Supporting Information for

Laevinoids A and B: Two Diterpenoids with an Unprecedented Backbone from *Croton laevigatus*

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Experimental Section

General experimental procedures. Melting points were obtained on a SGM X-4 apparatus (Shanghai Precision & Scientific Instrument Co., Ltd., P.R. China). Optical rotations were measured on a Perkin-Elmer 341 polarimeter at 296 K. IR spectra were recorded on a Perkin-Elmer 577 spectrometer with KBr disks. NMR experiments were performed in $CDCl_3$ on a Varian Mercury-500 spectrometer and were referenced to solvent peaks (δ_H at 7.26 and δ_C at 77.16 ppm). CD data were acquired on a JASCO 810 Spectrophotometer. ESI(±)MS and HRESI(±)MS experiments were carried out on a Esquire 3000plus LCMS and a Waters Q-TOF Ultima Global mass spectrometers, respectively. Semi-preparative HPLC was performed on a Waters 1525 pump equipped with a Waters 2489 detector (210 nm) and a YMC-Pack ODS-A column (250 × 10 mm, S-5 μm, 12 nm). HPLC analyses were carried out on an Agilent 1100 series LC instrument with a DAD detector and a symmetry C_{18} column (3.9 × 150 mm, 5 μm). Pre-coated silica gel GF254 plates (Qingdao Haiyang Chemical Co., Ltd., Qingdao, P. R. China) were used for TLC analyses. D101-macroporous absorption resin (Sinopharm Chemical Reagent Co., Ltd., Shanghai, P. R. China), MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Industries Ltd., Japan), Silica gel H (Qingdao Haiyang Chemical Co., Ltd., Qingdao, P. R. China), C18 reversed-phase silica gel (150–200 mesh, Merck, Germany), and Sephadex LH-20 gel (Amersham Biosciences, Sweden) were used for column chromatography (CC). All solvents used for CC were of at least analytical grade (Shanghai Chemical Reagents Co., Ltd., Shanghai, P. R. China), and solvents used for HPLC were of HPLC grade (J & K Scientific Ltd., Shanghai, P. R. China).

Plant material. The branches and leaves of *Croton laevigatus* Vahl. were collected in October 2010 on Hainan island, P. R. China, and were identified by Prof. S.-M. Huang of Department of Biology, Hainan University. A voucher specimen has been deposited in Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Deposition no.: CL-2010-1Y).

Extraction and isolation. The air-dried powder of the plant materials of *C. laevigatus* (5 Kg) was extracted with 95% EtOH at room temperature to afford a crude extract (230 g) which

was further partitioned between EtOAc (3 × 1.0 L) and water (1.0 L). The EtOAc partition (110 g) was then separated into three fractions (A, B, and C) via D101 macroporous absorption resin eluted with EtOH/H_2O (50%, 80%, and 95%). Fraction B (50.4 g) was subjected to MCI gel column eluted with MeOH/H_2O (50% to 90%) to give five sub-fractions (B1–B5). Fraction B3 was fractionated by silica gel CC eluted with petroleum ether/acetone (15:1 to 1:3) to furnish four fractions (B3a–B3d), the second fraction (B3b) of which was further purified by silica gel CC eluted with CH_3Cl/CH_3OH (200:1 to 10:1) to yield laevinoid A (**1**, 598 mg). Fraction B3c was subjected to silica gel CC (CH_3Cl/CH_3OH, 200:1 to 10:1) to give five fractions (B3c1-B3c5), and laevinoid B (**2**, 18 mg) was recovered from fraction B3c3 via purification on semi-preparative HPLC (3.0 mL/min, 50% CH_3CN/H_2O isocratic elution).

X-ray crystallographic analyses. Laevinoids A (**1**) and B (**2**) were crystallized from petroleum ether/acetone at room temperature. The X-ray crystallographic data of them were obtained on a Bruker SMART CCD detector employing graphite monochromated Cu-Kα radiation (λ = 1.54178 Å) at 140(2) and 143(2) K, respectively (operated in the ϕ-ω scan mode). The structures were solved by direct method using SHELXS-97 (Sheldrick 2008) and refined with full-matrix least-squares calculations on F2 using SHELXL-97 (Sheldrick 2008). All non-hydrogen atoms were refined anisotropically. The hydrogen atom positions were geometrically idealized and allowed to ride on their parent atoms.

Crystallographic data for **1** and **2** (key parameters see Tables S1 and S2, respectively) have been deposited at the Cambridge Crystallographic Data Centre (Deposition Nos.: CCDC 949414 and 949415, respectively). Copies of these data can be obtained free of charge via the internet at www.ccdc.cam.ac.uk/conts/retrieving.html or on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [Tel: (+44) 1223-336-408; Fax: (+44) 1223-336-033; E-mail: deposit@ccdc.cam.ac.uk].

Bioassays

^{3}**H-TdR assay**[1] **for immunosuppressive activity.** The immunosuppressive activity of laevinoids A (**1**) and B (**2**) against T Cell and B cell was evaluated by ^{3}H-TdR assay. Briefly, fresh spleen cells (5 × 10^{5} cell) which were obtained from BALB/c mice (female, 18–20 g, 7-9 week old) were seeded into 96-well plates (Falcon, CA). These cells were cultured with 5 mg/mL of concanavalin A (Con A) or 10 mg/mL of lipopolysaccharide (LPS) plus indicated concentrations of compounds at 37 °C for 48 h in 5% CO_2. Proliferation was assessed in terms of uptake of [^{3}H]thymidine during 8 h of pulsing with 25 μL of [^{3}H]thymidine (10 μ Ci/mL) for each well, and then the cells will be collected onto glass fiber filters by a Basic 96 harvester. The incorporated radioactivity was then counted using a Beta Scintillation Counter (MicroBeta Trilux, PerkinElmer).

CCK-8 assay.[2] Fresh spleen cells (5 × 10^{5} cell) were cultured in 96-well flat plates with 200 μL of RPMI 1640 media containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified, 37 °C, 5% CO_2-containing incubator for 48 h with or without various concentrations of tested compounds. To measure apoptosis, 10 μL of the CCK-8 solution (Cell Counting Kit-8 containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl) -5-(2,4-disulfophenyl)-2H-tetrazolium) was added to each well of the plate at the final 8 h of culture. Then, the absorbance was measured with an enzyme calibrator at 450 nm and 630 nm and the optical density (OD) values were measured by microplate reader (Bio-Red 550).

PTP1B inhibitory assay.[3] The PTP1B inhibitory activities of the two compounds were measured using pNPP as a substrate. In short, 50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA and 1 mM dithiothreitol (DTT) were added into the reaction buffer containing 2 mM pNPP and PTP1B (0.05–0.1 mg) dissolved in 10 % DMSO. Then the PTP1B enzyme was placed in each of 96 wells (final volume 100 μL) with or without tested compounds. Following incubation at 37 °C for 20 min, the reaction was terminated with the addition of 10 M NaOH. The amount of produced *p*-nitrophenol was estimated by measuring the absorbance

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[1] Wang, J.-X.; Tang, W.; Shi, L.-P.; Wan, J.; Zhou, R.; Ni, J.; Fu, Y.-F.; Yang, Y.-F.; Li, Y.; Zuo, J. P. *Br. J. Pharmacol.* **2007,** *150,* 651-661.
[2] Guo, Z.; Jin, X.; Jia, H. *J. Exp. Clin. Cancer Res.* **2013,** *32:* 26.
[3] Cui, L.; Na, M.K.; Oh, H.; Bae, E. Y.; Jeong, D. G.; Ryu, S. E.; S. Kim.; Kim, B. Y.; Oh, W. K.; Ahn, J. S. *Bioorg. Med. Chem. Lett.* **2006,** *16,* 1426-1429.

at 405 nm. The non-enzymatic hydrolysis of 2 mM pNPP was corrected for by measuring the increase in absorbance at 405 nm obtained in the absence of PTP1B enzyme.

SRB assay for A-549 cell Line.[4] The growth inhibitory effect of the compounds on the A-549 cell line was measured using the SRB (sulforhodamine B) assay. Briefly, A-549 cells were seeded into 96-well plates (Falcon, CA) and allowed 24 h to adhere. The cells were treated in triplicate with graded concentrations of the tested compounds at 37 °C for 72 h in 5% CO_2 in culture incubator, and then fixed with 10% trichloroacetic acid in 4 °C for 1 h. The culture plates were washed and dried before stained with SRB solution (0.4 wt %/vol in 1% acetic acid) for 15 min. After 5 washings using 1% acetum and dried in air, sulforhodamine B was dissolved in 150 μL buffer containing 10 mM Tris-base. The optical density of each well was recorded by plate reader at a wavelength of 515 nm. The results were expressed as IC_{50} as calculated by the Logit method.

MTT assay for HL-60 cell Line.[5] Cytotoxic activity of the compounds against the HL-60 cell line was evaluated using the MTT method (microculture tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Briefly, optimal amount of cells in 90 μL of culture medium, which were treated in triplicate with graded concentrations of the tested compounds at 37 °C for 72 h in 5% CO_2, were plated in each well of 96-well plates (Falcon, CA). And then a 20 μL aliquot of MTT solution (5 mg/mL in saline solution) was put directly into the wells. After 4 h incubation, 100 μL of “triplex solution” (10% SDS / 5% isobutanol / 10 mM HCl) was added, and the plates were incubated at 37 °C in 5% CO_2 overnight and then measured using a plate reader at 570 nm (VERSA Max, Molecular Devices).

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[4] Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990,** *82,* 1107-1112.

[5] Tao, Z.; Zhou, Y.; Lu, J.; Duan, W.; Qin, Y.; He, X.; Lin, L.; Ding, J. *Cancer Biol. Ther.* **2007,** *6,* 691-696.

