Enicostemins A and B, New Secoiridoids from *Enicostemma verticillatum*

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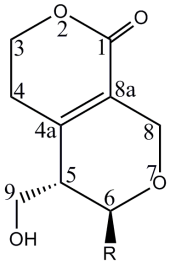
The new secoiridoids Enicostemins A (**1**) and B (**2**) were isolated from the *n*-butanol-soluble fraction of *Enicostemma verticillatum* along with gentiocrucine (**3**) and rutin (**4**), which were isolated for the first time from the genus *Verticillatum*. Their structures were assigned based on spectroscopic studies.

Key words: Enicostemma verticillatum, Secoiridoids, Enicostemins A and B

Introduction

The genus *Enicostemma* belonging to the family Gentianaceae comprises four species [1, 2]. One of these is *Enicostemma verticillatum*, which is widely distributed in South America, Africa, and Asia [1]. In Pakistan, it is mainly found in Thatta, Badin, Hyderabad, Mirpur, Gharo, and Manghopir [1]. *E. verticillatum* is a bitter tonic and is used as a substitute for chirayita as a blood purifier. The literature survey revealed that only a flavone *C*-glucoside has so far been reported from this species [3]. Herein, we report the isolation and structure elucidation of two new secoiridoids named as enicostemins A (**1**) and B (**2**) (Fig. 1) along with gentiocrucine (**3**) [4] and rutin (**4**) [5], which have been isolated for the first time from this species.



1. R = CH_2OH
2. R = *O*–β-D-glucosyl

Fig. 1. Structures of enicostemins A (**1**) and B (**2**).

Results and Discussion

Enicostemin A (**1**) was obtained as a colorless gummy solid. The high-resolution EI-MS of **1** exhibited an $[M]^+$ peak at m/z = 214.0841 corresponding to the molecular formula $C_{10}H_{14}O_5$ (calcd. 214.0838). The IR spectrum indicated the presence of hydroxyl groups (3400 cm^{-1}), a conjugated carbonyl function (1701 cm^{-1}) and conjugated double bonds (1635 cm^{-1}). The UV spectrum showed a strong absorption at 240 nm which is characteristic of secoiridoids [6].

The ^{1}H NMR spectrum showed the multiplet of an oxymethine proton at δ_H = 3.69, and oxymethylene protons were observed at δ_H = 4.41 (m, 1H, H-3), 4.38 (m, 1H, H-8), 4.25 (d, J = 16.0 Hz, 1H, H-8), 4.02 (m. 1H, H-3), 3.78 (dd, J = 11.0, 7.0 Hz, 1H, H-10), 3.71 (m, 2H, H-9), and 3.64 (dd, J = 11.0, 4.5 Hz, 1H, H-10).

The broad band (BB) and distortionless enhancement by polarization transfer (DEPT) ^{13}C NMR spectra showed 10 signals comprising 5 methylene, 2 methine and 3 quaternary carbons. The most downfield signal at δ_C = 165.4 (C-1) was attributed to an α,β-unsaturated ester while signals of conjugated olefinic carbons were observed at δ_C = 153.8 (C-4a) and 125.0 (C-8a). The oxymethine carbon gave a signal at δ_C = 77.8 (C-6), and four oxymethylene carbons resonated at δ_C = 67.6 (C-8), 65.9 (C-3), 63.3 (C-10), and 60.5 (C-9). Both the ^{1}H and ^{13}C NMR data showed close resemblance to those of 5,6-dihydro-5-hydroxymethyl-6-methyl-1*H*,3*H*-pyrano[3,4-c]-pyran-1-one [7, 8]. However, the absence of signals due to an olefinic proton indicated the presence

of a tetra-substituted double bond which could be located between C-4a and C-8a. Compound **1** also differs from 5,6-dihydro-5-hydroxymethyl-6-methyl-1*H*,3*H*-pyrano[3,4-c]-pyran-1-one in having a hydroxymethyl moiety at C-6 instead of a methyl group. The presence of hydroxymethyl groups at both C-5 and C-6 could also be confirmed through ^{1}H-^{1}H correlated spectroscopy (COSY) as well as heteronuclear multiple-bond correlation spectroscopy (HMBC), as illustrated in Fig. 2. Upon irradiation of the oxymethylene protons of C-9 at δ_H = 3.71 the multiplet of H-5 collapsed into a doublet (J = 2.7 Hz). On the other hand, irradiation of H-10$_a$ at δ_H = 3.78 changed the multiplet of H-6 into a double doublet (J = 2.7 and 7.0 Hz), and irradiation of H-10$_b$ at δ_H = 3.64 converted the multiplet of H-6 into a double doublet (J = 2.7 and 4.5 Hz). The smaller coupling constant between H-5 and H-6 was quite similar to the ones of gentiopicroside [9] and 5,6-dihydro-5-hydroxymethyl-6-methyl-1*H*,3*H*-pyrano[3,4-c]-pyran-1-one [7], and therefore both the protons at C-5 and C-6 are equatorial, and the hydroxymethyl substituents at C-5 and C-6 are *trans*-oriented. Conclusive evidence was provided by nuclear Overhauser enhancement spectroscopy (NOESY) which showed a correlation between H-6 and the oxymethylene protons at H-9. Thus enicostemin A (**1**) could be assigned the structure 5,6-bis(hydroxymethyl)-4,5,6,8-tetrahydro-1*H*,3*H*-pyrano [3,4-*c*]pyran-1-one (Fig. 1).

