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Ryanodane diterpenes from two *Erythroxylum* species

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Abstract

Ryanodane diterpenes, named 14-O-methyl-ryanodanol and ryanodanol, were isolated from ripe fruit of *Erythroxylum passerinum*. Compound **2** was also found in the leaves of this species, while **1** was obtained from the leaves of *E. nummularia*. Compound **1** showed insecticidal activity against *Aedes aegypti* larvae.

Keywords: *Erythroxylum passerimum*; *Erythroxylum numularia*; Erythroxylaceae; Ryanodane diterpenes; Larvicidal activity

1. Introduction

The *Erythroxylum* genus contains tropane alkaloids in its leaves and fruits (Zuanazzi et al., 2001), and comprises approximately 97% of the species of the Erythroxylaceae family, which are spread throughout tropical and subtropical regions (Plowman and Hensold, 2004; Evans et al., 1981). However, the species collected in the State of Bahia, Brazil, usually contains only fatty acid esters of triterpenes (Chávez et al., 1996). There are many endemic species in Brazil, however especially along the coast in "restinga" and sandy soil forests. It is estimated (Plowman, 1987) that within the Neotropics, the number of species per unit area in the State of Bahia can be matched only by Venezuela. This paper thus describes the isolation of two new ryanodane diterpenes, 6α,11α-epoxy-14α-methoxy-ryanodane-1α,5β,7β,11β,13β-pentaol (**1**) and 6α,11α-epoxyryanodane-1α,5β,7β,11β,13β,14α-hexaol (**2**), as well as known flavonoids, from the ripe fruit of *E. passerinum* Mart, and **2** as well as known flavonoids from its leaves. In addition, *E. nummularia* Peyr. contained known flavonoids and compound **1**. Several ryanodane diterpenes have been shown to have insecticidal properties (Fraga et al., 2001; Hübner et al., 2001). An evaluation of the larvicidal activity of the *Erythroxylum* spp extracts and of compound **1** showed that this compound is effective against *Aedes aegypti* (L.) larvae, even though the extracts do not exhibit activity. The natural occurrence of ryanodane diterpenes appears to be restricted to *Cinnamomum* and *Persea* (Lauraceae) members (Isogai et al., 1976; Gonzalez-Coloma et al., 1996), and as alkaloid diterpenes in *Ryania* (Flacourtiaceae) (Cabras et al., 2001) and *Spigelia* (Loganiaceae) (González-Coloma et al., 1999).

2. Results and discussion

Ethyl acetate extracts of the leaves and ripe fruits of *E. passerinum*, and chloroform and ethyl acetate extracts

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of *E. nummularia* leaves were examined by chromatographic procedures. The *E. passerinum* ripe fruits yielded the new ryanodane diterpenes **1** and **2**, but only **2** and three known flavonoids (quercetin, quercetin-3-*O*-D-glucopyranosyl, and *epi*catechin) were isolated from the leaves. On the other hand, the ethyl acetate extract of *E. nummularia* leaves yielded the above flavonoids while 4′,7-dimethyl-quercetin (ombuin) and **1** were isolated from the chloroform extracts. The known flavonoids were identified by comparing their spectroscopic (IR, MS, ^{1}H and ^{13}C NMR) data and α_D values to those reported in the literature (Harborne and Williams, 2000; Inigo and Pomilio, 1985; Agrawal and Bansal, 1989).

The negative FAB-MS of compound **1** showed a *quasi* molecular ion $\{M-1\}^+$ at *m*/*z* 397 and the EIMS of **2** registered a molecular ion $[M]^+$. at *m*/*z* 384. The fragments observed in these spectra, in conjunction with the IR spectra, established the presence of hydroxyl groups in the molecules. The ^{13}C and ^{1}H NMR spectra (Tables 1 and 2) indicated 21 carbon and 29 hydrogen atoms for **1**, and 20 carbon and 26 hydrogen atoms for **2**. On the basis of these data, together with the elemental analysis (C, 60.50% and H 8.41% for **1**; C, 56.06% and H, 7.88% for **2**), the molecular formulae were deduced to be $C_{21}H_{34}O_7 \cdot H_2O$ (**1**) and $C_{20}H_{32}O_7 \cdot 2H_2O$ (**2**).

