Isolation of Montecristin, a Key Metabolite in Biogenesis of Acetogenins from *Annona muricata* and Its Structure Elucidation by Using Tandem Mass Spectrometry†

C. Gleye, A. Laurens,* R. Hocquemiller, and A. Cavé

Laboratoire de Pharmacognosie, URA 1843 CNRS (BIOCIS), Faculté de Pharmacie, Université Paris XI, 92296 Châtenay-Malabry cedex, France

O. Laprévote and L. Serani

Laboratoire de Spectrométrie de Masse, Institut de Chimie des Substances Naturelles, CNRS, 91198 Gif-sur-Yvette

Received May 15, 1996 (Revised Manuscript Received November 19, 1996®)

During the course of our continuing search for acetogenins from Annonaceae, a new metabolite, montecristin, possibly involved in the biogenesis of acetogenins, was isolated from the roots of *Annona muricata*. Its structure was elucidated on the basis of UV, IR, ^{1}H and ^{13}C NMR, and mass spectrometry. The identification of the main stuctural features of montecristin (**1**) was obtained from the NMR spectra whereas their locations on the alkyl chain were evidenced by using mass spectrometry. The attribution of each carbon and location of substituents on the alkyl chain of this fatty acid γ-lactone was evidenced by using tandem mass spectrometry (MS/MS) and high-energy collisional activation of $[M + Li]^+$ lithium complexes. Finally, the structure determination of montecristin was strengthened by epoxidation and transformation leading to a known adjacent bis-tetrahydrofuran acetogenin.

Introduction

Annona muricata L. (Annonaceae) is a small tropical tree whose edible fruits are used commercially for the production of juice, candy, and sherbets.[1] Intensive chemical investigation of the seeds and leaves of *Annona muricata* led to the isolation of acetogenins which display some interesting biological activities such as cytotoxic, antitumor, antiparasitic, and pesticidal properties.[2] The general structure of these acetogenins is characterized by a long aliphatic chain bearing a terminal methyl-substituted unsatured γ-lactone and one or two tetrahydrofuran units sometimes replaced by epoxy rings and (or) double bonds.[3]

Roots of this plant, used in traditional medicine for their antiparasitic and pesticidal properties,[1] have been studied only for their alkaloidal content.[4] Since acetogenins are known to possess various biological activities, it occurred to us that their presence in the roots contribute to the medicinal properties. We investigated the acetogenin contents of the roots. Besides the known acetogenins annonacin,[5] and the epomuricenins-A and -B,[6] previously isolated from the seeds, a new compound, montecristin (**1**), was obtained. This compound was devoid of any tetrahydrofuran or epoxy ring, but contained two double bonds and a vicinal diol system. The localization of the two double bonds and the two hydroxyls on the alkyl chain was facilitated by the use of tandem mass spectrometry (MS/MS) and high-energy collisional activation of $[M + Li]^+$ complexes.

Results and Discussion

The dried and powdered roots were extracted with methanol. The MeOH extract, after concentration under vacuum, was partitioned between water and chloroform. The organic layer was dried and submitted to successive fractionations by column chromatography. Preparative HPLC led to the isolation of montecristin (**1**), a white wax, mp 62−65 °C, $[\alpha]_D$ +25° (c = 0.1; MeOH).

The molecular weight of montecristin (574) was determined on the basis of CIMS which exhibited a protonated molecule $[M + H]^+$ at m/z 575 corresponding to a molecular formula $C_{37}H_{66}O_4$ HRCIMS (obsd 575.5058 (MH^+), calcd 575.5039).

IR and UV spectra of **1** along with a positive Kedde's reaction[7] revealed the presence of an α,β-unsatured γ-lactone, and this was confirmed by ^{1}H NMR spectroscopy[8] (Table 1). In addition, the ^{1}H and ^{13}C NMR spectra revealed, by the absence of their characteristic chemical shifts, that montecristin does not possess any THF ring.[3]

A two-proton multiplet at δ 3.42 was consistent with the overlapping methine resonances of a vicinal diol system, and this was supported by the presence of two methine carbon resonances at δ 73.93 and 74.35. The presence of two isolated double bonds was evidenced by multiple resonances due to four protons at δ 5.37−5.41 and by four carbon peaks at δ 128.78, 129.20, 130.04, 130.43. The selective irradiation at 400 MHz of the α

† Part 48 in the series "Acetogenins from Annonaceae". For part 47 see Franck, X.; Figadère, B.; Cavé, A. *Tetrahedron Lett.* **1996**, *37*, 1593−1594.