Table S1. X-ray crystallographic data for laevinoid A (**1**)[a]

Empirical formula	$C_{20}H_{22}O_5$
Formula weight	342.38
Temperature	140 (2) K
Wavelength	1.54178 Å
Crystal system	Monoclinic
Space group	P2 (1)
Unit cell dimensions	*a* = 8.0200 (2) Å, α = 90°
	b = 9.1995 (2) Å, β = 97.3900 (10)°
	c = 12.0639 (2) Å, γ = 90°
Volume	882.68 (5) $Å^3$
Z	2
Calculated density	1.288 Mg/m^3
Absorption coefficient	0.755 mm^{-1}
F(000)	364
Crystal size	0.30 × 0.25 × 0.20 mm^3
Theta range for data collection	3.69 to 67.44°
Index ranges	–9<=h<=7, –11<=k<=11, –14<=l<=14
Reflections collected	7809
Independent reflections	2976 [R(int) = 0.0320]
Completeness to theta = 66.33°	96.4 %
Absorption correction	Semi-empirical
Max. and min. transmission	0.8637 and 0.8052
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	2976 / 1 / 229
Goodness-of-fit on F^2	1.009
Final R indices [I>2σ(I)]	R1 = 0.0325, wR2 = 0.0803
R indices (all data)	R1 = 0.0327, wR2 = 0.0811
Absolute structure parameter	–0.02 (14)
Largest diff. peak and hole	0.141 and –0.238 e. $Å^{-3}$

[a] Colorless crystals of **1** were obtained in petroleum ether/acetone

Table S2. X-ray crystallographic data for laevinoid B (**2**) [a]

Empirical formula	$C_{43}H_{52}Cl_2O_{11}$
Formula weight	815.75
Temperature	143 (2) K
Wavelength	1.54178 Å
Crystal system	orthorhombic
Space group	P2 (1) 2 (1) 2 (1)
Unit cell dimensions	a = 11.0308 (2) Å, α = 90.00°
	b = 11.6168 (2) Å, β = 90.00°
	c = 30.5185 (4) Å, γ = 90.00°
Volume	3910.72 (11) $Å^3$
Z	4
Calculated density	1.386 Mg/m^3
Absorption coefficient	2.017 mm^{-1}
F(000)	1728
Crystal size	0.25 × 0.22 × 0.12 mm^3
Theta range for data collection	2.90 to 69.73°
Index ranges	−13<=h<=11, −14<=k<=13, −36<=l<=36
Reflections collected	33746
Independent reflections	7194 [R(int) = 0.0292]
Completeness to theta = 69.87°	98.8 %
Absorption correction	Semi-empirical
Max. and min. transmission	0.7938 and 0.6325
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	7194 / 0 / 515
Goodness-of-fit on F^2	1.043
Final R indices [I>2σ(I)]	R1 = 0.0309, wR2 = 0.0814
R indices (all data)	R1 = 0.0316, wR2 = 0.0820
Absolute structure parameter	0.020 (8)
Largest diff. peak and hole	0.294 and −0.235 e. $Å^{-3}$

