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Glycolipid ester-type heterodimers from *Ipomoea tyrianthina* and their pharmacological activity

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ABSTRACT

Tyrianthins A (**1**) and B (**2**), two new partially acylated glycolipid ester-type heterodimers were isolated from *Ipomoea tyrianthina*. Scammonic acid A was determined as the glycosidic acid in both monomeric units. Tyrianthin A (**1**) (IC_{50} 0.24 ± 0.09 μM and E_{max} 81.80 ± 0.98%), and tyrianthin B (**2**) (IC_{50} 0.14 ± 0.08 μM and E_{max} 87.68 ± 0.72%) showed significant in vitro relaxant effect on aortic rat rings, in endothelium- and concentration-dependent manners. Also, these compounds were able to increase the release of GABA and glutamic acid in brain cortex, and displayed weak antimycobacterial activity.

Ipomoea tyrianthina Lindley (syn. *I. orizabensis* Pelletan, Lebed. ex Steud., Convolvulaceae) is a perennial twining herb with a large root. A decoction of the root of *I. tyrianthina* prepared with a small section of the root to a liter of water is drunk along the day to alleviate abdominal pains and as a purgative.[1] Intraperitoneal administration to mice of glycolipids from the dichloromethane-soluble extract of *I. tyrianthina* resulted in antidepressant activity, protective effects against pentylenetetrazole-induced seizures, and relaxant effects on spontaneous contractions in isolated rat ileum.[2] The chemical components of the resin glycosides isolated from this plant have been characterized as tetrasaccharides of 11-hydroxyhexadecanoic acid.[2–5]

In a continuing investigation on secondary metabolites with biological activity from *Ipomoea* species, we have studied the resin glycosidic content of the methanol-soluble extract from the root of *I. tyrianthina*. We report herein on the isolation and characterization of two new glycolipid ester type heterodimers, tyrianthins A (**1**) and B (**2**), as well as their in vitro pharmacological activities as vasorelaxant, release of GABA and glutamic acid, and antimycobacterial activity.

The methanol-soluble extract of *I. tyrianthina* was subjected to a series of chromatographic separations, leading to the separation of two chromatographic fractions. The less polar chromatographic fraction was subjected to preparative HPLC in the reversed-phase mode, resulting in the isolation of two new glycolipid ester-type heterodimers named tyrianthins A (**1**) and B (**2**).[6] These two compounds were hydrolyzed in an aqueous/ethanolic alkaline medium by separated, producing a water-soluble glycosidic acid derivative and an organic solvent-soluble acidic fraction.[7] The structure of the glycosidic acid was the same for both reaction products, and assigned as a tetrasasaccharide 11-hydroxyhexadecanoic acid, identified as scammonic acid A by comparison of physical and spectroscopic data, which has been previously identified in resin glycosides of *Convolvulus elongatus*,[8] *I. stans*,[9] and *I. orizabensis*.[2–5]

Compound **1** (Fig. 1) was isolated as a white amorphous solid, with negative optical rotation $[\alpha]_D^{25}$ −22.0. The IR spectrum of **1** indicated the presence of hydroxyl and carbonyl groups, aliphatic and ether linkages. The molecular formula $C_{100}H_{168}O_{45}$ was assigned for **1** from mass spectral analysis (*m/z* 2091.3775 $[M+H]^+$ in HRMS and *m/z* 2091 $[M+H]^+$ in FABMS). The negative-ion FABMS

