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Full Papers

Cohibins C and D, Two Important Metabolites in the Biogenesis of Acetogenins from *Annona muricata* and *Annona nutans*[1]

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Two new annonaceous acetogenins, cohibins C (**1a**) and D (**1b**), have been isolated by extensive chromatography of a hexane extract of *Annona muricata* seeds and a cyclohexane extract of *Annona nutans* root bark. Their structures have been established on the basis of spectral evidence (NMR, MS) and confirmed by chemical transformation into a pair of monotetrahydrofuran (mono-THF) acetogenins. The role of these compounds in the biogenesis of mono-THF acetogenins is discussed.

The numerous bioactivities of the annonaceous acetogenins as cytotoxic, immunosuppressive, pesticidal, antiparasitic, and antimicrobial agents[2,3] have attracted increasing interest in these compounds. Recently, we have isolated some early precursors of annonaceous acetogenins from *Annona muricata* L. and *Annona nutans* R. E. Fr. (Annonaceae) with only double bonds on an aliphatic chain.[4] In a continued search for key biogenetic precursors of acetogenins,[5–8] this paper reports the isolation and identification of two new acetogenins, cohibins C (**1a**) and D (**1b**), from the seeds of *A. muricata* and the root bark of *A. nutans*. These compounds are annonaceous acetogenins without either a tetrahydrofuran (THF) or an epoxide ring within the aliphatic chain, but they contain an isolated double bond and a vicinal diol moiety at the appropriate position, suggesting that they are biogenetic precursors of acetogenins with epoxide or THF rings.[9] Two known compounds, cohibins A and B, were also isolated[7] from plants investigated in this study.

Results and Discussion

The dried and powdered seeds of *A. muricata* and the root bark of *A. nutans* were extracted with methanol. The MeOH extracts, after concentration under a vacuum, were partitioned between water/hexane and water/cyclohexane, respectively, for *A. muricata* and *A. nutans*. The organic layers were dried and submitted to successive fractionation by column chromatography (Si gel and Sephadex LH-20), followed by repeated reversed-phase HPLC, which led to the isolation of a mixture of cohibins C and D (**1a** + **1b**), in both cases.

The mass peak at *m/z* 577 $[M + H]^+$ in the CIMS of **1a** + **1b** indicated a molecular weight of 576, suggesting a molecular formula of $C_{37}H_{68}O_4$. A weak UV λ_{max} at 213.6 nm and a strong IR absorption at 1741 cm^{-1} indicated the presence of an α,β-unsaturated γ-methyl γ-lactone moiety, characteristic for acetogenins of subtype 1.[2] This structural feature was confirmed by typical resonances in the ^1H NMR and ^{13}C NMR spectra (Table 1), also indicating the absence of an OH group at C-4.[2]

The lack of any THF ether proton and the corresponding carbon signals in the NMR spectra indicated the absence of a THF ring along the aliphatic chain. However, the existence of an isolated double bond was discerned in the ^1H NMR spectrum of **1a** + **1b** by a two-proton multiple resonance around δ 5.35–5.41 and confirmed by two carbon resonances at δ 128.9 and 131.5 in the ^{13}C NMR spectrum. The chemical shifts observed for the methylene carbons allylic to the double bond at δ 23.3 and 27.3 were consistent with a cis geometry of the double bond.[10] A vicinal diol moiety was also evidenced by a ^1H NMR signal at δ 3.42 for two carbinol methine protons, and corresponding signals in the ^{13}C NMR spectra for two oxygenated carbons were observed at δ 74.4 and 74.7. Examination of the ^1H–^1H COSY spectrum revealed that the double bond and vicinal diol moieties were separated by two methylene units (Figure 1).

To determine the position of the vicinal diol and the double bond along the hydrocarbon chain in **1a** + **1b**, an EIMS study was undertaken (Figure 2). The most significant ions resulted from fragmentations at the vicinal diol site. They corresponded to pairs of fragment-ion peaks at *m/z* 323/295 (transannular cleavage) and *m/z* 305/277 (transannular cleavage with loss of H_2O) and to a pair at *m/z* 293/265 (α-cleavage). Fragment-ion peaks shifted by 28 amu and suggested the presence of two isomeric compounds. Taking into account the very few fragmentations observed in the EIMS of these linear acetogenins, a high-energy collision-induced dissociation (CID) of $[M + Li]^+$ mass spectrum was obtained.

