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Characterization of a xylose containing oligosaccharide, an inhibitor of multidrug resistance in *Staphylococcus aureus*, from *Ipomoea pes-caprae*

Carolina Escobedo-Martínez [a], Sara Cruz-Morales [a], Mabel Fragoso-Serrano [a], M. Mukhlesur Rahman [b], Simon Gibbons [b], Rogelio Pereda-Miranda [a,*]

[a] Departamento de Farmacia, Facultad de Química, Universidad Nacional Autónoma de México, Ciudad Universitaria, Mexico City 04510, DF, Mexico
[b] Centre for Anti-infective Research, The School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, UK

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ABSTRACT

Pescaprein XVIII (**1**), a type of bacterial efflux pump inhibitor, was obtained from the $CHCl_3$-soluble resin glycosides of beach morning glory (*Ipomoea pes-caprae*). The glycosidation sequence for pescaproside C, the glycosidic acid core of the lipophilic macrolactone **1** containing D-xylose and L-rhamnose, was characterized by means of several NMR techniques and FAB mass spectrometry. Recycling HPLC also yielded eight non-cytotoxic bacterial resistance modifiers, the two pescapreins XIX (**2**) and XX (**3**) as well as the known murucoidin VI (**4**), pecapreins II (**6**) and III (**7**), and stoloniferins III (**5**), IX (**8**) and X (**9**), all of which contain simonic acid B as their oligosaccharide core. Compounds **1**–**9** were tested for *in vitro* antibacterial and resistance-modifying activity against strains of *Staphylococcus aureus* possessing multidrug resistance efflux mechanisms. All of the pescapreins potentiated the action of norfloxacin against the NorA over-expressing strain by 4-fold (8 μg/mL from 32 μg/mL) at a concentration of 25 μg/mL.

1. Introduction

The genus *Ipomoea* (Convolvulaceae) is a widely known source of complex resin glycosides possessing interesting biological effects (Pereda-Miranda and Bah, 2003; Pereda-Miranda et al., 2010). In Mexico, the herbal drug *Ipomoea pes-caprae* is called "riñonina" and comes from the Spanish word "riñón", which means kidney and reflects the belief of traditional healers that it moderates the "heat" of an infected kidney (Pereda-Miranda et al., 2005). Worldwide, *I. pes-caprae*, commonly known as railroad vine or beach morning glory, is used as an infusion for urinary or kidney complaints, hypertension, skin infections caused by *Mycobacterium tuberculosis*, and in decoctions to treat functional digestive disorders, internal pain, colic, lumbago, dysentery, arthritis, rheumatism, and other inflammatory conditions (De Souza et al., 2000; Pongprayoon et al., 1992).

Previous to this investigation, seven pentasaccharides (pescapreins I–IV and VII–IX) and two tetrasaccharides (pescapreins V and VI) of jalapinolic acid were first reported as a result of the chemical analysis of an herbal store sample of this crude drug (Pereda-Miranda et al., 2005; Escobedo-Martínez and Pereda-Miranda, 2007). A Chinese investigation using wild root samples collected in the Hainan province yielded eight new pentasaccharides, pescaprein X–XVII (Tao et al., 2008). Locally, it is used as an oral decoction to cure rubella as well as to alleviate jellyfish-sting pruritus and applied externally to treat pain and bedsores. The above mentioned investigations have proved that the presence of congeners among the pescaprein series is a result of variations in the type of acylating groups at C-2 of the third saccharide unit (Rha′), and C-2, C-3 and C-4 of the fifth saccharide (Rha‴) in the oligosaccharide core (simonic acid B). It was also reported that this diastereoisomerism at positions C-2 or C-3 could be a consequence of a transesterification via an *ortho*-acid ester intermediate in slightly acidic and neutral aqueous solution (Tao et al., 2008).

Convolvulaceous oligosaccharides have been shown to exert a potentiation effect of norfloxacin against the NorA over-expressing *Staphylococcus aureus* strain SA-1199B (Chérigo et al., 2008, 2009; Pereda-Miranda et al., 2006b). They have demonstrated good activity at low concentrations (10 mM or less) being more active than reserpine, a known efflux pump inhibitor used as a reference control. Therefore, they could be further developed to provide more potent inhibitors of the NorA multidrug efflux pump (Stavri et al., 2007). Departing from this point, the present work was undertaken to emphasize the chemical diversity among the pescaprein series, which indicates their potential to further explore resistance-modifying activity against *S. aureus*. A wild plant collection was used for this investigation because it showed differences in its resin glycoside composition from that of a commercial sample

* Corresponding author. Tel.: +52 55 5622 5288; fax: +52 55 5622 5329.
E-mail address: pereda@unam.mx (R. Pereda-Miranda).

