Unusual C25 Steroid Isomers with Bicyclo[4.4.1]A/B Rings from a Volcano Ash-Derived Fungus *Penicillium citrinum*

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Eleven new unusual C25 steroid isomers with bicyclo[4.4.1]A/B rings, 24-*epi*-cyclocitrinol (**1**), 20-*O*-methyl-24-*epi*-cyclocitrinol (**3**), 20-*O*-methylcyclocitrinol (**4**), 24-oxocyclocitrinol (**7**), 12*R*-hydroxycyclocitrinol (**8**), neocyclocitrinols B (**10**) and D (**12**), *erythro*-23-*O*-methylneocyclocitrinol (**13**), *threo*-23-*O*-methylneocyclocitrinol (**14**), isocyclocitrinol B (**15**), and precyclocitrinol B (**18**), and five known steroids, cyclocitrinol (**2**), neocyclocitrinols A (**9**) and C (**11**), isocyclocitrinol A (**16**), and 22-*O*-acetylisocyclocitrinol A (**17**), were characterized from cultures of the volcanic ash-derived fungus *Penicillium citrinum* HGY1-5. Their structures and absolute configurations were established by spectroscopic and chemical methods together with X-ray diffraction analysis. Compounds **3**, **4**, and **10**−**14** were determined to be artifacts on the basis of acidic transformation of **1**−**4**. The biosynthetic origin of these steroids derived from ergosterol was investigated by feeding ^{13}C-labeled acetates to the growing cultures of *P. citrinum* HGY1-5. The biological activities of all 16 steroids were tested using the cAMP assay on GPR12-CHO and WT-CHO cells. The results showed that compounds **1**, **2**, **10**, **11**, and **14** could induce the production of cAMP in GPR12-transfected CHO cells.

C25 steroids with bicyclo[4.4.1]A/B rings are a series of very unusual steroids. Only four examples, cyclocitrinol,[1] isocyclocitrinol A (**16**), 22-acetylisocyclocitrinol A (**17**),[2] and neocyclocitrinol,[3] have been reported. The first compound of this type, cyclocitrinol, was isolated from a terrestrial *Penicillium citrinum* and reported as a new sesterterpenoid.[1] Afterward, its structure was revised to a bicyclo[4.4.1] A/B ring steroid by X-ray structure analysis and modified Mosher's method.[2] Recently, neocyclocitrinol was reported as a mixture of 23,24-epimers from a plant-derived *Penicillium janthinellum*, and the configuration of the 20,22-double bond was not determined.[3] A biosynthesis route originating from ergosterol was proposed to explain the origins of the bicyclic system and the side chain.[3] Isocyclocitrinol A (**16**) and 22-*O*-acetylisocyclocitrinol A (**17**) showed weak antibacterial activity against *Staphylococcus epidermidis* and *Enterococcus durans*.[2] Our recent investigation of secondary metabolites of a volcano ash isolate of *P. citrinum* led to the discovery of a series of C25 steroids with bicyclo[4.4.1]A/B rings. Details of the isolation, structural elucidation, absolute configuration determination, biosynthesis pathway, and the effects on GPR12 activation are described in this paper.

Results and Discussion

Penicillium citrinum HGY1-5, isolated from the crater ash collected from the extinct volcano Huguangyan in Guangdong, China, was cultured in 20 L of liquid medium for 9 days, and the metabolites were extracted with EtOAc. The crude extracts (22 g) were repeatedly chromatographed on Si gel columns and extensive reversed-phase semipreparative HPLC to afford 24-*epi*-cyclocitrinol (**1**), cyclocitrinol (**2**),[1] 20-*O*-methyl-24-*epi*-cyclocitrinol (**3**), 20-*O*-methylcyclocitrinol (**4**), isocyclocitrinol A (**16**),[2] and 22-*O*-acetylisocyclocitrinol A (**17**).[2] Some minor constituents were also detected on HPLC analysis. In order to isolate these minor analogues, the strain was refermented in 40 L of liquid medium for 15 days to afford 24-oxocyclocitrinol (**7**), 12*R*-hydroxycyclocitrinol (**8**), neocyclocitrinols A−D (**9**−**12**), *erythro*-23-*O*-methylneocyclocitrinol (**13**), *threo*-23-*O*-methylneocyclocitrinol (**14**), isocyclocitrinol B (**15**), and precyclocitrinol B (**18**).

Compound **1** was obtained as colorless needles following crystallization from MeOH. Its molecular formula, $C_{25}H_{36}O_4$, was determined by HRESIMS, indicating eight degrees of unsaturation. Its IR spectrum exhibited strong absorptions at 3421 and 1649 cm^{-1}, indicative of hydroxy and conjugated carbonyl groups. Analysis of the ^{13}C NMR data for **1** revealed one carbonyl, four quaternary carbons, 10 methines, seven methylenes, and three methyls. Comparison of the ^{1}H and ^{13}C NMR data (Tables 1 and 2) with those of the known compound cyclocitrinol[1] indicated they had the same constitution. The key ^{1}H−^{1}H COSY correlation of H-18α with H-5 and the HMBC correlations from H-18α to C-1, C-4, C-5, C-6, C-9, and C-10, from H-4α to C-6, from H-1 to C-9 and C-18, from H-9 to C-1, C-10, and C-18, and from H-7 to C-5 (Figure 1a) proved the existence of the bicyclo[4.4.1] system of the A/B rings.

Compound **2** was obtained as colorless needles following crystallization from MeOH, and its formula $C_{25}H_{36}O_4$ was determined by HRESIMS. Comparison of the ^{1}H and ^{13}C NMR data (Tables 1 and 2) with those of **1** showed that their ^{1}H NMR data were similar except for small discrepancies of the coupling patterns of H-22 (doublet for **1**, doublet of doublets for **2**), coupling constants of $J_{23,24}$ (5.9 Hz for **1**, 5.5 Hz for **2**), and the chemical shifts of the side-chain carbons (C-20 to C-24) in the ^{13}C NMR spectrum. This indicated that **2** was an epimer of **1** with different stereogenicity on the side chain. The X-ray crystallographic analysis of their *p*-bromobenzoates (**5**, **6**) established the absolute configuration of **1** and **2**. They were C-24 epimers, i.e., 24*S* (**1**) and 24*R* (**2**), respectively (Figure 2). Compound **2** was thus the known compound cyclocitrinol,[1] as they had the same coupling patterns of H-22 (doublet of doublets) and the same strong hydroxy absorption at 3405 cm^{-1} in the IR spectra.

Compounds **3** and **4**, obtained as colorless needles following crystallization from MeOH, had the same molecular formula, $C_{26}H_{38}O_4$, according to the HRESIMS. Except for the C-20 *O*-methyl resonance, their 1D NMR data were similar to those of **1** and **2** (Tables 1 and 2). The key HMBC correlation of CH_3O− (δ 3.12) with C-20 indicated that **3** and **4** were 20-*O*-methyl derivatives of cyclocitrinols (Figure 1a). Compounds **1** and **3** could interconvert like **2** and **4** experienced in acidic solution (Figure 5,

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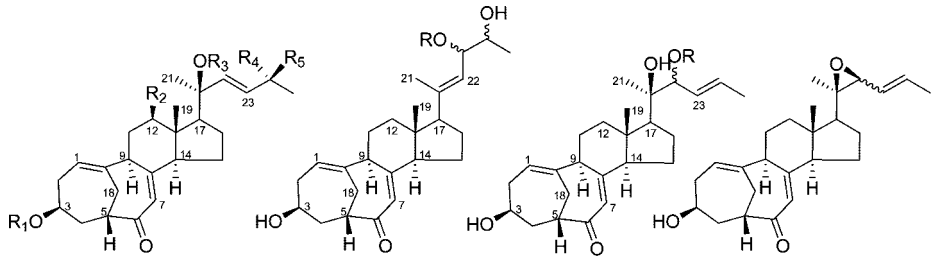
Chart 1



1: $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = OH$
2: $R_1 = R_2 = R_3 = R_5 = H$, $R_4 = OH$
3: $R_1 = R_2 = R_4 = H$, $R_3 = Me$, $R_5 = OH$
4: $R_1 = R_2 = R_5 = H$, $R_3 = Me$, $R_4 = OH$
5: $R_1 = p\text{-}BrC_6H_4CO$, $R_2 = R_3 = R_4 = H$, $R_5 = OH$
6: $R_1 = p\text{-}BrC_6H_4CO$, $R_2 = R_3 = R_5 = H$, $R_4 = OH$
7: $R_1 = R_2 = R_3 = H$, $R_4R_5 = O$
8: $R_1 = R_3 = R_5 = H$, $R_2 = R_4 = OH$
8a: $R_1 = R_3 = R_5 = H$, $R_2 = R_4 =$ (*S*)-MTPA
8b: $R_1 = R_3 = R_5 = H$, $R_2 = R_4 =$ (*R*)-MTPA

9: 23*R*, 24*R*, R = H
10: 23*S*, 24*S*, R = H
11: 23*R*, 24*S*, R = H
12: 23*S*, 24*R*, R = H
13: 23, 24-*erythro*, R = Me
14: 23, 24-*threo*, R = Me

15: 22*R*, R = H
16: 22*S*, R = H
17: 22*S*, R = Ac

18: 22*R* or 22*S*

Scheme 1). The absolute configurations of **3** and **4** were determined as 24*S* and 24*R*, respectively.

