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Vitepyrroloids A–D, 2-Cyanopyrrole-Containing Labdane Diterpenoid Alkaloids from the Leaves of *Vitex trifolia*

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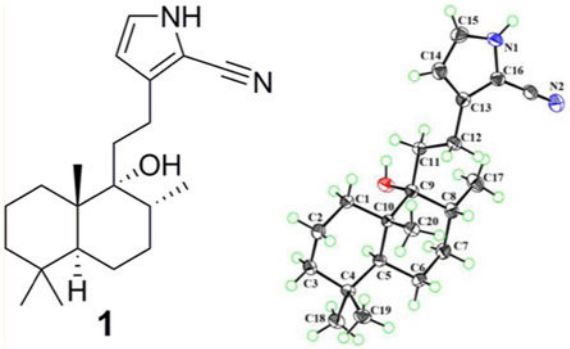
Abstract

Vitepyrroloids A–D (**1–4**), four new 2-cyano-substituted pyrrole-ring-containing labdane diterpenoids, were isolated from the leaves of *Vitex trifolia*. Their structures were elucidated based on spectroscopic data analysis. The absolute configuration of compound **1** was determined by X-ray diffraction. Compounds **1–4** are unprecedented labdane diterpenoids featuring a 2-cyano-substituted pyrrole ring. Compound **1** showed cytotoxic activity against a human nasopharyngeal carcinoma cell line (CNE1) with an IC_{50} value of 8.7 μM.

Graphical abstract



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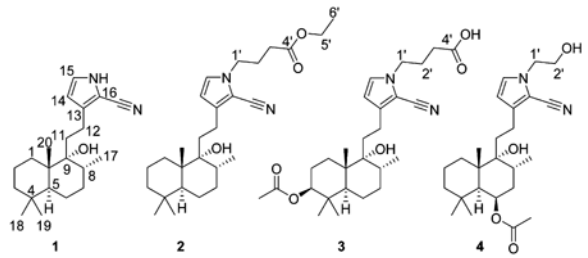
IR and NMR data for compounds **1–4**, EIMS of compound **1**, HREIMS for compounds **1** and **2**, HRESIMS for **3** and **4**, LC-HRESIMS for methanol extract of *V. trifolia*, and cytotoxicity for **1–4** (PDF) Crystallographic data (CIF)

Notes: The authors declare no competing financial interest.

Vitex trifolia L. (Verbenaceae), a small deciduous shrub, is widely distributed in Fujian, Guangdong, Guangxi, and Yunnan provinces in mainland China. The fruits of *V. trifolia* have been traditionally used for the treatment of colds, migraine, headache, and rheumatism.[1] The leaf extract has been reported to show cytotoxic activity.[2] Previous studies on this plant have focused on the isolation of various diterpenoids, especially the labdane diterpenoids.[3–5] Labdane-type diterpenes possess significant biological properties, such as cytotoxic,[6] antibacterial,[7] and anti-inflammatory activities.[8] Herein, four novel labdane diterpenoid alkaloids, vitepyrroloids A–D (**1–4**), were isolated from the leaves of *V. trifolia*. Compounds **1–4** are unprecedented labdane diterpenoid alkaloids containing a cyano-substituted pyrrole cyclic system. In compounds **2–4**, the pyrrole amino proton is replaced by ethyl butanoate, butanoic acid, and hydroxyethyl groups, respectively. The structures of compounds **1–4** were elucidated via spectroscopic data analysis, and the absolute configuration of **1** was determined by X-ray crystallographic analysis. Compound **1** was evaluated for cytotoxic activity against a well-differentiated human nasopharyngeal carcinoma cell line (CNE1) and a poorly differentiated human nasopharyngeal carcinoma (CNE2) cell line.