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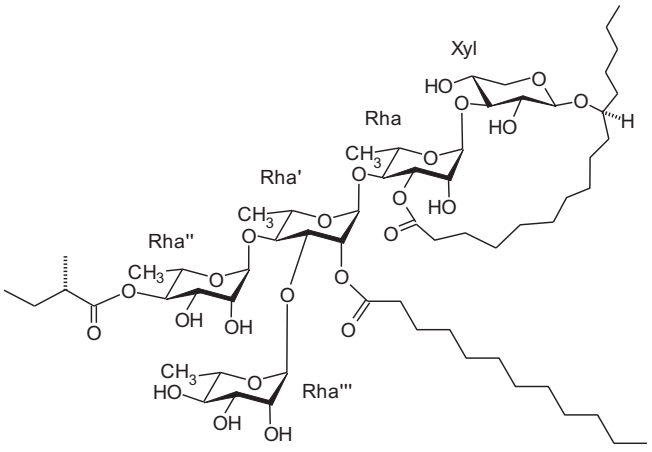
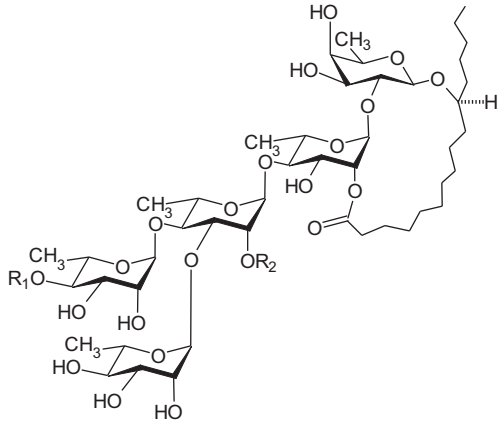
previously analyzed (Escobedo-Martínez and Pereda-Miranda, 2007; Pereda-Miranda et al., 2005). HPLC isolation of nine pentasaccharides (**1**–**9**) from the $CHCl_3$-soluble resin glycoside mixture is described herein. Several NMR techniques and FABMS were used to characterize the glycosidation sequence of compound **1**, a novel glycosidic acid from the Convolvulaceae family and trivially named pescaproside C.



1



	R_1	R_2
2	methylpropanoyl = iba	*n*-dodecanoyl = dodeca
3	*n*-hexanoyl = hexa	dodeca
8	(2*S*)-methylbutanoyl = mba	*n*-decanoyl = deca
9	mba	dodeca

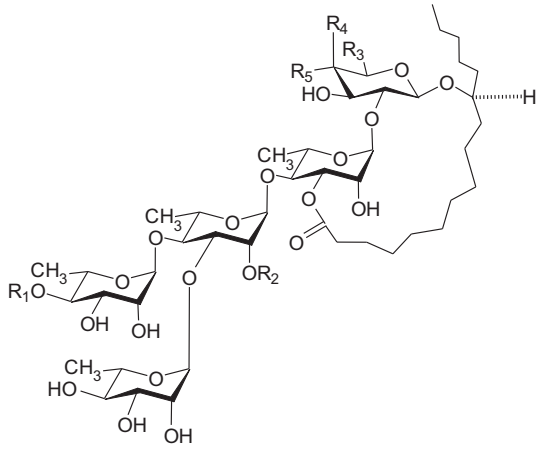
2. Results and discussion

$CHCl_3$-soluble extracts of the crude drug "riñonina" were fractionated by column chromatography on silica gel. The major fractions, rich in low-polarity resin glycosides, were purified by recycling preparative-scale HPLC (Pereda-Miranda and Hernández-Carlos, 2002), resulting in the isolation of nine glycolipids (**1**–**9**). These compounds showed similar ^1H and ^{13}C NMR spectra and displayed diagnostic signals common for the pescaprein series (Escobedo-Martínez and Pereda-Miranda, 2007; Pereda-Miranda et al., 2005). All compounds displayed the same negative-ion FABMS fragmentation pattern previously described for related resin glycosides, and the resulting diagnostic peaks were useful to confirm the nature of each of their individual pentasaccharide cores (Escobedo-Martínez and Pereda-Miranda, 2007; Pereda-Miranda et al., 2005). For example, compound **1** afforded a pseudomolecular ion $[M-H]^-$ at *m/z* 1235 ($C_{62}H_{107}O_{24}$) in contrast to the ion at *m/z* 1249 for its homologue **7** (pescaprein III), indicating a difference of one methylene group between these compounds which resulted from the presence of the aldopentose xylose instead of a methylpentose, e.g., fucose (Zhou et al., 2007). This 14 mass unit difference was also observed for all common fragment peaks produced by glycosidic cleavage of each sugar moiety at *m/z* 1005, 823, 531, and 403 in compound **1**. This compound exhibited the initial loss of two esterifying groups at *m/z* 1151 $[M-H-C_5H_8O]^-$ and 1053 $[M-H-C_{12}H_{22}O]^-$ which represented the elimination of one unit of α-methylbutyric acid and one of dodecanoic acid, respectively (Chérigo et al., 2008; Escobedo-Martínez and Pereda-Miranda, 2007; Pereda-Miranda et al., 2005).