The molecular formula of **7**, assigned as $C_{25}H_{34}O_4$ by HRESIMS, was consistent with both ^{13}C and ^1H NMR spectroscopic data (Tables 1 and 2). Analysis of the 1D NMR data revealed **7** was an analogue of **1**. The carbonyl (δ_C 198.5) substitution for the oxygenated methine (δ_H 4.10/δ_C 66.6) and the obvious chemical shift effects for C-22/H-22 (+16.4/+1.27 ppm), C-23/H-23 (−4.2/+0.79 ppm), and C-25/H-25 (+3.8/+1.19 ppm) were observed. The H-25 resonance was changed to a singlet in the ^1H NMR spectrum of **7**. Thus, the structure of **7** was established as 24-oxocyclocitrinol.

Compound **8** analyzed by HRESIMS for the molecular formula $C_{25}H_{36}O_5$. The physical data of **8** were similar to those of **1** and **2** (Tables 1 and 2). Examination of the ^1H and ^{13}C NMR data revealed that a methylene on the C ring of the steroid nucleus was replaced by an oxygenated methine. $^1H-^1H$ COSY correlations from H-12 (δ_H 3.52) to H-11 and from H-11 to H-9, and the NOESY coupling between H-12 and H-9 (Figure 1a), revealed that the additional hydroxy group was located at C-12, and the orientation of H-12 was further supported by $J_{11,12}$ (7.3 and 8.7 Hz). The absolute configuration of C-24 was determined as *R* by application of the modified Mosher's method (Figure 1b).[2] The structure of **8** was thus elucidated as 12*R*-hydroxycyclocitrinol.

HRESIMS at *m/z* 399.2544 $[M - H]^-$ established the molecular formula of compound **9** as $C_{25}H_{36}O_4$, implying **9** was an isomer of **1**. ^1H NMR and ^{13}C NMR data revealed they had the same steroid nucleus but different side chains. $^1H-^1H$ COSY correlations indicated one $=CH-CH(OH)-CH(OH)-CH_3$ moiety in the side chain of **9** (Figure 1a). The NOE association between Me-21 (δ_H 1.66) and H-23 (δ_H 3.95) (Figure 1a) established the *E*-configuration of the 20,22-double bond. Compounds **10**−**12** shared the same formula, $C_{25}H_{36}O_4$, with **9** established by their HRESIMS data, and comparison of their spectroscopic data with those of **9** suggested that they were 23,24-diastereomers. Searching the structure on SciFinder Scholar 2006, neocyclocitrinol, reported as a mixture,[3] was hit. By comparing the physical data, the structures of compounds **9**−**12** were established as shown. The absolute configuration of the stereogenic centers of the side chain of **9** was determined to be 23*R*, 24*R* by X-ray crystallographic analysis (Figure 2). Interpretation of the ^1H and ^{13}C NMR spectra (Tables 1 and 2) of **9**−**12** with emphasis on the $J_{23,24}$ values[4] indicated that **9** and **10** were the 23,24-*threo*-isomers, while **11** and **12** were the 23,24-*erythro*-isomers. The resonances of *threo* H-23 and H-24 were upfield compared to those of the *erythro*-isomers due to steric effects (Figure 3). Comparing the ^{13}C NMR data (Table 2), the chemical shifts of C-21 and C-22 in **9** and **11** were consistent. This indicated they should have the same configuration at C-23. Similar analysis indicated that compounds **10** and **12** should also be C-24 epimers. Consequently the absolute configurations of **10**−**12** were assigned as 23*S*,24*S*, 23*R*,24*S*, and 23*S*,24*R*, respectively. This determination from the NMR data was further supported by the acid-catalyzed isomerizations (Figure 5, Scheme 1). By comparison of the ^1H and ^{13}C NMR data of **9**−**12** measured in CD_3OD with those of the known neocyclocitrinol[3] (Supporting Information, T7 and T8), the reported compound comprises a mixture of **9** and **11**.

Compound **13** was obtained as an amorphous powder that gave a pseudomolecular ion peak at *m/z* 413.2678 $[M - H]^-$ in the HRESIMS, consistent with the molecular formula $C_{26}H_{38}O_4$. The ^1H and ^{13}C NMR data of **13** revealed that it was a mixture of two epimers. Further comparing the data with those of **9**−**12**, combined with the key HMBC correlation between MeO (δ_H 3.15/3.16) and C-23 (δ_C 81.39/81.32) (Figure 1a), indicated that **13** was a mixture of the 23-*O*-methyl derivatives of **11** and **12**. Compound **14** had the same molecular formula, $C_{26}H_{38}O_4$, established by HRESIMS at *m/z* 415.2851 $[M + H]^+$ (calcd 415.2848), and similar ^1H and ^{13}C NMR data to those of **13**. It was also obtained as a mixture, and the $J_{23,24}$ value (6.4 Hz) and the upper field shifts of H-23 and H-24 in **14** indicated a *threo*-configuration.

The molecular formula of **15** was determined as $C_{25}H_{36}O_4$ from HRESIMS. Its ^1H and ^{13}C NMR data were similar to those of the known isocyclocitrinol A (**16**),[2] except for minor differences at C-17, C-20, C-21, and C-22 (Tables 1 and 2). HMBC correlations from H-21 to C-17, C-20, and C-22, from H-22 to C-20, C-21, C-23, and C-24, from H-23 to C-20 and C-25, from H-24 to C-22 and C-25, and from H-25 to C-24 (Figure 1a) confirmed **15** as the 20- or 22-epimer of **16**. Comparing the ^{13}C NMR data with those of **16**, a downfield shift of C-17 and an upfield shift of C-21 were observed in **15**, probably due to the steric effects of 22*R*-OH (Figure 3), which was confirmed by the subsequent epoxide opening of **18** (Figure 4). Thus, the structure of **15** was deduced as the 22-epimer of **16**.

The molecular formula of **18** was identified as $C_{25}H_{34}O_3$ on the basis of HRESIMS. Its ^1H NMR spectrum was similar to those of **15** and **16** except for the obvious shift effects of H-17, H-19, and H-21−H-24 (Table 1), indicating **18** could be the epoxide precursor of **15** or **16**, i.e., precyclocitrinol. Subjected to acidic hydrolysis in MeOH and H_2O, **18** transformed into **1**, **2**, **15**, **16**, and the corresponding *O*-methyl derivatives **1′**−**4′** (Figure 4). Compounds **1**−**4** transformed into **10**, **11**, **13a**, and **14a**; **9**, **12**, **13b**, and **14b**; **1**, **10**, **11**, **13a**, and **14a**; and **2**, **9**, **12**, **13b**, and **14b**, respectively, after similar treatment (Figure 5), while **9** and **15** resisted isomerization under the same conditions.

These results provided significant support for further elucidating the configuration and analyzing the origin of the side chains of compounds **1**−**4** and **7**−**18** (Schemes 1 and 2). According to Andrey's assumption of the biosynthesis pathway of the side chain,[3] (1*Z*,3*E*)-1-methylpenta-1,3-dien-1-yl or (1*E*,3*E*)-1-methylpenta-1,3-dien-1-yl (**a**) was formed first and then transformed into **18**. As

Table 1. ^{1}H NMR Data for Compound **1**−**4** and **7**−**18** (600 MHz, TMS, δ ppm, J in Hz)