[a] Colorless crystals of **2** were obtained in petroleum ether/acetone

Figure S1. ^{1}H NMR spectrum of laevinoid A (**1**) in $CDCl_3$

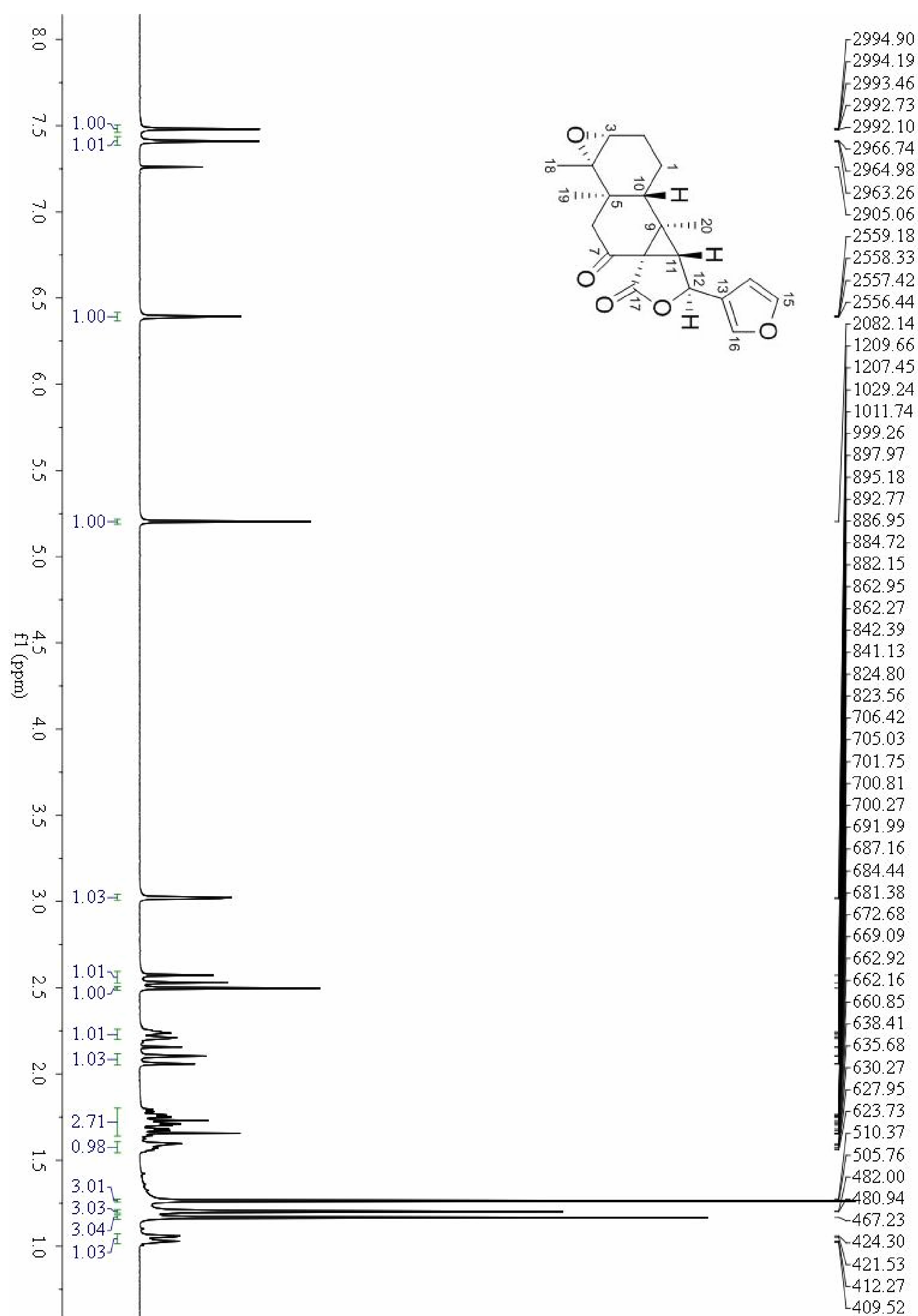

Figure S2. ^{13}C NMR spectrum of laevinoid A (**1**) in $CDCl_3$

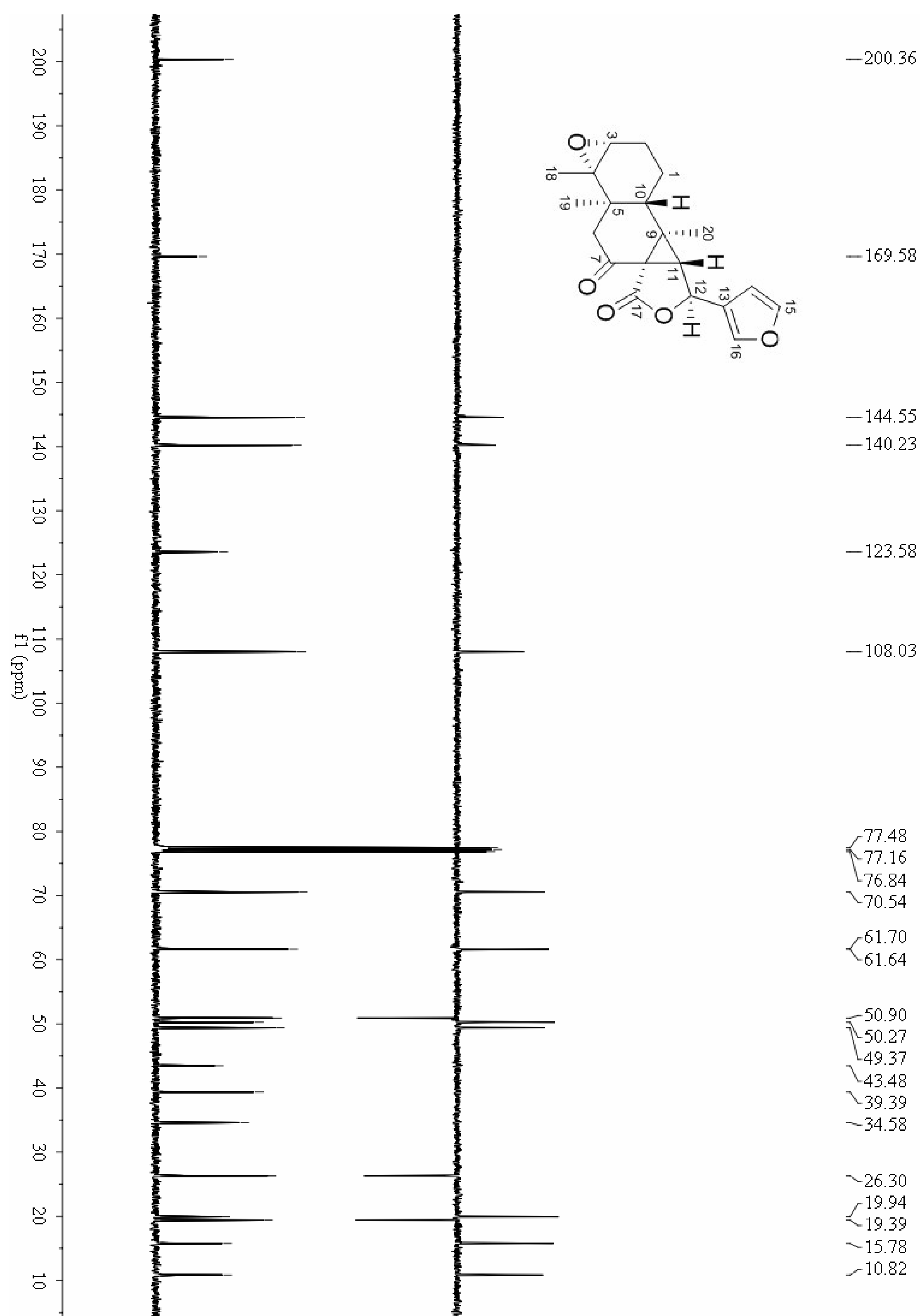

Figure S3. ^{1}H–^{1}H COSY spectrum of laevinoid A (**1**) in $CDCl_3$

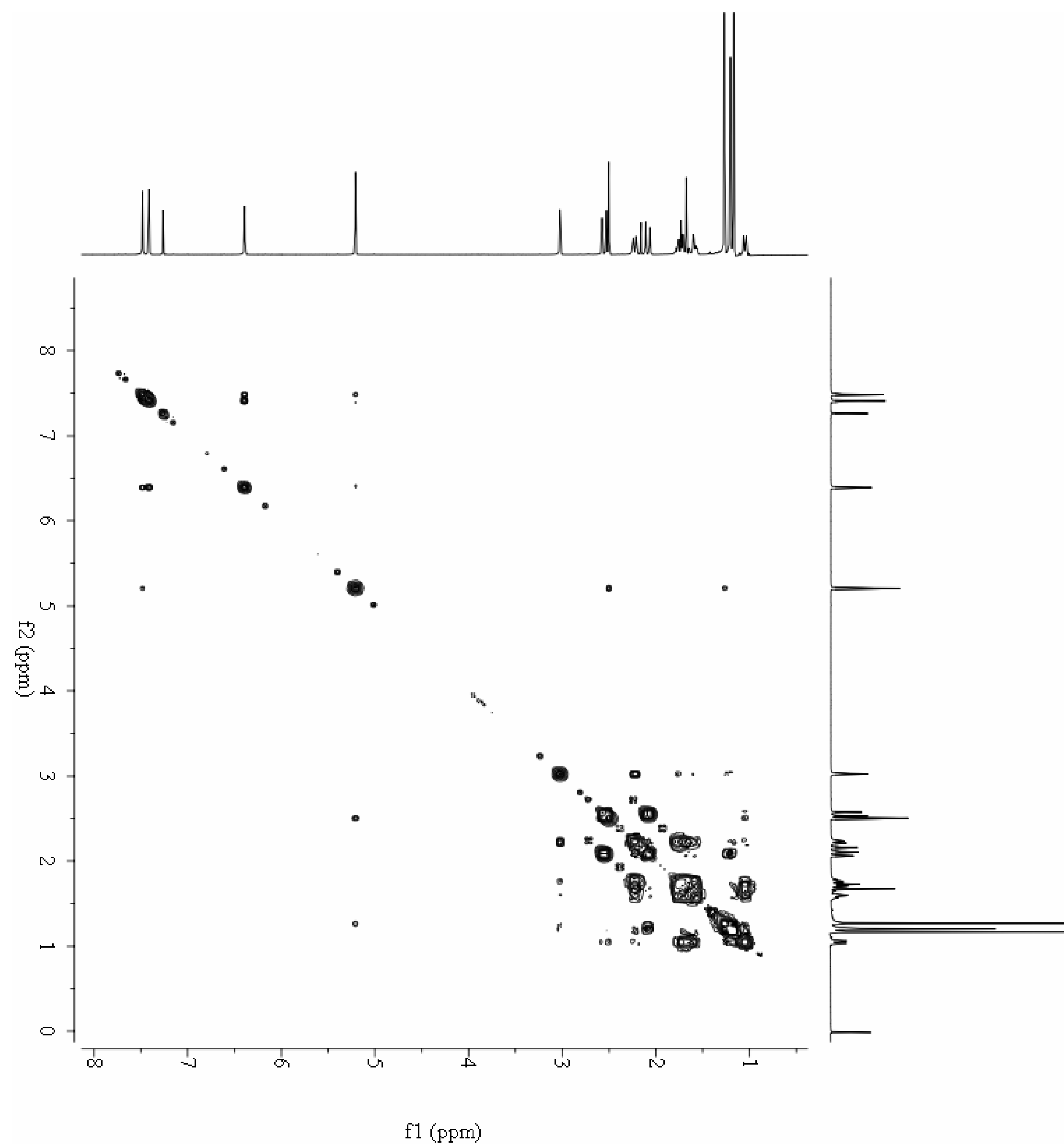

Figure S4. HSQC spectrum of laevinoid A (**1**) in $CDCl_3$

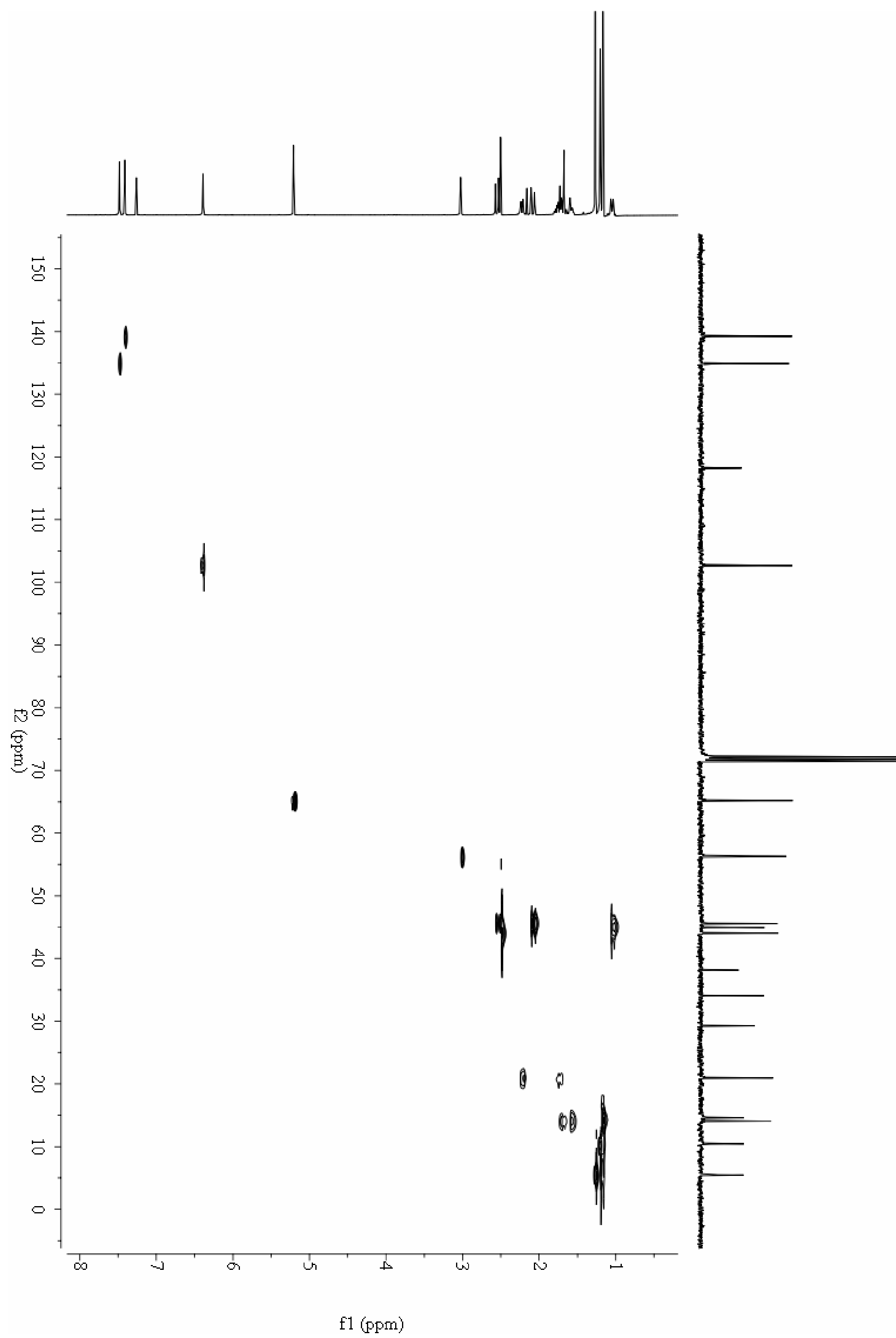

Figure S5. HMBC spectrum of laevinoid A (**1**) in $CDCl_3$

