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A New Type of Monoterpenoid Indole Alkaloid Precursor from *Alstonia rostrata*

Xiang-Hai Cai,*,† Mei-Fen Bao,† Yu Zhang,† Chun-Xia Zeng,‡ Ya-Ping Liu,† and Xiao-Dong Luo*,†

State Key Laboratory of Phytochemistry and Plant Resources in West China and Southwest China Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China

xhcai@mail.kib.ac.cn; xdluo@mail.kib.ac.cn

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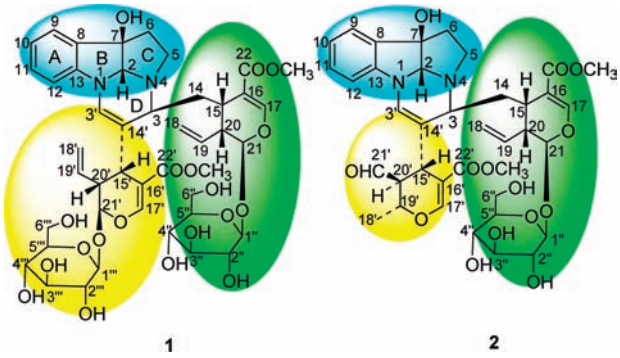
ABSTRACT



Currently, all monoterpenoid indole alkaloids (MIAs) have been derived from strictosidine, which originates from the condensation of tryptophan with secologanin in a 1:1 ratio. However, our phytochemical research on *Alstonia rostrata* revealed a potential new precursor for these compounds. We isolated the alstrostines A and B, and it was determined that they were derived from tryptophan and secologanin in a 1:2 ratio, which supported the presence of a new type of MIA precursor.

Alkaloids represent a highly diverse group of compounds that are related solely by the presence of a nitrogen atom in a heterocyclic ring. Plants are estimated to produce approximately 12 000 different alkaloids, and they can be organized into groups according to their carbon skeletal structures.[1] Among them, monoterpenoid indole alkaloids (MIAs) might account for a quarter of this large group of natural products. Both their highly complex chemical structures and pronounced pharmacological activity have made research on alkaloids, including elucidation of their biosynthetic pathways, very attractive for many decades.[2] MIAs have been proposed to arise from strictosidine, which itself originates from the condensation of tryptophan with secologanin in a 1:1 ratio.[3] Currently, strictosidine is the only key precursor recognized in the biosynthesis of MIAs.[4] However, additional ratios of tryptophan and secologanin occur in nature (e.g., 1:2, 2:1), and this led us to investigate whether these ratios may play a role in MIA biosynthesis. Plants from the genera *Alstonia* and *Melodinus* represent excellent systems for this work in that they both accumulate high levels of MIAs.[5] Here we describe

† State Key Laboratory of Phytochemistry and Plant Resources in West China.

‡ Southwest China Germplasm Bank of Wild Species.

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the structural determination and proposed biosynthesis of a new kind of MIA precursor from *A. rostrata* (Figure 1).

The dried and powdered leaves of *A. rostrata* (8.0 kg)[6] were extracted with MeOH (20 L × 3) at room temperature, and the solvent was evaporated *in vacuo*. The residue was dissolved in 1% HCl, and the solution was subsequently basified to pH 8−9, using ammonia. The basic solution was partitioned with EtOAc, affording a two-phase mixture including the aqueous phase and EtOAc phase (total alkaloids). The total alkaloid fraction (78 g) was collected and then dissolved in MeOH and was subjected to column chromatography over silica gel eluting with $CHCl_3$−MeOH [from $CHCl_3$ to $CHCl_3$−MeOH (1:1)] to afford six fractions (I−VI). Fraction IV (6.5 g) was separated utilizing a preparative reversed-phase C_{18}-MPLC column with a gradient flow of 30−70% (v/v) aqueous MeOH to yield seven subfractions IV-1−7. Subfraction IV-6 (214 mg) was further separated by chromotography on silica gel using $CHCl_3$−MeOH (6:1) as an eluent to give alstrostine B (**2**, 11 mg). Fraction VI (5.9 g) was purified using a preparative reversed-phase C_{18}-MPLC column with a gradient flow of 20−50% (v/v) aqueous MeOH to yield six subfractions VI-1−6. Alstrostine A (**1**, 5 mg) was subsequently isolated from subfraction VI-6.