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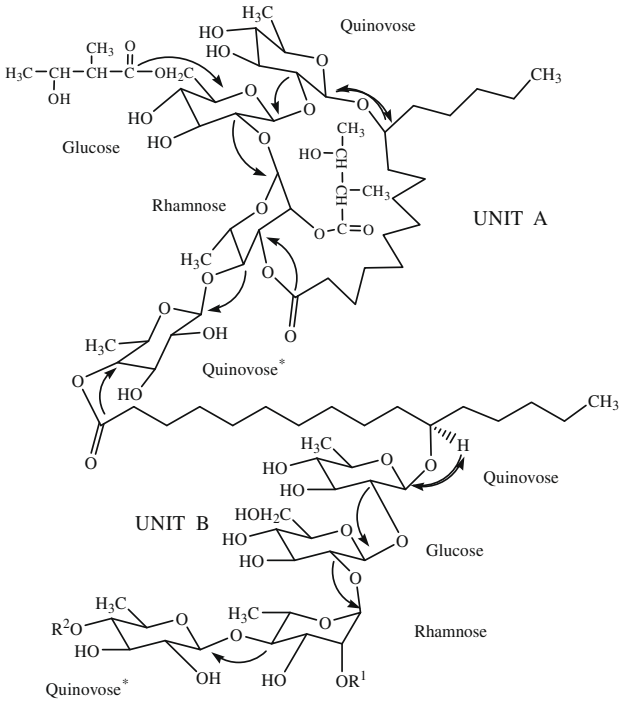

1: R^1= niloyl; R^2= 2-methylbutanoyl
2: R^1= H; R^2= butanoyl

Figure 1. Selected HMBC (⟶) and ROESY (⟷) of tyrianthin A (**1**) and tyrianthin B (**2**).

of **1** showed: its quasimolecular ion peak due to $[M–H]^-$ at *m/z* 2089, the high-mass fragment ion at *m/z* 1055 which represents the fragmentation of the macrocyclic moiety (unit A) and the glycosidic moiety (unit B) of the ester-type heterodimer linkage (Fig. 1),[10] fragments due to elimination of niloyl residues in unit A at *m/z* 955 [*m/z* 1055−$C_5H_8O_2$]$^-$ and *m/z* 871 [*m/z* 955−C_5H_8O]$^-$, and fragmentation due to glycosidic cleavage of sugar moieties at *m/z* 707 [*m/z* 871−$C_6H_{10}O_4$ (methylpentose)]$^-$, 561 [*m/z* 707−$C_6H_{10}O_4$ (methylpentose)]$^-$, 417 [*m/z* 561−$C_6H_8O_4$ (hexose)]$^-$, and 271 [*m/z* 417−$C_6H_{10}O_5$ (methylpentose)]$^-$.

The ^{1}H NMR spectrum of **1** displayed the following representative signals: two 11-hydroxyhexadecanoic acid (aglycon) methyl proton at δ_H 0.86, methyl protons of 6-deoxyhexoses in the range 1.5–1.6 ppm, methyl protons of short chain acids ca. 0.9–1.1 ppm, eight anomeric protons at 4.81, 4.88, 4.97, 5.02, 5.33, 5.34, 5.95, and 5.08 ppm. In the ^{13}C NMR spectrum of compound **1** it was possible to determine eight signals in the diagnostic anomeric region at 105.9, 105.8, 103.1, 102.5, 102.0, 101.5, 101.1, and 101.0 ppm (Table 1) corroborating the presence of eight saccharide units in **1**. Acid hydrolysis of scammonic acid A yielded D-quinovose, D-glucose, and L-rhamnose (identified by coelution in HPLC with authentic samples), and 11-hydroxyhexadecanoic acid.[11] COSY and TOCSY experiments revealed that each tetrasaccharide core of **1** was composed of two quinovoses, one glucose, and one rhamnose moiety. TOCSY spectra were obtained with different mixing times, and edition of ^{1}H NMR subspectra for each resolved signal facilitated the assignment of all the signals in units A and B (Fig. 1). The anomeric configurations for the sugar moieties were assigned as β for glucopyranosyl and quinovopyranosyl, and α for rhamnopyranosyl, from their $^3J_{H1,H2}$ coupling constants (7.7, 7.6, and 1.4 Hz, respectively). The $^1J_{CH}$ between H-1 and C-1 determined in the coupled HSQC experiment, of 170 for rhamnose and 160 Hz for quinovose and glucose, supported these assignments.[12] The sugar sequence for each tetrasaccharide core was evidenced from 2D-NMR CIGAR spectrum of **1**, in which long range correlations were observed between quinovose H-2/C-1 glucose, glucose H-2/C-1 rhamnose, and rhamnose H-4/C-1 quinovose*. The use of the NMR experiments HSQC and HSQC-TOCSY processed with forward linear prediction and zero filling,[13] allowed assignment of all protonated carbons in units A and B.