Enicostemin B (**2**) was obtained as a colorless gummy solid. The molecular formula $C_{15}H_{22}O_{10}$ was established by HR-FAB-MS showing an $[M–H]^-$ peak at m/z = 361.1134 (calcd. for $C_{15}H_{21}O_{10}$, 361.1129). The IR and UV spectra were very similar to those of **1**.

The ^{1}H NMR spectrum was also similar to that of **1** except for the downfield shift of H-6 to δ_H = 5.01. The anomeric proton was observed at δ_H = 5.90 as a doublet (J = 7.4 Hz, H-1′). Further oxymethine protons of the hexose moiety were observed in the range δ_H = 3.91 – 3.53 while the oxymethylene protons were observed at δ_H = 3.72 (dd, J = 11.0, 5.5 Hz, 1H, H-6′) and 3.62 (dd, J = 11.0, 4.1 Hz, 1H, H-6′). The larger coupling constant of the anomeric proton indicated a β-glycosidic linkage. Enzymatic hydrolysis provided an aglycone which could not be worked up due to paucity of material. The glycone could be identified as D-glucose through co-TLC with an authentic sample and the sign of its optical rotation. The downfield shift of C-6 indicated the presence of a β-D-glucopyranosyloxy moiety at this position.

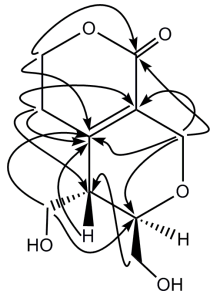


Fig. 2. Important HMBC correlations of enicostemin A (**1**).

The ^{13}C NMR spectrum showed 15 signals comprising 5 methylene, 7 methine and 3 quaternary carbons. It showed common features to those of **1** except for the signal of C-6 being shifted downfield to δ_C = 98.7 (C-6) due to the presence of two vicinal oxygen atoms. In addition, the signals of a hexose moiety were observed at δ_C = 103.9 (C-1′), 78.1 (C-5′), 77.6 (C-3′), 73.0 (C-2′), 71.6 (C-4′), and 62.8 (C-6′). Conclusive evidence was provided by the HMBC spectrum showing a ^3J correlation of the anomeric proton at δ_H = 5.90 with C-6 (δ_C = 98.7). Similarly H-6 at δ_H = 5.01 showed a ^3J correlation with the anomeric carbon (δ_C = 103.9). Further HMBC and NOESY correlations were similar to those of **1** allowing us to assign the structure of **2** as 5-(hydroxymethyl)-6-{[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl]-oxy}-4,5,6,8-tetrahydro-1*H*,3*H*-pyrano[3,4-*c*]-pyran-1-one (Fig. 1).

Experimental Section

General experimental procedures

The UV and IR spectra were recorded on Hitachi UV-3200 and JASCO 302-A spectrometers. ^{1}H, ^{13}C NMR, and 2D NMR spectra were recorded on a Bruker AM-400 spectrometer. Chemical shifts (δ) are expressed in ppm relative to TMS as the internal standard, and coupling constants (J) are given in Hz. The EI-MS and HR-EI-MS were measured on a JEOL JMS-HX-110 mass spectrometer. Silica gel 230 – 400 mesh (E. Merck. Darmstadt, Germany) was used for column chromatography. Diaion HP-20 ion exchange resin (Nippon Rensui Co., Mitsubishi Chemical Corporation, Tokyo, Japan) was employed for ion exchange chromatography, and silica gel plates Si 60 F_{254} (E. Merck Darmstadt, Germany) for TLC. Preparative high-performance liquid chromatography (HPLC) was used for the final purification *via* recycling preparative HPLC (LC-908W-C-60, Japan Analytical Industry Co. Ltd, Tokyo, Japan) using an ODS-M-80 column (4 μM, 250 × 200 mm^2; Japan Analytical Industry, Co. Ltd).

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of compounds **1** and **2** (in $CDCl_3$; δ in ppm and J in Hz).