The MS/MS spectrum of the $[M + Li]^+$ ion of **1a** + **1b** at *m/z* 583 displayed a number of fragment ion peaks among which two different ion series could be distinguished,

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Table 1. NMR Assignments for Cohibin C (1a) (400 MHz in $CDCl_3$)[a]

position	^1H	^{13}C
1		173.3
2		134.0
3	2.26 t (7.1)	25.3
4	1.55 m	27.2[b]
5–15	1.23–1.32	25.3–29.5
16	1.41 m	33.6
17	3.42 m	74.4[c]
18	3.42 m	74.7[c]
19	1.55 m	33.6
20	2.20 m	23.3
21	5.41 m	128.9
22	5.35 m	131.5
23	2.05 m	27.3[b]
24	1.29 m	25.3–29.5
25–31	1.23–1.32	25.3–29.5
32	1.23–1.32	31.9
33	1.30 m	22.6
34	0.86 t (7.0)	14.3
35	6.98 d (1.5)	148.6
36	4.99 dq (6.8; 1.5)	77.4
37	1.41 d (6.8)	19.3

[a] Chemical shifts (δ) are in ppm relative to TMS. Coupling constants (J) are in Hz. [b,c] Interchangeable signals.

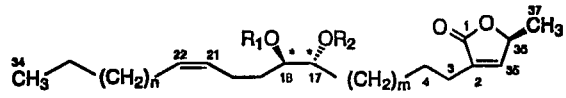


Figure 1. Chemical structures of 1a and 1b.

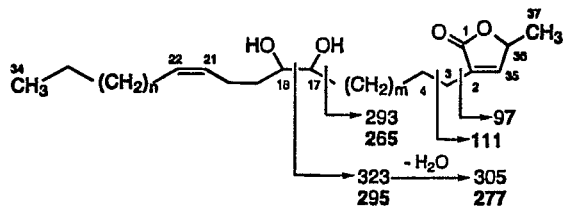


Figure 2. EIMS fragmentations of cohibins C (1a) (m = 12, n = 10; numbering) and D (1b) (m = 10, n = 12). Bold type refers to compound 1b only.

depending on whether they possess the terminal lactone ring. The first ion series (A series), containing the lactone moiety, was formed by remote charge fragmentation[11] of the whole aliphatic chain leading to the typical pattern of successive ion peaks separated by 14 amu (Figure 3). This ion series was interrupted at substitution or unsaturation sites, thus allowing their location on the alkyl chain. Fragment ion peaks at *m/z* 413 and 373 were indicative of a double bond at C-21/C-22 or C-20/C-21. Furthermore, the loss of H_2O from the fragment ions at *m/z* 373 accounted for the location of the hydroxyl groups between the double bond and the lactone ring. Their vicinal C-17 and C-18 positions were established by the presence of three ion peaks separated by 30 amu (*m/z* 299, 329, and 359). Because of the presence of two methylene units between the diol and the double bond, the unsaturation was then placed at position C-21/C-22. A close study of the A series showed another ion series of weak intensity, shifted by 28 amu and connected with the main fragment peaks series. These fragment-ion peaks at *m/z* 345 and *m/z* 385 for the double bond C-19/C-20 and *m/z* 271, 301, and 331 for the

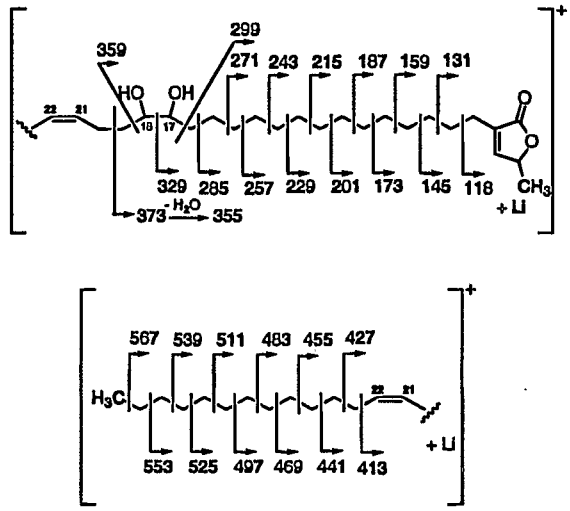


Figure 3. MS/MS fragmentations (*m/z*) of cohibin C (1a), corresponding to the A series.