position	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**7**[b]	**8**[a]	**9**[a]
1	5.53 dd (6.6, 8.1)	5.53 dd (6.8, 8.7)	5.56 dd (6.2, 8.4)	5.56 dd (6.2, 8.4)	5.57 dd (5.5, 8.2)	5.54 m	5.55 dd (6.4, 8.2)
2α	2.07 ddt (2.2, 8.1, 13.2)	2.07 ddt (2.1, 8.2, 13.3)	2.24 ddt (2.2, 8.4, 13.2)	2.24 ddt (2.6, 8.4, 13.2)	2.25 ddt (2.2, 8.7, 13.2)	2.06 ddt (2.3, 8.7, 13.2)	2.07 m
2β	2.33 ddd (6.6, 11.0, 13.2)	2.33 ddd (6.4, 11.0, 13.3)	2.48 ddd (6.2, 11.3, 13.2)	2.48 ddd (6.2, 11.3, 13.2)	2.48 ddd (5.9, 11.9, 13.2)	2.33 ddd (6.4, 11.0, 13.2)	2.34 m
3	3.10 m	3.11 m	3.49 m	3.50 m	3.50 m	3.11 m	3.12 m
4α	2.61 brd (13.2)	2.62 brd (13.2)	2.89 brd (12.8)	2.89 brd (12.8)	2.89 brd (12.8)	2.61 brd (12.8)	2.63 brd (12.8)
4β	1.51 dd (3.7, 13.2)	1.51 dd (4.0, 13.2)	1.67 m	1.67 m	1.68 m	1.51 m	1.52 m
5	2.66 m	2.67 m	2.74 m	2.74 m	2.74 m	2.67 m	2.68 m
7	5.37 s	5.38 s	5.56 s	5.56 s	5.57 s	5.41 s	5.42 s
9	2.78 dd (5.8, 12.5)	2.78 dd (5.5, 12.4)	2.76 dd (5.8, 12.4)	2.76 dd (5.8, 12.4)	2.77 dd (5.9, 12.4)	2.93 brt (8.7, 9.7)	2.84 dd (6.0, 12.4)
11α	1.49 m	1.49 m	1.58 m	1.58 m	1.61 m	1.58 m	1.54 m
11β	1.74 m	1.75 m	1.67 m	1.67 m	1.80 m	1.59 m	1.76 m
12α	1.42 td (4.4, 13.2)	1.42 m	1.43 m	1.43 m	1.51 m	3.52 brt (7.3, 8.7)	1.43 td (4.4, 12.8)
12β	2.13 m	2.13 m	2.19 m	2.19 m	2.20 m		1.74 m
14	2.10 ddd (1.7, 6.6, 12.1)	2.10 m	2.07 q (12.1)	2.07 q (12.1)	2.11 ddd (12.3, 6.9)	2.12 m	2.22 brt (8.7)
15α	1.38 m	1.38 m	1.47 m	1.47 m	1.63 m	1.46 m	1.57 m (8.7)
15β	1.46 m	1.46 m	1.58 m	1.58 m	1.55 m	1.56 m	1.52 m (8.7)
16α	1.56 m	1.56 m	1.68 m	1.68 m	1.82 m	1.54 m	1.82 m
16β	1.66 m	1.66 m	1.73 m	1.73 m	1.73 m	1.74 m	1.68 m
17	1.66 m	1.66 m	1.84m	1.84 m	1.87 t (10.0)	1.84 t (9.6)	2.27 brt (10.1)
18α	2.46 brs	2.47 brs	2.56 m	2.56 m	2.53 m	2.41 dd (6.4, 7.8)	2.47 brs
18β	2.50 m	2.50 m	2.57 m	2.57 m	2.55 m	2.47 brs	2.47 brs
19	0.69 s	0.71 s	0.74 s	0.74 s	0.78 s	0.55 s	0.53 s
21	1.20 s	1.20 s	1.29 s	1.29 s	1.41 s	1.12 s	1.66 s
22	5.62 d (15.3)	5.62 dd (1.3, 15.6)	5.67 d (15.7)	5.68 d (15.9)	6.89 d (15.5)	5.77 dd (1.3, 15.6)	5.15 d (8.7)
23	5.49 dd (5.9, 15.3)	5.49 dd (5.5, 15.6)	5.57 dd (6.2, 15.7)	5.57 dd (6.2, 15.9)	6.28 d (15.5)	5.53 dd (5.5, 15.6)	3.95 ddd (4.0, 6.6, 8.7)
24	4.10 m	4.10 m	4.35 m	4.35 m		4.11 m	3.40 m
25	1.08 d (5.9)	1.08 d (6.4)	1.29 d (6.2)	1.29 d (6.2)	2.27 s	1.09 d (6.4)	0.96 d (6.4)
MeO−			3.14 s	3.14 s			
3-OH	4.60 d (4.4)	4.61 d (4.5)				4.61 d (4.1)	4.62 d (4.6)
12-OH						6.03 d (1.8)	
23-OH							4.47 d (4.0)
24-OH	4.57 d (4.4)	4.57 d (4.5)				4.58 d (4.6)	4.38 d (4.0)

position	**10**[a]	**11**[a]	**12**[a]	**13**[a]	**14**[a]	**15**[b]	**18**[b]
1	5.55 dd (6.4, 8.2)	5.55 dd (6.4, 8.2)	5.55 dd (6.6, 8.2)	5.55 dd (6.4, 8.2)	5.55 dd (6.4, 8.2)	5.57 brt (7.3)	5.57 dd[c]
2α	2.07 m	2.07 m	2.07 m	2.07 m	2.07 m	2.25 ddt (13.3,8.2)	2.25 ddt (13.3, 8.2, 2.2)
2β	2.34 m	2.34 m	2.34 m	2.34 m	2.34 m	2.48 ddd (13.3,11.4,6.4)	2.49 ddd (13.3, 11.4, 6.0)
3	3.12 m	3.12 m	3.12 m	3.12 m	3.12 m	3.50 brt (11.0)	3.51 brt (11.4)
4α	2.63 brd (13.3)	2.63 brd (12.8)	2.63 brd (12.8)	2.63 brd (12.7)	2.62 brd (13.4)	2.89 brd (12.8)	2.89 brd (13.1)
4β	1.52 m	1.52 m	1.52 m	1.52 m	1.52 m	1.68 m	1.68 m
5	2.68 m	2.68 m	2.68 m	2.67 m	2.67 m	2.75 m	2.75 m
7	5.42 s	5.42 s	5.42 s	5.42 s	5.42 s	5.59 s	5.59 s[c]
9	2.84 dd (5.5, 11.9)	2.84 dd (5.9, 11.5)	2.84 dd (5.5, 12.0)	2.84 dd (5.5, 11.9)	2.84 dd (5.9, 11.9)	2.78 dd (12.8, 6.0)	2.78 dd (12.4, 5.9)
11α	1.54 m	1.54 m	1.54 m	1.54 m	1.54 m	1.61 m	1.61 m
11β	1.80 m	1.79 m	1.80 m	1.80 m	1.80 m	1.85 m	1.89 m
12α	1.47 td (4.6, 12.8)	1.43 td (4.6, 12.4)	1.47 td (4.2, 12.4)	1.47m	1.47m	1.48 m	1.51 m
12β	1.74 m	1.74 m	1.74 m	1.74 m	1.74 m	2.17 m	2.15 m
14	2.25 brt	2.22 brt (8.8)	2.25 brt (8.7)	2.25 brt (8.7)	2.25 brt (8.7)	2.09 ddd (12.3, 6.4)	2.14 m
15α	1.57 m	1.57 m	1.54 m	1.54 m	1.54 m	1.62 m	1.63 m
15β	1.52 m	1.52 m	1.49 m	1.49 m	1.49 m	1.54 m	1.54 m
16α	1.84 m	1.82 m	1.82 m	1.82 m	1.82 m	1.94 m	1.68 m
16β	1.65 m	1.68 m	1.68 m	1.68 m	1.68 m	1.74 m	1.62 m
17	2.23 brt (9.6)	2.26 brt (9.6)	2.23 brt (9.6)	2.31 brt (9.6)	2.31 brt (9.6)	1.74 m	1.85 t (9.6)
18α	2.47 brs	2.48 brs	2.47 brs	2.47 brs	2.47 brs	2.55 d (13.3)	2.57 d (13.2)
18β	2.47 brs	2.48 brs	2.47 brs	2.47 brs	2.47 brs	2.59 dd (13.3, 6.0)	2.59 dd (13.2, 6.4)
19	0.50 s	0.53 s	0.50 s	0.52/0.53 s	0.52/0.54 s	0.86 s	0.78 s
21	1.66 s	1.64 s	1.65 s	1.68 s	1.71 s	1.26 s	1.33 s
22	5.16 d (8.7)	5.22 d (8.7)	5.22 d (8.2)	5.11 d (9.1)	5.01 d (9.6)	3.91 d (7.8)	3.09 d (7.4)
23	3.95 ddd (4.0, 6.6, 8.7)	4.05 ddd (4.6, 4.6, 8.7)	4.09 ddd (4.6, 5.0, 8.2)	3.78/3.77 dd (4.1, 9.1)	3.71 dd (6.4, 9.6)	5.41 ddq (15.1, 8.2, 1.4)	5.37 ddq (15.4, 7.4, 1.3)
24	3.40 m	3.50 m	3.49 m	3.60 m	3.53 m	5.78 dqd (15.1, 6.4)	5.88 dqd (15.4, 6.8)
25	0.94 d (6.4)	0.97 d (6.4)	0.96 d (6.4)	0.96/0.98 d (6.4)	0.93/0.95 d (6.6)	1.74 dd (6.4, 0.9)	1.76 dd (6.8, 1.3)
MeO−				3.15/3.16 s	3.14/3.15 s		
3-OH	4.62 d (4.6)	4.62 d (4.6)	4.62 d (4.6)	4.62 d (4.0)	4.62 d (4.5)		
12-OH							
23-OH	4.49 d (4.0)	4.38 d (4.6)	4.41 d (5.0)				
24-OH	4.37 d (4.0)	4.29 d (5.0)	4.29 d (4.6)	4.42 d (5.0)	4.44/4.43 d (4.1)		

[a] Spectra recorded at 600 MHz in DMSO-d_6. [b] Spectra recorded at 600 MHz in $CDCl_3$. [c] Overlapping signals.

Table 2. ^{13}C NMR Data for Compound **1**−**4** and **7**−**17** (150 MHz, TMS, δ ppm)