The observed optical rotation ($[\alpha]_D^{25}$ +0.42) for 11-hydroxyhexadecanoic acid obtained from acid hydrolysis of scammonic acid A was closely comparable to that reported for *S* enantiomer ($[\alpha]_D^{25}$ +0.45).[9] The position of the aglycon moieties in the tetrasaccharide core of units A and B was determined by correlation between H-11 aglycon/H-1 quinovose in a 2D-NMR ROESY spectrum. The 2D-NMR CIGAR experiment of compound **1** allowed to locate the position of lactonization on unit A, by the long range correlation between ^{13}C=O (176.3 ppm) aglycon/H-3 (5.08 ppm) rhamnose, H-2, H-2* (2.40, 2.82 ppm) aglycon. The position of esterification of acyclic unit B in the macrocyclic unit A, was determined by the long range correlation between ^{13}C=O (176.2 ppm) aglycon of unit B/H-4 (4.72 ppm) of quinovose* of unit A.

The analysis by GC–MS of the organic solvent-soluble acidic fraction from the alkaline hydrolysis of tyrianthin A (**1**), allowed 2-methylbutanoic acid and 2-methy-3-hydroxylbutanoic acid (nilic acid) to be identified (identified by coelution in GC with authentic samples and mass spectra analysis).[14] The sites of esterification by these acids in the tetrasaccharide cores were determined in the 2D-NMR CIGAR spectrum of **1**, by the long-range correlation in unit A between ^{13}C=O (173.5 ppm) of niloyl/H-6 (4.30 ppm) of glucose, ^{13}C=O (173.5 ppm) of niloyl*/H-2 (5.60 ppm) of rhamnose, and in unit B between ^{13}C=O (173.6 ppm) niloyl/H-2 (5.58 ppm) of rhamnose, ^{13}C=O (175.5 ppm) 2-methylbutanoyl/H-4 (4.32 ppm) of quinovose*.

Compound **2** (Fig. 1) was isolated as a white amorphous solid, with negative optical rotation $[\alpha]_D^{25}$ −20.1. The IR spectrum of **2** indicated the presence of hydroxyl, aliphatic, carbonyl, and ether groups. The molecular formula of **2** $C_{94}H_{158}O_{43}$ was determined from mass spectral analysis (*m/z* 1977.2354 in HRMS and *m/z* 1977 in FABMS). The negative-ion FABMS of **2** showed: its quasimolecular ion $[M–H]^-$ at *m/z* 1975, the high-mass fragment ion [(M−(unit B)]$^-$ at *m/z* 1055, and fragmentation peaks due to cleavage of sugar moieties at *m/z* 707, 561, 417, and 271.[10]

The ^{1}H NMR spectrum of **1** displayed the following representative signals: eight anomeric protons at 4.81, 4.88, 4.96, 5.02, 5.34, 5.35, 5.91, and 6.07 ppm, two aglycon methyl proton (δ_H 0.86), methyl protons of 6-deoxyhexoses (1.5–1.6 ppm), and methyl protons of short chain acids (ca. 1.1 ppm). The ^{13}C NMR spectrum of **2** showed eight anomeric signals at 105.8, 105.7, 103.0, 102.4, 102.0, 101.6, 101.1, and 101.0 ppm (Table 1) corroborating the presence of eight saccharide units in **2**. Acid hydrolysis of **2** yielded D-quinovose, D-glucose, and L-rhamnose (identified by coelution in HPLC with authentic samples), and 11-hydroxyhexadecanoic acid. A combination of ^{1}H NMR spectrum and 2D-NMR experiments (COSY, TOCSY, and HSQC-TOCSY) allowed us to determine that each tetrasaccharide core of **2** was composed of two quinovoses, one glucose, and one rhamnose moiety. The anomeric configurations for the sugar moieties were assigned as β for glucopyranosyl and quinovopyranosyl, and α for rhamnopyranosyl, from their $^3J_{H1,H2}$ and $^1J_{CH}$ (H-1/C-1) coupling constants. The sugar sequence for each tetrasaccharide core in **2** was evidenced from long range correlations in the 2D-NMR CIGAR spectrum between quinovose H-2/C-1 glucose, glucose H-2/C-1 rhamnose, and rhamnose H-4/C-1 quinovose*. The use of the NMR experiments HSQC and HSQC-TOCSY allowed assignment of all the resonances of protonated carbons in units A and B (Table 1).