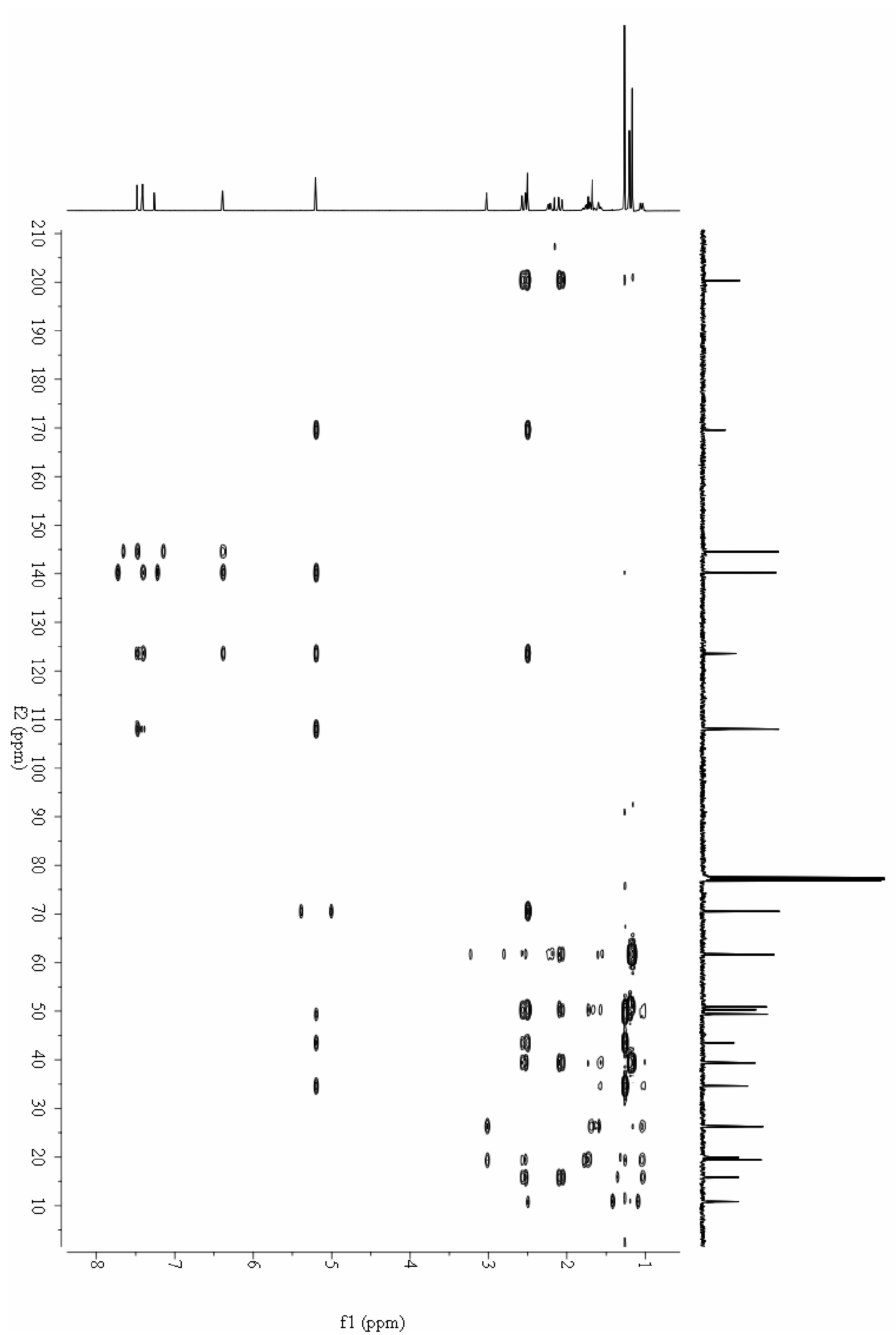

Figure S6. ROESY spectrum of laevinoid A (**1**) in $CDCl_3$

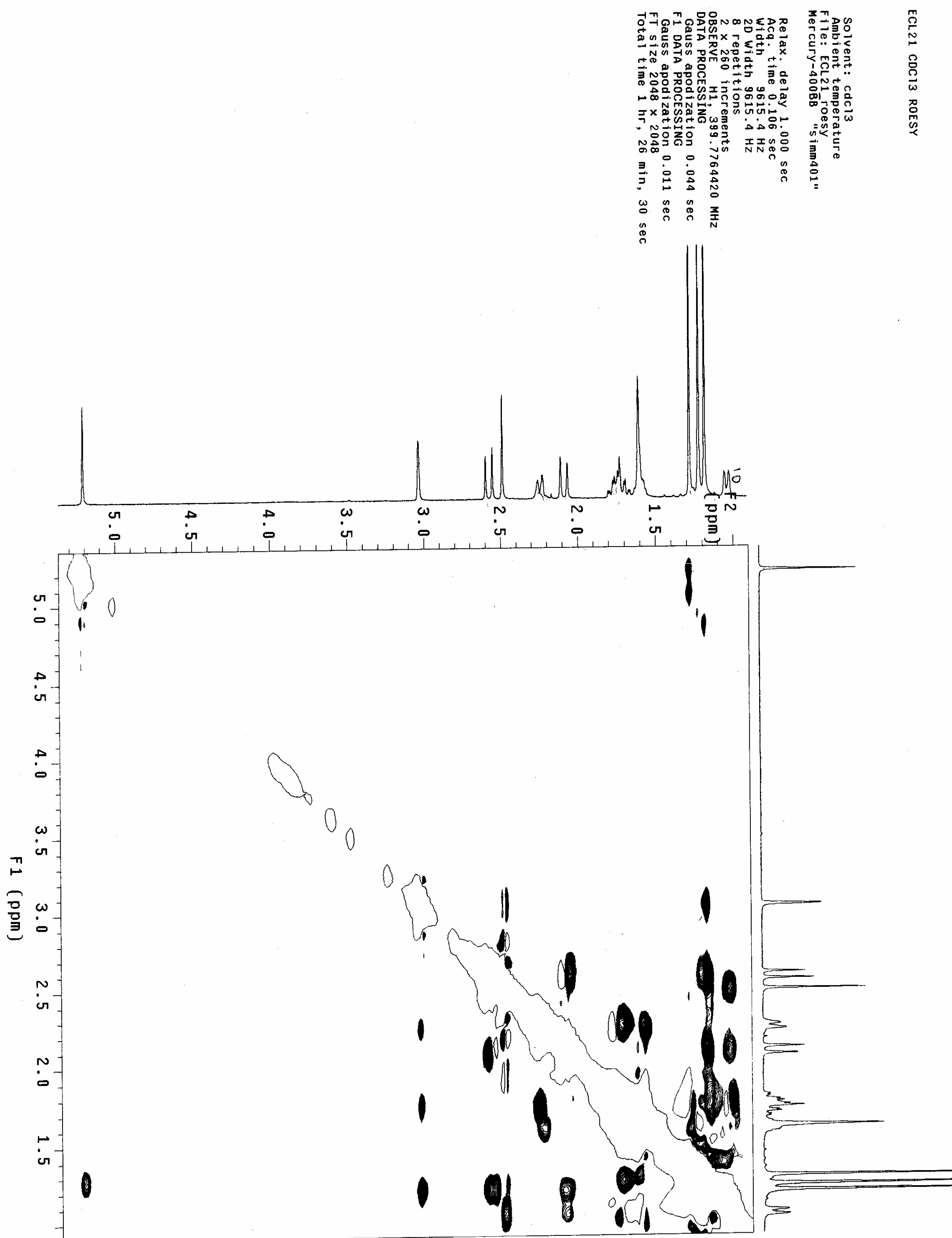

Figure S7. ESI(+)MS spectrum of laevinoid A (**1**)

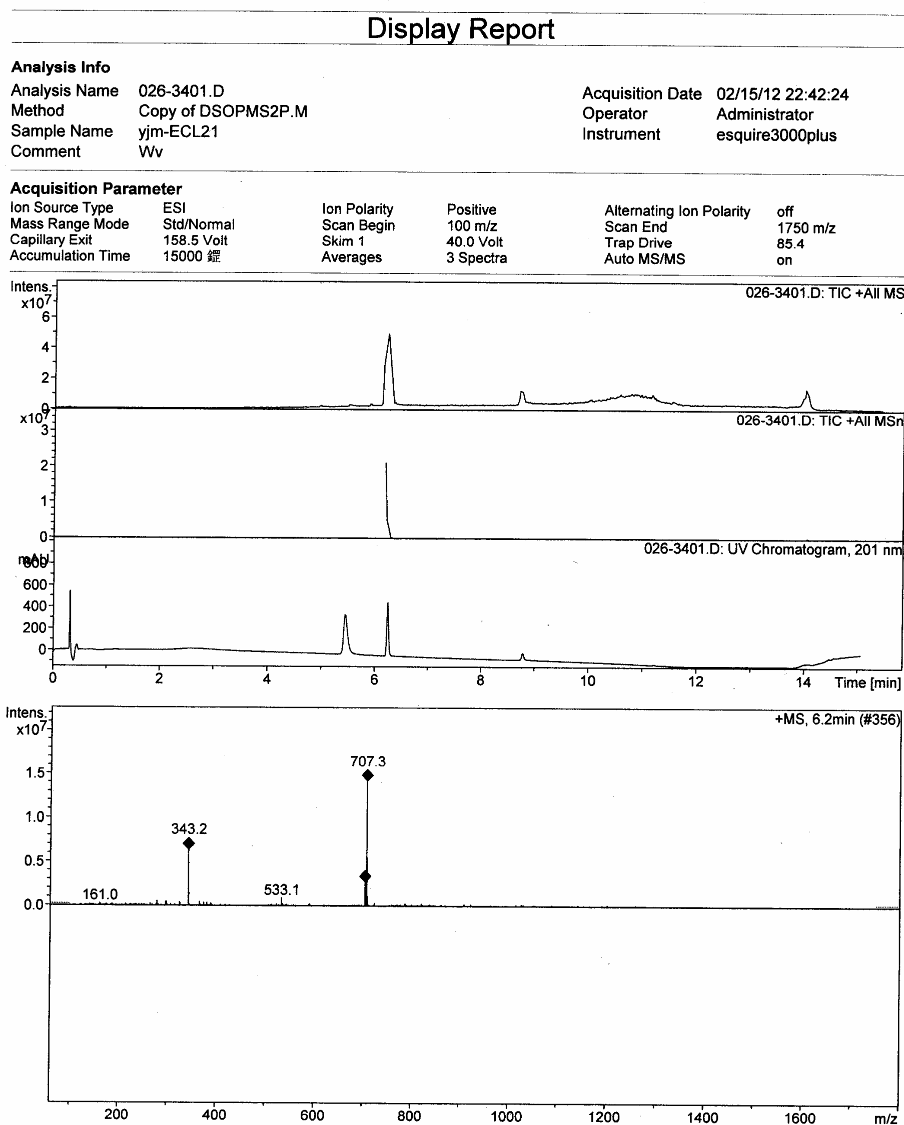

Figure S8. ESI(−)MS spectrum of laevinoid A (**1**)

Display Report

Analysis Info

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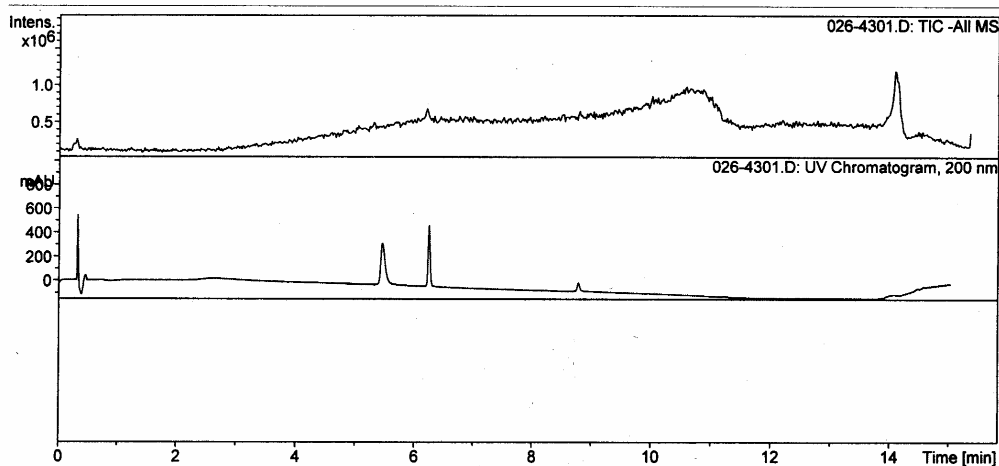
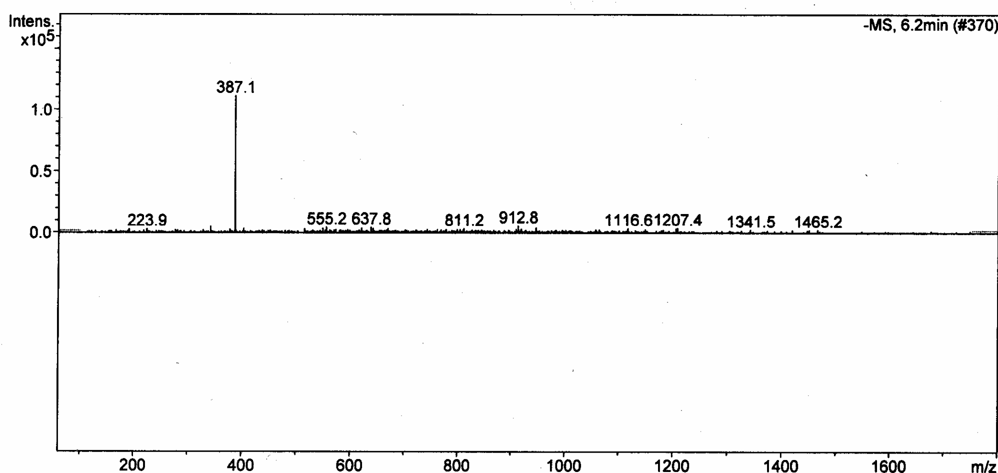
Method	Copy of DSOPMS2N.M	Operator	Administrator
Sample Name	yjm-ECL21	Instrument	esquire3000plus
Comment	Wv		

Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Negative	Alternating Ion Polarity	off
Mass Range Mode	Std/Normal	Scan Begin	100 m/z	Scan End	1750 m/z
Capillary Exit	-158.5 Volt	Skim 1	-40.0 Volt	Trap Drive	92.9
Accumulation Time	14766 鋸	Averages	3 Spectra	Auto MS/MS	on





Bruker Daltonics DataAnalysis 3.1 printed: 02/16/12 13:28:22 Page 1 of 1

Figure S9. HRESI(–)MS spectrum of laevinoid A (**1**)

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 3.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
75 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 6-60 H: 2-110 O: 0-30
ESL21 LCT PXE KE324 31-Aug-2012 16:06:22
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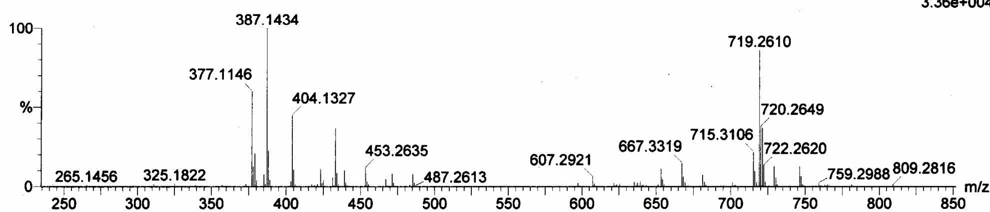

Minimum:				-1.5			
Maximum:		3.0	3.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
387.1434	387.1444	-1.0	-2.6	10.5	136.3	0.0	C21 H23 O7

Figure S10. IR spectrum of laevinoid A (**1**)