In the new pescapreins XVIII–XX (**1**–**3**), COSY, TOCSY, and HSQC techniques were used to assign the important ^1H and ^{13}C chemical shifts of each sugar unit (Tables 1 and 2). ROESY and HMBC correlations completed the linkage within the pentasaccharide cores (Duus et al., 2000; Pereda-Miranda et al., 2010). For example, the carbon signals at δ_C 102.1, 75.3, 79.3, 71.5 and 67.2 in compound **1** were assigned to C-1, C-2, C-3, C-4, and C-5 of the xylose, respectively (Zhou et al., 2007). The observed interaction between C-5 (CH_2, δ_C 67.2) and its two attached hydrogens (δ_H 3.65 and 4.30) was the critical HMQC correlation for the assignment of this aldopentose. The ^1H NMR coupling constant for the anomeric proton H-1 ($^3J_{1,2}$ = 7 Hz) was consistent with the β-configuration for D-xylose in the $^4C_1$ conformation (Pereda-Miranda and Bah, 2003). The long-range correlation between H-1 (δ_H 4.89) of this saccharide and C-11 (δ_C 79.5) of the lipidic aglycone in the HMBC spectrum indicated that this unit was the first in the oligosaccharide core. Additional long-range heteronuclear coupling correlations ($^3J_{CH}$) were observed between the following proton and carbon signals: H-1 (δ_H 6.47) of the second saccharide (Rha) and C-3 (δ_C 79.3) of xylose; H-1 (δ_H 5.68) of the second rhamnose (Rha′) and C-4 (δ_C 78.2) of the first rhamnose (Rha); H-1 (δ_H 6.47) of the fourth saccharide (Rha‴) and C-3 (δ_C 80.3) of second rhamnose (Rha′); and H-1 (δ_H 6.47) of the fifth saccharide (Rha″) and C-4 (δ_C 79.8) of second rhamnose (Rha′). The anomeric α configuration in all the L-rhamnose units was deduced from a 2D $^1J_{CH}$ NMR experiment (Duus et al., 2000). These coupling constant values ($^1J_{CH}$ = 170–172 Hz) were 10 Hz higher than that registered for D-xylose ($^1J_{CH}$ = 160 Hz). In conclusion, this new oligosaccharide core for **1**, named pescaproside C, consisted of D-xylose as the first monosaccharide instead of a D-fucose as found in simonic acid B for compounds **2–9** (Escobedo-Martínez and Pereda-Miranda, 2007; Pereda-Miranda et al., 2005). Its structure was determined to be (11*S*)-jalapinolic acid 11-*O*-α-L-rhamnopyranosyl-(1 → 3)-*O*-[α-L-rhamnopyranosyl-(1 → 4)]-*O*-α-L-rhamnopyranosyl-(1 → 4)-*O*-α-L-rhamnopyran- osyl-(1 → 3)-*O*-β-D-xylopyranoside.

Three acylation sites were identified at H-3 of Rha, H-2 of Rha′ and H-4 of Rha″ in the ^1H NMR spectrum of **1**. HMBC studies using long-range heteronuclear coupling correlation located the acylating substituents through the observed connectivities between a specific carbonyl ester group with their vicinal proton resonance ($^2J_{CH}$), in addition to the pyranose ring proton at the site of the esterification ($^3J_{CH}$). Lactonization by the aglycone ($\delta_{C\text{-}1}$ 174.9) was placed at C-3 of the second saccharide (Rha) (δ_H 5.61). The methylbutyroyl group ($\delta_{C\text{-}1}$ 176.3) was located at C-4 of Rha″ (δ_H 5.78); and the additional acyl substituent (*n*-dodecanoic acid; $\delta_{C\text{-}1}$ 173.2) exhibited a $^3J_{CH}$ coupling with the H-2 signal of Rha′ (δ_H 5.84).