Table 1
NMR spectral data of compounds **1** and **2** (400 MHz ^{1}H NMR, 100 ^{13}C NMR in C_5D_5N)

Position	**1**				**2**			
	Unit A		Unit B		Unit A		Unit B	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
Qui								
1	4.88 d (7.6)	102.5	4.81 d (7.6)	103.1	4.88 d (7.6)	102.4	4.81 d (7.6)	103.0
2	4.27*	79.2	4.26*	79.1	4.26*	79.2	4.26*	79.3
3	4.71 dd (9.0, 9.0)	78.7	4.70 dd (9.0, 9.0)	78.1	4.70 dd (9.0, 9.0)	78.7	4.71 dd (9.0, 9.0)	78.0
4	3.78 dd (8.9, 9.0)	76.4	2.95 dd (8.9, 9.0)	76.5	2.96 dd (8.9, 9.0)	76.3	2.98 dd (8.9, 9.0)	76.2
5	3.84*	72.7	3.87*	72.6	3.83*	72.6	3.85*	72.5
6	1.52 d (7.0)	18.2	1.52 d (7.0)	18.0	1.50 d (7.0)	18.1	1.50 d (7.0)	18.2
Glc								
1	5.02 d (7.7)	101.5	4.97 d (7.7)	102.0	5.02 d (7.7)	101.6	4.96 d (7.7)	101.9
2	4.71 dd (9.1, 7.7)	79.2	3.90 dd (9.1, 7.7)	79.1	4.70 dd (9.1, 7.7)	79.1	3.89 dd (9.1, 7.7)	79.0
3	4.22 dd (9.0, 9.1)	78.3	4.18 dd (9.0, 9.1)	78.1	4.22 dd (9.0, 9.1)	78.2	4.17 dd (9.0, 9.1)	78.0
4	4.16 dd (9.0, 9.0)	70.4	4.11 dd (9.0, 9.0)	69.9	4.14 dd (9.0, 9.0)	70.2	4.12 dd (9.0, 9.0)	69.8
5	4.02*	77.4	4.03*	77.5	4.03*	77.4	4.044*	74.5
6	4.72 dd (11.5, 6.5)	64.8	4.23 dd (11.5, 6.5)	62.5	4.71 dd (11.5, 6.5)	64.2	4.23m	62.5
6*	4.95 dd (11.5, 6.5)		4.45 dd (11.5, 6.5)		4.95 dd (11.5, 6.5)		4.44 dd (11.5, 6.5)	
Rha								
1	6.08 d (1.4)	101.1	5.95 d (1.4)	101.0	6.07 d (1.4)	101.1	5.91 d (1.4)	101.0
2	6.41 dd (3.2, 1.4)	75.0	5.88 dd (3.2, 1.4)	74.9	6.45 dd (3.2, 1.4)	75.1	5.45 dd (3.2, 1.4)	69.2
3	5.59 dd (9.3, 3.2)	71.2	4.01 dd (9.3, 3.2)	72.3	5.59 dd (9.3, 3.2)	71.2	3.98 dd (9.3, 3.2)	72.2
4	4.32 dd (9.0, 9.3)	78.8	4.29 dd (9.0, 9.3)	78.6	4.33 dd (9.0, 9.3)	78.8	4.29 dd (9.0, 9.3)	78.6
5	4.42 dd (6.5, 9.0)	69.3	4.41 dd (6.5, 9.0)	69.3	4.43 dd (6.5, 9.0)	69.4	4.42 dd (6.5, 9.0)	69.4
6	1.58 d (6.5)	18.9	1.57 d (6.5)	18.8	1.54 d (6.5)	18.9	1.52 d (6.5)	18.8
*Qui**								
1	5.34 d (7.6)	105.9	5.33 d (7.6)	105.8	5.34 d (7.6)	105.8	5.35 d (7.6)	105.7
2	4.08 dd (9.0, 7.6)	73.2	3.18 dd (9.0, 7.6)	76.1	3.21 dd (9.0, 7.6)	73.1	3.18 dd (9.0, 7.6)	76.1
3	3.64 dd (9.0, 9.0)	77.3	3.63 dd (9.0, 9.0)	77.2	3.61 (9.0, 9.0)	77.0	3.60 (9.0, 9.0)	77.1
4	4.72 dd (9.1, 9.0)	78.3	4.32 dd (9.1, 9.0)	70.3	4.72 dd (9.1, 9.0)	78.2	4.33 dd (9.1, 9.0)	70.2
5	3.69 dd (6.4, 9.1)	75.2	3.70 dd (6.4, 9.1)	70.3	3.66 dd (6.4, 9.1)	75.4	3.67 dd (6.4, 9.1)	70.5
6	1.59 d (6.4)	18.9	1.60 d (6.4)	19.0	1.61 d (6.4)	18.9	1.60 d (6.4)	18.8
Jal								
1		176.3		176.2		176.3		176.2
2	2.40*	35.7	2.40*	35.3	2.39*	35.7	2.39*	35.5
2*	2.82*		2.82*		2.81*		2.81*	
11	3.62*	82.6	3.60*	83.5	3.62*	82.3	3.60*	83.6
16	0.86 t (7.0)	14.6	0.86 t (6.9)	14.7	0.86 t (7.0)	14.6	0.86 t (6.9)	14.7
nil								
1		173.5		173.6		173.5		
2	2.88*	48.6	2.88*	48.9	2.89*	48.6		
2-Me	1.37 d (7.4)	13.7	1.35 d (7.4)	13.7	1.34 d (7.4)	13.7		
3	4.42*	69.8	4.31*	70.1	4.41*	69.9		
3-Me	1.30 d (7.2)	13.8	1.30 d (7.2)	13.8	1.30 d (7.2)	13.8		
*nil**								
1		173.5				173.5		
2	2.90*	48.6			2.91*	48.8		
2-Me	1.38 d (7.4)	13.7			1.38 d (7.4)	13.7		
3	4.41*	69.6			4.40*	69.8		
3-Me	1.30 d (7.2)	13.8			1.30 d (7.2)	13.8		
mba								
1				175.5				
2			2.43*	41.6				
2-Me			0.95 d (7.2)	12.0				
3			1.75*	27.4				
3*			1.51*					
3-Me			0.92 t (7.2)	12.1				
but								
1								175.5
2							2.55 t (7.0)	41.6
3							1.68*	25.8
4							1.11 t (7.3)	17.4