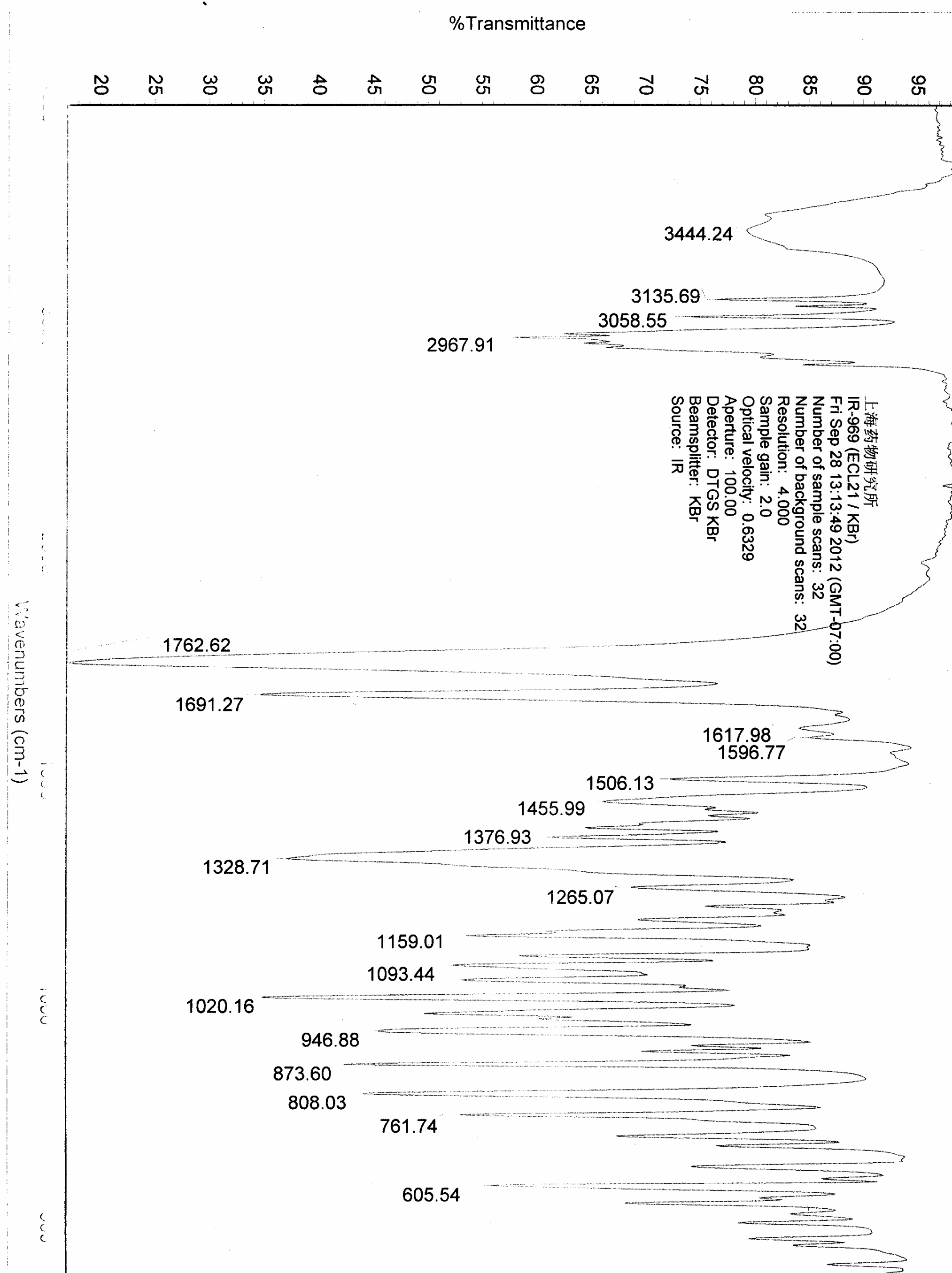

Figure S11. ^{1}H NMR spectrum of laevinoid B (**2**) in $CDCl_3$

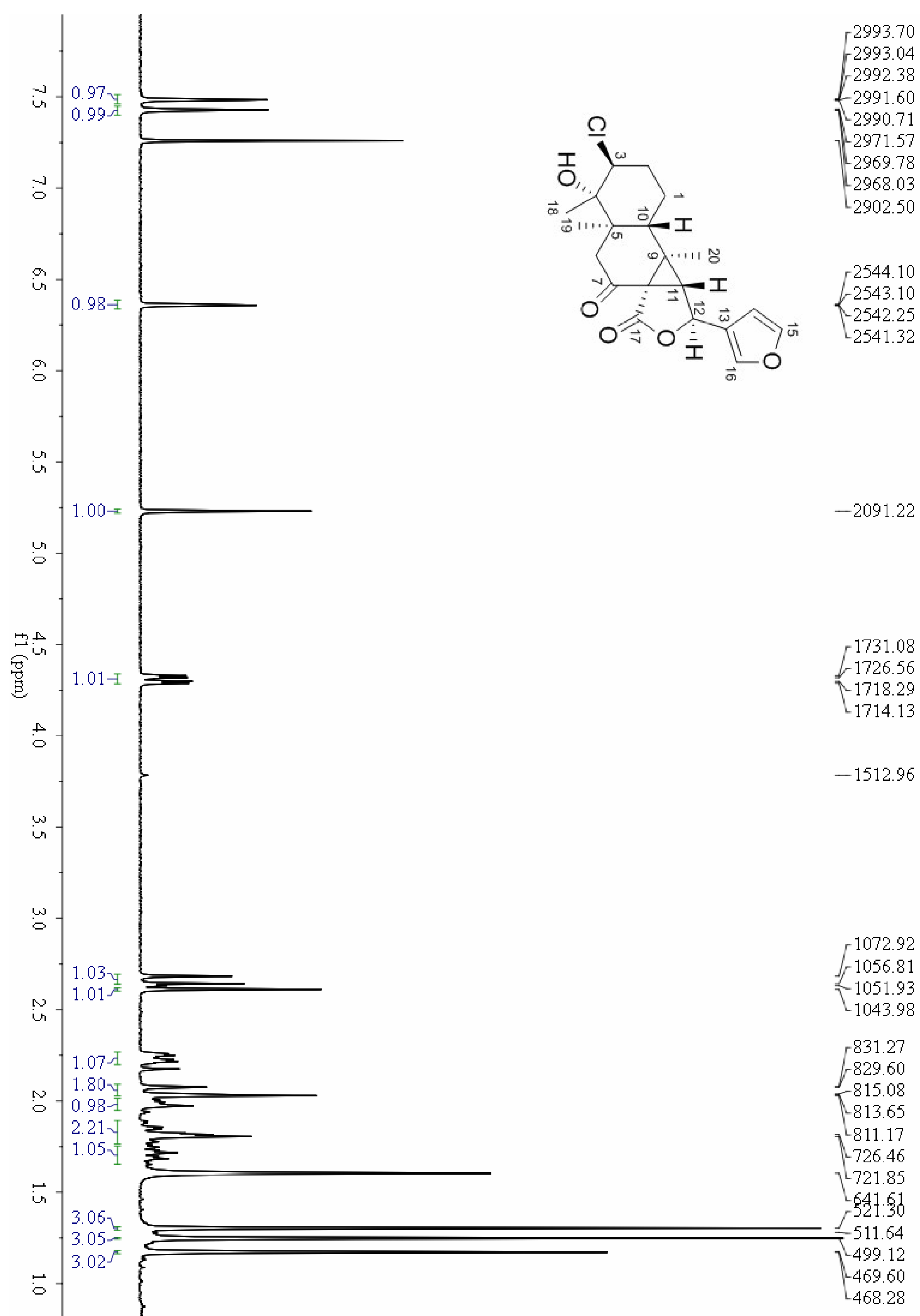

Figure S12. ^{13}C NMR spectrum of laevinoid B (**2**) in $CDCl_3$

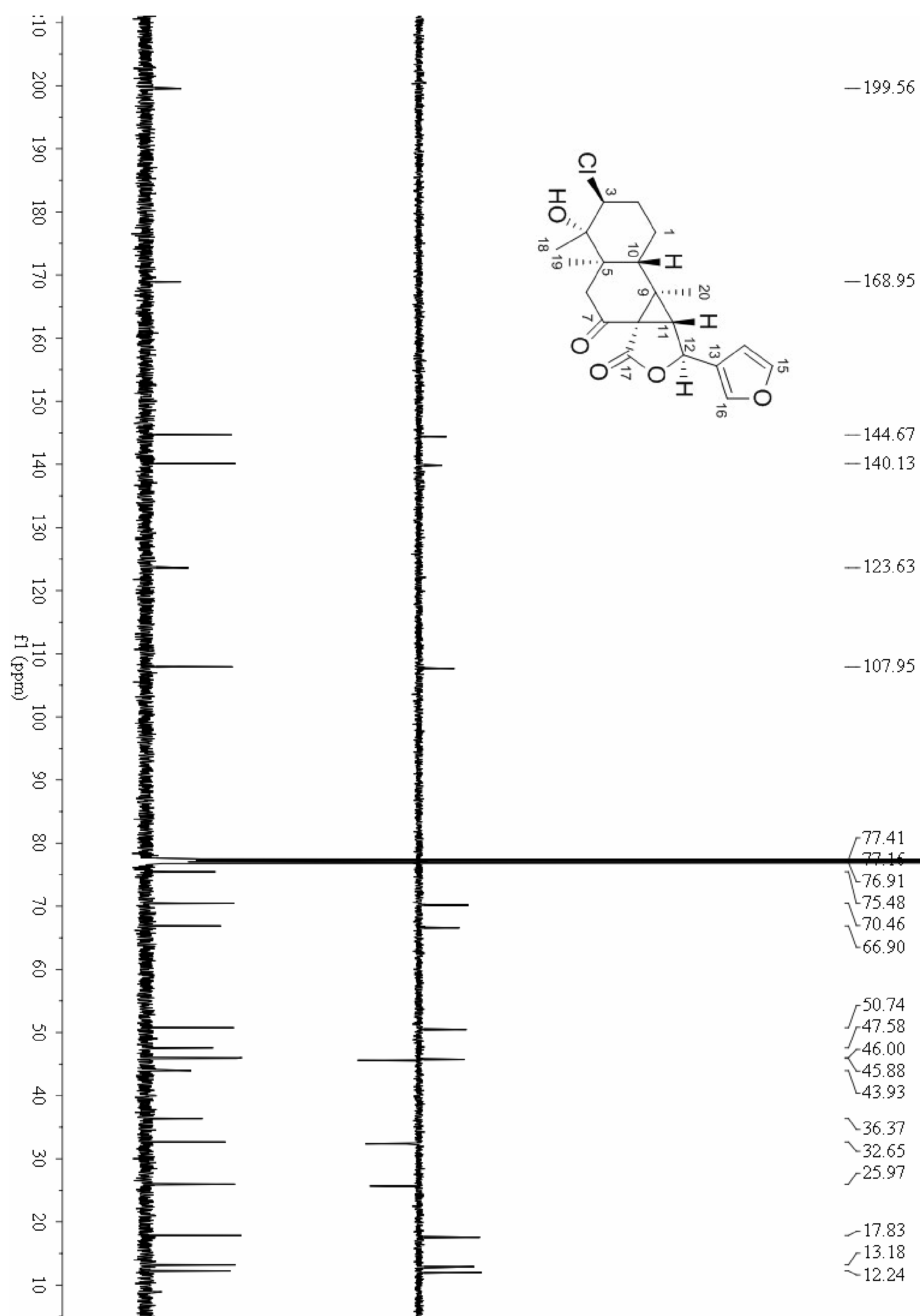

Figure S13. HSQC spectrum of laevinoid B (**2**) in $CDCl_3$

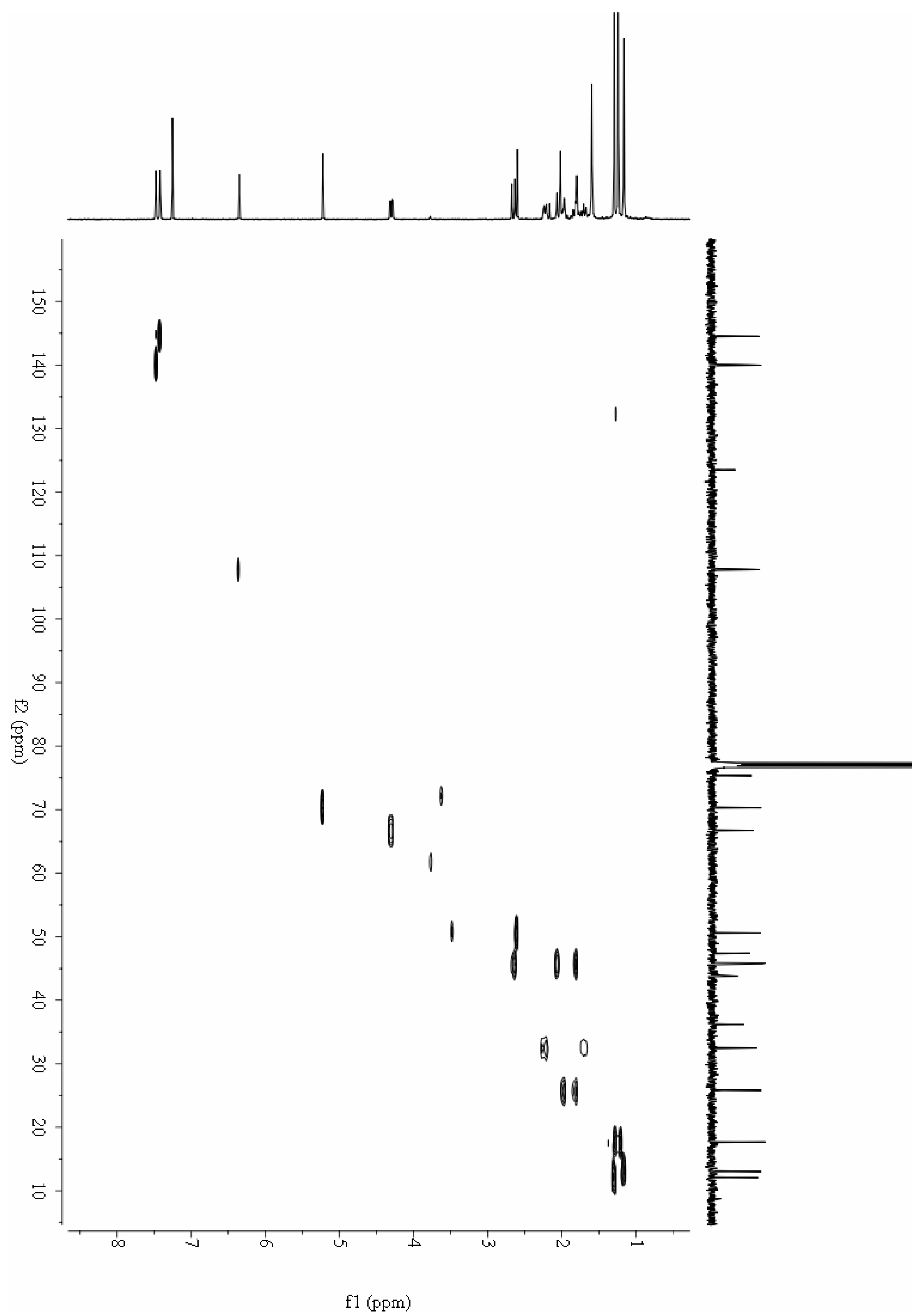

Figure S14. HMBC spectrum of laevinoid B (**2**) in $CDCl_3$

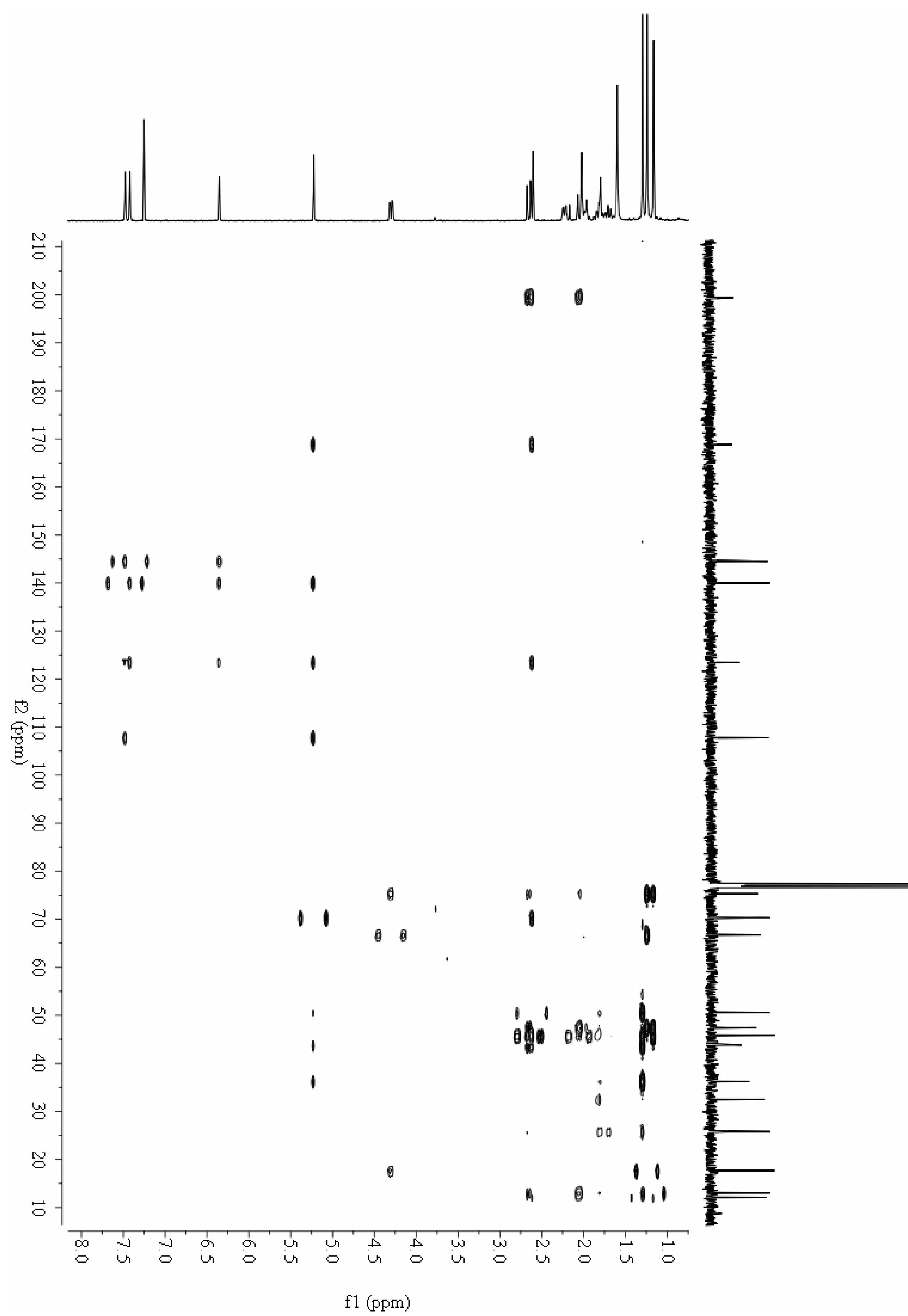

Figure S15. ROESY spectrum of laevinoid B (**2**) in $CDCl_3$

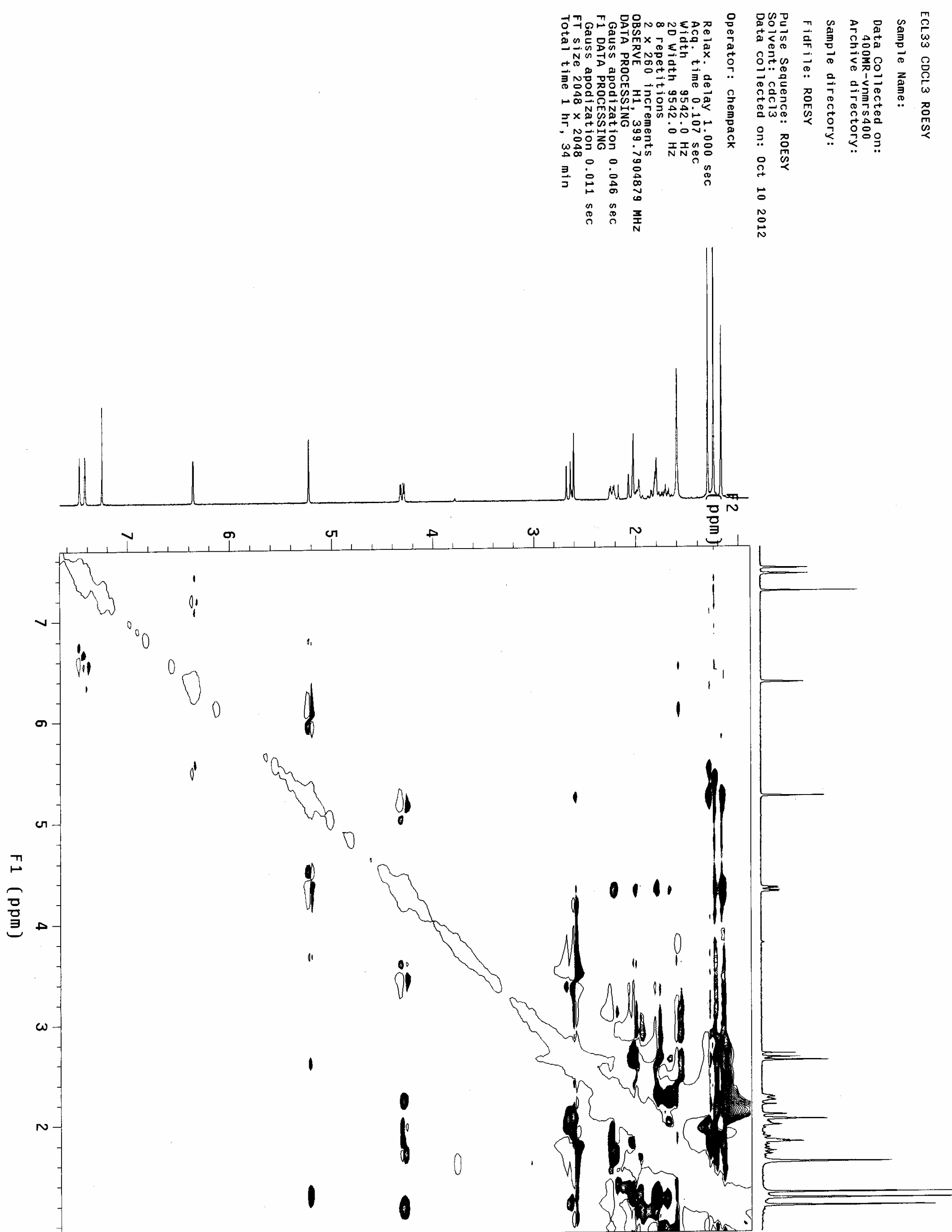

Figure S16. ESI(+)MS spectrum of laevinoid B (**2**)

Display Report

Analysis Info

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Sample Name	yjm-ECL-33	Instrument	esquire3000plus
Comment	?□		

Acquisition Parameter

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Mass Range Mode	Std/Normal	Scan Begin	100 m/z	Scan End	1750 m/z