Compound **1**[7] possessed a molecular formula of $C_{44}H_{56}N_2O_{19}$, as evidenced by a high resolution electron spray ionization mass spectra (HRESIMS) at m/z 917.3549 $[M + H]^+$, in combination with ^1H, ^{13}C NMR, DEPT spectra, and appropriate for 18 degrees of unsaturation. The UV spectrum of compound **1** demonstrated the presence of conjugated groups by presenting maximum absorptions at 211, 231, 279, and 314 nm. Its IR spectrum indicated the presence of hydroxyls (3430 cm^{-1}), carbonyls (1703 cm^{-1}), and olefin groups (1632 cm^{-1}). The ^1H NMR spectrum presented two doublets (δ_H 7.25, 6.67 ppm) and two triplets (δ_H 7.13, 6.79 ppm), displaying the signals for an unsubstituted indole alkaloid.[8] In the HMBC spectrum of **1**, crosspeaks between the signal (δ_H 7.25 ppm, H-9) with carbon resonances [δ_C 149.5 (s, C-13), 130.7 (s, C-11), and 89.1 (s) ppm], allowed the assignment of the later carbon signal to C-7. Similarly, HMBC correlations from the signal at δ_C 4.90 (s) ppm to C-7/C-13 suggested the proton signal was attributed to H-2. The presence of two methylene signals (δ_C 50.9, 43.6 ppm) that were correlated [δ_H 2.82 (1H, m)/δ_H 2.28 (1H, m) and 2.58 (1H, m)/δ_H 1.97 (1H, m)] in the $^1H-^1H$ COSY spectrum and showed HMBC correlations to C-7/C-2 (δ_C 81.7, s) indicated the existence of a five-membered pyrrolidine C ring joined to the hydroxy-indoline framework.[9] The two signals were thus assigned to C-5 and C-6, respectively. This presumption was confirmed by HMBC correlations from H-2 to C-5/6. In addition, the remaining 34 ^{13}C NMR signals of **1** showed the presence of two terminal double bonds [δ_C 120.2 (t), 135.7 (d), 119.1 (t), 137.0 (d) ppm], two α, β-unsaturated carboxylic acid methyl esters [δ_C 153.0 (d), 110.6 (s), 169.6 (s), 51.9 (q), 154.0 (d), 112.5 (s), 169.0 (s), 51.9 (q) ppm], and two glucoses [δ_C 100.2 (d), 101.1 (d), 62.8 (t), 61.8 (t) ppm, and eight sp^3 methines between δ_C 78.5 and 70.9 ppm]. These data support the existence of two secologanin moieties.[10,3b] Both secologanin moieties were subsitituted by condensation of their respective aldehyde groups, as shown by the absence of aldehyde spectral signals. Additionally, HMBC correlations between δ_H 3.29 (H-3) with C-2, and between δ_H 6.30 (s, H-3′) with C-2/C-13, revealed that the two secologanins were jointed to N-1 and N-4, respectively. Correlations from H-3′ to C-3 (δ_C 56.1 d) and C-15′ (δ_C 37.8, d); from H-3 to C-14 (δ_C 33.0, t),

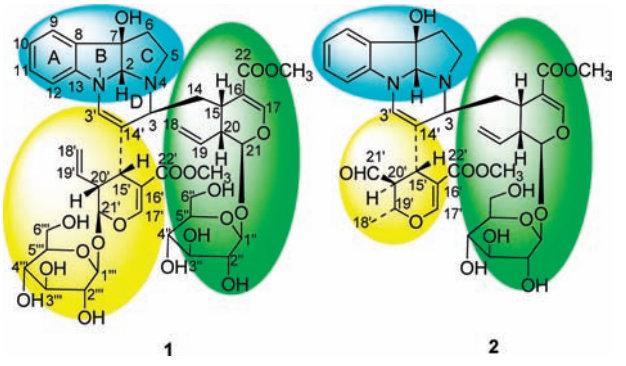

Figure 1. Structure of compounds **1**−**2**.

C-14′ (δ_C 115.2 s), and C-3′; and from H-15′ (δ_H 3.22) to C-3 (δ_C 56.1, d), C-3′, and C-14′ in the HMBC spectrum revealed a new six-membered ring D. The compound numbering system corresponded in biogenetic origin to the monoterpenoid indole alkaloids (Figure 1). Its full assignments of ^1H, ^{13}C, spectroscopic data were determined by an HMBC, HSQC $^1H-^1H$ COSY spectrum (Table 1).