position	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**7**[b]	**8**[a]	**9**[a]	**10**[a]	**11**[a]	**12**[a]	**13**[a]	**14**[a]	**15**[b]
1 (CH)	121.9	121.9	121.8	121.8	122.1	122.3	122.1	122.1	122.1	122.1	122.1	122.1	122.0
2 (CH_2)	35.9	35.9	35.7	35.7	35.6	35.9	35.9	35.9	35.9	35.9	35.9	35.9	35.6
3 (CH)	63.1	63.1	64.6	64.6	64.5	62.9	63.1	63.1	63.1	63.1	63.1	63.1	64.5
4 (CH_2)	41.3	41.3	41.7	41.6	41.5	41.2	41.4	41.3	41.4	41.3	41.3	41.3	41.6
5 (CH)	48.1	48.1	48.5	48.5	48.5	48.2	48.1	48.1	48.1	48.1	48.1	48.1	48.5
6 (qC)	204.1	204.1	205.2	205.2	205.1	204.2	204.1	204.1	204.1	204.1	204.0	204.1	205.3
7 (CH)	124.5	124.5	125.0	125.0	125.2	125.3	124.3	124.3	124.3	124.3	124.3	124.3	125.2
8 (qC)	157.1	157.1	157.7	157.7	156.7	155.1	156.9	156.9	157.0	157.0	156.83/156.86	156.8	157.3
9 (CH)	53.2	53.2	54.1	54.1	53.9	51.6	53.3	53.2	53.3	53.2	53.3	53.2	54.0
10 (qC)	145.7	145.7	146.0	146.0	145.6	144.7	145.5	145.5	145.5	145.5	145.5	145.5	145.9
11 (CH_2)	27.5	27.5	27.7	27.7	27.6	34.6	27.4	27.5	27.4	27.5	27.4	27.4	27.6
12 (CH_2)	38.8	38.8	39.4	39.4	39.3	75.0[c]	36.9	37.3	36.9	37.2	37.13/37.08	37.13/37.07	39.3
13 (qC)	45.9	45.9	46.4	46.3	46.3	50.4	46.9	46.6	46.9	46.6	46.7	46.73/46.82	46.4
14 (CH)	55.2	55.2	55.9	55.9	55.7	52.8	54.3	54.5	54.3	54.4	54.4	54.3	55.8
15 (CH_2)	22.1	22.1	22.5	22.5	22.7	21.8	22.4	22.2	22.4	22.3	22.36/22.33	22.3	22.8
16 (CH_2)	22.3	22.3	22.5	22.5	22.5	23.4	23.7	23.8	23.7	23.8	23.8	23.85/23.74	21.2
17 (CH)	60.0	60.1	60.2	60.3	60.0	62.8	58.9	58.6	58.9	58.6	58.91/58.85	58.9	55.2
18 (CH_2)	27.1	27.1	27.6	27.6	27.6	27.0	27.2	27.2	27.2	27.2	27.2	27.2	27.5
19 (CH_3)	14.3	14.3	14.8	14.8	14.6	9.8	13.4	13.3	13.4	13.2	13.44/13.40	13.47/13.50	14.1
20 (qC)	73.2	73.3	79.4	79.6	75.3	73.0	135.8	135.8	135.0	134.9	138.62/138.74	139.5	77.0
21 (CH_3)	28.9	28.9	21.7	21.7	29.0	30.2	17.3	18.9	17.3	18.7	18.24/18.01	18.53/18.16	20.6
22 (CH)	136.2	136.0	134.5	134.5	152.6	132.4	128.1	127.0	128.2	127.3	124.30/124.43	124.32/124.08	77.5
23 (CH)	130.8	130.8	134.1	134.2	126.6	131.5	72.0	72.2	71.5	71.6	81.39/81.32	81.94/81.85	129.3
24 (CH)	66.6	66.3	68.8	68.7	198.5	66.3	70.3	70.3	69.7	69.7	68.36/68.31	68.7	130.2
25 (CH_3)	24.1	24.0	23.6	23.6	27.9	23.7	18.9	19.1	18.5	18.3	18.72/18.89	18.9	18.0
MeO-(CH_3)			49.7	49.7							55.4	55.41/55.37	

[a] Spectra recorded at 150 MHz in DMSO-d_6. [b] Spectra recorded at 150 MHz in $CDCl_3$. [c] CH.

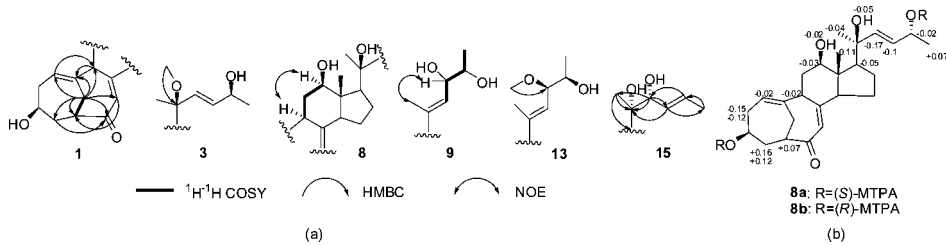


Figure 1. (a) Selected HMBC, $^1H-^1H$ COSY, and NOE correlations of compounds **1**, **3**, **8**, **9**, **13**, and **15**. (b) Proton chemical-shift differences ($\Delta\delta = \delta_S - \delta_R$) between the (*R*)- and (*S*)-MTPA esters **8a** and **8b** of 12*R*-hydroxylcyclocitrinol B (**8**), expressed in ppm.

the acidic condition induced by the mass production of the acidic compound citrinin[5] (yield ~100 mg/L), the intermediate product **18** was rapidly transformed into compounds **1**, **2**, **15**, and **16** by a nonenzymatic process in the fermentation broth. The absolute configuration of C-22 in **18** is still unsolved due to the lack of a pure sample. Compounds **3**, **4**, and **9**−**14** were all authenticated as artifacts (Figure 5, Schemes 1 and 2).

Andrey[3] suggested a plausible biochemical route to the bicyclic system originated from ergosterol. We also isolated ergosterol from the gum of this strain. Ergosterol is the major sterol in the more advanced ascomycetes and basidiomycetes.[6] The study of the biosynthetic origin of the des-A-ergostane type steroid blazeispirol A[7] indicated that ergosterol might be an active precursor for novel steroid skeletons. Trying to prove the hypothesis, cultures of the fungus *P. citrinum* were supplemented with stable isotope-labeled precursors [1,2-$^{13}C_2$]-acetate and [2-^{13}C]-acetate and the incorporation patterns of the enriched compound **2** were measured by ^{13}C NMR spectroscopy. Thirteen of the 25 carbon atoms were predominantly labeled from the methyl group of acetate by feeding of sodium [2-^{13}C]-acetates, and the result of the sodium [1,2-$^{13}C_2$]-acetate feeding experiment revealed the incorporation of eight intact acetate units in **2** by strong coupling of the following pairs: C-2/C-3, C-5/C-6, C-9/C-11, C-10/C-18, C-12/C-13, C-16/C-17, C-20/C-21, and C-23/C-24 (Table 3 and Figure 6). The labeling pattern of **2** was identical to that previously observed for ergosterol[7] and consistent with Andrey's hypothetical biosynthetic scheme.[3]

The biological activities of compounds **1**−**4** and **7**−**17** were evaluated using the cAMP assay[8,9] in GPR12-CHO and WT-CHO cells. Cyclic AMP regulates multiple neuronal functions, including neurite outgrowth and axonal regeneration. GPR12 is highly expressed in the central nervous system, and its expression in various cell lines results in constitutive stimulation of cAMP production. On the basis of the present findings, up-regulating GPR12 in damaged neurons may hold potential as a therapeutic strategy to treat various neurological disorders, including spinal cord injuries and stroke.[10] The results showed that compounds **1**, **2**, **10**, **11**, and **14** could induce the production of cAMP in GPR12-transfected CHO cells at 10 μM (Figure 7A). The induction of cAMP generation is receptor dependent, since no cAMP was detected in wild-type CHO cells (Figure 7B). Further pharmacological analysis should be performed to determine whether these compounds could be specific agonists for GPR12.

Experimental Section

General Experimental Procedures. Melting points were measured using a Yanaco MP-500D micromelting point apparatus and are uncorrected. Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were recorded on a Nicolet Nexus 470 spectrophotometer using KBr discs. ^1H and ^{13}C NMR, DEPT, and 2D NMR spectra were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as internal standard, and chemical shifts were recorded as δ values. ESIMS were measured on a Q-TOF Ultima Global GAA076 LC mass spectrometer. Semiprepartive HPLC was performed using an ODS column [Shin-pak ODS (H), 20 × 250 mm, 5 μm, 4 mL/min].

Fermentation, Extraction, and Purification. *P. citrinum* HGY1-5 was isolated from the crater ash collected from the extinct volcano