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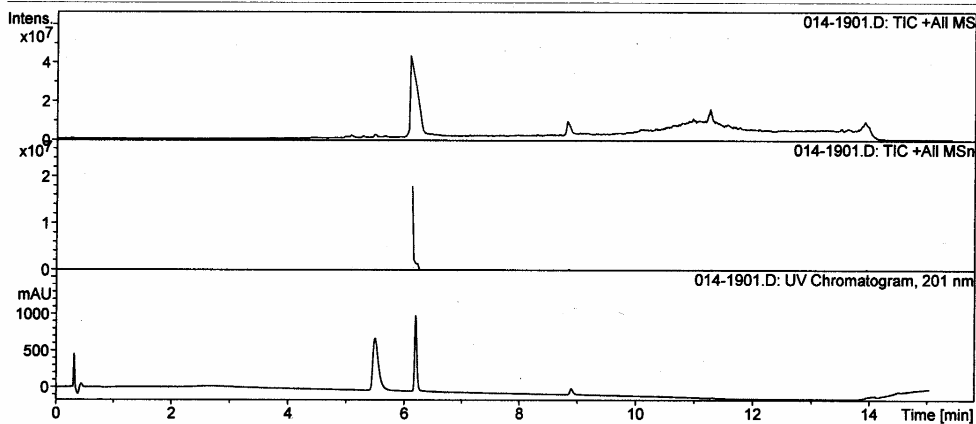
Accumulation Time	11837 鏢	Averages	3 Spectra	Auto MS/MS	on



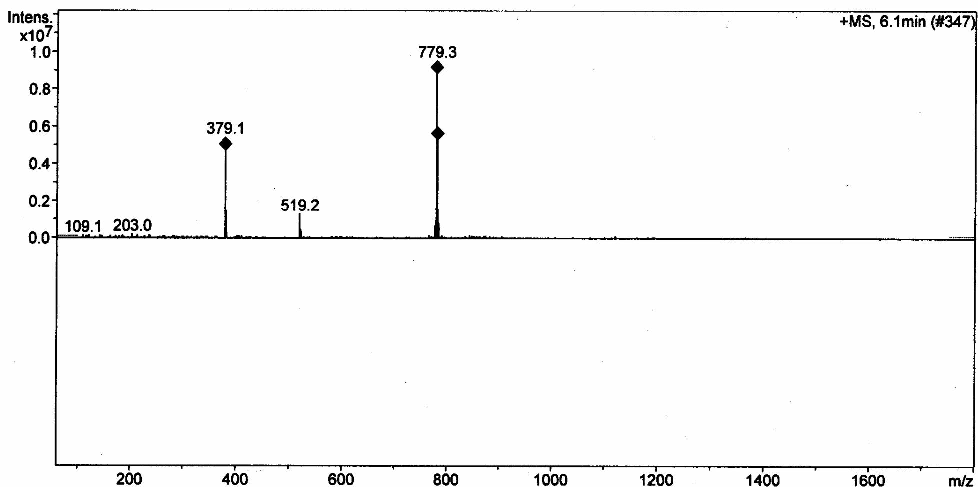


Figure S17. HRESI(+)MS spectrum of laevinoid B (**2**)

Elemental Composition Report

Page 1

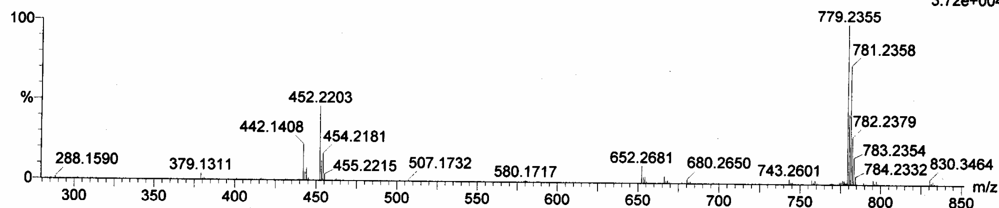
Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
132 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 6-60 H: 2-110 O: 0-30 Cl: 0-1

ESL33 LCT PXE KE324 31-Aug-2012 15:59:41

ESL33_20120831 18 (0.372) AM2 (Ar,10000.0,0.00,1.00); ABS; Cm (6:33) 1: TOF MS ES+ 3.72e+004



Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
Minimum:				-1.5			
Maximum:		3.0	5.0	50.0			
379.1311	379.1312	-0.1	-0.3	8.5	32.0	0.0	C20 H24 O5 Cl

Figure S18. IR spectrum of laevinoid B (**2**)