Compound **2**[11] had a molecular formula of $C_{38}H_{46}N_2O_{14}$ according to HRESIMS (m/z 753.2886, $[M - H]^-$), with an absence of a glucose fraction to **1**. The UV absorption bands (211, 233, 277, and 315 nm) and IR spectrum (3429, 1713, and 1630 cm^{-1}) of compound **2** demonstrated that it was the same type of compound as **1**. The ^1H and ^{13}C NMR spectra of **2** were very similar to those of **1**, except that **2** had the signals of δ_C 18.1 (q), 70.0

(6) Leaves and twigs of *A. rostrata* collected in Apr. 2010 in Simao of Yunnan Province, P. R. China, and identified by Dr. Chun-Xia Zeng. Voucher specimen (Cai100613) deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, the Chinese Academy of Sciences.

(7) Alstrostine A: white powder; $[\alpha]_D^{20} = -257°$ (*c*, 0.09, CH_3OH); UV (CH_3OH) λ_{max} 211 (ε 3310), 231(ε 3096), 279 (ε 2032), and 314 (ε 662) nm; IR (KBr) ν_{max} 3430, 1703, and 1632 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; positive ESIMS m/z $[M + H]^+$ 917 (100); HRESIMS m/z 917.3549 $[M + H]^+$ (calcd for $C_{44}H_{56}N_2O_{19}$, 917.3555).

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(11) Alstrostine B: white powder; $[\alpha]_D^{20} = -290°$ (*c*, 0.13, CH_3OH); UV (CH_3OH) λ_{max} 211(ε 3203), 233 (ε 3295), 277 (ε 2846), 315 (ε 708) nm; IR (KBr) ν_{max} 3429, 1713, and 1630 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; positive ESIMS m/z $[M - H]^-$ 753 (100); HRESIMS m/z 753.2886 $[M - H]^-$ (calcd for $C_{38}H_{45}N_2O_{14}$, 753.2870).

Table 1. ^{1}H and ^{13}C NMR Assignments of Compounds **1** and **2**[a]

entry	**1** δ_C	**2** δ_C[b]	**1** δ_H (J in Hz)	**2** δ_H (J in Hz)[b]
2	81.7 d	81.7 d	4.90 (1H, s)	5.00 (1H, s)
3	56.1 d	53.1 d	3.29 (1H, m)	3.29 (1H, m)
5	50.9 t	49.9 t	2.82 (1H, m)	2.90 (1H, m)
			2.58 (1H, m)	2.33 (1H, m)
6	43.6 t	43.1 t	2.28 (1H, m)	2.33 (1H, m)
			1.97 (1H, m)	1.91 (1H, m)
7	89.1 s	88.6 s		
8	135.7 s	136.2 s		
9	125.0 d	124.4 d	7.25 (1H, d, 6.8)	7.24 (1H, d, 6.8)
10	121.4 d	120.9 d	6.79 (1H, t, 6.8)	6.76 (1H, t, 6.8)
11	130.7 d	129.9 d	7.13 (1H, t, 6.8	7.09 (1H, t, 6.8)
12	108.6 d	108.6 d	6.67 (1H, d, 6.8)	6.73 (1H, d, 6.8)
13	149.5 s	148.4 s		
14	33.0 t	32.4 t	2.08 (1H, m)	1.87 (1H, m)
			1.97 (1H, m)	2.28 (1H, m)
15	26.4 d	27.0 d	3.27 (1H, m)	3.33 (1H, m)
16	110.6 s	111.7 s		
17	153.0 d	152.0 d	7.44 (1H, s)	7.40 (1H, s)
18	120.2 t	119.7 t	5.32 (1H, d, 15.0)	5.35 (1H, d, 15.0)
			5.27 (1H, d, 12.0)	5.20 (1H, d, 12.0)
19	135.7 d	135.8 d	5.80 (1H, m)	5.78 (1H, m)
20	45.0 d	44.1 d	2.72 (1H, m)	2.80 (1H, m)
21	98.5 d	97.8 d	5.55 (1H, d, 3.6)	5.49 (1H, d, 3.6)
22	169.6 s	167.9 s		
22-OMe	51.9 q	51.3 q	3.70 (3H, s)	3.65 (3H, s)
7-OH			5.85 (1H, s)[c]	5.08 (1H, s)
3′	126.1 d	125.1 d	6.30 (1H, s)	6.29 (1H, s)
14′	115.2 s	115.1 s		
15′	37.8 d	33.1 d	3.22 (1H, d, 3.6)	3.48 (1H, s)
16′	112.5 s	105.8 s		
17′	154.0 d	156.8 d	7.59 (1H, s)	7.69 (1H, s)
18′	119.1 t	18.1 q	5.17 (1H, d, 15)	1.47 (1H, d, 6.8)
			5.12 (1H, d, 12)	
19′	137.0 d	70.0 d	5.65 (1H, m)	4.21 (1H, q, 6.8)
20′	47.1 d	51.6 d	2.54 (1H, m)	2.49 (1H, s)
21′	98.0 d	201.6 d	5.48 (1H, d, 6.4)	9.67 (1H, s)
1″	100.2 d	100.0 d	4.68 (1H, d, 7.2)	4.68 (1H, d, 7.2)
2″	74.7 d	74.3 d	3.18 (1H, m)	3.25 (1H, m)
3″	78.5 d	77.9 d	3.34 (1H, m)	3.40 (1H, m)
4″	71.6 d	71.4 d	3.29 (1H, m)	3.36 (1H, m)
5″	78.3 d	77.9 d	3.43 (1H, m)	3.40 (1H, m)
6″	62.8 t	62.8 t	3.90 (1H, m)	3.88 (1H, m)
			3.68 (1H, m)	3.66 (1H, m)
1‴	101.1 d		4.63 (1H, d, 7.8)	
2‴	74.6 d		3.15 (1H, m)	
3‴	78.1 d		3.08 (1H, m)	
4‴	70.9 d		3.23 (1H, m)	
5‴	78.0 d		3.34 (1H, m)	
6‴	61.8 t		3.48 (1H, m)	
			3.20 (m)	
22′	169.0 s	167.3 s		
22′-OMe	51.9 q	51.3 q	3.66 (3H, s)	3.63 (3H, s)