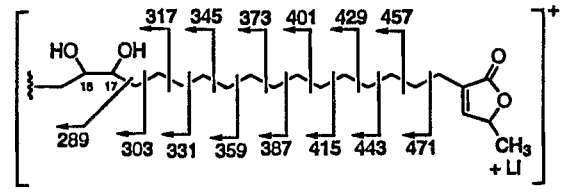


Figure 4. MS/MS fragmentations (*m/z*) of cohibin C (1a), corresponding to the B series.

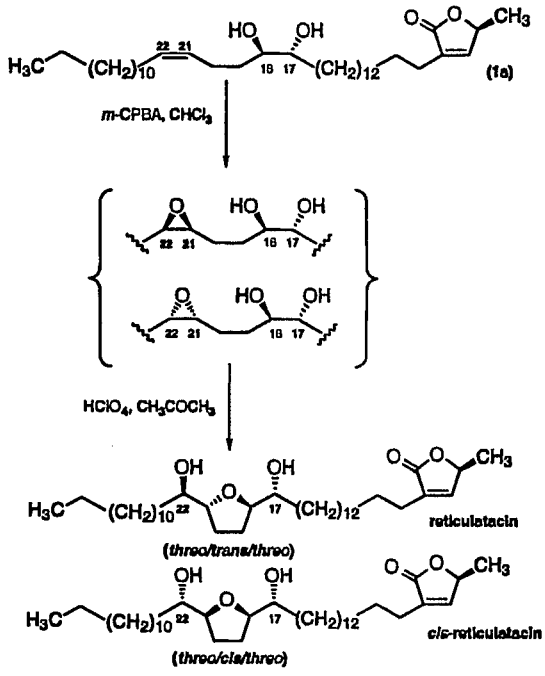
diol system C-15/C-16 were assigned to the minor isomer cohibin D (1b). No other modifications were observed below *m/z* 271.

The second ion series (B series) corresponded to ions containing the methyl-terminal side chain of the lithiated molecule. The sequential remote charge fragmentations of the aliphatic chain between the lactone and the diol led to successive ion peaks in the range *m/z* 471–289 (1a) and *m/z* 471–317 (1b) (Figure 4).

To confirm the double-bond location related to the diol motif, a chemical degradative process followed by GC analysis was used. Oxidative degradation of the mixture of compounds 1a and 1b was performed by treatment with $KMnO_4/NaIO_4$.[12] The double bonds of the aliphatic chain of 1a and 1b were cleaved, affording the corresponding carboxylic acids. The crude mixture of the oxidative reaction was analyzed by GC after trimethylsilylation. A major fragment was characterized as tridecanoic acid, by comparison with an authentic sample, and a minor carboxylic acid, pentadecanoic acid, was also detected. This finding is in full agreement with the presence of two compounds: the major one, cohibin C (1a), with the vicinal diol located at the C-17/C-18 positions and the double bond at the C-21/C-22 positions; and the minor one, cohibin D (1b), with the vicinal diol at the C-15/C-16 positions and the double bond at the C-19/C-20 positions along the hydrocarbon chain.