DEPT 135° and 90°, and *g*HMBC investigation of **1** indicated the presence of one methoxy, five methyl, three methylene, and five methine groups, and seven tetra-substituted sp^3 carbon atoms. Moreover, the presence of an isopropyl group in the molecule was detected and confirmed by the ion at *m*/*z* 355 $[M\text{-}CH(CH_3)_2]^+$ observed in the ESI-MS and NMR spectroscopic signals (δ_H 0.92, 1.06, 1.98; δ_C 18.6, 18.7, 34.2). The *g*HMBC experiments showed correlations between the methyl hydrogen (H_3-19 and H_3-20) of the isopropyl group and the carbinolic carbon at δ 85.5 (C-13), which indicates the position of this group This spectrum allowed us to assign correlations between the methine carbon (C-8) and H-14 (δ 3.34), and between H-14 and C-7 (δ 98.5), C-9 (δ 46.8), and CH_3O (δ 56.6). The last correlation indicates that the methoxy group is linked to C-14. The hydrogen atoms of the methyl group (3H-17) showed correlations with C-13, C-7, C-11 (δ 103.6), and C-12 (δ 65.5), as well as between 3H-16 and C-10 (δ 41.8), C-8 (δ 59.6), C-9 (δ 46.8), and C-5 (δ 83.5). These bidimensional correlations were essential for confirming the positions of oxygenated carbon atoms and methyl groups, and they identified most of the substituents of the ryanodane skeleton of **1**. The ^{1}H–^{1}H COSY and ^{1}H selective irradiation NMR experiments for **1** showed geminal and W couplings of H-10*ax*/H-10*eq* and H-10*ax*/H-8. This long-range coupling constant observed for H-8 and H-10*ax* allowed us to establish the relative stereochemistry of bridges formed by the sequential carbon atoms C-9, C-10, C-11 and C-12, as well as between the atoms C-6–O–C-11–C-12, indicating a *syn* orientation of these carbon atoms. Moreover, these experiments showed coupling between 2H-4 (δ 2.10, 1.29), and 2H-3 (δ 1.56), as well as between H-2 (δ 1.82) and 2H-3, H-1 (δ 3.77), and 3H-15 (δ 1.03), which led to the establishment of the atom sequence (C-5 to C-1) of the molecule. This sequence was confirmed by 1D-TOCSY experiments. The magnitude of the coupling constants for H-2 and H-1 ($J_{1,2} = 10.0$ Hz) is consistent with*trans* di-axial orientation for these hydrogen atoms and hence a diequatorial arrangement of Me-15 and OH-1. All these data, along with the correlations observed by *g*-HMBC between H-8 and the carbon atoms C-6 and

Table 1
^{13}C NMR spectroscopic data for compounds **1** and **2** (MeOH-d_4, 126 MHz)[a]

Compound **1**			Compound **2**	
	δ	*g*HMBC	δ	*g*HMBC
1	72.8	3H-15, H-2	72.8	H-2, 3H-15
2	35.2	3H-15	35.2	H-1, 3H-15
3	30.0	3H-15, H-1	30.0	3H-15, H-1
4	26.8		26.7	
5	83.5	H-10*eq*, H-4β, 3H-16, H-3	83.3	H-10*eq*, H-4β, 3H-16, H-3
6	86.9		86.8	H-8
7	98.5	H-14, 3H-17	98.4	H-14, H-8, 3H-17
8	59.6	3H-16, H-10	64.3	H-10*ax*, 3H-16
9	46.8	H-14, 3H-16	47.0	H-14, H-8, H-10*eq*,*ax*, 3H-16
10	41.8	3H-16	42.0	H-8, 3H-16
11	103.6	3H-17	103.1	H-10*ax*, H-10*eq*, 3H-17
12	65.5	3H-17	65.3	H-8, 3H-17
13	85.5	3H-17, 3H-20, 3H-19	85.2	H-8, 3H-17, 3H-19, 3H-20
14	87.0	-OCH_3	77.1	H-8
15	18.9	H-1	18.8	H-1
16	15.1	H-8, H-10*eq*,*ax*	15.2	H-8, H-10*eq*,*ax*
17	9.1	H-12	9.4	
18	34.2	H-14, 3H-19, 3H-20	33.9	H-14, 3H-19, 3H-20
19	18.6	3H-20	18.6	H-18, 3H-20
20	18.7	3H-19	18.7	H-18, 3H-19
–O$\underline{CH_3}$	56.6	H-14		