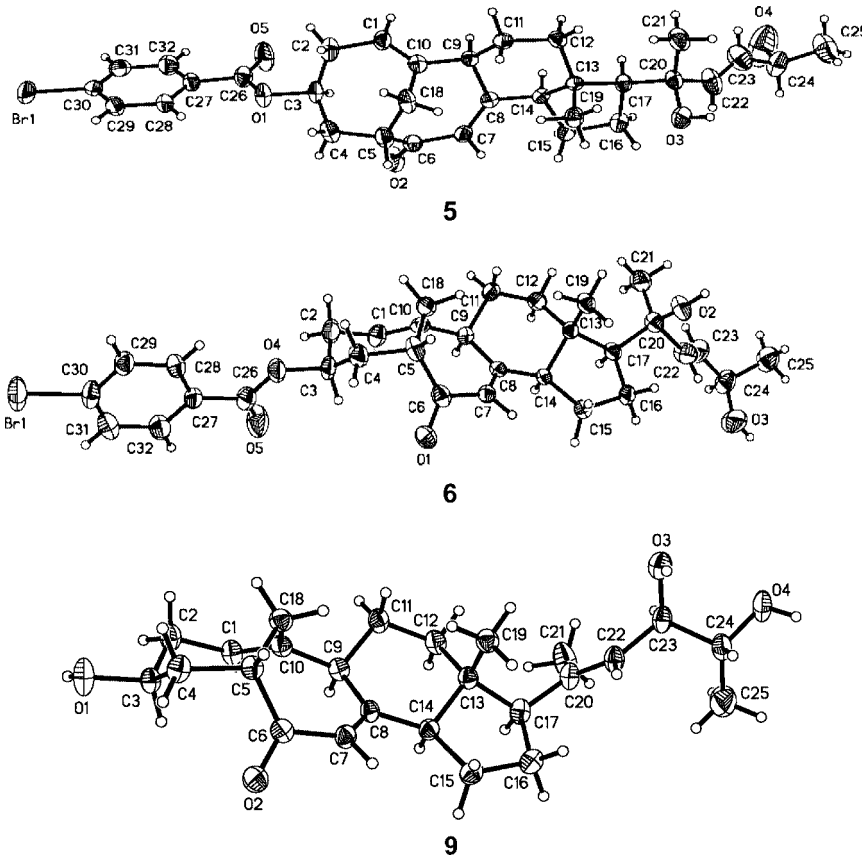
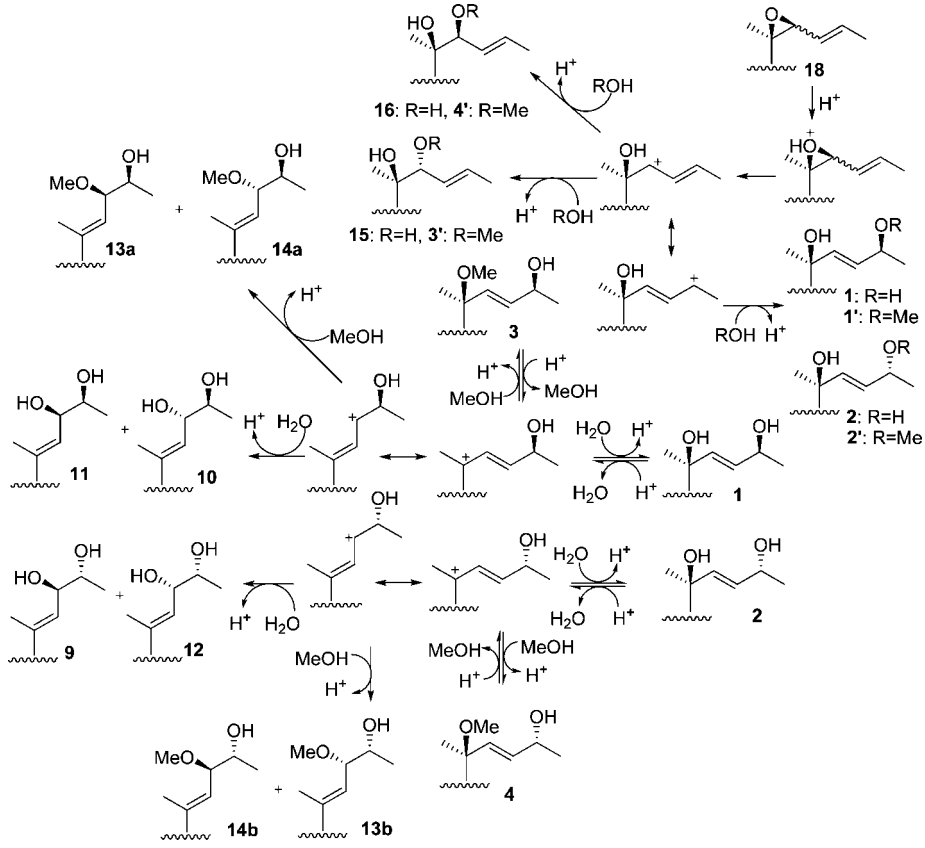


Figure 2. X-ray structures of compounds **5**, **6**, and **9**.

Scheme 1. Possible Mechanisms for the Reactions of the Side Chains of Compounds **1**−**4** and **18** with H_2O and MeOH in AcOH



Huguangyan in Guangdong, China. It was identified according to its morphological characteristics and 18S RNA by Prof. Li Tian, the First Institute of Oceanography, SOA, Qingdao, China. A voucher specimen is deposited in our laboratory at −80 °C. The working strain was prepared on potato dextrose agar slants and stored at 4 °C. Fermentation was carried out as follows: Spores were directly inoculated into 500

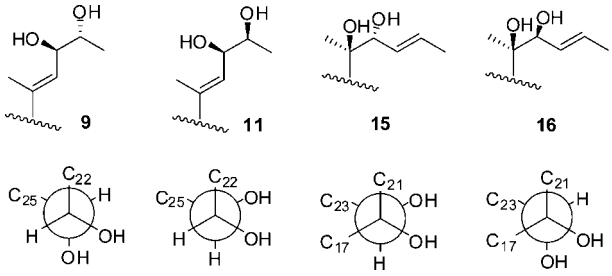


Figure 3. Preferred conformations of compounds **9**, **11**, **15**, and **16**.

mL Erlenmeyer flasks containing 100 mL of fermentation media (mannitol 20 g, maltose 20 g, glucose 10 g, monosodium glutamate 10 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, yeast extract 3 g, and corn steep liquor 1 g, dissolved in 1 L of water, pH 6.5). The flasks were incubated on a rotatory shaker at 165 rpm at 28 °C. After 9 days of cultivation, 20 L of whole broth was filtered through cheesecloth to separate the broth supernatant and mycelia. The former was extracted with EtOAc, while the latter was extracted with acetone. The acetone extract was evaporated under reduced pressure to afford an aqueous solution and then extracted with EtOAc. The two EtOAc extracts were combined and concentrated in vacuo to give a crude gum (20 g). The crude gum was subjected to Si gel column chromatography (CC, $CHCl_3$/MeOH, v/v, gradient), and the fraction eluted with the solvent $CHCl_3$/MeOH (20:1) was subjected to repeated chromatography on Sephadex LH-20 CC ($CHCl_3$/MeOH, 1:1). Subfraction 3-2-3 was further purified by HPLC (MeOH/H_2O, 6:4) to give compounds **1** (39.4 mg) and **2** (17.3 mg). Subfraction 2-5-4 was further purified by HPLC (MeOH/H_2O, 7:3) to give compounds **3** (5.2 mg) and **4** (3.2 mg). Similar purification procedures were applied to subfraction 3-2-5 (HPLC eluted with CH_3CN/H_2O, 4:6) and subfraction 3-2-4 (HPLC eluted with CH_3CN/H_2O, 45:55) to afford compounds **16** (1.5 mg) and **17** (3.2 mg), respectively. The strain was refermented in 40 L of liquid medium for 15 days under the same fermentation and extraction conditions to give a crude gum (88 g). The gum was subjected to similar CC procedures and separated into several subfractions. Subfraction 2-1-6 was separated on HPLC eluted with MeOH/H_2O, 55:45, to yield compounds **9** (40 mg), **10** (10 mg), **11** (45 mg), and **12** (15 mg). Compounds **13** (121 mg) and **14** (201 mg) were separated form subfraction 2-1-2 by HPLC eluted with MeOH/H_2O, 65:35. Similar purification of subfractions 2-1-2-2 (HPLC eluted with CH_3OH/H_2O, 60:40), 2-1-9 (HPLC eluted with MeOH/H_2O, 45:55), 2-1-4 (HPLC eluted with CH_3OH/H_2O, 65:35), and 1-1-4 (HPLC eluted with CH_3OH/H_2O, 90:10), gave compounds **7** (13 mg), **8** (11 mg), **15** (14 mg), and **18** (1.1 mg), respectively.

Preparation of *p*-Bromobenzoates (5 and 6). To a stirred suspension of 12 mg of **1** in 2 mL of dry CH_2Cl_2 was added 1 mL of Et_3N and 25 mg of *p*-BrC_6H_4COCl at room temperature. Four hours later 8 mg of DMAP was added and allowed to react another 2 h, and then the reaction was quenched by adding 2 mL of H_2O. The mixture was extracted with 3 × 5 mL of EtOAc, and the EtOAc solution was dried on anhydrous Na_2SO_4 and evaporated at reduced pressure. The residue was subjected to flash CC over Si gel (petroleum ether/acetone, 65:35) and HPLC (92% MeOH/H_2O) to give **5** (3.3 mg, 18.9% yield). The same procedure was applied to **2** (12 mg) to afford **6** (2.0 mg, 10.9% yield).

3-*p*-Bromobenzoylepicyclocitrinol (5): colorless needles (v/v 1:1 of petroleum ether and acetone); $C_{32}H_{39}BrO_5$; mp 180−181 °C; $[\alpha]^{20}_D$ +33.2 (*c* 0.17,$CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.66 (1H, dd, H-1), 2.44 (1H, m, H-2α), 2.59 (1H, m, H-2β), 4.76 (1H, m, H-3), 2.97 (1H, d, H-4α), 2.83 (1H, m, H-5), 5.62 (1H, s, H-7), 2.82 (1H, m, H-9), 2.21 (1H, m, H-12β), 2.12 (1H, m, H-14), 1.96 (1H, m, H-17), 2.63 (2H, m, H-18), 0.80 (3H, s, CH_3-19), 1.36 (3H, s, CH_3−21), 5.80

