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New Unusual Iridoids from the Leaves of Noni (*Morinda citrifolia* L.) Show Inhibitory Effect on Ultraviolet B-Induced Transcriptional Activator Protein-1 (AP-1) Activity

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Abstract—A novel iridoid dimer in whose structure the two iridoid units are connected by a rare ether group, together with two new unusual iridoids showing significant inhibition of UVB-induced Activator Protein-1 (AP-1) activity in cell cultures, have been isolated from the leaves of noni (*Morinda citrifolia* L.). Their structures were determined on the basis of detailed high-field 1D and 2D spectral analysis. Their inhibitory effect on UVB-induced transcriptional Activator Protein-1 (AP-1) activity are also discussed.

Introduction

Iridoids are monoterpenes based on the cyclopenta[*c*]pyranoid skeleton represented by iridane (*cis*-2-oxabicyclo[4.3.0]nonane).[1] They are found as natural constituents in a large number of plant families, usually as glucosides.[2–4] The name iridoid is a generic term derived from the names iridomyrmecin, iridolactone and iridodial, compounds isolated from some species of *Iridomyrmex*, a genus of ants, in which they occur as defensive secretions.[5] This class of compounds displays various biological activities,[6] such as cardiovascular activity, antitumor activity, antiviral activity and immunomodulator activity. The family Rubiaceae is well known for the constituents of iridoid.[2,3] In our investigation for bioactive iridoids from plants, we studied the leaves of *Morinda citrifolia* L. (Rubiaceae), also known as noni, native to the Indian Ocean, which grows in the open coastal regions and in forest areas up to about 1300 feet above sea level. The plant is a small evergreen tree. The bark, stem, root, leaf and fruit have been used traditionally as a folk remedy for many diseases including diabetes, hypertension and cancer.[7,8] Our preliminary communications[9,10] have reported the characterization of citrifolinin **A** (**1**) and citrifolinoside (**2**), which feature the presence of a unique substitute, a rearranged ferulic acid moiety, and have shown significant inhibition of UVB-induced Transcriptional Activator Protein-1 (AP-1) activity. This paper reports the isolation and inhibitory effect on UVB-induced AP-1 of these two novel metabolites, as well as the isolation and characterisation of a new unusual bis-iridoid, named citrifolinin **A-1** (**3**). In the structure of citrifolinin **A-1**, the two iridoid units are not connected by an ester group as in general bis-iridoids, but by a rare ether group directly between C-10 positions. However, citrifolinin **A-1** didn't display the inhibition of UVB-induced Transcriptional Activator Protein-1 (AP-1) activity.

Results and Discussion

The butanol fraction of the ethanol extract of the dried noni leaves (5 kg) was subjected successively on Diaion HP-20, silica gel, and RP-18 silica gel to afford compound **1**–**3**. They structures were established by interpretation and full assignments of 1D and 2D NMR spectroscopic data.