	1		**2**	
No.	δ_H	δ_C	δ_H	δ_C
1	–	165.4	–	165.4
2	–	–	–	–
3	4.02 (m)	65.9	4.00 (m)	65.9
	4.41 (m)		4.40 (m)	
4	2.48 (m)	28.8	2.47 (m)	28.6
4a	–	153.8	–	153.5
5	2.38 (m)	44.2	2.61 (m)	41.2
6	3.69 (m)	77.8	5.01 (d, 1.5)	98.7
7	–	–	–	–
8	4.25 (d, 16.0)	67.6	4.27 (d, 16.0)	66.8
	4.38 (m)		4.37 (m)	
8a	–	125.0	–	125.0
9	3.71 (m)	60.5	3.71 (m)	60.7
10	3.78 (dd, 7.0, 11.0)	63.3	–	–
	3.64 (dd, 4.5, 11.0)		–	–
1′			5.90 (d, 7.4)	103.9
2′			3.91 (m)	73.0
3′			3.70 (m)	77.6
4′			3.53 (m)	71.6
5′			3.65 (m)	78.1
6′			3.62 (dd, 4.1, 11.0)	62.8
			3.72 (dd, 5.5, 11.0)	

Plant material

The whole plant material of *E. verticillatum* (Gentianaceae) was collected from Thatta region (Sindh, Pakistan) and identified by Prof. Dr. Surraiya Khatoon, Plant Taxonomist, Department of Botany, University of Karachi, Karachi, Pakistan, where a voucher specimen (No. 15013) has been deposited in the herbarium.

Extraction and isolation

The shade-dried plant material (30 kg) was extracted with MeOH (3 × 1 L) at r.t. The residue from the methanolic extract was suspended in water and successively extracted with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. The *n*-BuOH-soluble fraction (130 g) was subjected to chromatography over a Diaion HP-20 column eluting with mixtures of MeOH and water in decreasing order of polarity. The fraction eluted with MeOH-H_2O (3 : 1) was rechromatographed over silica gel and eluted with mixtures of CH_2Cl_2 and MeOH in increasing order of polarity. The fraction eluted with CH_2Cl_2-MeOH (9.6 : 0.4) was a binary mixture of compounds which was separated by preparative HPLC eluting with MeOH-H_2O (1 : 1). Compound **1** was obtained as a colorless gummy solid (18 mg; t_R = 22 min). Compound **2** was also obtained as a colorless gummy solid (15 mg; t_R = 41 min).

*Enzymatic hydrolysis of enicostemin B (**2**)*

Compound **2** (4 mg) was dissolved in H_2O (2 mL), to which β-glycosidase from almond (To-yobo, Japan) (2 mg) had been added, and the solution was kept at 37 °C for 22 h. After addition of H_2O (2 mL), the solution was extracted with EtOAc (5 mL). The residue recovered from the organic phase could not be worked up due to paucity of material. The aqueous layer was concentrated *in vacuo*, and the residue was purified by column chromatography over silica gel eluting with $CHCl_3$-MeOH with an increasing amount of MeOH to give D-glucose (2 mg) which was identified by co-TLC over silica gel with an authentic sample [solvent: *n*-BuOH-Me_2CO/H_2O (4 : 5 : 1, t_R = 0.35), $[\alpha]_D^{20}$ = +50.1 (c = 0.1, H_2O)].

*Enicostemin A (**1**)*

Colorless gummy solid. – $[\alpha]_D^{20}$ = −155 (c = 0.02, MeOH). – UV ($CHCl_3$): λ_{max} = 240 (4.32) nm. – IR (KBr) ν_{max}: = 3400 (OH), 1701 (ester) and 1635 cm^{-1} (conjugated C=C). – HRMS ((+)-EI): m/z = 214.0841 $[M]^+$ (calcd. 214.0838 for $C_{10}H_{14}O_5$). – EIMS: m/z (rel. int., %) = 214 (22) $[M]^+$, 196 (15), 183 (32), 178 (21), 165 (19), 153 (100). – ^{1}H and ^{13}C NMR spectral data: see Table 1.

*Enicostemin B (**2**)*

Colorless gummy solid. – $[\alpha]_D^{20}$ = −118 (c = 0.02, MeOH). – UV ($CHCl_3$): λ_{max} = 240 (4.32) nm. – IR (KBr) ν_{max}: = 3400 (OH), 1701 (ester) and 1635 cm^{-1} (conjugated C=C). – Negative HRMS ((–)-FAB): m/z = 361.1134 $[M–H]^-$ (calcd. 361.1129 for $C_{15}H_{21}O_{10}$). – ^{1}H and ^{13}C NMR spectral data: see Table 1.

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