[a] ^{13}C NMR data were assigned through DEPT 135° and 90°, *g*HMBC, and *g*HMBC experiments.

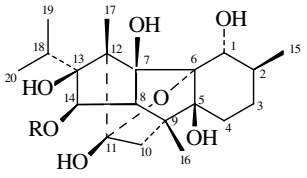
Table 2
^{1}H NMR spectroscopic data for compounds **1** and **2** (MeOH-d_4, 500 MHz, J in Hz)[a]

H	**1**		**2**	
	δ	1D- and 2D-*g*NOESY	δ	1D-*g*NOESY
1	3.77 (*d*, 10.0)	H-3, 3H-15	3.75 (*d*, 10.5)	H-3, 3H-15
2	1.82 (*ddquint*, 11.0, 10.0, 6.5)	3H-15	1.81 (*ddquint*, 11.0, 10.5, 6.5)	H-3, H-1, 3H-15
3	1.56 (*m*, 10.5)	3H-15	1.56 (*m*)	
4α	2.10 (*ddd*, 13.0, 12.0, 6.5)	H-4β, H-3, H-2, H-10*ax*	2.06 (*ddd*, 13.0, 10.0, 6.5)	H-4β, H-3, H-2, H-10*ax*
4β	1.29 (*ddd*, 13.0, 6.5, 2.5)	H-4α, H-3,	1.26 (*ddd*, 13.0, 6.5, 2.5)	
8	2.66 (*d*, 2.0)	H-14, 3H-16, -OCH_3	2.55 (*d*, 2.0)	H-14, 3H-16
10*eq*	1.55 (*d*, 14.0)	H-19, H-18	1.55 (*d*, 14.0)	
10*ax*	1.75 (*dd*, 14.0, 2.0)	H-4α, H-10*eq*	1.72 (*dd*, 14.0, 2.0)	H-4α, H-10*eq*, 3H-16
14	3.34 (*s*)		3.81 (*s*)	H-8, H-18, H-10*eq*, 3H-19, 3H-16
15	1.03 (*d*, 6.5)	H-3, H-2, H-1	1.01 (*d*, 6.5)	H-3, H-2,H-1
16	0.94 (*s*)	H-14, H-4β, H-10*ax*	0.92 (*s*)	
17	1.33 (*s*)	3H-20	1.33 (*s*)	3H-20
18	1.97 (*qq*, 7.0, 6.5 Hz)	H-14, 3H-20	1.98 (*qq,* 7.0, 6.5)	H-14, H-18, H-10*eq*, 3H-19, 3H-20
19	0.92 (*d*, 7.0)	H-14, -OCH_3	0.96 (*d*, 6.5)	H-14, H-18, 3H-20
20	1.06 (*d*, 6.5)	H-18, 3H-17, 3H-19	1.07 (*d*, 7.0)	H-18, 3H-17, 3H-19
–OCH_3	3.37 (*s*)	H-10*eq*, 3H-19, 3H-20		

[a] Multiplicities were determined through ^{1}H–^{1}H COSY and TOCSY experiments.