Compound **1** was obtained as colorless crystals with a molecular formula of $C_{21}H_{32}N_2O$ based on the HREIMS ion at *m/z* 328.2507 $[M]^+$ (calcd for $C_{21}H_{32}N_2O$, 328.2509), suggesting seven degrees of unsaturation. The IR spectrum showed absorptions typical for hydroxy (3348 cm^{-1}), amino (3601 cm^{-1}), and C≡N (2210 cm^{-1}) groups. The ^1H NMR spectrum indicated the presence of three methyl singlets [δ_H 0.82 (3H, s), 0.86 (3H, s), 0.93 (3H, s)] and one methyl doublet [δ_H 0.94 (3H, d, J = 6.6 Hz)]. A secondary amide group was revealed by the broad singlet at δ_H 8.74 (1H, br s). The ^{13}C NMR spectrum (Table 1) of **1** exhibited 21 carbon signals, including four methyls (δ_C 16.6, 34.0, 22.2, 16.4), seven sp^3 methylenes (δ_C 32.1, 18.9, 42.0, 21.9, 31.5, 36.0, 23.5), two sp^3 methines (δ_C 46.6, 36.8), two sp^2 methines (δ_C 110.2, 123.5), an oxygenated tertiary carbon (δ_C 77.3), two sp^3 quaternary carbons (δ_C 33.6, 43.5), and three carbons without hydrogens at low field (δ_C 138.1, 99.3, 114.6). The aforementioned data further confirmed the presence of a cyano group, which was implied by the IR spectrum (2210 cm^{-1}). ^1H–^1H COSY correlations of NH/H-15/H-14 (Figure 1) indicated a –NH–CH–CH– spin system. The small coupling constants for H-14 (J = 2.8 Hz) and H-15 (J = 2.8 Hz) suggested a pyrrole cyclic system. Four olefinic carbons, one C≡N bond, and one pyrrole cyclic system accounted for five out of the seven degrees of unsaturation, suggesting a dicyclic diterpenoid skeleton for **1**. Detailed analysis of the 2D NMR spectrum suggested compound **1** is a labdane-type diterpenoid alkaloid.[10] The HMBC cross-peaks of H-14/C-12, C-16; H-12/C-13, C-16; and H-15/C-13, C-16 and the weak cross-peak from H-15 to the cyano carbon indicated the presence of a 2-cyano-substituted pyrrole ring, which was confirmed by the base peak *m/z* 105 in the EIMS (Figure S2, Supporting Information). The HMBC cross-peaks of H-12/C-9 and H-11/C-13 (Figure 1) revealed the 2D structure of 1 as shown. Key NOESY correlations of H_3-19/H_3-20, H_3-20/H-8, H_3-20/H-11, and H_3-18/H-5 suggested that CH_3-19, CH_3-20, and H-8 are cofacial, and these were assigned as β-oriented, with CH_3-17 and CH_3-18 having an α-orientation. Crystals of **1** were obtained from MeOH and subjected to X-ray diffraction using Cu Kα radiation (Figure 2). Thus, the absolute configuration of compound **1**, given the trivial name vitepyrroloid A, was defined as 5*S*, 8*R*, 9*R*, and 10*S*.



Compound **2** exhibited a molecular formula of $C_{27}H_{42}N_2O_3$ as determined by the HREIMS ion at *m/z* 442.3188 $[M]^+$ (calcd for $C_{27}H_{42}N_2O_3$, 442.3190). Its IR spectrum showed absorptions for hydroxy (3394 cm^{-1}) and cyano (2210 cm^{-1}) groups. The NMR data of **2** were closely similar to those of **1**, except for the presence of three methylenes, an ethoxy group, and an ester carbonyl group. Moreover, the amino proton observed in the ^1H NMR spectrum of **1** was absent from the spectrum **2**, indicating the occurrence of a substituent on the pyrrole nitrogen of **2**. The COSY cross-peaks of H-1′/H-2′/H-3′ and H-5′/H-6′ revealed the presence of $–CH_2–CH_2–CH_2–$ and $–OCH_2CH_3$ moieties (Figure S14, Supporting Information), with the correlations of H-3′ and H-5′ with C-4′ in the HMBC spectrum indicating a $–(CH_2)_3COOC_2H_5$ moiety. Furthermore, the observation of HMBC cross-peaks of H-1′/C-15, C-16 and of H-15/C-1′ suggested that the ethyl butyrate moiety is connected to the nitrogen atom (Figure S16, Supporting Information). The relative configuration of **2** was assigned as being the same as that of **1** based on a detailed NOESY analysis (Figure S17, Supporting Information). Biogenetically, the structure of compound **2** should have the same absolute configuration as that of **1**. Thus, compound **2** (vitepyrroloid B) was determined as 5*S*, 8*R*, 9*R*, and 10*S*.