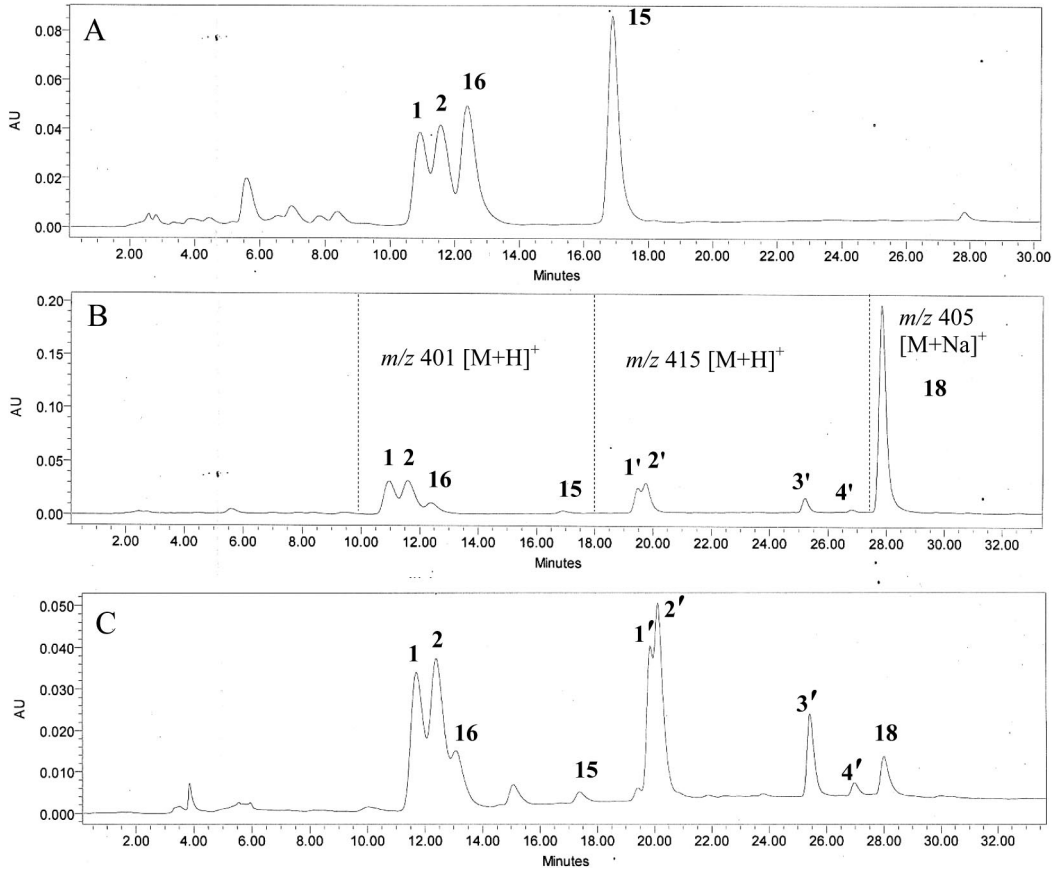


Figure 4. HPLC and LC-MS analysis of the ring-opening products of compound **18**. Conditions: 0−5 min, 60% MeOH/H_2O; 5−20 min, 60% MeOH/H_2O to 80% MeOH/H_2O, gradient; after 20 min, 80% MeOH/H_2O; 242 nm; 1 mL/min. (A) HPLC profile of the mixture of compounds **1**, **2**, **15**, and **16**. (B) HPLC-MS profile of compound **18** (the pure compound had been stored in MeOH and H_2O at room temperature for 30 days). (C) HPLC profile of the acid-catalyzed ring-opening products of **18** in the mixture of MeOH, H_2O, and AcOH.

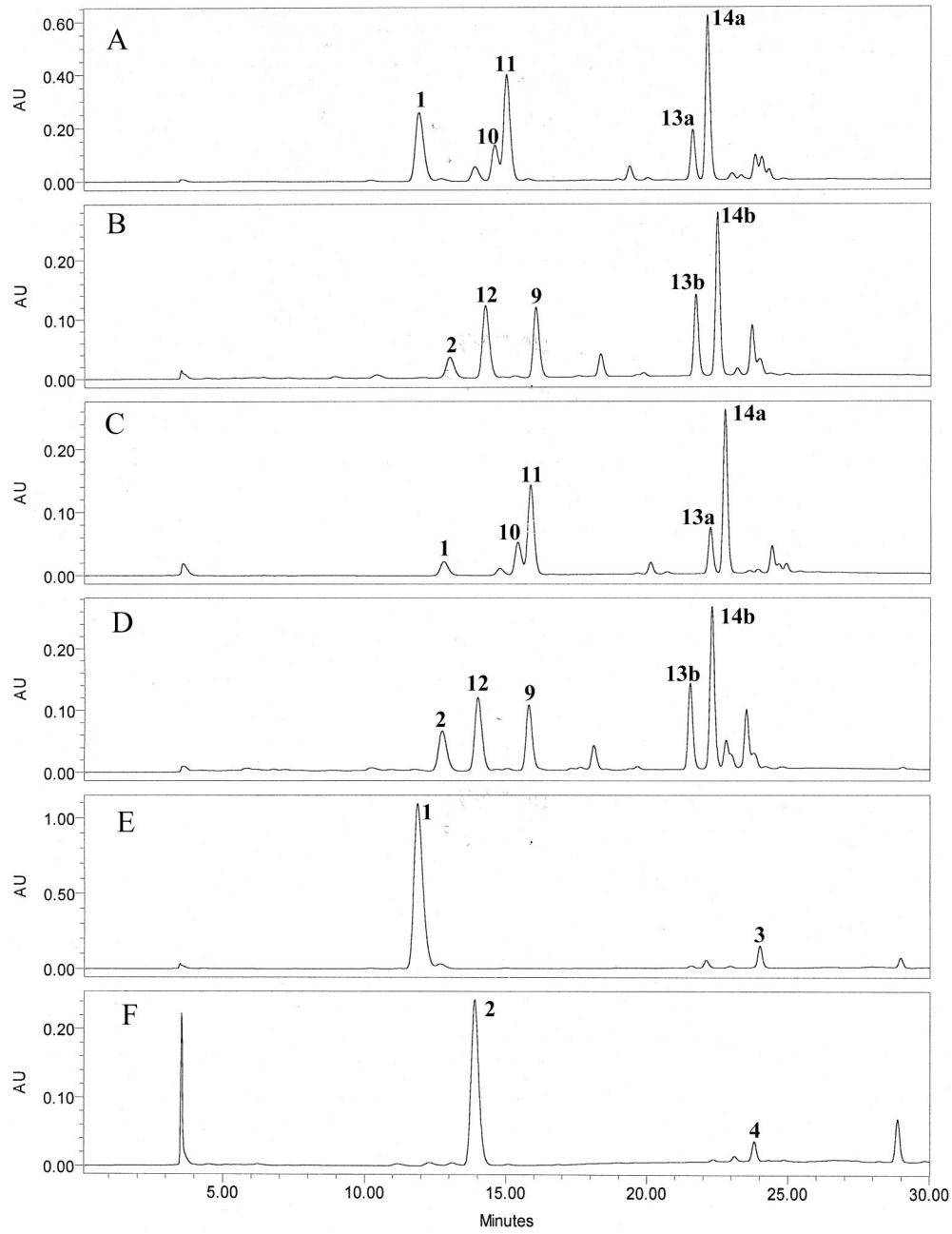


Figure 5. HPLC analysis of the acid-catalyzed isomerization and alcoholysis products of compounds **1**−**4**. Conditions: 0−5 min, 60% MeOH/H_2O; 5−20 min, 60% MeOH/H_2O to 80% MeOH/H_2O, gradient; after 20 min, 80% MeOH/H_2O; 242 nm; 1 mL/min. A−D show the HPLC profiles of isomerization products of compounds **1**−**4** at 30 °C for 24 h in MeOH/H_2O/HOAc (1:1:0.1), respectively. E and F show the HPLC profiles of alcoholysis products of compounds **1** and **2** at 4 °C for 48 h in MeOH/HOAc (1:0.05), respectively. Standards were used to verify the identities of the peaks when retention times changed due to the unstable HPLC conditions (Supporting Information, Figures F47−F52).

Scheme 2. Postulated Biosynthetic Pathway of Compounds **1**, **2**, **7**, **8**, **15**, and **18**

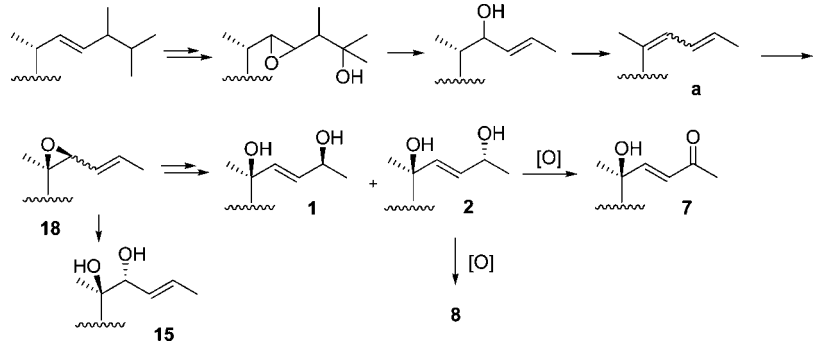


Table 3. ^{13}C NMR Data of Compound **2** in DMSO-d_6 Together with Specific Incorporations and Coupling Constants after Feeding [2-^{13}C]-Acetate (I) and [1,2-$^{13}C_2$]-acetate (II)

carbon	δ_C (ppm)	enriched factor (I)[a]	J_{CC} (Hz) (II)
1 (CH)	121.9	1.85	
2 (CH_2)	35.9	0.91	38.9
3 (CH)	63.1	1.83	38.9
4 (CH_2)	41.3	0.98	
5 (CH)	48.1	1.99	36.6
6 (qC)	204.1	1.06	36.6
7 (CH)	124.5	1.55	
8 (qC)	157.1	1.13	
9 (CH)	53.2	1.74	34.3
10 (qC)	145.7	0.99	41.2
11 (CH_2)	27.5	0.92	34.3
12 (CH_2)	38.8	1.15	38.9
13 (qC)	45.9	1.60	38.9
14 (CH)	55.2	-[a]	
15 (CH_2)	22.1	1.46	
16 (CH_2)	22.3	0.89	29.8
17 (CH)	60.1	1.71	29.8
18 (CH_2)	27.1	2.09	41.2
19 (CH_3)	14.3	1.40	
20 (qC)	73.3	0.84	41.2
21 (CH_3)	28.9	1.75	41.2
22 (CH)	136.0	2.14	
23 (CH)	130.8	1.05	50.4
24 (CH)	66.3	2.22	50.4
25 (CH_3)	24.0	1.07	

[a] Mean of two independent experiments. Determined as the ratio of enriched to natural ^{13}C NMR intensity normalized to carbon at 55.2 ppm (C-14).