Qui = quinovopyranosyl, Glc = glucopyranosyl, Rha = rhamnopyranosyl, Jal = 11-hydroxyhexadecanoyl, nil = 3-hydroxy-2-methylbutanoyl, mba = 2-methylbutanoyl, and but = butanoyl. Chemical shifts marked with asterisk (*) indicate overlapped signals.

The observed optical rotation ($[\alpha]_D^{25}$ +0.42) for aglycon moiety in **2** corroborated its *S* configuration.[9] The position of the aglycon moiety in each one of the oligosaccharide cores of **2** was determined at C-1 of quinovose by correlations in the ROESY NMR spectrum between H-11 aglycon/H-1 quinovose. The analysis by GC–MS the organic solvent-soluble acidic fraction from the alkaline hydrolysis of **2** revealed the presence of butanoic acid and nilic acid (identified by coelution in GC with authentic samples and mass

spectra analysis).[14] The long range correlations in the 2D-NMR CIGAR experiment were used to locate: two niloyl groups at C-6 of glucose and C-2 of rhamnose in unit A, and a butanoyl group at C-4 of quinovose* in unit B; the site of lactonization was determined by the correlation between $^{13}C{=}O$ of aglycon/H-3 of rhamnose in unit A; and the site of esterification of both units by the correlation between $^{13}C{=}O$ of aglycon unit B/H-4 of quinovose* of unit B.