The relative configuration of the vicinal hydroxyl groups was suggested as threo by comparing the ^1H NMR signal at δ 3.42 with those of other vicinal diol-containing acetogenins.[13] To confirm this configuration, an acetonide derivative (2) was prepared.[14] The CIMS (CH_4) of 2 showed a protonated peak at *m/z* 617 $[MH]^+$. The ^1H NMR spectrum displayed a single peak at δ 1.37 (6H) for the

Scheme 1. Oxidation of Cohibin C (1a) Leading to Corresponding Acetogenins via Two Diastereomeric Epoxides[a]



[a] Absolute configurations of vicinal diols, epoxides, and THF rings may be inverted.

acetonyl methyl groups, while an overlapping multiplet at δ 3.59 was consistent with trans stereochemistry for the ring. Therefore, the configuration of the diol was determined to be threo, since the trans configuration in **2** could only be derived from a vicinal diol with a threo configuration. The absolute configuration at C-36 of the components of the mixture was established by an enzymatic method developed by our laboratory,[15] indicating that the two acetogenins **1a** + **1b** have the same 36*S* configuration.

To confirm the existence of two isomers, a biomimetic semisynthesis of corresponding acetogenins was performed by epoxidation of **1a** + **1b** with *m*-chloroperbenzoic acid[16] (*m*-CPBA) followed by treatment with perchloric acid ($HClO_4$).[17] This procedure led to a mixture of four mono-THF acetogenins (**3**) (Scheme 1 for **1a**). The ^1H NMR and ^{13}C NMR spectra of **3** corresponded to mono-THF acetogenin stereoisomers.[18] The EIMS data were in agreement with those of *cis*-reticulatacin (mono-THF pattern between C-17/C-22) and *cis*-uvariamicin-I (mono-THF pattern between C-15/C-20), reported in an earlier study from the roots of *A. muricata*.[19]

These results are in agreement with the hypothetical biogenetic pathway of the mono-THF acetogenins, which presumably involves the epoxidation of *cis*-dienes followed by the ring opening of the epoxide moieties and ring closure to form the THF-ring flanking hydroxyls.[10] On the basis of this biogenetic proposal, cohibin C (**1a**) must be derived from a $\Delta^{17,21}$ diunsaturated precursor, named muridienin-4, which was reported in an earlier study[4] (Scheme 2). An oxidation step would lead to the unsaturated epoxide, epomusenin A, isolated from the seeds of *Rollinia mucosa*[20] and *A. muricata* (Gleye, C; Ph D Thesis, Université Paris XI, France; unpublished results, 1998). A ring opening of the epoxide group by attack of H_2O could lead to the vicinal diol of cohibin C. The threo configuration between the vicinal diol group of cohibin C is in agreement with the cis configuration of the epoxide in epomusenin A and supports this biogenetic proposition. In this way, we can postulate that acid–base rearrangement occurs with inversion of configuration due to its SN_2 mechanism.[2] An oxidation of the double bond could lead to a hypothetical syn or anti epoxide–diol because the oxidation can occur on both sides of the double bond. The syn epoxide–diol could rearrange to the dihydroxylated *threo/trans/threo*-reticulatacin previously described,[21] and the anti epoxide–diol would lead to the diastereoisomer, *cis*-reticulatacin.[19] Similarly, cohibin D (**1b**), involving in this case muridienin 2 as isolated from roots[5] and seeds of *A. muricata* (Gleye, C; Ph D Thesis, Université Paris XI, France; unpublished results, 1998), could lead to uvariamicin I[22] (threo/trans/threo) and the diastereoisomer *cis*-uvariamicin I.[19]

Consequently, cohibins C and D (**1a** + **1b**) seem to emerge as possible links in the biogenetic pathway of mono-THF acetogenins. Cohibins C and D (**1a** + **1b**) are homologues of cohibins A and B[7] and are a rare example of acetogenins with a double bond–vicinal diol system.[23–25]