® Abstract published in *Advance ACS Abstracts,* January 1, 1997.

(1) Kerharo, J. *La pharmacopée sénégalaise traditionelle*; Vigot frères: Paris, 1974; pp 144−146.
(2) Fang, X. P.; Rieser, M. J.; Gu, Z. H.; Zhao, G. X.; McLaughlin, J. L. *Phytochem. Anal.* **1993**, *4*, 27−48.
(3) Cavé, A.; Figadère, B.; Laurens, A.; Cortes, D. In *Progress in the Chemistry of Organic Natural Products*; Hertz, W., Ed.; Springer Verlag: New York, 1996; Vol. 70, pp 81−288.
(4) Leboeuf, M.; Legueut, C.; Cavé, A.; Desconclois, J. F.; Forgacs, P.; Jacquemin H. *Planta Med.* **1981**, *42*, 37−44.
(5) Rieser, M. J.; Fang, X. P.; Rupprecht, J. K.; Hui, Y. H.; Smith, D. L.; McLaughlin, J. L. *Planta Med.* **1993**, *59*, 91−92.
(6) Roblot, F.; Laugel, T.; Leboeuf, M.; Cavé, A.; Laprévote, O. *Phytochemistry* **1993**, *34*, 281−285.
(7) Kirchner, J. G. In *Technique of organic chemistry;* Perry, E. S., Weissberger, A., Eds.; Interscience: New York, 1967; Vol. 12, pp 147−186.
(8) Schmitz, F. J.; Kraus, K. W.; Ciereszko, L. S.; Sifford, D. H.; Weinheimer, A. J. *Tetrahedron Lett.* **1966**, *1*, 97−103.

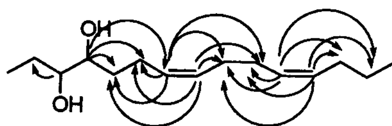
S0022-3263(96)00901-2 CCC: $14.00 © 1997 American Chemical Society

Table 1. NMR Assignments of 1 ($CDCl_3$, δ)

atom no.	^{1}H NMR	^{13}C NMR
1	–	176.83
2	–	134.30
3	2.26 t	25.10
4	1.55 m	27.33
5/11–25/31	1.19–1.36	22.52–29.19
12	1.49 m	33.53
13	3.42 m	73.93^{a}
14	3.42 m	74.35^{a}
15	1.55 m	33.35
16	2.20 m	23.40
17	5.41 m	128.78^{b}
18	5.37 m	130.43^{c}
19	2.11 m	27.23
20	2.11 m	27.23
21	5.41 m	129.20^{b}
22	5.37 m	130.04^{c}
23	2.03 m	27.23
24	1.29 m	29.60
32	1.26 m	31.88
33	1.20 m	22.66
34	0.87 t	13.95
35	6.98 d	148.72
36	5.00 dq	77.48
37	1.41 d	19.04

$^{a-c}$ Interchangeables. J_{3-4} = 7.16 Hz; J_{33-34} = 7.03 Hz; J_{35-36} = 1.76 Hz; J_{36-37} = 6.80 Hz.