The molecular formula calculated from the mass spectra for pescaprein XIX (**2**; $C_{62}H_{107}O_{24}$) and that of pescaprein XX (**3**; $C_{64}H_{111}O_{24}$) indicated that both were diastereosiomers of the known pescaprein II (**6**) and pescaprein IV (**10**), respectively, where by means of 2D NMR analysis the lactonization position was found to be at C-2 of the second saccharide unit instead of at C-3



	R_1	R_2	R_3	R_4	R_5
4	mba	dodeca	CH_2OH	H	OH
5	mba	deca	CH_3	OH	H
6	iba	dodeca	CH_3	OH	H
7	mba	dodeca	CH_3	OH	H
10	hexa	dodeca	CH_3	OH	H

(Escobedo-Martínez and Pereda-Miranda, 2007; Pereda-Miranda et al., 2005).

In vitro antibacterial and resistance-modifying activity against strains of *Staphylococcus aureus* possessing multidrug resistance efflux mechanisms indicated that the non-cytotoxic pescapreins **1**–**9** potentiated the action of norfloxacin against the NorA overexpressing strain. They exerted an effect which increased the activity of norfloxacin 4-fold (8 μg/mL from 32 μg/mL) at a concentration of 25 μg/mL (Table 3).

3. Concluding remarks

The novel glycosidation sequence of compound **1** is similar to that of simonic acid B, a commonly found glycosidic acid among the members of the morning glory family (Convolvulaceae), with the only difference being the presence of D-xylopyranose as the first monosaccharide in the oligosaccharide core instead of D-fucose (Pereda-Miranda et al., 2010). In this family, xylose has been reported three times: as part of the tetrasaccharides cuscutic acid B and operculinic acid F from *Cuscuta chinensis* (Du et al., 1998) and *I. operculata* (Noda et al., 1990), respectively, as well as in the pentasaccharide operculinic acid D from *I. operculata* (Ono et al., 1989).

In addition to the results previously reported for other lipophilic oligosaccharides, e.g., the murucoidins (Chérigo et al., 2008, 2009) and orizabins (Pereda-Miranda et al., 2006b), the susceptibility of *Staphylococcus aureus* to the non-cytotoxic pescapreins (**1**–**9**) seems to correlate to the acylation degree of the oligosaccharide core. The lipophilic properties would seem to be an important structural requirement to facilitate cellular uptake to its MDR pump target. Therefore, this type of amphipathic oligosaccharides could be further developed to provide more potent inhibitors of this multidrug efflux pump and facilitate the reintroduction of ineffective antibiotics into clinical use for the treatment of refractive infections.

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. Negative-ion LRFABMS and HRFABMS 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) 600E multisolvent delivery system equipped with a refractive index detector (Waters 410). GC–MS was performed on a Hewlett–Packard 5890-II instrument coupled to a JEOL

Table 1
^1H (500 MHz) NMR spectroscopic data of compounds **1**–**3** (pyridine-d_5).[a]