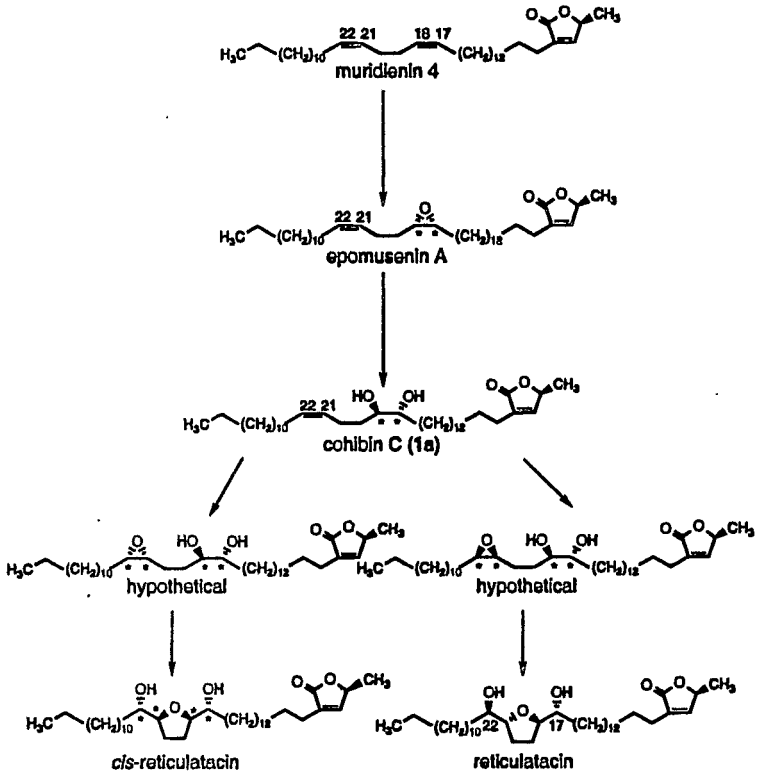
Experimental Section

General Experimental Procedures. UV spectra were determined in MeOH on a Philips PU 8720 spectrophotometer. IR spectra were recorded on a Perkin-Elmer 257 spectrophotometer. The ^1H NMR spectra were obtained with Bruker AC-400 (at 400 MHz) and AC-200 (at 200 MHz) NMR spectrometer. The ^{13}C NMR spectra were obtained with the Bruker AC-200 instrument at 50 MHz. EIMS (40 eV) and CIMS (CH_4) were registered with a Nermag spectrometer R10-10C. MS/MS were obtained using a ZabSpec-T five-sector tandem mass spectrometer (Micromass, Manchester, UK). The first analyzer (MS1) comprised a ZabSpec triple-sector ($E_1B_1E_2$) instrument, and the second mass spectrometer (MS2) consisted of a double-sector instrument (B_2E_3) of reverse Mattauch-Herzog geometry focusing the ion beam on a focal plane. Precursor ions $[M + Li]^+$ were generated by cesium ion bombardment at 30 keV (matrix: *m*-nitrobenzyl alcohol + LiCl). HPLC separations were performed with a Waters 590 pump connected with a SSV Waters injector, a Waters 84 UV detector (detection at 214 nm), and a Waters semipreparative μBondapak C_{18} column (10 μm, 250 × 20 mm). GC was carried out with a Hewlett-Packard 5890 chromatograph, injector at 300 °C, FID detector at 350 °C, and a HT-5 capillary column (5% phenyl polycarborane–siloxane phase, length 25 m, i.d. 0.22 mm, film thickness 0.1 μm, SGE International, Ringwood, Victoria 3134, Australia) with N_2 as the carrier gas. The average linear gas velocity was 1.4 mL/min at 200 °C. The temperature program was 100 °C, increasing 5 °C/min to 270 °C. TLC was performed on precoated 0.25-mm thick Merck plates of Si gel 60 F_{254}. Column chromatography was carried out on Si gel 60 Merck and Si gel 60 H Merck. Exclusion chromatography was run using Sephadex LH-20 Pharmacia.

Plant Material. Seeds of *A. muricata* were collected in September 1994, in Paraiba, Brazil. A voucher specimen is deposited at the herbarium of the Laboratorio de Tecnologia Farmaceutica, Universidade Federal da Paraiba, Paraiba, Brazil (JPB 18342). The root bark of *A. nutans* was collected in Piribebuy, Paraguay, in October 1995. A voucher specimen is deposited at the herbarium of the Faculty of Chemical Sciences, Universidad National de Asuncion, Asuncion, Paraguay (AF 929).