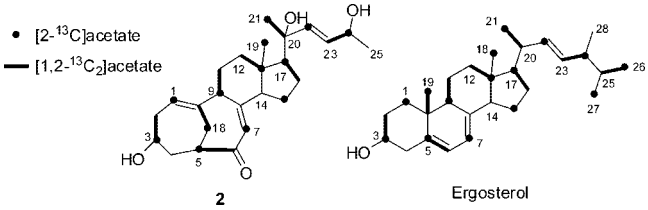


Figure 6. Stable isotope labeled **2** formed after feeding [2-^{13}C]-acetate, and [1,2-$^{13}C_2$]-acetate.

(1H, d, H-22), 5.66 (1H, dd, H-23), 4.33 (1H, m, H-24), 1.27 (3H, d, CH_3−25), 7.56 (2H, d), 7.87 (2H, d), 1.49−1.89 (8H, H-4β, H-11α, H-11β, H-12α, H-15α, H-15β, H-16α, H-16β).

3-*p*-Bromobenzoylcyclocitrinol (6): colorless needles (v/v 1:1 of petroleum ether and acetone); $C_{32}H_{39}BrO_5$; mp 170−171 °C; $[\alpha]^{20}_D$ +94.1 (*c* 0.10, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$) δ 5.66 (1H, dd, H-1), 2.44 (1H, m, H-2α), 2.59 (1H, m, H-2β), 4.76 (1H, m, H-3), 2.97 (1H, d, H-4α), 2.83 (1H, m, H-5), 5.62 (1H, s, H-7), 2.82 (1H, m, H-9), 2.22 (1H, m, H-12β), 2.13 (1H, m, H-14), 1.96 (1H, m, H-17), 2.63 (2H, m, H-18), 0.81 (3H, s, CH_3-19), 1.36 (3H, s, CH_3−21), 5.82 (1H, dd, H-22), 5.68 (1H, dd, H-23), 4.34 (1H, m, H-24), 1.27 (3H, d, CH_3−25), 7.56 (2H, d), 7.87 (2H, d), 1.49−1.89 (8H, H-4β, H-11α, H-11β, H-12α, H-15α, H-15β, H-16α, H-16β).

(*S*)-MTPA Ester (8a) of Compound 8. To a solution of **8** (3 mg) in pyridine (1 mL) were added (*R*)-(−)-MTPACl (8 μL) and DMAP (50 μg). The mixture was allowed to stand at 20 °C for 12 h. After addition of H_2O (1 mL) and extraction with $CHCl_3$, the extract was evaporated and the residue was subjected to HPLC (10:90, H_2O/MeOH) to afford the (*S*)-MTPA ester (**8a**, 5 mg) of **8**. **8a**: colorless oil; ^1H NMR (600 MHz, DMSO-d_6) δ 5.60 (1H, m, H-1), 2.11 (1H, m, H-2α), 2.59 (1H, m, H-2β), 4.66 (1H, m, H-3), 2.71 (1H, d, H-4α), 1.90 (1H, m, H-4β), 2.90 (1H, m, H-5), 5.46 (1H, s, H-7), 3.00 (1H, dd, H-9), 3.52 (1H, m, H-12), 2.13 (1H, m, H-14), 1.71 (1H, m, H-16β), 1.84 (1H, t, H-17), 2.41 (1H, dd, H-18α), 2.62 (1H, d, H-18β), 0.36 (3H, s, CH_3-19), 1.07 (3H, s, CH_3-21), 5.98 (1H, d, H-22), 5.59 (1H, dd, H-23), 5.59 (1H, m, H-24), 1.35 (3H, d, CH_3-25), 6.24 (1H, s, HO-20), 5.99 (1H, d, HO-12), 1.40−1.60 (5H, H-11α, H-11β, H-15α, H-15β, H-16α), 3.50 (6H), 7.44−7.51 (10H).

(*R*)-MTPA Ester (8b) of Compound 8. Compound **8** (3 mg) was treated with (*S*)-(+)-MTPACl (8 μL) by the same procedure described above to afford the (*R*)-MTPA ester (**8b**, 5 mg) of **8**. **8b**: colorless oil; ^1H NMR (600 MHz, DMSO-d_6) δ 5.62 (1H, m, H-1), 2.26 (1H, m, H-2α), 2.71 (1H, m, H-2β), 4.66 (1H, m, H-3), 2.59 (1H, d, H-4α), 1.74 (1H, m, H-4β), 2.83 (1H, m, H-5), 5.46 (1H, s, H-7), 3.02 (1H, dd, H-9), 3.55 (1H, m, H-12), 2.16 (1H, m, H-14), 1.77 (1H, m, H-16β),1.89 (1H, t, H-17), 2.40 (H, dd, H-18α), 2.62 (H, d, H-18β), 0.47 (3H, s, CH_3-19), 1.11 (3H, s, CH_3-21), 6.15 (1H, d, H-22), 5.69 (1H, dd, H-23), 5.61 (1H, m, H-24), 1.28 (3H, d, CH_3-25), 6.29 (1H, s, HO-20), 6.01 (1H, d, HO-12), 1.48−1.64 (5H, H-11α, H-11β, H-15α, H-15β, H-16α), 3.46 (3H), 3.48 (3H), 7.43−7.51 (10H).

X-ray Diffraction Analysis of 5, 6, and 9. X-ray crystal structure analysis of compound **5**: colorless block crystal; space group $P2_12_12_1$, a = 9.801(4) Å, b = 11.207(4) Å, c = 26.272(10) Å, V = 2885.8(19) Å^3, Z = 4, crystal size 0.43 × 0.40 × 0.35 mm^3. A total of 5027 unique reflections (2θ < 50.02°) were collected using graphite-monochromated Mo Kα (λ = 0.71073 Å) on a CCD area detector diffractometer. The structure was solved by direct methods (SHELXS-97) and expanded using Fourier techniques (SHELXS-97). The final cycle of full-matrix least-squares refinement was based on 5027 unique reflections (2θ < 50.02°) and 343 variable parameters and converged with unweighted and weighted agreement factors of R_1 = 0.043 and R_w = 0.081 for $I > 2.0\sigma(I)$ data.

X-ray crystal structure analysis of compound **6**: colorless block crystal; space group $P2_12_12_1$, a = 9.772(4) Å, b = 11.108(5) Å, c = 26.280(11) Å, V = 2853(2) Å^3, Z = 4, crystal size 0.20 × 0.17 × 0.08 mm^3. A total of 5008 unique reflections (2θ < 50.00°) were collected using graphite-monochromated Mo Kα (λ = 0.71073 Å) on a CCD area detector diffractometer. The structure was solved by direct methods (SHELXS-97) and expanded using Fourier techniques (SHELXS-97). The final cycle of full-matrix least-squares refinement was based on 5008 unique reflections (2θ < 50.00°) and 343 variable parameters and converged with unweighted and weighted agreement factors of R_1 = 0.046 and R_w = 0.111 for $I > 2.0\sigma(I)$ data.

X-ray crystal structure analysis of compound **9**: colorless block crystal; space group $P2_12_12_1$, a = 6.6828(13) Å, b = 10.1709(16) Å, c = 33.541(3) Å, V = 2279.8(6) Å^3, Z = 4, crystal size 0.65 × 0.34 × 0.04 mm^3. A total of 2339 unique reflections (2θ < 50.02°) were collected using graphite-monochromated Mo Kα (λ = 0.71073 Å) on a CCD area detector diffractometer. The structure was solved by direct methods (SHELXS-97) and expanded using Fourier techniques (SHELXS-97). The final cycle of full-matrix least-squares refinement was based on 2339 unique reflections (2θ < 50.02°) and 262 variable parameters and converged with unweighted and weighted agreement factors of R_1 = 0.049 and R_w = 0.110 for $I > 2.0\sigma(I)$ data.

Hydrolysis of Precyclocitrinol B (18). Compound **18** was stirred with MeOH (500 μL), H_2O (500 μL), and AcOH (50 μL) for 2 h at room temperature. The products were identified as **1**, **2**, **15**, **16**, and the corresponding *O*-methyl derivatives **1′**−**4′** by HPLC (HPLC conditions: phase A: H_2O; phase B: MeOH; 0−5 min, 60% MeOH/H_2O; 5−20 min, 60%MeOH/H_2O to 80% MeOH/H_2O, gradient; after 20 min, 80% MeOH/H_2O; 242 nm; 1 mL/min) and LC-MS (positive ESIMS).

Acidic Isomerization and Alcoholysis of Compounds 1−4. Compounds **1**−**4** were separately stirred in MeOH (500 μL), H_2O (500 μL), and AcOH (50 μL) at 30 °C for 24 h. HPLC analysis revealed the products as **10**, **11**, **13a**, and **14a**; **9**, **12**, **13b**, and **14b**; **1**, **10**, **11**, **13a**, and **14a**; and **2**, **9**, **12**, **13b**, and **14b**, respectively. Compounds **1** and **2** were also stirred respectively in MeOH (1000 μL) and HOAc (50 μL) at 4 °C for 48 h, whose products were identified as **3** and **4**, respectively, by HPLC analysis (HPLC conditions: phase A: H_2O; phase B: MeOH; 0−5 min, 60% MeOH/H_2O; 5−20 min, 60% MeOH/H_2O to 80% MeOH/H_2O, gradient; after 20 min, 80% MeOH/H_2O; 242 nm; 1 mL/min).