Tyrianthins A (**1**) and B (**2**) showed significantly vasorelaxant effect on endothelium-intact (E+) aortic rings[15] (IC_{50} = 0.24 ± 0.09 μM and E_{max} = 81.80 ± 0.98% and IC_{50} = 0.14 ± 0.08 μM and E_{max} = 87.68 ± 0.72%, respectively). When endothelium was removed (E−), the relaxant activity was abolished ($p < 0.05$) as shown in Figure 2. These results indicated that tyrianthins A and B produced their vasorelaxant effect through endothelium derived factors as cyclooxygenase, endothelium dependent hyperpolarization factor (EDHF) or nitric oxide synthase pathways.[16]

In a Central Nervous System bioassay,[17] the administration of tyrianthin A (**1**) and tyrianthin B (**2**) to mouse brain slices induced an increment of GABA at 30 s. The release of GABA by compound **1** remained until 180 s, but for compound **2** release of GABA diminished with time (Fig. 3). Tyrianthin A (**1**) released glutamic acid up to 100 pmol/mg at 180 s, but tyrianthin B (**2**) released glutamate up to 180 pmol/mg at 180 s. This behavior of GABA and glutamic acid releasing is similar to that observed for glycolipid tetrasaccharides isolated from *I. tyrianthina* and *I. stans*[5,18] (Fig. 4)

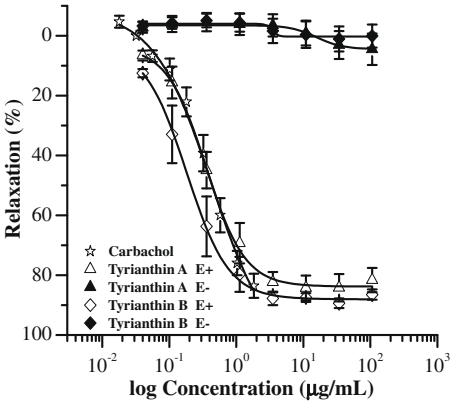

Figure 2. Concentration–response curves showing the vasorelaxant effect of tyrianthins A (**1**) and B (**2**), on rat aortic rings pre-contracted with noradrenaline (1×10^{-7} M). Values are expressed as the percentages of inhibition of contractile responses calculated as the mean from six data ± SE ($p < 0.05$). E+ = intact endothelium, E− = removed endothelium.

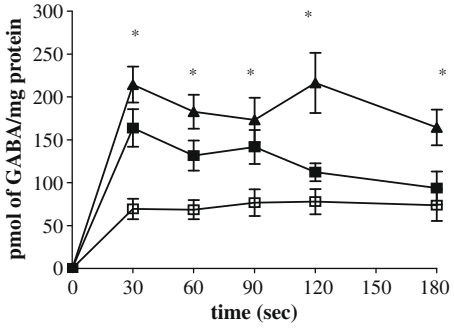

Figure 3. Release of GABA evoked by 20 μg/mL of tyrianthin A (▲) and tyrianthin B (■). The experiment in the absence of compound was carried out as control (□). Values are expressed as pmol of GABA released/mg of protein and are the mean ± SEM of 3 independent experiments. * $p < 0.05$.

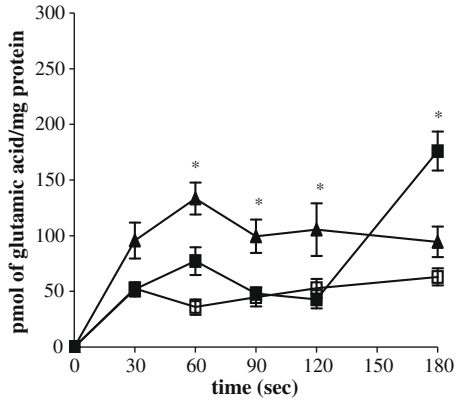

Figure 4. . Release of glutamic acid evoked by 20 μg/mL of tyrianthin A (▲) or tyrianthin B (■). The experiment in the absence of tyrianthins was carried out as control (□). Values are expressed as pmol of glutamic acid released/mg of protein and are the mean ± SEM of 3 independent experiments. * $p < 0.05$.