C-10, suggested that compound **1** was a pentacyclic ryanodane diterpenoid. An HMBC correlation between 2H-3 and C-5 is consistent with this conclusion and suggested that C-5 was bound to C-4, and therefore, C-1 should be bound to C-6. This proposition is supported by 1D- and 2D-*g*NOESY experiments, which showed spatial correlations of H-4α, H-10*ax* and H-2. Likewise, these spectra revealed spatial correlations between H-10*eq*, H-18, and 3H-19, as well as between the methyl group (C-20) and 3H-17, which helped to determine the relative configuration of the molecule. The multiplicity of H-8 (d, $J_{w8,10ax} = 2$ Hz) suggested an angle of 90° between H-8 and H-14 (s, δ 3.34), which made it possible to establish the relative configuration of the methoxyl group at C-14. Thus the C-9/C-10/C-11/C-12 bridge and the isopropyl group are on the α face of the AB ring system while H-8 is on the β face. Finally, the strong *n*Oe increments observed between 3H-20 and 3H-17, as well as those between 3H-16 and H-4β, were in accordance with the *rel* (1*R*, 2*S*, 5*S*, 6*S*, 7*S*, 8*R*, 9*R*, 11*S*, 13*S*, 14*S*) configuration proposed for **1**, named 14-O-methyl ryanodanol or 6α,11α-epoxy-14α-methoxy-ryanodane-1α,5β,7β,11β, 13β-pentaol.

The mass and NMR spectra of **2** confirmed the presence of an isopropyl group, two methyl groups bound to quaternary carbon atoms, a methyl group linked to a tertiary carbon, along with three methylene and two methane groups, and six tetra-substituted sp^3 carbon atoms, which suggested that **2** was a ryanodane diterpene. The main difference between the NMR spectra of **2** and **1** is the absence of signals for the methoxyl group at C-3, which affected significantly the chemical shifts of C-14 ($\Delta\delta = -9.9$) and C-8 ($\Delta\delta = +4.7$), as well as those of H-14 ($\Delta\delta = 0.47$) and H-8 ($\Delta\delta = -0.11$). These findings allowed us to propose that a hydroxyl group should be placed at C-14. Since the multiplicities and the magnitude of the coupling constants determined for all the other hydrogen atoms observed in **2** were not affected significantly by this replacement, the structure of **2**, including its relative configuration, has been deduced to be the same as that of **1**, except for the substituent at C-14. An analysis similar to that described for **1**, which included an evaluation of ^{1}H and ^{13}C NMR, DEPT 135° and 90°, ^{1}H–^{1}H COSY, *g*HMQC, *g*HMBC, and 1D-*g*NOESY data (Tables 1 and 2, Fig. 1) supported the relative configuration (1*R*, 2*S*, 5*S*, 6*S*, 7*S*, 8*R*, 9*R*, 11*S*, 13*S*, 14*S*) determined for **2**, named ryanodanol or 6α,11α-epoxyryanodane-1α,5β,7β,11β,13β,14α-hexaol. The relative configurations of compounds **1** and **2** are in accordance with those of ryanodane diterpenes (Gonzalez-Coloma et al., 1996; Isogai et al., 1976) in the literature.



1: R = CH_3
2: R = H

Fig. 1. Ryanodanes isolated from *Erythroxylum passerinum* and *E. nummularia*.

3. Concluding remarks

Ryanodane diterpenes have been found in Lauraceae species, especially in the *Cinnamomum* and *Persea* genera, and as alkaloidal diterpenes in the Loganiaceae family. However, this is the first report of the ryanodane diterpenes **1** and **2** in species of the Erythroxylaceae family.

Extracts and the isolated **1** were tested for larvicidal and molluscicidal activities as well as brine shrimp lethality

Table 3
Toxicity of EtOAc extract from *Erythroxylum passerinum* (EP), MeOH extract of *Erythroxylum nummularia* (EN), and compound **1**

	LC_{50} (ppm)	
	Aedes aegypti larvae	Brine shrimp test
EP	500	100
EN	500	>1000
1	82	21.8

(Table 3). The extracts proved to be inactive in the evaluation tests, but compound **1** exhibited activity in BST (LC_{50} 21.8 ppm) and slight activity in the larvicidal test (LC_{50} 82 ppm).