Scheme 1 ^{1}H–^{1}H Magnetization Transfers in the HOHAHA NMR Spectrum of 1



methylene at δ 2.20 afforded a doublet for one of the olefinic protons, with the resulting coupling constant of 10.99 Hz. Similarly, irradiation of the α methylene at 2.03 afforded a doublet for the other olefinic protons with a coupling constant of 11.17 Hz. These coupling constants were consistent with a *Z* geometry for both double bonds.[9] This stereochemistry is in agreement with the ^{13}C NMR chemical shifts of the α methylene carbons at δ 23.40 and 27.23.[10]

The presence of two adjacent methylene groups between the diol system and a double bond was evidenced by COSY ^{1}H–^{1}H correlations. The HOHAHA correlation spectrum of **1** confirmed the presence of two methylene groups between the diol system and the nearest double bond. Further, the resonance of two methylene groups at δ 2.11 integrated for 4 protons and correlated only with the olefinic protons. The absence of a correlation between these methylene groups and other highfield methylene groups indicated that they are between the double bonds. In total these results are in agreement with the partial structure (Scheme 1) but do not allow the location of the diol system in relation to the lactone ring or the terminal methyl. This structural problem was solved by mass spectrometric methods.

The liquid secondary ion mass spectrum (LSIMS) obtained by fast cesium ion bombardment, using a *m*-nitrobenzyl alcohol matrix laced with lithium chloride, displayed a very intense [M + Li]$^{+}$ ion peak at *m/z* 581. The lack of any structurally significant fragment ion

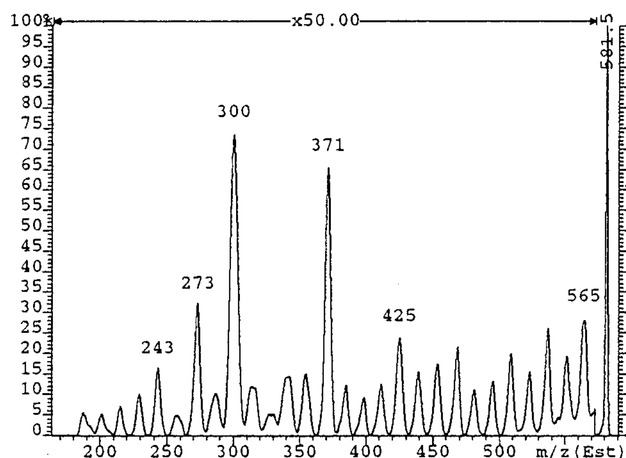


Figure 1. CID-MIKE spectrum of the [M + Li]$^{+}$ ion at *m/z* 581 from montecristin (**1**).

peaks in the mass spectrum made it necessary to use high-energy collision-induced dissociation (CID).

The mass-analyzed ion kinetic energy (MIKE) spectrum of the precursor [M + Li]$^{+}$ ion was recorded by using the first mass spectrometer (MS1) of a five-sector tandem instrument. The $E_1B_1E_2$ geometry of the MS1 (B and E being magnetic and electrostatic analyzers, respectively) allows the selection of the chosen precursor ion by the first two sectors (E_1B) and the collisional activation against helium in a collision cell located in the third field-free region. The fragmentations arising from the CID processes were monitored by scanning the second electrostatic analyzer (E_2).

The CID-MIKE spectrum of the [M + Li]$^{+}$ ion displayed a series of product ions characteristic of remote-charge type fragmentations[11] (Figure 1). Among them, two ions at *m/z* 425 and 371, could be attributed to allylic cleavages on both sides of a double bond, as previously described by Gross *et al.*[12,13] Two other peaks at *m/z* 243 and 273 indicate the presence of a hydroxy group between the two corresponding cleavage sites, but the poor resolution inherent in this technique did not allow an unambiguous peak assignment, thus justifying MS/MS experiments.

The CID-MS/MS spectrum of the [M + Li]$^{+}$ ion at *m/z* 581 was obtained by selecting the precursor ion with the first mass spectrometer ($E_1B_1E_2$), followed by collision-induced dissociation in the fourth field-free region, and scanning the second mass spectrometer (B_2E_3 geometry) at unit mass resolution. A high-sensitivity multichannel array detector was used in this experiment.