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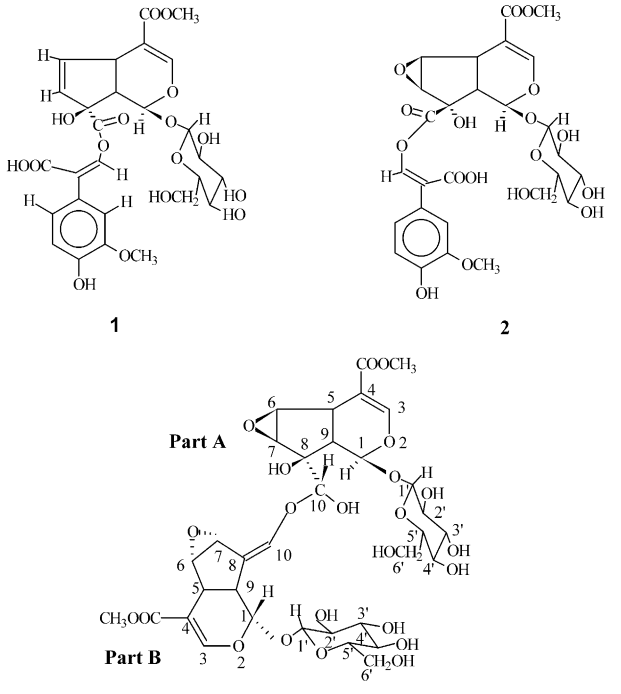
Compound **3** proved to have the molecular formula $C_{34}H_{42}O_{23}$, as seen from the negative-ion APCI-MS (*m*/*z* 817 $[M-H]^-$) combined with ^{13}C NMR and DEPT data (Table 1). On the basis of its NMR data, the structure of a bis-iridoid glycoside was hypothesized for compound **3**. In fact, among the signals observed in its ^1H and ^{13}C NMR spectra, the resonance of two distinct parts, indicated as parts A and B, could be identified. In the ^1H NMR spectrum, signals very similar in coupling patterns to those of the protons in 6β,7β-epoxysplendoside[11] type appeared in pairs as follows: δ 7.57 (d, $J=2.0$ Hz; H-3A); 7.55 (d, $J=1.2$ Hz; H-3B); 6.04 (s, H-1A); 5.32 (s, H-1B); 3.95 (d, $J=2.8$ Hz, H-6A); 3.94 (d, $J=2.8$ Hz, H-6B); 3.70 (d, $J=2.8$ Hz, H-7A); 3.44 (d, $J=2.8$ Hz, H-7B); 3.34 (d, $J=9.6$ Hz, H-5A); 3.28 (d, $J=9.2$ Hz, H-5B); 2.35 (d, $J=9.6$ Hz, H-9A); 2.54 (d, $J=9.2$ Hz, H-9B). The only significant differences between parts A and B were the NMR data of position 8 and 10. In part A, the proton and carbon signals at δ_H 5.70 (d, $J=2.8$ Hz, H-10) and δ_C 81.1 (s, C-8), 107.6 (d, C-10) suggest that C-8 is a quaternary carbon with an oxygen substitution; C-10 exists as a hemiacetal group (Fig. 1). However, in part B, the proton and carbon signals at δ_H 7.38 (d, $J=2.8$ Hz, H-10) and δ_C 98.3 (s, C-8), 164.4 (d, C-10) reveal the presence of an enol-ether system (Fig. 1). In addition, the signals in the region of δ_H 3.10–5.00 comprised two anomeric proton resonances at δ_H 4.60 (d, $J=8.0$ Hz) and 4.54 (d, $J=7.6$ Hz), suggesting the presence of two β-glucopyranoside units, which support the proposed dimeric structure. The dimeric feature was also confirmed by the ^{13}C NMR data (Table 1). The HMBC correlation between C-1 (δ 100.1) and H-1′ (δ 4.60, d, $J=8.0$ Hz) for part A, and C-1 (δ 99.8) and H-1′ (δ 4.54, d, $J=7.6$ Hz) for part B, as well as C-1′ and H-1 for both iridoid moieties led to the assignments of the glucose units of each, parts A and B (Fig. 2). The HMBC cross peaks between H-10A and C-10B, H-10B and C-10A, and the NOESY correlation between H-10A and H-10B clearly establish the connectivity between the two iridoids, parts A and B, to be a rare ether linkage (Fig. 1).

Table 1. δ_H (600 MHz) and δ_C (150 MHz) NMR spectra data of compound **3** ($CDOD_3$) (δ in ppm, *J* in Hz)

	Part A		Part B	
	δ_H	δ_C	δ_H	δ_C
1	6.04 s	94.2 d	5.32 s	93.8 d
3	7.57 d, 2.0	154.7 d	7.55 d, 1.2	153.9 d
4		108.3 s		107.2 s
5	3.34 d 9.6	33.7 d	3.28 d 9.2	32.6 d
6	3.95 d, 2.8	58.2 d	3.94 d, 2.8	57.6 d
7	3.70 d, 2.8	61.1 d	3.44 d, 2.8	58.2 d
8		81.1 s		98.3 s
9	2.35 d, 9.6	47.2 d	2.54 d, 9.2	44.9 d
10	5.70 d, 2.8	107.6 d	7.38 d, 2.8	164.4 d
11		168.5 s		168.2 s
OCH_3	3.77 s	52.0 q	3.75 s	51.9 q
1′	4.60 d, 8.0	100.1 d	4.54 d, 7.6	99.8 d
2′	3.13 t, 8.8	74.5 d	3.12 t, 9.2	74.3 d
3′	3.27 m	78.2 d	3.23 m	78.1 d
4′	3.23 m	71.3 d	3.20 m	71.3 d
5′	3.31 m	77.8 d	3.29 m	77.8 d
6′	3.66 m 3.87 dd, 1.2, 12.0	62.6 t	3.66 m 3.87 dd, 1.2, 12.0	62.4 t



1

2

3

Figure 1. Structures of compounds **1**–**3**.