Compound **3** was obtained as an amorphous powder and gave the molecular formula $C_{27}H_{40}N_2O_5$ based on its sodiated molecular ion peak in the HRESIMS at *m/z* 495.2824 $[M + Na]^+$ (calcd 495.2829). Its IR spectrum exhibited characteristic absorptions for hydroxy (3398 cm^{-1}) and cyano (2210 cm^{-1}) groups. In comparison to **2**, the ^1H and ^{13}C NMR data of **3** (Table 1) did not show signals of an ethoxy group, but it did contain those for an acetoxy group. The HMBC cross-peaks from the oxygenated methine proton (δ_H 4.44) to C-18 (δ_C 28.5), C-19 (δ_C 16.7), and the acetoxy carbon (δ_C 171.4) suggested that the acetoxy group is located at C-3 (Figure 3). The COSY cross-peaks of H-1′/H-2′/H-3′ in combination with the correlations from H-1′ to C-15 and C-16 in the HMBC spectrum indicated a butyric acid moiety to be connected to the pyrrole nitrogen. Key NOESY correlations of H_3-19/H_3-20, H_3-20/H-8, H_3-20/H-11, H_3-18/H-5, H_3-18/H-3, and H-3/H-5 (Figure 3) suggested that CH_3-19, CH_3-20, and H-8 are cofacial, and they were assigned as β-oriented, with H-3, CH_3-17, and CH_3-18 α-oriented. On the basis of comparison with **1**, compound **3** (vitepyrroloid C) was established as 3*S*, 5*S*, 8*R*, 9*R*, and 10*S*. Moreover, it is worth mentioning that the protonated molecular ion peak at *m/z* 473.3009 $[M + H]^+$ for compound **3** was observed in an LC-HRESIMS experiment on the original MeOH extract of *V. trifolia* (Supporting Information, Figure S34). This result clearly indicated that compounds featuring a 2-cyano-substituted pyrrole ring exist in the crude product of the plant and, thus, are not artifact products.

The molecular formula of compound **4** was determined as $C_{25}H_{38}N_2O_4$ from the sodiated HRESIMS ion at *m/z* 453.2717 $[M + Na]^+$ (calcd for $C_{25}H_{38}N_2O_4Na$, 453.2724). The ^1H and ^{13}C NMR data (Table 1) indicated **4** to be closely similar to **3** structurally, except for the absence of two sp^3 CH_2 units and a carbonyl carbon and the presence of an oxygenated methylene group. The COSY cross-peaks from H-5 (δ_H 1.63) to the oxygenated methine proton H-6 (δ_H 5.37) suggested that the acetoxy group in **4** is connected to C-6 (Figure S30, Supporting Information). The COSY correlations of H-1′/H-2′ and the HMBC cross-peaks of H-1′/C-15 and H-1′/C-16 indicated that a $–CH_2CH_2OH$ moiety is located at the nitrogen atom of the pyrrole ring (Figures S30 and S32, Supporting Information). The NOESY correlations of H_3-19/H_3-20, H_3-20/H-8, H_3-20/H-11, H_3-18/H-5, H_3-18/H-6, and H-6/H-5 were consistent with CH_3-19, CH_3-20, and H-8 being cofacial with β-orientations, with H-6, CH_3-17, and CH_3-18 assigned as α-oriented (Figure S33, Supporting Information). Accordingly, the structure of compound **4** (vitepyrroloid D) was determined as 5*S*, 6*R*, 8*R*, 9*R*, and 10*S*.

Compounds **1–4** were evaluated for in vitro cytotoxicity against the CNE1 and CNE2 cell lines. As shown in Table S1 in the Supporting Information, compound **1** was the most active isolated compound against these two cell lines, with IC_{50} values of 8.7 and >10 μM, respectively. Compound **1** is an unprecedented labdane diterpenoid alkaloid containing a cyano moiety and a pyrrole ring. The N–H group may be necessary for manifestation of cytotoxic activity, because the less active compounds **2–4** are derivatives of **1**, but are substituted at the pyrrole ring nitrogen atom. Compounds **1–4** may be derived from the precursors of geranylgeranyl pyrophosphate (GGPP), ammonia, and an amino acid as shown in Figure 4. Protonation of GGPP can initiate a concerted cyclization sequence, terminated by loss of a proton from a methyl, yielding (+)-copayl PP. Next, sequential oxidation and reduction could produce intermediate **i**. A subsequent attack on intermediate **i** by ammonia (NH_3) or an amino acid (L-glutamic acid or L-serine) followed by decarboxylation would give intermediate **ii**, which could be converted to intermediate **iii** via Schiff base and Mannich reactions. Finally, oxidation of the intermediate **iii** followed by the loss of CO_2 and H_2O leads to intermediate **v** or compound **1**. Oxidation and acetylation of the intermediate **v** gave compounds **3** and **4**. Compound **2** could be yielded from the intermediated **v** via estenification using ethanol (Figure 4).[11–14]