4. Experimental section

4.1. General experimental procedures

The ^1H and ^{13}C NMR, DEPT, COSY, and HETERO-COSY ($J = 140$ and 9 Hz) spectra were obtained on Varian Gemini 2000 and INOVA instruments using $CDCl_3$ and CD_3OD as solvent and reference, respectively. The UV and FTIR spectra were recorded on a Varian Cary 320 and on a JASCO spectrophotometer mod. Valor III, respectively. The EIMS was recorded with an HP mod. 5988A mass detector. Melting points were measured on a Microquimica MIAPF 301 apparatus and are uncorrected. Column chromatography (CC) used silica gel 60 and, the fractions were monitored by Si gel TLC. The spots were developed using iodine fumes, $FeCl_3$ spray reagent and UV light (254/366 nm).

4.2. Plant material

E. passerinum fruit and leaves were collected on sandy soil at Reserva do Parque da Lagoa do Pituaçu, Salvador, BA, Brazil in the Spring of 1998 and identified by Professor Maria L.S. Guedes of Herbarium Alexandre Leal Costa, where a voucher specimen (#043630) is deposited. *E. nummularia* leaves were collected on sandy soil at Reserva do Campus da Universidade Estadual de Feira de Santana (UEFS), Feira de Santana, BA, Brazil in the Summer of 1999 and identified by Professor L.P. Queiróz of Herbarium of UEFS, where a voucher specimen (#10442) is deposited.

4.3. Extraction and isolation

Dried powdered fruit (680 g) and leaves (2.6 kg) of *E. passerinum*, and leaves (1.2 kg) of *E. nummularia* were extracted twice with MeOH at room temperature for 48 h. The combined methanol extracts of the fruits (65.8 g) and leaves (93.4 g) of *E. passerinum* were partitioned successively with EtOAc and H_2O, and yielded 859.0 g and 1.61 g of EtOAc extracts, respectively. The methanol extract of the leaves (203.3 g) of *E. nummularia* gave ethyl acetate (2.3 g) extracts after similar solvent partition.

Both 14-O-methyl-ryanodanol (**1**, 102.3 mg) and ryanodanol (**2**, 93.4 mg) were obtained from EtOAc extract of the fruit of *E. passerinum* after CC on SiO_2 gel eluted with mixtures of $CHCl_3$:EtOAc (1:1 and 7:3, respectively). The EtOAc extract of *E. passerimum* leaves was submitted to CC over SiO_2 (60H) as adsorbent and eluted with $CHCl_3$, and mixtures of $CHCl_3$/MeOH (9:1 and 4:1). The fraction eluted with $CHCl_3$/MeOH (9:1) was purified sequentially on Sephadex LH-20 using CH_2Cl_2/MeOH (1:1). This procedure yielded **2** (12.4 mg) and quercetin (9.3 mg).

The $CHCl_3$/MeOH (8:2) fraction was submitted to flash CC with SiO_2 60H, with mixtures of $CHCl_3$:MeOH (8:2 and 7:3) and it yielded *epi*catechin (68.3 mg) and quercetin-3-*O*-D-glucopyranosyl (25.0 mg).

The EtOAc extract of *E. nummularia* leaves was submitted to CC on SiO_2 gel using $CHCl_3$ and $CHCl_3$/MeOH (9:1). 4′,7-Dimethyl-quercetin (5.5 mg) was obtained from the former fraction after being purified on Sephadex LH-20 eluted with CH_2Cl_2/MeOH (1:1). The 14-methoxy ryanodanol (**1**, 21.0 mg) was obtained from the fraction eluted with $CHCl_3$/MeOH (9:1) followed by purification by flash CC with SiO_2 (60H) using $CHCl_3$/MeOH (9:1) as eluent. The flavonoids quercetin, *epi*catechin and quercetin-3-*O*-D-glucopyranosyl were isolated from the EtOAc extracts of both *E. nummularia* and *E. passerinum* leaves after the chromatographic procedures.