Proton[b]	**1**	2	3
fuc-1		4.73 d (7.5)	4.76 d (7.5)
2		4.16 dd (9.5, 7.5)	4.18 dd (9.4, 7.5)
3		4.07 dd (9.5, 2.5)	4.11 dd (9.4, 3.0)
4		3.99 d (2.5)	4.02 d (3.0)
5		3.77 q (6.5)	3.70 q (6.5)
6		1.51 d (6.5)	1.52 d (6.5)
xyl-1	4.89 d (8.0)		
2	4.25 dd (9.0, 8.0)		
3	4.30 dd (9.0, 8.5)		
4	4.12 ddd (10.5, 8.5, 5.5)		
5	3.65 t (10.8) 4.30 dd (11.0, 5.5)		
rha-1	6.47 d (1.5)	6.16 bs	6.20 d (1.5)
2	5.32 (2.5, 1.5)	6.00 dd (3.0, 1.5)	6.04 dd (3.0, 1.5)
3	5.61 dd (9.5, 2.5)	4.59 dd (9.0, 3.0)	4.61 dd (9.0, 3.0)
4	4.68 dd (9.5, 9.5)	4.31 dd (9.0, 9.0)	4.32 dd (8.5, 8.5)
5	5.07 dq (9.5, 6.0)	4.33–4.36*	4.35–4.41*
6	1.71 d (6.0)	1.65 d (6.0)	1.66 d (6.0)
rha′-1	5.68 d (1.5)	5.48 d (1.5)	5.50 (1.5)
2	5.84 dd (2.5, 1.5)	5.95 dd (3.0, 1.5)	5.99 dd (3.0, 1.5)
3	4.52 dd (9.5, 2.5)	5.01 dd (9.0, 3.0)	5.08 dd (9.0, 3.0)
4	4.23 dd (9.5, 9.5)	4.42–4.45*	4.45–4.50*
5	4.30 dq (9.5, 6.0)	4.21–4.24*	4.31–4.39
6	1.62 d (6.0)	1.61 d (6.0)	1.63 d (6.2)
rha″-1	5.93 bs	5.92 bs	5.97 bs
2	4.63 brs	4.68 brs	4.72 brs
3	4.42 dd (9.5, 3.5)	4.48 dd (9.5, 3.0)	4.53 dd (9.1, 3.5)
4	5.78 dd (9.5, 9.5)	5.78 dd (9.5, 9.5)	5.83 dd (9.6, 9.6)
5	4.36 dq (9.5, 6.0)	4.33–4.36*	4.35–4.41*
6	1.40 d (6.0)	1.38 d (6.0)	1.40 d (6.2)
rha‴-1	5.58 d (1.5)	5.60 d (2.0)	5.63 d (1.5)
2	4.80 brs	4.81 brs	4.86 brs
3	4.53 dd (9.0, 3.0)	4.42–4.45*	4.45–4.50*
4	4.25 dd (9.0, 9.0)	4.21–4.24*	4.31–4.39
5	4.28 dq (9.0, 6.5)	4.29 t (9.5)	4.32 t (9.0)
6	1.72 d (6.0)	1.60 d (6.0)	1.62 d (6.0)
jal-2a	2.26 ddd (15.0, 7.0, 3.0)	2.24 ddd (14.5, 8.0, 4.0)	2.25 ddd (14.0, 7.8, 4.0)
2b	2.95 t (12.0)	2.40 ddd (14.7, 8.5, 3.7)	2.41 ddd (14.5, 8.2, 4.2)
11	3.86 m	3.85 m	3.87 m
16	0.87 t (7.0)	0.87 t (7.5)	0.87 (7.0)
Iba-2		2.63 sept (7.0)	
3		1.16 d (7.0)	
3́		1.19 d (7.0)	
mba-2	2.50 tq (7.0, 6.5)		
2-Me	1.20 d (7.0)		
3-Me	0.94 t (7.5)		
hexa-2			2.32 t (7.5)
6			0.78 t (7.0)
dodeca-2	2.38 t (7.5)	2.30 t (7.5)	2.30 t (7.5)
12	0.93 t (7.0)	0.93 t (7.5)	0.93 t (7.3)

[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, bs = broad singlet; brs = broad signal, d = doublet, t = triplet, m = multiplet, q = quartet, sept = septet.

[b] Abbreviations: fuc = fucose; xyl = xylose; rha = rhamnose; jal = 11-hydroxyhexadecanoyl; iba = 2-methylpropanoyl; mba = 2-methylbutanoyl; hexa = hexanoyl; deca = decanoyl; dodeca = dodecanoyl.

Table 2
^{13}C (125 MHz) NMR spectroscopic data of compounds **1–3** (pyridine-d_5).[a]

Carbon[b]	1	2	3
fuc-1		104.4	104.3
2		80.2	80.2
3		73.3	73.3
4		73.0	73.0
5		70.8	70.8
6		17.4	17.4
xyl-1	102.1		
2	75.3		
3	79.3		
4	71.5		
5	67.2		
rha-1	100.4	99.1	99.2
2	69.7	73.2	73.2
3	77.8	79.9	79.9
4	78.2	68.4	68.5
5	68.1	70.8	70.5
6	18.2	18.8	18.8
rha′-1	99.2	98.8	98.8
2	73.0	73.9	73.9
3	80.3	79.9	80.0
4	79.8	79.8	79.6
5	67.2	68.6	68.6
6	18.8	18.6	18.6
rha″-1	103.7	103.6	103.6
2	72.7	72.7	72.7
3	70.2	72.6	72.6
4	74.8	74.9	74.8
5	68.3	68.2	68.2
6	17.0	17.9	17.9
rha‴-1	104.4	104.7	104.6
2	72.7	72.6	72.6
3	72.5	70.2	70.2
4	73.7	73.6	73.6
5	70.8	70.7	70.4
6	19.3	19.0	18.4
jal-1	174.9	173.0	173.1
2	33.7	34.2	34.3
11	79.5	82.4	82.3
16	14.3	14.3	14.3
iba-1		176.3	
2		41.5	
3		11.8	
3′		17.0	
mba-1	176.3		
2	41.6		
2-Me	11.8		
3-Me	17.0		
hexa-1			173.1
2			34.2
6			14.3
dodeca-1	173.0	172.9	173.0
2	34.4	34.5	34.2
12	14.4	14.3	14.3