The configuration of C6, C7-epoxy was confirmed as β by $J_{H1,9} < 1$ Hz, $\Delta\delta$ C_3–$C_4 = 46.4$ (part A) and 46.7 (part B) < 47 ppm, and $\delta_{C1} = 94.2$ (part A) and 93.8 (part B) < 99 ppm.[12,13] In order to determine the stereochemistry of C-8 and C-10 for part A, NOESY measurements were carried out on **3**. In the NOESY spectrum, the presence of a strong cross peak between H-1 and H-10 indicat that the linkage between C-8 and C-10 is α orientation, H-10 is in the β position. This is proved by the three-dimensional structure model. Thus, compound **3** was determined as shown (Fig. 1), named as citrifolinin **A-1**. Full assignments of the ^1H and ^{13}C NMR signals were accomplished using HMBC, HMQC, ^1H–^1H COSY, TOCSY, and NOESY experiments (Table 1).

It is well known that UVB irradiation plays a major role in the development of human skin cancer,[14,15] acting both as a tumor initiator and tumor promoter. Transactivation of AP-1 plays a key role in tumor promotion.[16,17] Inhibition of AP-1 activity has been shown to lead to suppression of cell transformation.[17] It has been reported that some chemopreventive agents, such as aspirin and tea polyphenols, can inhibit cell transformation and tumor promotion and were also found to suppress AP-1 transactivation.[17–20] All of these results strongly indicated that the inhibition of AP-1 activity leads to the suppression of tumor promotion. Because of this we investigated the inhibitory activity of UVB-induced AP-1 for these three compounds. It was shown

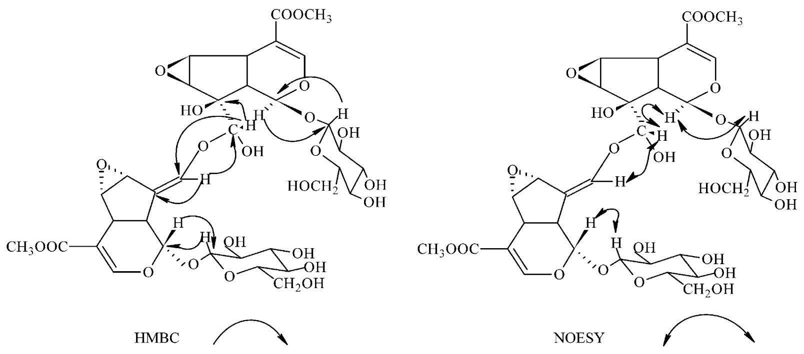


Figure 2. Significant HMBC (H→C) and NOESY correlation of compound 3.

that citrifolinin **A** (1) and citrifolinoside (2) displayed significant inhibitory effect with an IC_{50} of 69.6 and 29.0 μM respectively (Fig. 3). However, citrifolinin **A-1** (3) did not show the inhibition on UVB-induced AP-1 activity. Since AP-1 has an important role in tumorigenesis, the results of this investigation may provide new insight into the function of iridoids as chemopreventive agents, the mechanism of noni leaves in tumor suppression and the possibility for its application in tumor prevention and treatment.

Experimental

General experimental procedures

^1H (600 MHz), ^{13}C (150 MHz), and 2D NMR spectra were obtained on a Varian AM-600 spectrometer with TMS as internal reference. APCI MS was obtained on a Fisons/VG Platform II mass spectrometer. Prepared Thin-layer chromatography was performed on Sigma-Aldrich TLC plates (1000 μm thickness, 2–25 μm particle size). Thin-layer chromatography was performed on Sigma-Aldrich TLC plates (250 μm thickness, 2–25 μm particle size), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in ethanol solution.

Plant material

The dried leaves of *Morinda citrifolia* were collected from Bengal, India in 1999 and were identified by Dr. Vladimir Badmaev at the Sarbinsa Corporation. A voucher specimen (HS16) was deposited in the Department of Food Science, Cook College, Rutgers University.