Experimental Section

General Experimental Procedures

The melting point was measured on an X-5 melting point instrument and was uncorrected. Optical rotations were obtained with a PerkinElmer 341 automatic polarimeter. UV spectra were recorded with MeOH as the solvent using a Shimadzu UV-2450 spectrophotometer. IR spectra were determined on a Bruker Tensor 37 infrared spectrophotometer with KBr pellets. The 1D and 2D NMR spectra were obtained on a Bruker AM-400 NMR spectrometer with tetramethylsilane (TMS) as an internal reference. HREIMS were measured on a Thermo MAT95XP high-resolution mass spectrometer, and EIMS on a Thermo DSQ EIMS spectrometer. HRESIMS were acquired on a Shimadzu LCMS-IT-TOF instrument, and the ESIMS on an Agilent 1200 series LC-MS/MS system. RP-C_{18} silica gel (Fuji, 40–75 μm),

MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan), silica gel (200–300 Mesh Marine Chemical Ltd., Qingdao, People's Republic of China), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden) were used for column chromatography (CC). Semipreparative HPLC separations were carried out on a LC-20AT Shimadzu liquid chromatography system with a YMC-Pack ODS-A column and an Agilent SB-C_{18} column (250 × 9.4 mm, 5 μm) connected with an SPD-M20A diode array detector.

Plant Material

The leaves of *Vitex trifolia* were collected in September 2014 from Xishuangbanna in Yunnan Province, People's Republic of China, and were authenticated by Prof. Xinxin Zhou of the School of Chinese Materia Medica, Guangzhou University of Chinese Medicine. A voucher specimen (XG-2014002) has been deposited at the School of Pharmacy Sciences, Sun Yat-sen University.

Extraction and Isolation

The air-dried, powdered leaves of *V. trifolia* (30 kg) were percolated four times with 95% EtOH at room temperature for 3 days. After removing the organic solvent, the residue was partitioned with EtOAc–H_2O. The EtOAc extract (1.5 kg) was subjected to silica gel CC using petroleum ether–EtOAc mixtures (1:0, 10:1, 3:1, 1:1, 1:4, 0:1 v/v) to obtain fractions A–F. Fraction C (100 g) was subjected to silica gel CC eluting with CH_2Cl_2–EtOAc (from 200:1 to 1:1) to provide six fractions, C1–C6. C3 (23 g) was loaded onto a Sephadex LH-20 column and eluted with CH_2Cl_2–MeOH (1:1) to yield two subfractions, C3a and C3b. Fraction C3a was further subjected to silica gel CC (CH_2Cl_2–MeOH, 300:1 to 95:5) to give four fractions, C3a1–C3a4. Subfraction C3a2 was purified by semipreparative HPLC (80% CH_3CN in H_2O, 1.5 mL/min) to yield **3** (5 mg) and **4** (4.6 mg). Fraction B (234 g) was further subjected to silica gel CC, by eluting with CH_2Cl_2–MeOH (from 300:1 to 30:1), to obtain three fractions, B1–B3. Fraction B1 (100 g) was separated using silica gel CC to give fraction B1a, which was further fractionated over a RP-C_{18} column (MeOH–H_2O, 40% to 90%, v/v) to yield three fractions, B1a1–B1a3. Fraction B1a2 was subjected to silica gel CC (cyclohexane–EtOAc, 10:1 to 1:1) to give three subfractions, B1a2-1–B1a2-3. Subfraction B1a2-1 was separated by semipreparative HPLC (80% MeOH–H_2O, 1.5 mL/min) to yield compounds **1** (8.5 mg) and **2** (7.4 mg).

Vitepyrroloid A (1): colorless crystals (MeOH); mp 145–146°C; $[\alpha]^{25}_D$ +10 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 247 (4.09) nm; IR (KBr) ν_{max} 3601, 3348, 2922, 2848, 2210, 1645, 1462, 1410, 1385, 1255, 1119, 901, 758, 665, 579, 499 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HREIMS *m/z* 328.2507 $[M]^+$ (calcd for $C_{21}H_{32}N_2O$, 328.2509).