[a] Chemical shifts (δ) are in ppm relative to TMS.
[b] Abbreviations: fuc = fucose; xyl = xylose ; rha = rhamnose; jal = 11-hydroxyhexadecanoyl; iba = 2-methylpropanoyl; mba = 2-methylbutanoyl; hexa = hexanoyl; deca = decanoyl; dodeca = dodecanoyl.

SX-102A spectrometer. GC conditions: HP-5MS (5%-phenyl)-methylpolysiloxane column (30 m × 0.25 mm, film thickness 0.25 μm); He, linear velocity 30 cm/s; 50 °C isothermal for 3 min, linear gradient to 300 °C at 20 °C/min; final temperature hold, 10 min. MS conditions: ionization energy, 70 eV; ion source temperature, 280 °C; interface temperature, 300 °C; scan speed, 2 scans s^{-1}; mass range, 33–880 amu.

4.2. Plant material

The herbal drug "riñonina" was collected in dunes along an upper beach in Las Salinas, Chamela Bay, Jalisco, Mexico in October 2005. A small sample (50 g) was archived at the Departamento de Farmacia, Facultad de Química, UNAM. Macroscopic anatomical features enable the drug to be identified by one of the authors (R.P.-M.) as *Ipomoea pes-caprae* through comparison with a voucher specimen collected at the same location by Dr. Robert Bye (voucher specimen R. Bye 17707) in November 1989 and deposited in the Ethnobotanical Collection of the National Herbarium (MEXU), Instituto de Biología, UNAM.

4.3. Extraction and isolation of compounds *1–9*

The whole plant (1 kg) was powdered and extracted exhaustively by maceration at room temperature with $CHCl_3$ to give, after removal of the solvent, a dark-green syrup (15.8 g). The total extract was absorbed on silica gel (25 g) and subjected to column chromatography (CC) over the same normal phase (630 g) with $CHCl_3$ in hexane (1:1 and 1:0), Me_2CO in $CHCl_3$ (1:9, 3:7, and 1:1), Me_2CO in MeOH (1:0 and 1:1) and MeOH. The composition of the 200 fractions obtained (250 mL each) was monitored by TLC (silica gel 60 F254 aluminum sheets, $CHCl_3$–MeOH, 4:1), and identical fractions were combined in eight pools of resin glycosides mixtures (fractions 102–185). These were compared by C_{18} reversed-phase HPLC (Waters column 5 μm, 4.6 × 250 mm) with reference solutions of the previously isolated resin glycosides from this species by an isocratic elution of CH_3CN-MeOH (9:1) at a 0.7 mL/min flow rate, and a sample injection of 10 μL (1 mg/mL). The analysis confirmed a higher complexity in the composition of the less lipophilic fractions 152–165 (pool VII) and 168–185 (pool VIII) eluted with MeOH and differing from that observed for the reference solutions (Escobedo-Martínez and Pereda-Miranda, 2007). The preparative HPLC separations were carried out using a Symmetry C_{18} column (Waters; 7 μm, 19 × 300 mm), using a flow rate of 9 mL/min. The elution was isocratic with CH_3CN–H_2O (4:1) and a sample injection of 500 μL (fraction concentration: 50 mg/mL). The crude fraction VII (150 mg) yielded four major peaks with t_R values of 19.11 (peak I, 16.4 mg), 21.89 (peak II, 14.0 mg), 33.85 (peak III, 30.4 mg), and 43.58 min (peak IV, 24.3 mg) which were collected by the technique of "heart cutting" and independently re-injected to be recycled in order to achieve total homogeneity after twenty consecutive cycles employing an isocratic elution of CH_3CN–MeOH (9:1) with a flow rate of 9 mL/min for all the peaks. Peak I afforded pure major compound **4** (9.7 mg). Peak II afforded compound **5** (7.2 mg). Peak III was split into two peaks during the recycling process to afford pure compounds **1** (14.3 mg) and **6** (12.3 mg). Peak IV yielded compound **7** (22.3 mg). Fraction VIII (200 mg) was subjected to preparative HPLC on the same C_{18} column using an elution with CH_3CN–MeOH (3:7). Eluates across the peaks with t_R values of 23.7 (**8**, 50 mg), 27.5 (**2**, 10 mg), 32.8 (**9**, 40 mg), and 36.8 min (**3**, 4 mg) were collected, independently re-injected, and recycled by 15 consecutive cycles to achieved total purification. All known compounds (**4–9**) were identified by comparison of NMR spectroscopic data with published values (Chérigo et al., 2008; Noda et al., 1994, 1998; Pereda-Miranda et al., 2005).