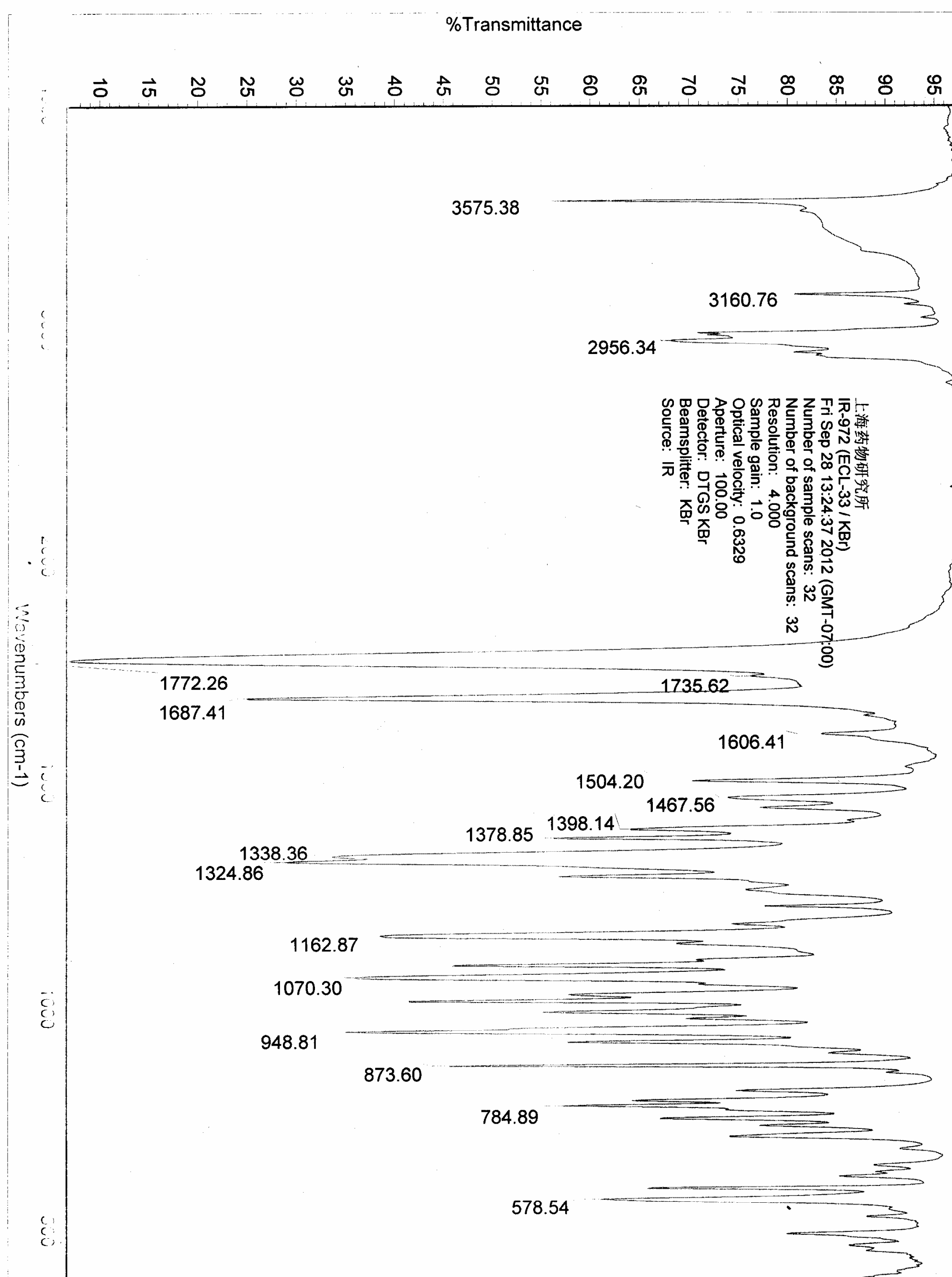


Figure S19. Key 2D NMR correlations for laevinoid B (**2**)

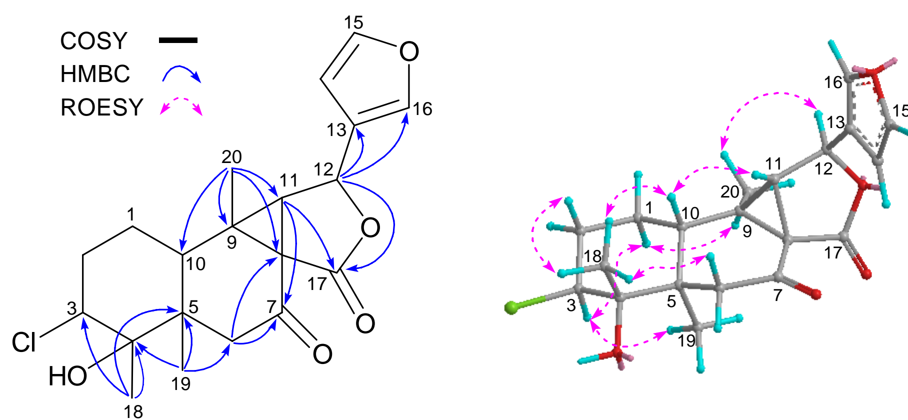
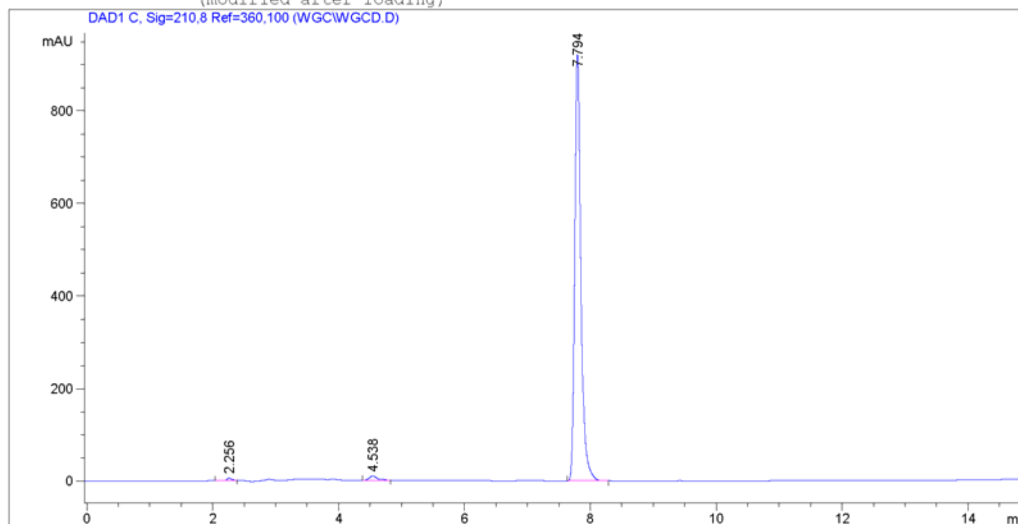


Figure S20. Purity report of laevinoid A (**1**) from HPLC analyses (1.0 mL/min, 50–100% MeCN/H_2O over 15 min)

```
Data File D:\DATA\WGC\WGCD.D                                                    Sample Name: ecl21

    Std MeCN/H2O method

    =====================================================================
    Injection Date  : 8/29/2013 6:00:41 PM
    Sample Name     : ecl21                          Location : Vial 11
    Acq. Operator   : wgc
    Acq. Instrument : Instrument 1                  Inj Volume : 5 µl
    Acq. Method     : C:\HPCHEM\1\METHODS\DEF_LC.M
    Last changed    : 8/29/2013 5:30:33 PM by wgc
                      (modified after loading)
    Analysis Method : C:\HPCHEM\1\METHODS\DEF_LC.M
    Last changed    : 8/30/2013 8:39:18 AM by wgc
                      (modified after loading)
```



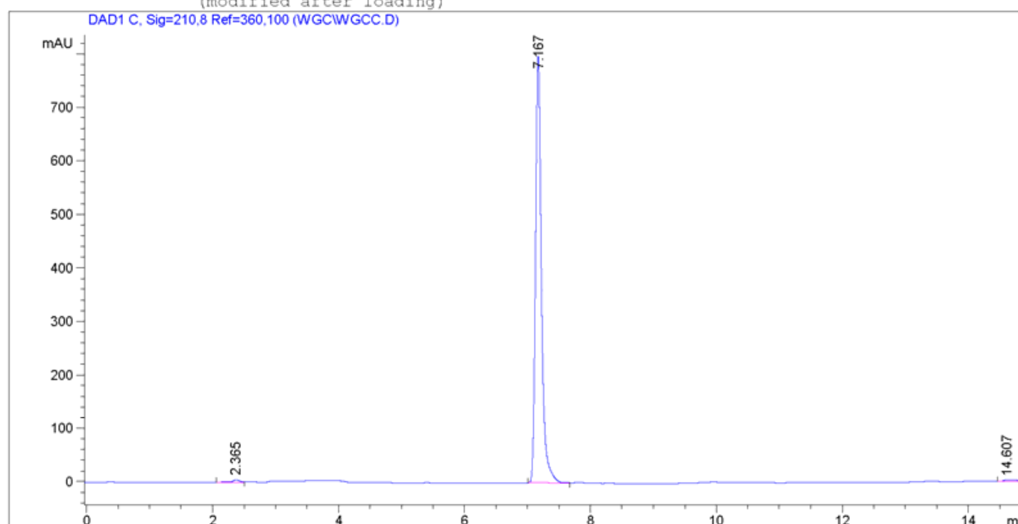

```
    =====================================================================
                             Area Percent Report
    =====================================================================

    Sorted By             :      Signal
    Multiplier            :      1.0000
    Dilution              :      1.0000
    Sample Amount         :      1.00000  [ng/ul]   (not used in calc.)
    Use Multiplier & Dilution Factor with ISTDs


    Signal 1: DAD1 C, Sig=210,8 Ref=360,100

    Peak RetTime Type  Width     Area      Height     Area
      #   [min]        [min]   [mAU*s]     [mAU]        %
    ----|-------|----|-------|----------|----------|--------|
       1   2.256 BB    0.0951   44.07250    6.50275   0.6885
       2   4.538 BB    0.1377   93.65614    9.95739   1.4630
       3   7.794 BB    0.1030 6263.72314  924.68567  97.8485

    Totals :                  6401.45179  941.14581

     Results obtained with enhanced integrator!
    =====================================================================
                              *** End of Report ***


Instrument 1 8/30/2013 9:01:24 AM wgc                                              Page 1 of 1
```

Figure S21. Purity report of laevinoid B (**2**) from HPLC analyses (1.0 mL/min, 50–100% MeCN/H_2O over 15 min)

Data File D:\DATA\WGC\WGCC.D Sample Name: ecl33

Std MeCN/H2O method

```
=====================================================================
Injection Date  : 8/29/2013 5:38:09 PM
Sample Name     : ecl33                          Location : Vial 12
Acq. Operator   : wgc
Acq. Instrument : Instrument 1                  Inj Volume : 5 µl
Acq. Method     : C:\HPCHEM\1\METHODS\DEF_LC.M
Last changed    : 8/29/2013 5:30:33 PM by wgc
                  (modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\DEF_LC.M
Last changed    : 8/30/2013 8:39:18 AM by wgc
                  (modified after loading)
```




```
=====================================================================
                          Area Percent Report
=====================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Sample Amount         :      1.00000  [ng/ul]   (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 C, Sig=210,8 Ref=360,100
```

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.365	BV	0.1241	46.01462	5.26011	0.8534
2	7.167	BB	0.0995	5302.72803	798.36829	98.3508
3	14.607	BV	0.1869	42.90329	3.07214	0.7957

Totals : 5391.64594 806.70053

Results obtained with enhanced integrator!

```
=====================================================================
                         *** End of Report ***
```

Instrument 1 8/30/2013 9:09:27 AM wgc Page 1 of 1