Vitepyrroloid B (2): amorphous powder; $[\alpha]^{25}_D$ +9 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 249 (3.96) nm; IR (KBr) ν_{max} 3394, 2920, 2850, 2210, 1731, 1643, 1460, 1414, 1375, 1254, 1188, 1026, 752, 648, 499 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HREIMS *m/z* 442.3188 $[M]^+$ (calcd for $C_{27}H_{42}N_2O_3$, 442.3190).

Vitepyrroloid C (3): amorphous powder; $[\alpha]^{25}_D$ +18 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 249 (3.61) nm; IR (KBr) ν_{max} 3398, 3192, 2926, 2854, 2210, 1732, 1643, 1468, 1419,

1373, 1255, 1028, 970, 800, 648, 496 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 495.2824 $[M + Na]^+$ (calcd for $C_{27}H_{40}N_2O_5Na$, 495.2829).

Vitepyrroloid D (4): amorphous powder; $[\alpha]^{25}_D$ –2 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 250 (3.87) nm; IR (KBr) ν_{max} 3396, 3192, 2924, 2848, 2212, 1728, 1645, 1466, 1414, 1375, 1057, 1020, 955, 750, 613, 499 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 453.2717 $[M + Na]^+$ (calcd for $C_{25}H_{38}N_2O_4Na$, 453.2724).

X-ray Crystallographic Analysis of Compound 1

The crystal structure and absolute configuration of 1 were determined by single-crystal X-ray diffraction analysis. A suitable crystal was selected and tested on an Xcalibur, Onyx, Nova diffractometer with the temperature kept at 103(2) K during data collection. The structure was solved and refined, using the programs XS and olex2.refine, respectively. The program X-Seed was used as an interface to the SHELX programs and to prepare the figures. Crystal data for compound **1**: $C_{21}H_{32}N_2O$ (*M* = 328.50); orthorhombic, space group $P2_12_12_1$ (no. 19), *a* = 7.45094(6) Å, *b* = 9.21543(8) Å, *c* = 26.7264(2) Å, *V* = 1835.14(3) $Å^3$; Z = 4, *T* = 103(2) K, μ(Cu Kα) = 0.558 mm^{-1}, D_{calc} = 1.1889 g/cm^3, 13 373 reflections measured, 3577 unique (R_{int} = 0.0278, R_{sigma} = 0.0238), which were used in all calculations. The final R_1 was 0.0298 and wR_2 was 0.0738 (all data). Flack parameter = 0.2(2). Crystallographic data for **1** have been deposited at the Cambridge Crystallographic Data Centre under the reference number CCDC 1519552.

Cytotoxicity Assay

The well-differentiated human nasopharyngeal carcinoma (CNE1) and the poorly differentiated human nasopharyngeal carcinoma (CNE2) cell lines were obtained from Sun Yat-sen University Cancer Center and cultured on RPMI-1640 medium at 37°C in a humidified atmosphere with 5% CO_2. The cells were seeded at 2000–4000 cells/well in 96-well plates. For the test compound treatment experiments, the cells were treated in triplicate with graded concentrations of each substance (predissolved in DMSO) for a period of 3 days. At the end of the compound treatment period, MTT solution (20 μL, 5 mg/mL) in PBS (PBS without MTT as the blank) was fed to each well of the culture plate (containing 100 μL of medium). After 4 h of incubation, the formazan crystals that formed in the well were dissolved with 100 μL of DMSO for optical density reading at 492 nm.[15] All IC_{50} values were calculated by nonlinear regression analysis (GraphPad Prism).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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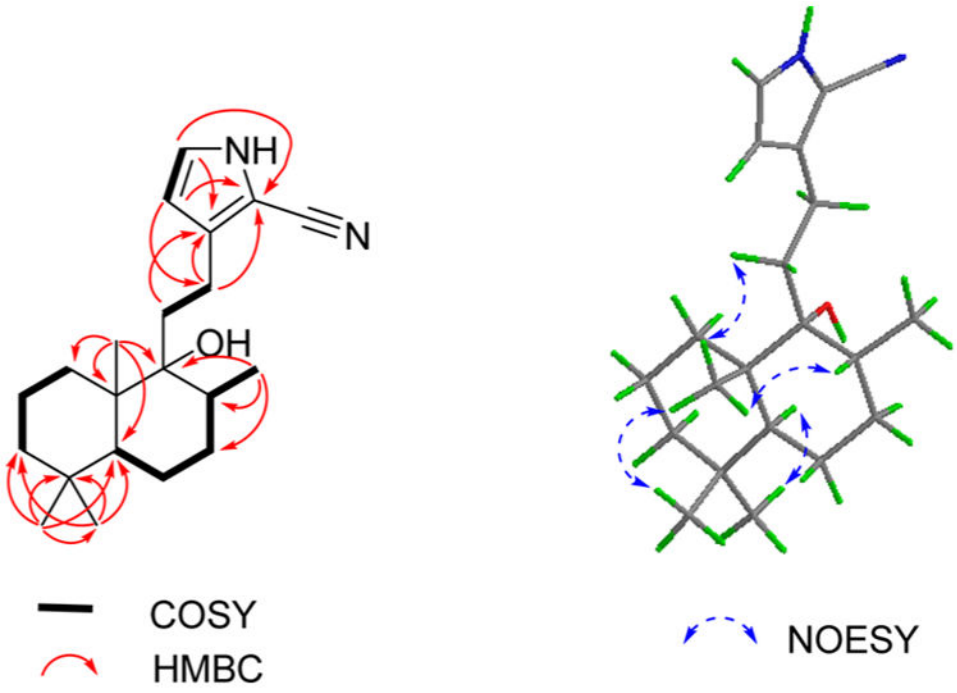