4.4. Compound characterization

4.4.1. Pescaprein XVIII (*1*)

White powder; mp 77–79 °C; $[\alpha]_D$ −42 (*c* 0.1 MeOH); for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; negative FABS *m/z* 1235 $[M-H]^-$, 1151 $[M-H-C_5H_8O]^-$, 1053 $[M-H-C_{12}H_{22}O]^-$, 1005 $[1151-C_6H_{10}O_4]^-$, 823 $[1053-C_6H_{10}O_4-C_5H_8O]^-$, 531[823–2 × 146 $(C_6H_{10}O_4)]^-$, 403 [531–128 $(C_6H_{10}O_4-H_2O]^-$, 271 $[Jal-H]^-$; HRFAB–MS *m/z*: 1235.7155 $[M-H]^-$ (calcd for $C_{62}H_{107}O_{24}$ requires 1235.7152).

4.4.2. Pescaprein XIX (*2*)

White powder; mp 121–123 °C; $[\alpha]_D$ −32 (*c* 0.5 MeOH); for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; negative FABS

Table 3
Susceptibility of *Staphylococcus aureus* to pescapreins XVIII–XX (**1**–**3**) and known compounds (**4**–**9**) and their cytotoxicity.[a]

Compound	ED_{50} (μg/mL)			MIC (μg/mL)				
	HCT-15	MCF-7	HeLa	ATCC 25923	XU-212	EMRSA-15	SA-1199B[b]	
							Nor (−)	Nor (+)
1	>20	>20	>20	>512	>512	>512	512	8
2	>20	>20	>20	>256	>256	>256	512	8
3	>20	>20	>20	>256	>256	>256	512	8
4	>20	>20	>20	>512	>512	>512	512	8
5	>20	>20	>20	>512	>512	>512	512	8
6	13.4	19.0	12.3	>512	>512	>512	512	8
7	10.0	>20	19.6	>512	>512	>512	512	8
8	>20	>20	>20	>256	>256	>256	512	8
9	>20	>20	>20	>256	>256	>256	512	8
Tetracycline	–	–	–	0.125	64	0.125	0.25	–
Norfloxacin	–	–	–	0.5	8	0.25	–	32
Reserpine	–	–	–	–	–	–	–	8[c]
Vinblastine	0.003	0.007	0.008	–	–	–	–	–

[a] Abbreviations: HCT-15 = colon carcinoma; MCF-7 = breast 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 20 μg/mL of reserpine which was used as positive control for an efflux pump inhibitor.

m/z 1235 $[M-H]^-$, 1165 $[M-H-C_4H_6O]^-$, 837 $[1165-C_{12}H_{22}O-C_6H_{10}O_4]^-$, 673, 545, 417, 271; HRFAB-MS *m/z*: 1235.7153 $[M-H]^-$ (calcd for $C_{62}H_{107}O_{24}$ requires 1235.7152).

4.4.3. Pescaprein XX (**3**)

White powder; mp 120–122 °C; $[\alpha]_D$ −60 (*c* 0.8 MeOH); for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; negative FABS *m/z* 1263 $[M-H]^-$, 1165 $[M-H-C_6H_{10}O]^-$, 1081 $[M-H-C_{12}H_{22}O]^-$, 837 $[1165-C_{12}H_{22}O-C_6H_{10}O_4]^-$, 673, 545, 417, 271; HRFAB-MS *m/z*: 1263.7452 $[M-H]^-$ (calcd for $C_{64}H_{111}O_{24}$ requires 1263.7465).