The MS/MS spectrum displayed a number of fragment ion peaks among which two different ion series can be distinguished in the spectrum, depending on whether or not they possess the terminal lactone ring. The first ion series (series A), containing the lactone moiety, was formed by remote charge fragmentations of the entire aliphatic chain leading to the typical pattern of successive ion peaks separated by 14 mass units. This ion series was interrupted at the substitution or unsaturation sites, thus allowing their location on the alkyl chain (Scheme 2). Fragment ion peaks at *m/z* 411, 385, 371, 357, 331,

(9) Gaudemer, A. In *Stereochemistry*; Kagan, H. B., Ed.; Thieme: Stuggart, 1977; Vol. 1, pp 44–136.
(10) Gu, Z. H.; Zhao, G. X.; Oberlies, N. H.; Zeng, L.; McLaughlin J. L. In *Phytochemistry of Medicinal Plants*; Amason, J. T., Ed.; Plenum Press: New York, 1995; Vol. 29, pp 249–310.
(11) Adams, J.; Gross, M. L. *Anal. Chem.* **1987**, *59*, 1576–1582.
(12) Tomer K. B.; Jensen, N. J.; Gross, M. L. *Anal. Chem.* **1986**, *58*, 2429–2433.
(13) Jensen, N. J.; Tomer, K. B.; Gross, M. L. *J. Am. Chem. Soc.* **1985**, *107*, 1863–1868.

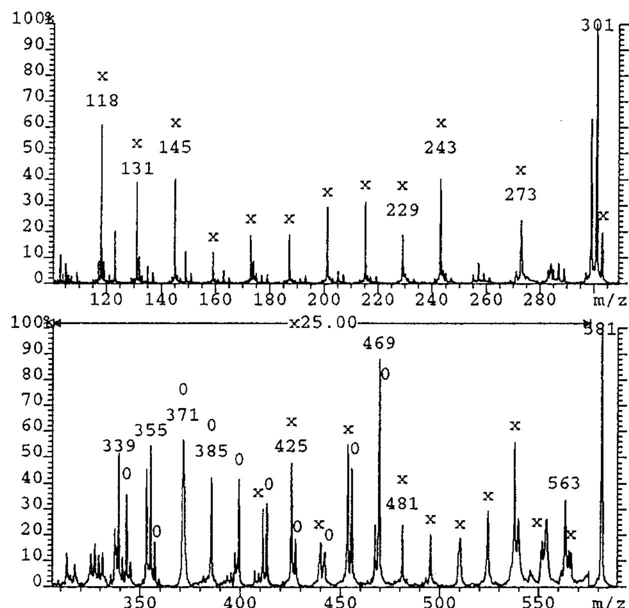
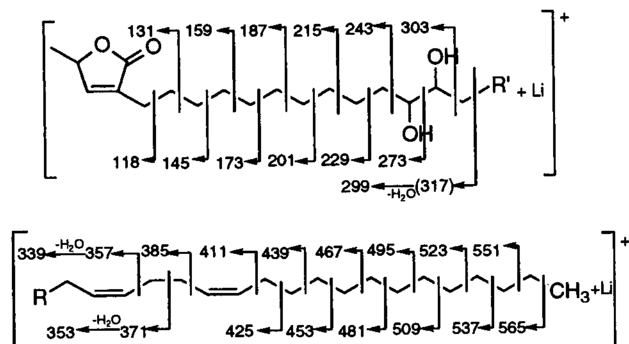


Figure 2. CID-MS/MS spectrum of the $[M + Li]^+$ ion at m/z 581 from montecristin (**1**). Fragment ion peaks belonging to the A and B series (see text) are labeled by x and o, respectively.

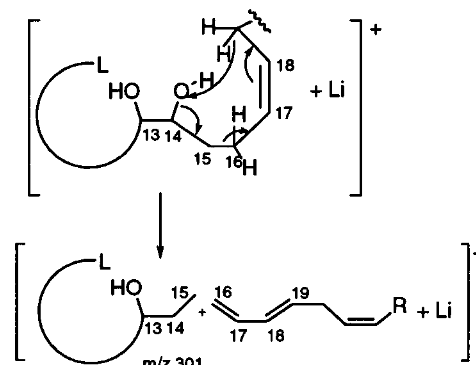
Scheme 2. MS/MS Fragmentations of 1 corresponding to A Series



and 317 were indicative of two double bonds (C21−C22 and C17−C18), in full agreement with the NMR data. Furthermore, the loss of a water molecule from the fragment ions at m/z 371, 357, and 317 accounted for the location of the hydroxy groups between the diene system and the lactone ring. Their vicinal C13 and C14 positions were established by the presence of three ion peaks separated by thirty mass units (m/z 303, 273, 243) (Figure 2). It is also noteworthy that the low mass end of the A series consisted of a radical ion at m/z 118, formed by a cleavage β to the lithiated lactone. The presence of a fragment ion at m/z 301 could be rationalized by the loss of a water molecule from the C14 hydroxyl group, accompanied by the formation of a neutral conjugated diene species according to Scheme 3.