Sodium [2-^{13}C]-Acetate and Sodium [1,2-$^{13}C_2$]-Acetate Feeding Experiments. Sodium [2-^{13}C]-acetate (500 mg) and sodium [1,2-$^{13}C_2$]-acetate (500 mg) were separately fed to 16 × 0.15 L cultures on days 1, 4, 7, and 10, and then both 2.4 L cultures were harvested on day 13. A total of 6.5 and 8.4 mg of the labeled **2** were respectively isolated from the crude organic extracts. The ^{13}C NMR data, percent relative enrichment, and J_{CC} coupling values in **2** are summarized in Table 3.

24-*epi*-Cyclocitrinol (1): colorless needles (methanol); mp 144−145 °C; $[\alpha]^{20}_D$ +144.0 (*c* 0.30, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.38), 242 (4.22); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 314.9 (−0.5), 247.1 (1.2), 216.9 (0.1), 192.9 (4.3); IR (film) ν_{max} 3421, 2943, 2866, 1649, 1459, 1366

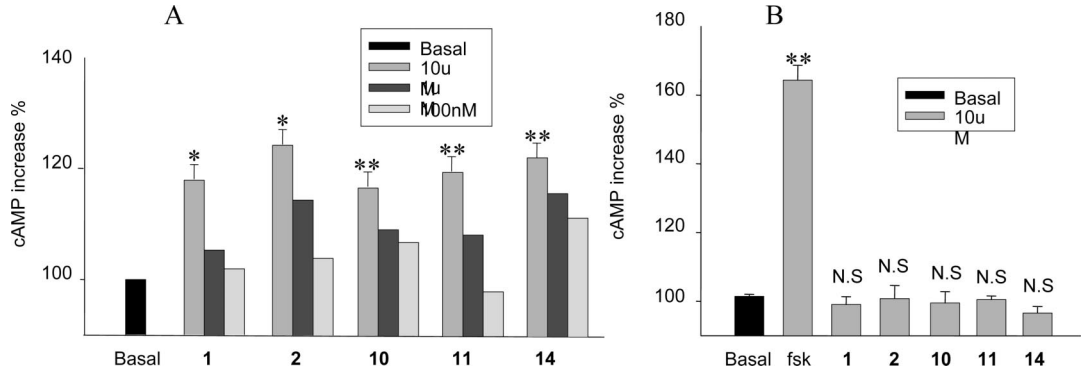


Figure 7. Characterization of compounds using cAMP assay in GPR12-CHO cells. (A) Dose−response analysis of compounds in GPR12-CHO cells. (B) Analysis of compounds in WT-CHO cells. Statistical analysis was performed by unpaired student test. Statistical significant is indicated by $*P < 0.05$, $**P < 0.005$, N.S. refers to no significant difference. Fsk: forskolin.

cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 399.2547 [M − H]$^-$ (calcd for $C_{25}H_{35}O_4$, 399.2535).

Cyclocitrinol (2): colorless needles (methanol); mp 182−184 °C; $[\alpha]^{20}_D$ +130.3 (*c* 0.30, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.27), 242 (4.14); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 317.0 (−0.6), 244.9 (1.2), 217.6 (0.1), 196.9 (3.1); IR (film) ν_{max} 3405, 2943, 2866, 1650, 1456, 1370 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 399.2537 [M − H]$^-$ (calcd for $C_{25}H_{35}O_4$, 399.2535).

20-*O*-Methyl-24-*epi*-cyclocitrinol (3): colorless needles (methanol); mp 122−124 °C; $[\alpha]^{20}_D$ +157.8 (*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.36), 241 (4.17); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 315.8 (−0.5), 247.2 (1.2), 217.3 (0.1), 196.1 (2.7); IR (film) ν_{max} 3413, 2940, 2862, 1654, 1457, 1368 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 413.2675 [M − H]$^-$ (calcd for $C_{26}H_{37}O_4$, 413.2692).

20-*O*-Methylcyclocitrinol (4): colorless needles (methanol); mp 99−101 °C; $[\alpha]^{20}_D$ +174.1 (*c* 0.22, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.43), 242 (4.14); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 313.7 (−0.6), 245.6 (1.4), 217.4 (0), 192.9 (3.7); IR (film) ν_{max} 3483, 3404, 2939, 2867, 1654, 1457, 1371 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 413.2693 [M − H]$^-$ (calcd for $C_{26}H_{37}O_4$, 413.2692).

24-Oxocyclocitrinol (7): colorless needles (methanol); mp 227−228 °C; $[\alpha]^{20}_D$ +153.5 (*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.15), 237 (4.24); IR (film) ν_{max} 3425, 3374, 2939, 2862, 1650, 1456, 1355, 1262, 1168, 1033 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 399.2530 [M + H]$^+$ (calcd for $C_{25}H_{35}O_4$, 399.2535).

12*R*-Hydroxycyclocitrinol (8): colorless needles (methanol); mp 147−148 °C; $[\alpha]^{20}_D$ +103.6 (*c* 0.27, MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.83), 238 (3.89); IR (film) ν_{max} 3421, 2955, 2869, 1646, 1522, 1456, 1366, 1165, 1067, 1025 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 417.2641 [M + H]$^+$ (calcd for $C_{25}H_{37}O_5$, 417.2641).

Neocyclocitrinol A (9): colorless needles (methanol); mp 205−206 °C; $[\alpha]^{20}_D$ +130.2 (*c* 0.065, MeOH); UV (MeOH) λ_{max} (log ε) 204 (5.12), 241 (4.31); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 315.4 (−0.4), 244.5 (0.8), 219.5 (0.2), 195.1 (3.1); IR (film) ν_{max} 3390, 2932, 2850, 1646, 1537, 1456, 1258, 1102, 1025 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 399.2544 [M − H]$^-$ (calcd for $C_{25}H_{35}O_4$, 399.2535).

Neocyclocitrinol B (10): colorless needles (methanol); mp 202−203 °C; $[\alpha]^{20}_D$ +125.9 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.02), 243 (3.88); IR (film) ν_{max} 3425, 2970, 2935, 1673, 1646, 1456, 1370, 1021 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 401.2698 [M + H]$^+$ (calcd for $C_{25}H_{37}O_4$, 401.2692).

Neocyclocitrinol C (11): colorless needles (methanol); mp 203−204 °C; $[\alpha]^{20}_D$ +94.2 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.30), 242 (4.19); IR (film) ν_{max} 3308, 2970, 2932, 2842, 1646, 1611, 1460, 1122, 1083, 1029 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 401.2702 [M + H]$^+$ (calcd for $C_{25}H_{37}O_4$, 401.2692).

Neocyclocitrinol D (12): colorless needles (methanol); mp 173−174 °C; $[\alpha]^{20}_D$ +98.3 (*c* 0.075, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.55), 242 (4.16); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 317.5 (−0.6), 243.3 (1.1), 218.4 (0.3), 194.1 (4.0); IR (film) ν_{max} 3417, 2928, 2873, 1642, 1545, 1456, 1363 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 401.2683 [M + H]$^+$ (calcd for $C_{25}H_{37}O_4$, 401.2692).

***erythro*-23-*O*-Methylneocyclocitrinol (13):** white powder; UV (MeOH) λ_{max}(log ε) 204 (5.12), 242 (4.26); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 315.8 (−0.5), 245.9 (0.8), 218.8 (0.3), 197.1 (3.3); IR (film) ν_{max} 3398, 2943, 2869, 1646, 1456, 1374, 1176, 1087 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 413.2678 [M − H]$^-$ (calcd for $C_{26}H_{37}O_4$, 413.2692).

***threo*-23-*O*-Methylneocyclocitrinol (14):** white powder; UV (MeOH) λ_{max} (log ε) 203 (4.47), 241 (4.08); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 315.9 (−0.5), 247.8 (0.9), 221.3 (0.4), 198.4 (3.5); IR (film) ν_{max} 3401, 2932, 2873, 1650, 1448, 1382, 1231 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 415.2851 [M + H]$^+$ (calcd for $C_{26}H_{39}O_4$, 415.2848).

Isocyclocitrinol B (15): colorless needles (methanol); mp 120−121 °C; $[\alpha]^{20}_D$ +184.1 (*c* 0.26, MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.94), 242 (3.98); IR (film) ν_{max} 3429, 2943, 2869, 1646, 1448, 1366, 1172, 1033 cm^{-1}; ^{1}H NMR and ^{13}C NMR data in Tables 1 and 2; HRESIMS *m/z* 401.2691 [M + H]$^+$ (calcd for $C_{25}H_{37}O_4$, 401.2692).

Precyclocitrinol B (18): colorless powder; ^{1}H NMR data in Table 1; HRESIMS *m/z* 405.2412 [M + Na]$^+$ (calcd for $C_{25}H_{34}O_3Na$, 405.2406).

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Supporting Information Available: The NMR spectra of compounds **1**−**18**, HPLC profiles of the products of acidic hydrolysis and alcoholysis of **1**−**4**, and CIF files of X-ray analysis of compounds **5**, **6**, and **9** are available free of charge via the Internet at http://pubs.acs.org.

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