Tyrianthin A (**1**) and tyrianthin B (**2**) were not active against *M. tuberculosis* (MIC = 100 μg/mL),[19] on difference to the behavior shown by glycolipid oligosaccharides isolated from *I. tyrianthina*,[5] *I. tricolor*,[20] and *I. leptophylla*[21] which showed moderate antimycobacterial activity (MICs 16–32 μg/mL). It seems that with increasing the molecular weight and complexity of glycolipids the antimycobacterial activity decreases.

In conclusion, tyrianthins A (**1**) and B (**2**) are the first glycolipid ester type heterodimers composed of two tetrasaccharide cores isolated from *I. tyrianthina*. Our results demonstrate the high vasorelaxant activity of **1** and **2** in concentration and endothelium dependent manners. Compounds **1** and **2** increased the release of GABA and glutamic acid from cortex tissue in mice in a time-dependent manner.

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6. Root of *I. tyrianthina* was collected in the state of Morelos (December 2004), Mexico. Botanical classification was carried out by Biol. M. Castro, Facultad de Ciencias, UNAM, and voucher specimen (Number 15077) is deposited at the Instituto Mexicano del Seguro Social Herbarium in Mexico City. Dried and ground roots (100 g) were extracted with *n*-hexane and later with CH_2Cl_2. The residual vegetal material was extracted exhaustively in methanol to give, after removal of the solvent, a brown solid (20 g). The methanolic extract was subjected to gravity column chromatography over with reverse phase (C_{18}) silica gel (50 g) using a gradient of CH_3OH in H_2O, leading to a brown resinous solid (10 g). The resinous material was subjected to separation by a column packed with Sephadex LH20 (20 g), and by preparative TLC, obtaining two

chromatographic fractions. The components of the less polar chromatographic fraction (1 g) were purified by preparative HPLC using an Ultrasil ODS column (10 mm i.d. × 300 mm, 5 μm, Altex), eluting with a mixture of CH_3CN–H_2O (8:2), at a flow rate of 1 mL/min at 25 °C, and detection with UV at 210 nm. Chromatographic peaks were collected and re-injected until pure. This technique afforded pure compounds **1** (400 mg, t_R 13.5 min) and **2** (100 mg, t_R 15.2 min). Tyrianthin A (**1**). Amorphous white powder; mp 146–148 °C; $[\alpha]_D^{25}$ −22.0 (*c* 1.1 CH_3OH); IR (KBr): 3376 (OH), 2985 (C–H), 1735 (C=O), 1090 (C–O) cm^{-1}; ^1H and ^{13}C NMR (C_5D_5N): Table 1; positive-ion FABMS *m/z* 2091 $[M+H]^+$; negative-ion FABMS *m/z* 2089 $[M-H]^-$, 1055 [M−unit B−H]$^-$, 955 $[1055-C_5H_8O_2]^-$, 855 $[955-C_5H_8O_2]^-$, 561, 417, and 271; HRMALDITOFMS *m/z* 2091.3775 $[M+H]^+$ (calcd for $C_{100}H_{168}O_{45}H^+$ 2091.3781). Tyrianthin B (**2**). Amorphous white powder; mp 140–142 °C; $[\alpha]_D^{25}$ −20.1 (*c* 1.3 CH_3OH); IR (KBr): 3376 (OH), 2985 (C–H), 1735 (C=O), 1090 (C–O) cm^{-1}; ^1H and ^{13}C NMR (C_5D_5N): Table 1; positive-ion FABMS *m/z* 1977 $[M+H]^+$; negative-ion FABMS *m/z* 1975 [M – H]$^-$, 1055 [M−unit B−H]$^-$, 955 $[1055-C_5H_8O_2]^-$, 855 $[955-C_5H_8O_2]^-$, 561, 417, and 271; HRMALDITOFMS *m/z* 1977.2354 $[M+H]^+$ (calcd for $C_{94}H_{158}O_{43}H^+$ 1977.2361).