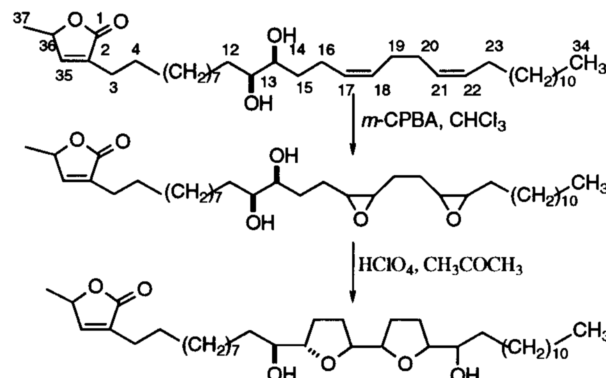
The B series corresponded to ions containing the methyl-terminal side chain of the lithiated molecule. The loss of the lactone ring occurred by a β-cleavage, with loss of 112 daltons from the precursor $[M + Li]^+$ ion (fragment-ion peak at m/z 469).[14] The sequential remote charge site fragmentations of the aliphatic chain between the lactone and the diol led to a series of ion peaks in

Scheme 3. MS/MS Fragmentations Induced by the Charge of lithiated OH



Scheme 4. MS/MS Fragmentations of 1 Corresponding to B Series

Scheme 5. Oxidation of 1 Leading to Corresponding Acetogenins *via* a Mixture of Diastereomeric Epoxides



the range m/z 469−343. The presence of a significant ion peak at m/z 313, albeit weak, confirmed the location of hydroxy groups at positions 13 and 14 (Scheme 4).

In order to determine the relative configuration of the vicinal OH groups, the acetonide derivative **2** was prepared.[15] The ^{1}H NMR spectrum of the latter (**2**) displayed a single peak at δ 1.38 (6H) for the acetonyl methyl groups while an overlapping multiplet at δ 3.58 fixed a *trans* stereochemistry for the ring. These observations allowed the assignment of a *threo* configuration of the vicinal diol moiety.

In order to further confirm the diene location related to the diol motif, epoxidation of **1** with *m*-chloroperbenzoic acid[16] followed by a treatment with $HClO_4$ were performed. It led to a mixture of bis-tetrahydrofuran acetogenins stereoisomers **3** (Scheme 5). The ^{1}H NMR spectrum of **3** corresponded to a bis-tetrahydrofuran acetogenin. Diagnostic peaks in the electron ionization mass spectrum[17] and constant-B/E linked scanning of the $[M + Li]^+$ ion, formed by fast-atom bombardment,[18]

(14) Laprévote, O.; Girard, C.; Das, B. C.; Laugel, T.; Roblot, F.; Leboeuf, M.; Cavé A. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 352−355.

(15) Sahpaz, S.; Laurens, A.; Hocquemiller, R.; Cavé, A.; Cortes, D. *Can. J. Chem.* **1994**, *72*, 1533−1536.

(16) Tam, V. T.; Pham, Q. C. H.; Chappe, B.; Roblot, F.; Laprévote, O.; Figadère, B.; Cavé, A. *Nat. Prod. Lett.* **1994**, *4*, 255−262.

corresponded to the known neoannonin B.[19] The MS experiments confirm the position of the bis-tetrahydrofuran pattern between 13 and 22. Therefore, this is in complete accord with a diene motif in one position between 13 and 22, and this further confirms the biogenesis of bis-tetrahydrofuran acetogenins.

