20 μg/ml) and incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. The cell concentration was determined by the NCI sulforhodamine method (Skehan et al., 1990) Results were expressed as the dose that inhibits 50% control growth after the incubation period (EC_{50}). The values were estimated from a semilog plot of the drug concentration (μg/ml) against the percentage of viable cells. Vinblastine was included as a positive drug control.

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