Extraction and Isolation. Air-dried and powdered seeds of *A. muricata* (10 kg) were extracted with MeOH. The MeOH extract was partitioned between H_2O and C_6H_{14} to yield 30 g of C_6H_{14}-soluble material. This extract was subjected to Si gel 60 column chromatography and eluted with C_6H_{14}/EtOAc (10:0 to 0:10) to yield 250 fractions of 250 mL each. Based on TLC similarities, identical fractions were combined to give a total of 16 fractions. Fraction 10 (2.8 g) was subjected successively to exclusion chromatography, eluted with CH_2Cl_2/MeOH (2:1)

Scheme 2. Biogenetic Pathway Proposed for *cis*-Reticulatacin and Reticulatacin[a]



[a] Absolute configurations may be inverted.

and Si gel 60 H column chromatography, eluted with C_6H_{14}/CH_2Cl_2/MeOH (90:40:5). A final purification using reversed-phase semipreparative HPLC procedure at a flow rate of 9 mL/min and the eluent CH_3OH/H_2O (95:5) led to 10 mg of a mixture of cohibins A and B[7] (t_R, 22.4 min) and 14 mg of a mixture of cohibins C and D (**1a** + **1b**) (t_R, 28.8 min).

The dried bark of *A. nutans* (400 g) was extracted with MeOH. Of this MeOH extract 37 g were partitioned between H_2O and C_6H_{12}, to yield 3.44 g of a C_6H_{12} extract. This extract was submitted to Si gel 60 column chromatography eluting with C_6H_{12}/CH_2Cl_2 (10:0 to 0:10) and CH_2Cl_2/EtOAc (10:0 to 0:10) gradients, which yielded 40 fractions of 50 mL each. Combined fractions 27–30 (305 mg) further were separated by exclusion chromatography eluting with CH_2Cl_2/MeOH (2:1) and Si gel 60 H column chromatography, eluted with CH_2Cl_2/EtOAc (8:2). Purification by semipreparative HPLC, at a flow rate of 10 mL/min and eluent CH_3OH/H_2O (94:6), led to 3.7 mg of a mixture of cohibins C and D (**1a** + **1b**) (t_R, 19.8 min). Combined fractions 31–33 (111 mg) were successively subjected to Si gel 60 H column chromatography, eluted with CH_2Cl_2/EtOAc (85:15) and reversed-phase semipreparative HPLC (Waters), with a flow rate of 10 mL/min and the eluent CH_3OH-H_2O (90:10). This process led to the isolation of 4.5 mg of a mixture of cohibins A and B[7] (t_R, 22.0 min).

Cohibins C and D (1a + 1b): white waxy solid (17.7 mg); UV (MeOH) λ_{max} (log ϵ) 213.6 (3.62) nm; IR ν_{max} (KBr) 3304, 2916, 2845, 1741, 1653, 1469, 1119, 1072, 1030 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 50 MHz), see Table 1; CIMS (CH_4) *m/z* 577 $[MH]^+$ (100%), 559 $[MH - H_2O]^+$, 541 $[MH - 2\ H_2O]^+$, 323, 305, 295, 293, 277, 265, 111, 97; EIMS (40 eV) *m/z* 323, 305, 295, 293, 277, 265, 111, 97 (see Figure 2); MS/MS of the *m/z* 583,6 $[M + Li]^+$ ion: *m/z* 567, 553, 539, 525, 511, 497, 483, 469, 455, 441, 427, 413, 399, 385, 373, 359, 355, 345, 331, 329, 327, 301, 299, 285, 271, 257, 243, 229, 215, 201, 187, 173, 159, 145, 131, 118 (see Figures 3 and 4).

Oxidative Degradation. A solution of 13 mg of $NaIO_4$ in 0.2 mL of water was treated with 0.2 mg of $KMnO_4$, stirred 30 min at 25 °C, and treated with 1 mg of anhydrous K_2CO_3 and 2 mg of the mixture **1a** + **1b**. The resulting reddish-purple suspension was stirred for 2 h at 25 °C, acidified to pH 4 with HCl, stirred again for 2 h, and extracted with EtOAc. The combined organic extracts were washed with brine and dried. The solvent was removed in vacuo to give 1 mg of residue. The crude mixture, after silylation (Sylon HTP, Supelco) was analyzed by GC. Two different peaks with retention times of 6.16 and 9.54 min were observed and identified, in turn, by comparison with authentic samples, as tridecanoic and pentadecanoic acids, respectively.