Conclusion

Montecristin is the first C_{37} acetogenin isolated from *Annona muricata*. It is the second example of acetogenin containing two double bonds in Annonaceae.[20] The complete identification of this structure used the classical analytical methods but the unambigous location of the double bonds on the chain in relation to diol and γ-lactone required the use of MS/MS method which proved to be very efficient. The proposed structure of montecristin is supported by the currently accepted biogenetic pathway of bis-tetrahydrofuran acetogenins.[21] Montecristin thus emerges as a key intermediate of acetogenins.

Experimental Section

The ^{1}H NMR spectra were recorded at 200 and 400 MHz. The ^{13}C NMR spectra were obtained at 50 MHz. EIMS (48 eV) and CIMS (CH_4) were registered with a Nermag spectrometer. FABMS and the constant-B/E linked scanning experiment were performed with an MS-80 double focusing mass spectrometer under the control of a DS 90 data system (xenon atom energy: 7 keV, matrix: *m*-nitrobenzyl alcohol + LiCl, collision gas: argon, collision energy: 4 keV). CID-MIKE and MS/MS spectra were obtained using a ZabSpec-T five-sector tandem mass spectrometer (Fisons Instruments, VG organic, Manchester, UK). The first analyzer (MS1) comprises a Zabspec triple sector ($E_1B_1E_2$) instrument, and the second mass spectrometer (MS2) consists of a double sector instrument (B_2E_3) of reverse Mattauch−Herzog geometry focusing the ion beam on a focal plane. $[M + Li]^+$ precursor ions were generated by cesium ion bombardment at 30 keV (Matrix: *m*-NBA + LiCl). The MIKE experiments were performed by setting E_1 and B_1 at fixed values corresponding to the chosen precursor ion accelerated at 8 keV. The fragmentations of the selected precursor ion occurring in the third field-free region were recorded by scanning E_2. The precursor ions submitted to MS/MS experiments were selected by MS1 set at approriate E and B values and then focused in a collision cell located in the fourth field-free region (between E_2 and B_2). Helium was introduced at a pressure leading to an attenuation of the precursor ion beam of almost 70%. The collision cell was floated at 4 kV so as to attain a collision energy of 4 keV. Fragment ions detection was achieved by use of the MCAD detector operating with a mass ratio of 1.225:1.0 at an angle of 30° with regard to the ion beam.[22−24] For each MS/MS acquisition, the mass scale between the precursor ion peak and the lowest mass end (m/z 50) was covered by successive overlapping exposures of 0.5 s.

Plant Material. Roots of *Annona muricata* (Annonaceae) were collected in Guinea (Conakry) in October 1993. A voucher specimen has been deposited at the faculty of medicine and pharmacy of Conakry.

Extraction and Isolation. The dried and powdered roots (600 g) were extracted with MeOH to give a brown extract (60 g). The bioactive MeOH extract (brine-shrimp test positive[3]) was partitioned between H_2O and CH_2Cl_2 to yield 45 g of CH_2Cl_2 extract. This extract was subjected to silica gel column chromatography (silica gel Merck 230−400 mesh) and eluted with hexane containing increasing amounts of EtOAc. The fractions collected were analyzed by TLC (silica gel Merck 60F254), on which basis they were grouped into 17 sets.

The solvent of the fraction number seven was evaporated off. The resulting residue (1.514 g) was subjected to silica gel column chromatography (silica gel Merck 60 H 70−230 mesh) eluted with CH_2Cl_2/EtOAc 10/1.5. Fractions containing **1** as judged from TLC were combined and purified by preparative HPLC procedure using a reversed phase μBondapak C_{18} 10 μm (250 × 20 mm) Waters preparative cartridge column, flow rate 9 mL/min, 20 mg/injection, and eluant CH_3OH/H_2O (92/8). A 14 mg amount of **1** was obtained.

Montecristin (1): white powder solid (14 mg); mp 62−65 °C; $[\alpha]_D$ +25° (c = 0.1; MeOH); IR (KBr) cm^{-1} 3300, 2900, 2840, 1740, 1650, 1470, 1120, 1080, 1030; UV λ_{max} (MeOH) nm (log ϵ) 211.7 (3.80); HRCIMS (CH_4) m/z 575.5058 (MH^+) calcd 575.5039 for $[C_{37}H_{66}O_4 + H]^+$; MS/MS: (Schemes 2−4); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 50 MHz): (Table 1); HOHAHA correlations: (Scheme 1).