7. *Alkaline hydrolysis*. The less polar chromatographic fraction (100 mg) was refluxed in 0.2 N NaOH (10 mL) for 60 min. The reaction mixture was acidified to pH 5 and extracted with CH_2Cl_2. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The aqueous layer was lyophilized, the residue was dissolved with methanol, and a white solid (glycosidic acid) was obtained after removal of solvent.
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11. *Acid hydrolysis*. The glycosidic acid was refluxed in 1.0 N HCl (5 mL of water–ethanol) for 1.0 h. The reaction mixture was taken to pH 5 with NaOH solution, and the solution extracted with CH_2Cl_2. An aliquot of the aqueous phase was subjected to HPLC on a Nucleosil 100 NH_2 column (Alltech; 5 μm, 250 × 4.6 mm), with an isocratic elution of CH_3CN–H_2O (8:2), at a flow rate of 1 mL/min, and a sample injection of 200 μL, affording three compounds which were identified by co-elution with standard α-L-rhamnose (1 mg t_R 7.9 min), 6-deoxy-β-D-glucose (quinovose 2 mg, t_R 8.9 min), and β-D-glucose (1 mg, t_R 15.2 min).
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14. The GC–MS system consisted of a HP 6890 gas chromatograph and a HP 5970 mass selective detector in the electron-ionization. GC conditions: 25 m × 0.2 mm HP-5 column; He, 1 mL/min; Oven temperature 40 °C, 2 min, 40–250 °C, Δ 15 °C/min, 250 °C 10 min; Injector temperature 300 °C; Interfase temperature 280 °C; split 1:40.
15. *Vasorelaxant assay*. Male Wistar rats (250–300 g of body weight) were sacrificed by exposure to diethyl ether. The thoracic aorta was cleaned of adhering connective tissue and was cut into 3–5 mm length rings. In some rings, the endothelium was removed. Then, tissue segments were mounted in stainless steel hooks, under an optimal tension of 3 g, in 10 mL organ baths containing warmed (37 °C) and oxygenated (O_2/CO_2, 19:1) Krebs solution (composition, mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; EDTA, 0.026 and glucose, 11.1, pH 7.4). Changes in tension were recorded by Grass-FT03 force transducers (Astromed, West Warwick, RI, USA) connected to a MP100 analyzer (Biopac Instruments, Santa Barbara, CA, USA). After equilibration, rings were contracted by noradrenaline (NA, 0.3 μM) and washed every 30 min for 2 h. The absence of endothelium was confirmed by the lack of a relaxing response to carbachol (1 μM). After precontraction with NA, the test samples (pure compounds, vehicle, and positive control) were added to the bath in a volume of 100 μL; then cumulative concentration–response curves were obtained for each ring. The relaxant effect of pure compounds and positive controls was determined by comparing the muscular tone of the contraction before and after addition of the test compounds.
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17. GABA and glutamate release in cortical brain slices. Mice were sacrificed by decapitation and anterior brain cortex was dissected out and slices (250–300 μm) were cut manually using a razor blade and a cover glass guide.[17] Brain slices were placed at 4 °C in 5 mL in a modified Krebs-Ringer medium (120.0 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.0 mM tris–HCl buffer (pH 7.4), and 10.0 mM glucose) pH 7.4, previously oxygenated with O_2 bubbling. Amino-oxyacetic acid at a concentration of 10.0 μM, was added to the medium to prevent GABA metabolism. After 5 min, slices were placed in 5 mL of the basal medium (Krebs-Ringer medium) in a well of microplate at 37 °C for 10 min. Next, pure compounds were added at a final concentration of 20.0 μg/mL and aliquots of 200 μL were taken out at 0.5, 1.0, 1.5, 2.0, and 3.0 min. At the end of the experiment, the content of GABA and glutamate into each collected aliquot was determined by HPLC, previous derivation with O-phthaldialdehyde. Protein content in brain slices was determined after its homogenization in 1 mL of water.
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19. *Antimycobacterial activity*. The in vitro antimycobacterial activity of compounds **1** and **2** was measured as the minimal inhibitory concentration (MIC), and carried out using the modified microplate Blue Alamar assay (MABA). The concentrations of pure compounds ranged from 100.00 to 3.13 μg/mL. Rifampin was used as positive controls. All evaluations were carried out in duplicate.
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