4.3.1. 6α,11α-Epoxy-14α-methoxy-ryanodane-1α,5β,7β,11β,13β-pentaol or 14-O-methyl ryanodanol (*1*)

Crystal; m.p. 197–198°C; $[\alpha]_D^{25} - 5.11$ (MeOH; c 0.99,); $C_{21}H_{34}0_7 \cdot H_2O$ (Found: C, 60.50; H, 8.41%. requires: C, 63.48 H, 8.82%); EIMS: 70 eV (rel. int.) *m/z*: 398 [M^+] (2); 355 (22), 303 (7), 337 (81), 277 (100); IR ν_{max} acetone (cm^{-1}): 3421 (OH), 2925, 1651, 1457; for ^{13}C NMR (300 MHz, CD_3OD) and ^{14}C NMR (75 MHz, $CDCl_3$) spectra, see Tables 1 and 2.

4.3.2. 6α,11α-Epoxyryanodane-1α,5β,7β,11β,13β,14α-hexaol or ryanodanol (*2*)

Yellow crystal; m.p. 216-217°C; $[\alpha]_D^{25} + 38.33$ (MeOH; c 0.77); $C_{20}H_{32}0_7 \cdot 2H_2O$ (Found: C, 56.06; H, 7.78%. requires: C, 62.50; H, 8.33%); EIMS: 70 eV (rel. int.) EIMS: 70 eV (rel. int.) *m/z*: 384 [$M]^+$ (2), 431 (20), 323 (43), 277 (100); IR ν_{max} acetone (cm^{-1}): 3414 (OH), 2926, 1651, 1457; for ^{13}C NMR (300 MHz, CD_3OD) and ^{14}C NMR (75 MHz, $CDCl_3$) spectra, see Tables 1 and 2.

4.3.3. 4′,7-Dimethyl-quercetin or ombuin

Yellow crystal; $C_{17}H_{14}0_7$; EIMS: 70 eV (rel. int.) *m/z*: 330 (100); 315 (34), 301 (7), 287 (11), 167 (22), 57 (55); UV λ_{max} nm (MeOH) 370, ($AlCl_3$) 425, 350, ($AlCl_3$ + HCl) 350, 425, (NaOAC) 395, (NaOMe) 345, 435; ^1H NMR (300 MHz, $CDCl_3$) δ 7.90 (1H, dd, J = ind, H-6′), 7.81 (1H, d, J = ind H-2′), 7.13 (1H, dd, $J = 8.0$ Hz, H-5′), 6.73 (1H, d, $J = 1.65$ Hz, H-8), 6.32 (1H, d, $J = 1.65$ Hz, H-6), 3.90 (6H, s, OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) in accordance with literature (Inigo and Pomilio, 1985).

4.4. Bioassays

4.4.1. Brine shrimp lethality test

Extracts and compound **1** were evaluated for lethality to brine shrimp using standard protocols (Meyer et al., 1982 and Serrano et al., 1996). A drop of DMSO was added to control and test substances in order to enhance their solubility.

4.4.2. Larvicidal test

This experiment was performed as described (Consolari and Oliveira, 1994). Briefly, larvae of the mosquito *A. eaegypti* that emerged from eggs in chlorine-free water were exposed to the extracts (500 ppm and 100 ppm) and to pure compounds in concentrations of 100 ppm, 75 ppm, 50 ppm and 10 ppm in water. A control was prepared as 0.5 ml of DMSO/ml of chlorine-free water. Next, 10 larvae were placed into each vial. The mortality rates of the mosquito larvae were evaluated after 24 h and 48 h. A series of at least 4 concentrations in triplicate was needed to obtain LC_{50} values. The results were analyzed by probit analysis.

4.4.3. Molluscicidal test

The extracts and compound **1** were evaluated for lethality to adults and embryos of the snail *Biomphalaria glabrata* using standard protocols (Marston and Hostettmann, 1991). A drop of DMSO was added to control and test substances in order to enhance their solubility. Niclosamide was used as a positive control.

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