Montecristin Acetonide (2). To 3.8 mg of **1** dissolved in 0.5 mL of C_6H_6 were added 2,2-dimethoxypropane (20 μL) and traces of *p*-toluenesulfonic acid. The mixture was stirred under reflux for 1.5 h. A 0.1 mg amount of K_2CO_3 was added, and the mixture was stirred for 4 h at rt. Extraction with CH_2Cl_2 gave **2** (2 mg): CIMS (CH_4) m/z 615 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 6.97 (1H, d, J = 1.80 Hz, H-35), 5.36 (4H, m, H-17,18,21,22), 5.00 (1H, dq, J = 1.80 and 6.70 Hz, H-36), 3.58 (2H, m, H-13,14), 2.26 (2H, t, J = 7.90 Hz, H-3), 1.42 (3H, d, J = 6.70 Hz, H-37), 1.38 (6H, s, acetonide), 0.87 (3H, t, J = 6.72 Hz, H-34).

Semisynthesis of Adjacent Bis-Tetrahydrofuran Acetogenins 3. Preparation of **3** was carried out according to reference 16. To 4 mg of **1** dissolved in 0.5 mL of $CHCl_3$ were added 7.33 mg of *m*-CPBA. The mixture was stirred for 8 h at rt, 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 μL), stirred 15 h at room temperature, and then evaporated to dryness under vacuum. CIMS (CH_4): m/z 607 $[M + H]^+$; ^{1}H NMR spectrum ($CDCl_3$, 200 MHz) δ 6.98 (1H, d, J = 1.5 Hz, H-35), 5.00 (1H, dq, J = 6.75 Hz, H-36), 3.81−3.88 (4H, m, H-14, 17, 18, 21), 3.39 (2H, m, H-13, 22), 2.26 (2H, t, J = 7.95 Hz, H-3), 1.41 (3H, d, J = 6.75 Hz, H-37), 0.87 (3H, t, J = 6.69 Hz, H-34).

Acknowledgment. This research was sponsored by the Direction de la Recherche et des Etudes Doctorales (DRED), through a biennal contract with the Réseau de Recherche "Pharmacochimie". Financial support from the association pour la recherche contre le cancer (ARC) is also gratefully acknoledged. The authors are grateful to J. Mahuteau and J. C. Jullian for NMR measurements, and T. Becue and S. de Barros (SAMM) for MS spectra.

JO960901J

(17) Cavé, A.; Cortes, D.; Figadère, B.; Hocquemiller, R.; Laprévote, O.; Laurens, A.; Leboeuf, M. In *Phytochemical Potential of Tropical Plants*; Downum, K. R., Romeo, J. T., Stafford, J. E., Eds.; Plenum Press: New York, 1993; Vol. 27, pp 167−202.

(18) Laprévote, O.; Das, B. C. *Tetrahedron* **1994**, *50*, 8479−8490.

(19) Zheng, X. C.; Yang, R. Z.; Quin, G. W.; Xu, R. S.; Fan, D. J. *Acta Bot. Sin.* **1995**, *37*, 238−243.

(20) Meneses Da Silva, E. L.; Roblot, F.; Mahuteau, J.; Cavé, A. *J. Nat. Prod.* **1996**, *59*, 528−530.

(21) Gromek, D.; Figadère, B.; Hocquemiller, R.; Cavé, A.; Cortes, D. *Tetrahedron* **1993**, *49*, 5247−5252.

(22) Ballard, K. D.; Gaskell, S. J.; Jennings, R. K. C.; Scrivens, J. H.; Vickers, R. G. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 553−559.

(23) Scrivens, J. H.; Rollins, K.; Jennings, R. K. C.; Bordoli, R. S.; Bateman, R. H. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 272−277.

(24) Bordoli, R. S.; Bateman, R. H. *Int. J. Mass. Spectrom. Ion Proc.* **1992**, *122*, 243−254.