[a] Data recorded in methanol-d_3 (1) and acetone-d_6 (2) on a Bruker Avance 600 MHz spectrometer (^{1}H, ^{13}C, HSQC, HMBC, ^{1}H−^{1}H COSY, and ROESY); chemical shifts (δ) in parts per million in reference to most downfield signal of methanol-d_3 (δ 3.31 ppm) and cetone-d_6 (δ 2.04 ppm) for ^{1}H and to center peak of downfield signal of methanol-d_3 (δ 49.0 ppm) and acetone-d_6 (δ 30.0 ppm) for ^{13}C. [b] Data were recorded on a Bruker AMD-400 MHz spectrometer. [c] Data were recorded in DMSO.

(d), and an aldhyede at δ_C 201.6 (d) instead of the terminal double bonds at C-18/C19 and signal of δ_C 98.0 (d, C-21) seen in **1**. This suggested a rearrangement occurred from C-21 to C-19 in **2**, and this was further supported by the HMBC spectrum, with correlations of δ_H 4.21 (H-19′) with δ_C 51.6 (C-20′), 33.1 (C-15′), 18.1(C-18′), and 201.6 (C-21′), and of δ_H 7.69 (s, H-17′) with δ_C 70.0 (d, C-19′), 33.1 (d, C-15′), 105.8 (s, C-16′), and 167.3 (s, C-22′). Other structural parts of **2** were identical to those **1**, as indicated by the HMBC spectra.

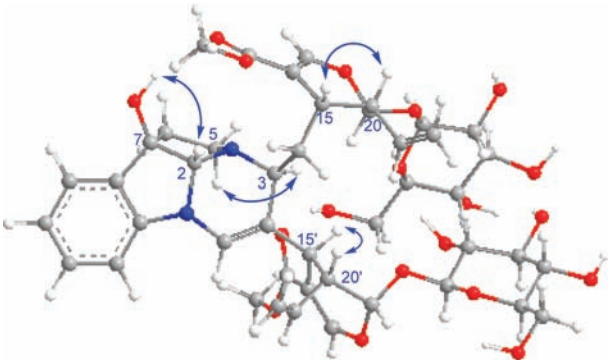

Figure 2. Key NOE correlations of energy-minimized conformer of **1**.

H-15(15′)/20(20′) of secologanin units in compound **1** were at the β-position and H-21(21′) at the α-position based on the biosynthesis[12] and the ROESY spectroscopic data.[10] However, ROESY correlation of H-19′/H-15′ in **2** placed H-19′ at the β-orientation, and its rearrangement from C-21′ to C-19′ resulted in inversion of configuration H-20′, α-orientation. The J values (J = 7.2, 7.8 Hz) of the two anomeric protons of the sugar moieties revealed a β-configuration of the glucose residues. D-Glucose was identified in the hydrolysate by TLC comparison with authentic samples and determination of their specific optical rotation (+ 24°).[13] Based on those data, the structures of **1** and **2** were elucidated as shown.