Figure 1.
^{1}H–^{1}H COSY (bold), selected HMBC (red arrows), and key NOESY (blue arrows) correlations for compound **1**.

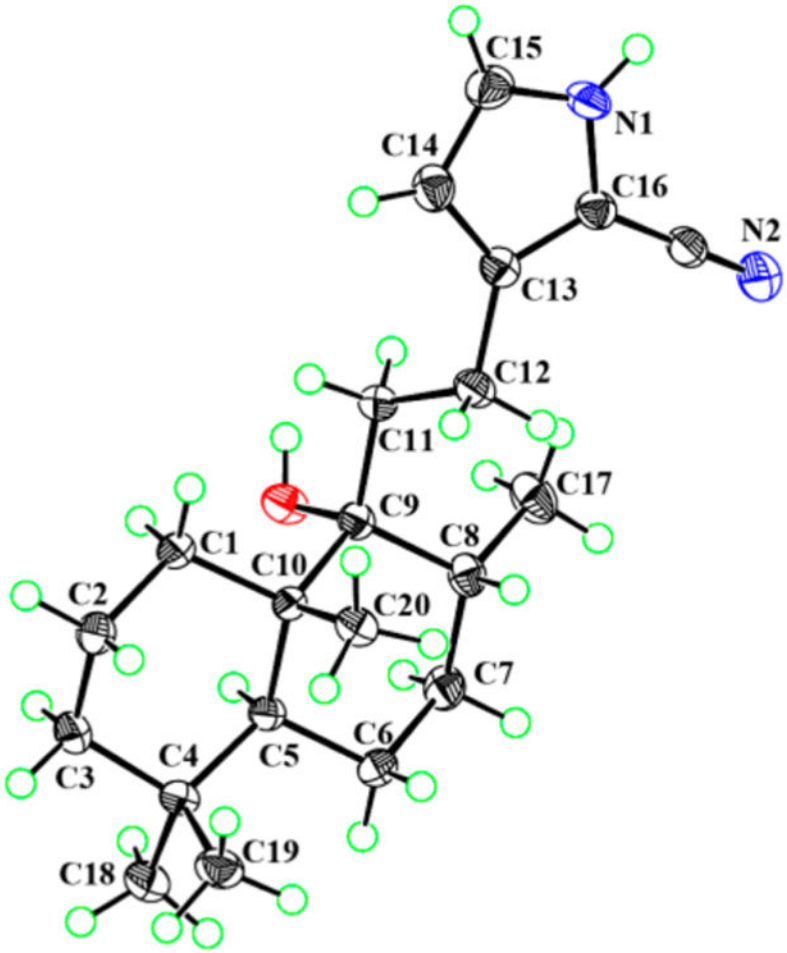

Figure 2.
PLATON drawing of compound **1**.