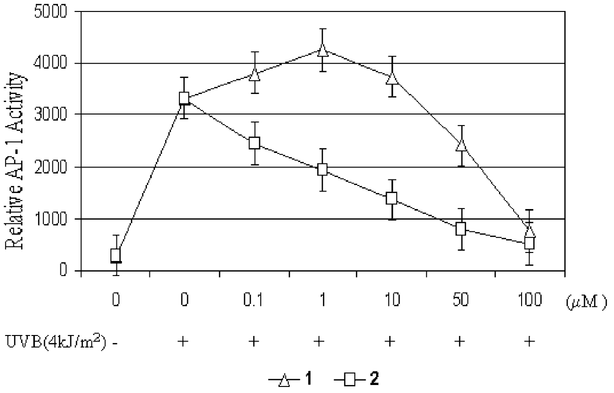


Figure 3. Inhibitory effect of 1 and 2 on UVB-induced AP-1 activity.

Extraction and isolation procedures

The dried noni leaves (5 kg) were extracted with 95% EtOH (4 L) at 50 °C for 1 day. The extract was concentrated to dryness under reduced pressure, and the residue was suspended in water (500 mL) and partitioned successively with hexane (3×500 mL), ethyl acetate (3×500 mL), and *n*-butanol (3×500 mL). The butanol fraction was subjected to a Diaion HP-20 column, eluted with a Water–EtOH (water, 30% EtOH, 70% EtOH, 95% EtOH) solvent system. The fraction (5 g) eluted by 30% EtOH was subjected to silica gel column chromatography with an ethyl acetate–MeOH–H_2O–hexane solvent system (10:1:1:0.5→5:1:1:0). A total of 10 fractions were collected. Fraction 2, eluted by ethyl acetate–MeOH–H_2O–hexane (10:1:1:0.5), was subjected to RP-18 silica gel column chromatography with 30% MeOH to give 3 fractions (I–III). Fractions II and III were rechromatographed on a Sephadex LH-20 eluted by 95% EtOH to give compounds **1** (200 mg) and **2** (110 mg) respectively. Fraction 9 eluted by ethyl acetate–MeOH–H_2O–hexane (5:1:1:0) was subjected to RP-18 silica gel column chromatography with 30% MeOH, and then Sephadex LH-20 eluted by 95% EtOH to give 46 mg compound **3.** Compound **3** was isolated as a white amorphous substance: ^1H NMR (CD_3OD, 600 MHz): see Table 1; ^{13}C NMR (CD_3OD, 150 MHz): see Table 1; negative APCI-MS *m*/*z* 817 $[M-H]^-$.

UV Irradiation

UVB irradiation was performed on serum-starved monolayer cell cultures by using a transluminator emitting UVB. The source of UVB was a bank of four Westinghouse F520 lamps (National Biological, Twinsburg, OH) at a fluence of 6J·S·m light in the UVB range. Approximately 10% of the remaining radiation from the F520 lamp was in the UVA range (320 nm). Although almost no UVC leakage occurred, the UVB irradiation was performed in a UVB exposure chamber fitted with a Kodak Kodacel K6808 filter that eliminated all wavelengths below 290 nm.[21] This lamp is one of the most frequently used UVB sources for the study of carcinogenesis. IARC refers to this lamp as a source emitting mainly UVB radiation for the studies of cancer induction in animals. UVB radiation was measured using the UVX radiometer from UVP (UVX-31).

Cell culture

JB6 P^+1-1 cell line was a stable transfectant with AP-1 luciferase reporter gene. P^+1-1 cells were cultured in monolayers at 37 °C, 5% CO_2 using minimum essential medium (MEM) containing 5% fetal bovin serum (FBS), 2 mM L-glutamine, and 25 μg/mL gentamycin. FBS and MEM were from Bio Whittaker, Inc. (Walkersville, MD); and the luciferase assay substrate was from Promega (Madison, WI).

Assay for AP-1 activity

JB6 P^+1-1 cells were used for assay of AP-1 activity. Viable cells (8×10^3), suspended in 100 μL of 5% FBS/MEM, were added to each well of a 96-well plate. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. 12–24 h later, cells were starved by culturing in 0.1% FBS/MEM for 24 h prior to being treated with or without noni leaf components (**1–3**) for 30 min and subsequently exposed to UVB (4 kJ/m^2) radiation and incubated for an additional 12 h. The cells were extracted with lysis buffer [0.1 M potassium phosphate buffer (pH 7.8); 1% Triton X-100; 1 mM DTT; 2 mM EDTA], and luciferase activity was measured as described previously using a luminometer (Monolight 2010).[20] The results are expressed as relative AP-1 activity (Fig. 3).

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