4.5. Sugar analysis

Compound **1** (8.5 mg) in 4 N HCl (5 mL) was heated at 90 °C for 2 h. The reaction mixture was diluted with H_2O (2.5 mL) and extracted with Et_2O (15 mL). The aqueous phase was neutralized with 1 N KOH, extracted with *n*-BuOH (20 mL), and concentrated to give a colorless solid. The residue was dissolved in CH_3CN–H_2O and directly analyzed by HPLC: Waters standard column for carbohydrate analysis (3.9 × 300 mm, 10 μm), using an isocratic elution of CH_3CN–H_2O (17:3), a flow rate of 1 mL/min, and a sample injection of 20 μL (sample concentration: 2 mg/mL). Co-elution experiments with standard carbohydrate samples allowed the identification of rhamnose (t_R = 6.9 min) and xylose (t_R = 8.3 min). Each of these eluates was individually collected, concentrated, and dissolved in H_2O. Optical activity was recorded after stirring the solutions for 3 h at room temperature and values were identical for those registered for commercially available samples: L-rhamnose $[\alpha]_D$ +8 (*c* 0.1, H_2O), control $[\alpha]_D$ +7.7 (*c* 0.1, H_2O); D-xylose $[\alpha]_D$ +19 (*c* 0.1, H_2O), control $[\alpha]_D$ +18.8 (*c* 0.1, H_2O). The organic phase was analyzed by GC–MS to allow the detection of two liberated esterifying residues which were identified as: 2-methylbutyric acid (t_R 5.1 min): *m/z* $[M]^+$ 102 (3), 87 (33), 74 (100), 57 (50), 41 (28), 39 (8); and *n*-dodecanoic acid (t_R 11.0 min): *m/z* $[M]^+$ 200 (15), 183 (2), 171 (18), 157 (40), 143 (10), 129 (48), 115 (20), 101 (15), 85 (33), 73 (100), 60 (80), 57 (30), 55 (47), 43 (44), 41 (30) by comparison of their retention times and spectra with those of authentic samples (Chérigo et al., 2008). Previously described methodology was used for the preparation and identification of 4-bromophenacyl (2*S*)-2-methylbutyrate: mp 40–42 °C; $[\alpha]_D$ +18 (*c* 1.0, MeOH) from the resin glycoside mixture pools VII and VIII (20 mg). This transesterification procedure has been used to confirm the absolute configuration for 2-methylbutyric acid (Bah et al., 2007).

4.6. Aglycon identification

A solution of the crude resin glycoside pools VII and VIII (10 mg each) in 5% KOH–H_2O (2.5 mL) was heated until reflux began (95 °C), this being maintained for 3 h. Each reaction mixture was acidified to pH 4.0 and extracted with $CHCl_3$ (20 mL). The aqueous phase was extracted with *n*-BuOH (20 mL), concentrated to dryness and submitted to the same acid-catalyzed hydrolysis described above for the sugar analysis. The organic phase-soluble product was methylated with CH_2N_2 to further perform its separations by HPLC from each of the two resin glycoside fraction as previously described to yield methyl (11*S*)-hydroxyhexadecanoate (jalapinolic acid methyl ester): t_R 16.4 min; mp 42–44 °C; $[\alpha]_D$ +7.3 (*c* 2, $CHCl_3$); ^{13}C NMR: 174.4, 72.0, 51.4, 37.5, 37.4, 34.1, 31.9, 29.6, 29.5, 29.4, 29.2, 29.1, 25.6, 25.3, 24.9, 22.6, 14.1. This aglycon (1 mg) was derivatized by treatment with Sigma Sil-A and analyzed by GC–MS analysis, (t_R 12.8 min): *m/z* $[M]^+$ 358 (0.3), 343 (0.5), 311 (10.5), 287 (59.7), 173 (100), 73 (46.3) (Pereda-Miranda et al., 2006a).

4.7. Biological assays

4.7.1. 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 Gibbons and Udo, 2000. SA-1199B, which over-expresses the NorA MDR efflux protein (Kaatz et al., 1993), was provided by Professor Glenn W. Kaatz and standard strain *S. aureus* ATCC 25923 was 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 Ca^{2+} (20 mg/L) and Mg^{2+} (10 mg/L), was used for susceptibility tests.

4.7.2. Susceptibility testing

Minimum inhibitory concentration values (MIC) were determined at least in duplicate by standard microdilution procedures, as recommended by the National Committee for Clinical

Laboratory Standards guidelines (1999). 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). Glycolipids **1–9** were tested at final concentrations ranging from 1 to 512 μg/mL prepared by serial twofold dilutions. All test compounds were dissolved in DMSO before dilution into MHB for use in MIC determinations. The inoculum (125 μL) was added into each well and the plate was incubated at 37 °C for 18 h. For the modulation assay, the pescapreins were tested at final concentrations of 25 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 as previously described (Pereda-Miranda et al., 2006b).

4.7.3. Cytotoxicity assay

Colon (HCT-15), cervix (HeLa), and breast carcinoma (MCF-7) 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.

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