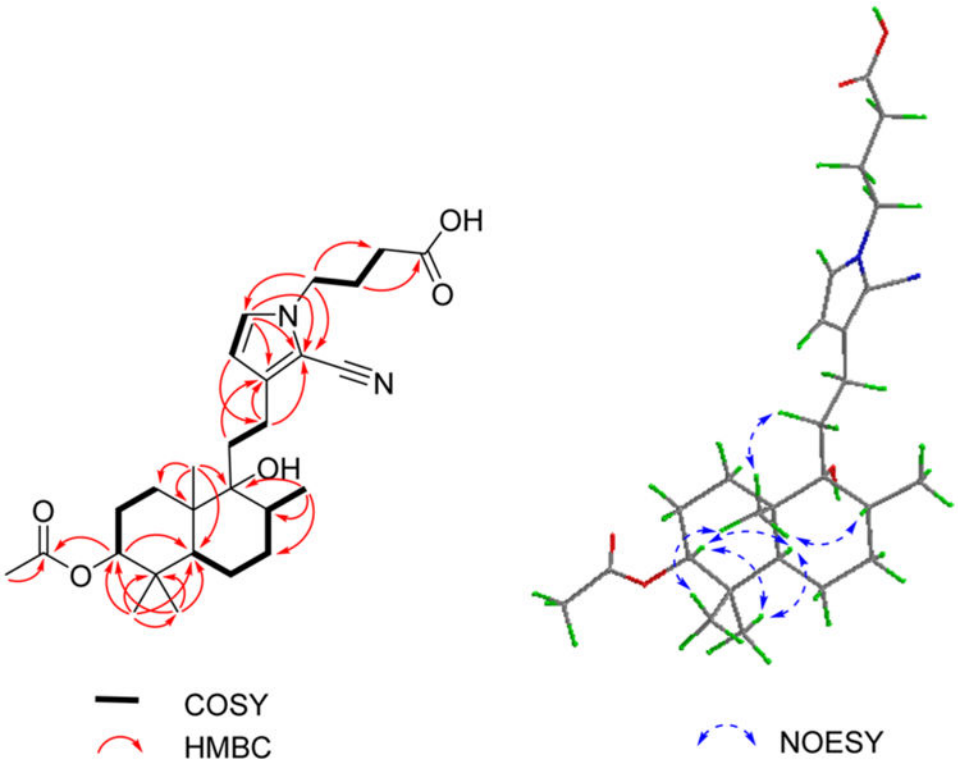

Figure 3.
^{1}H–H COSY (bold), selected HMBC (red arrows), and key NOESY (blue arrows) correlations for compound **3**.

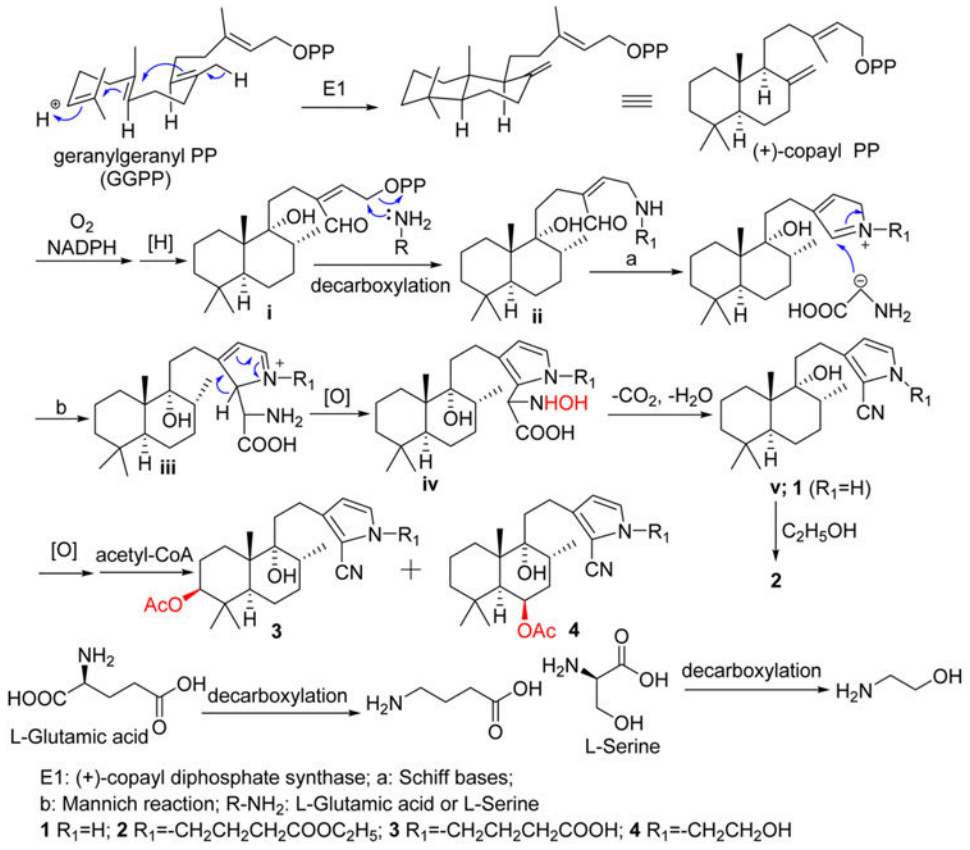

Figure 4.
Proposed biosynthetic origin of compounds **1–4**.