The relative configurations of H-2, H-3, and 7-OH of **1** and **2** were constructed from analysis of molecular models, and the energy was minimized using density functional theory (DFT) at the 6-31G (d,p) basis set level in Gaussian 03 overlaid with key correlations observed in the ROESY NMR spectrum (Figure 2).[14] Nuclear Overhauser Effect (NOE) correlation of H-2/7-OH (δ_H 4.74/δ_H 5.85 of **1**, and δ_H 5.00/δ_H 5.08 of **2**) in the ROESY spectrum indicated that both protons were on the same side. In addition, the ROESY correlations between H-3 with H-5 and their calculated interatomic distance of approximately 2.6 Å in the molecular model suggested the H-3 adopts another orientation. Based on these data, there were two isomers of **1** and **2**, respectively. Subsequently, for the assignment of the configuration of **1** and **2**, their optical rotation (OR)

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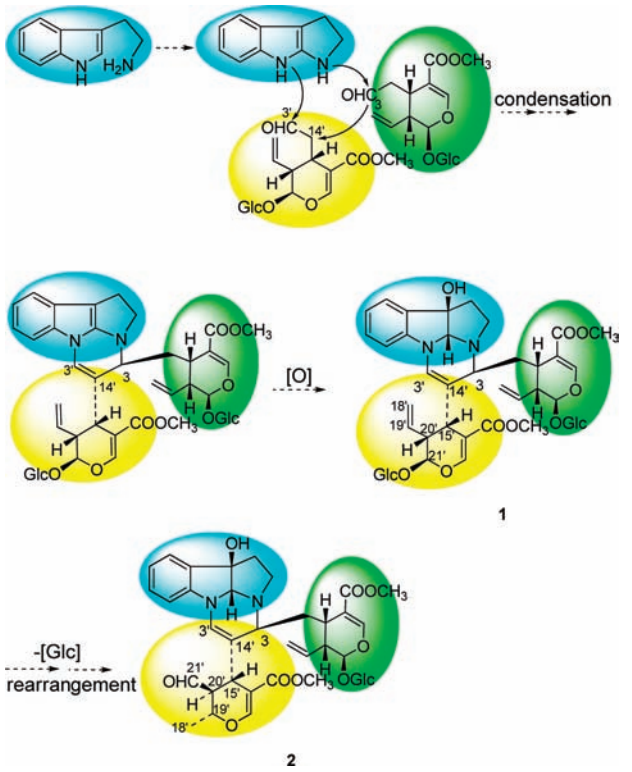

Figure 3. Proposed biosynthesis of **1**−**2**.

values were calculated by using the density functional theory (DFT) methods[15] in the Gaussian 03 program package.[16] The 'self-consistent reaction field' method (SCRF) was employed to perform the OR calculation of the most stable conformers of **1** and **2** with H-2 in the β-orientation in MeOH solution at the B3LYP/6-31G (d,p) level. The calculated OR values (−296° for **1** and −270° for **2**) were close to their experimental values (−257° for **1** and −290° for **2**), which supported that H-2/7-OH were β-oriented and H-3 was α-oriented in **1** and **2**.

In Figure 3, we propose a potential biosynthetic mechanism of compounds derived from a novel ratio of tryptophan and secologanins. Like biosynthesis of strictosidine,[3a] condensation of tryptophan and secologanins in a 1:2 ratio produced compound **1** with a 6/5/5/6 tetracyclic system (**1**). Further degulose and rearrangement from C-21 to C-19 of **1** formed **2**, which was common during biosynthesis of MIAs, such as the ajmalicine type.[17] The isolation of alstrostines A (**1**) and B (**2**) is the first evidence to support the presence of a new type of precursor of monoterpenoid indole alkaloids.

Compounds **1**−**2** were evaluated for their cytotoxicity against five human cancer cell lines, MCF-7 breast cancer, SMMC-7721 hepatocellular carcinoma, HL-60 myeloid leukemia, SW480 colon cancer, and A-549 lung cancer, using the MTT method reported previously[18] with minor revisions.[19] Unfortunately, none of them showed positive activity (IC_{50} > 40 μM).

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Supporting Information Available. 1-D and 2-D NMR data and optical rotation calculations of alstrostines A−B. This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) Cytotoxicity assay. All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA) in 5% CO2 at 37 °C. The cytotoxicity assay was performed according to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method in 96-well microplates. Briefly, 100 μL of adherent cells were seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition with an initial density of 1 × 10^5 cells/mL. Each tumor cell line was exposed to the test compound at concentrations of 0.0625, 0.32, 1.6, 8, and 40 μM in triplicates for 48 h, with cisplatin (Sigma, USA) as a positive control. After compound treatment, cell viability was detected, and the cell growth curve was graphed.