Cohibin Acetonide (2). The mixture **1a** + **1b** (5 mg) was dissolved in 200 μL of C_6H_6. To this solution were added 2,2-dimethoxypropane (10 μL) and a trace of *p*-toluenesulfonic acid. The mixture was stirred under reflux for 3 h, then a trace of K_2CO_3 was added and the mixture stirred for 3 h at room temperature. Extraction with CH_2Cl_2 gave **2** (3.8 mg): ^1H NMR ($CDCl_3$, 200 MHz) (numbering for acetogenin from cohibin C, **1a**) δ 0.88 (3H, t, J = 6.8 Hz, H-34), 1.37 (6H, s, acetonide), 1.41 (3H, d, J = 6.8 Hz, H-37), 2.26 (2H, t, J = 7.1 Hz, H-3), 3.59 (2H, m, H-17, H-18), 4.98 (1H, dq, J = 1.5, 6.8 Hz, H-36), 5.37–5.41 (2H, m, H-21, H-22), 6.98 (1H, d, J = 1.5 Hz, H-35); CIMS (CH_4) *m/z* 617 $[MH]^+$.

Semisynthesis of Mono-THF Acetogenin (3). To 4 mg of **1a** + **1b** dissolved in 0.5 mL of $CHCl_3$ was added 1 equivalent of *m*-CPBA. The mixture was stirred for 3 h at room temperature, washed with 1% $NaHCO_3$, extracted with CH_2Cl_2, and evaporated in vacuo. The residue was dissolved in acetone (0.5 mL), treated with 70% perchloric acid (10 mL), stirred 8 h at room temperature, and then evaporated to dryness. Purification by reversed-phase semipreparative HPLC at a flow rate of 9 mL/mn with the eluent CH_3OH/H_2O (95:5), gave **3** (3.1 mg): ^1H NMR ($CDCl_3$, 400 MHz) (numbering for acetogenin from cohibin C, **1a**) δ 0.88 (3H, t, J = 6.7 Hz, H-34), 1.25–1.34 (42H, m, H-5 to H-15, H-24 to H-33), 1.40–1.48 (4H, m, H-16, H-23), 1.41 (3H, d, J = 6.8 Hz, H-37), 1.54 (2H, m, H-4), 1.66–1.78 (2H, m, H-19$_a$, H-20$_a$), 1.92–2.01 (2H, m,

H-19$_b$, H-20$_b$), 2.26 (2H, t, J = 7.5 Hz, H-3), 3.41–3.42 (2H, m, H-17, H-22), 3.78–3.82 (2H, m, H-18, H-21), 4.98 (1H, dq, J = 1.7, 6.8 Hz, H-36), 6.98 (1H, d, J = 1.7 Hz, H-35); ^{13}C NMR ($CDCl_3$, 50 MHz) (numbering for acetogenin from cohibin C, **1a**) δ 14.0 (C-34), 19.3 (C-37), 22.0 (C-33), 25.0 (C-3), 25.2–29.9 (C-5 to C-15, C-24 to C-31), 27.3 (C-4), 28.1 and 28.8 (C-19, C-20), 31.9 (C-32), 33.4 and 34.0 (C-16, C-23), 74.0 and 74.3 (C-17, C-22), 77.5 (C-36), 82.7 (C-18, C-21), 134.0 (C-2), 148.7 (C-35), 173.2 (C-1); CIMS (CH_4) m/z 593 $[MH]^+$ (100%), 575 $[MH - H_2O]^+$, 557 $[MH - 2\ H_2O]^+$, 347, 323, 295, 269, 227, 199, 111, 97; EIMS (40 eV) m/z 323, 295, 227, 199, 181, 111, 97.

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