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Table 1

^{1}H and ^{13}C NMR Chemical Shifts (δ) of Compounds 1–4[a]

	1		2		3		4	
position	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
1a	1.50, overlap	32.1	1.48, overlap	32.0	1.67, overlap	29.9	1.49, overlap	33.8
1b	1.50, overlap		1.48, overlap		1.51, overlap		1.49, overlap	
2a	1.56, overlap	18.9	1.54, overlap	18.8	1.66, overlap	23.8	1.61, m	18.9
2b	1.48, overlap		1.47, overlap		1.66, overlap		1.49, m	
3a	1.33, overlap	42.0	1.32, overlap	41.9	4.44, dd (10.8, 4.0)	81.0	1.30, m	43.9
3b	1.14, td (12.9, 3.7)		1.13, td (12.9, 3.4)				1.15, td (13.3, 3.2)	
4		33.6		33.5		37.9		34.2
5	1.40, m	46.6	1.40, m	46.6	1.55, m	45.8	1.63, d (1.8)	47.7
6a	1.52, overlap	21.9	1.51, overlap	21.8	1.51, overlap	21.3	5.37, q-like (2.4)	70.4
6b	1.29, overlap		1.28, overlap		1.28, overlap			
7a	1.48, overlap	31.5	1.48, overlap	31.5	1.48, overlap	31.2	1.60, overlap	36.3
7b	1.28, overlap		1.27, overlap		1.32, overlap		1.55, overlap	
8	1.80, m	36.8	1.78, m	36.8	1.76, m	36.8	2.12, m	31.9
9		77.3		77.2		77.2		77.2
10		43.5		43.4		43.0		43.9
11a	1.92, ddd (14.4, 11.7, 6.3)	36.0	1.89, ddd (14.4, 11.8, 6.2)	35.9	1.90, ddd (14.6, 9.9, 7.7)	35.8	1.92, ddd (14.4, 11.9, 6.1)	35.8
11b	1.69, ddd (14.4, 11.3, 5.9)		1.67, ddd (14.4, 11.5, 5.9)		1.69, overlap		1.79, ddd (14.4, 11.5, 5.6)	
12	2.66, m	23.5	2.61, m	23.8	2.62, m	23.8	2.65, m	23.7
13		138.1		138.3		138.1		138.0
14	6.11, t (2.8)	110.2	6.00, d (2.6)	109.4	6.00, d (2.6)	109.6	6.03, d (2.6)	109.4
15	6.82, t (2.8)	123.5	6.70, d (2.6)	126.5	6.71, d (2.6)	126.7	6.83, d (2.6)	127.5
16		99.3		102.0		102.1		102.1
17	0.94, d (6.6)	16.6	0.93, d (6.8)	16.6	0.94, d (6.8)	16.4	0.96, d (6.8)	16.3
18	0.86, s	34.0	0.85, s	33.9	0.85, s	28.5	0.94, s	33.8
19	0.82, s	22.2	0.81, s	22.2	0.84, s	16.7	0.98, s	23.9
20	0.93, s	16.4	0.92, s	16.4	0.93, s	16.3	1.24, s	19.3
CN		114.6		114.0		114.0		114.1

	1		2		3		4	
position	**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**
1′			4.01, t (6.9)	48.0	4.04, t (7.0)	47.9	4.11, t (5.2)	51.3
2′			2.09, quintet (6.9)	26.4	2.10, m	26.2	3.91, t (5.2)	62.3
3′			2.26, t (6.9)	30.9	2.30, m	30.3		
4′				172.6		176.0		
5′			4.12, q (7.1)	60.9				
6′			1.24, t (7.1)	14.4				
OAc-3						171.4		
					2.03, s	21.6		
OAc-6								170.8
							2.03, s	22.2
NH	8.74, br s							

[a] ^{1}H NMR measured at 400 MHz, ^{13}C NMR measured at 100 MHz, and spectra obtained in $CDCl_3$ with TMS as internal standard. Assignments were supported with HSQC and HMBC NMR spectra.