DEGRADANT CHARACTERIZATION REPORT

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Name:	Structural Characterization of CC-00281		

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1.0 Abstract

A degradant named CC-00281 was formed upon heating an acidic aqueous solution of CC-00276 and was isolated from this solution. Spectrometric analysis of CC-00281, 13-methyl-16-oxo-17-norkauran-19-oic acid-[(2-*O* -β-D-glucopyranosyl -3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester], by NMR and MS allowed a full assignment of its structure. Evaluation of the data led to the conclusion that this degradant was produced from CC-00276 (Ref. 6.2) by the rearrangement of the aglycone with concomitant loss of the glycoside at C-13.

2.0 Background

CC-00276 has been degraded using the stress conditions described below, which generated a number of major degradation products. In order to identify these degradants, the compounds were isolated through a series of liquid chromatographic (LC) steps and then characterized by multiple nuclear magnetic resonance spectrometric (NMR) and mass spectrometric (MS) analyses.

3.0 Materials and Methods

Unless otherwise noted, all work was conducted at AMRI, Bothell Research Center, Bothell, Washington.

3.1 CC-00276. A sample of CC-00276, Lot VSPC-2973-6B, was obtained from Pure Circle, Malaysia.

3.2 LC-MS. Mass spectrometry was carried out on a Sciex API2000 triple quadrupole mass spectrometer with a TurboIonSpray ionization source operating in negative ion mode. A Sedere Sedex 75 ELS detector was used operating at 50 °C and 3.5 bar. Analysis of the samples was performed using the following method: Column: Phenomenex Synergi Hydro RP, 4.6 x 250 mm, 4 µm (p/n 00G-4375-E0); Column Temp: 55 °C; Mobile Phase A: H_2O (0.0284% NH_4OAc, 0.0116% HOAc); Mobile Phase B: Acetonitrile; Flow Rate: 1.0 mL/min; Injection volume: 50 µL. Detection was by UV (210 nm), ELSD, and MSD (+ESI *m/z* 200-1450).

Gradient:

Time (min)	%A	%B
0.0	75	25
8.5	75	25
10.0	71	29
16.5	70	30
18.5	66	34
24.5	66	34
26.5	48	52
29.0	48	52
31.0	30	70

37.0	30	70
37.1	75	25
45.0	75	25

Isolation of CC-00281 by HPLC. The method (HPLC Method 1) used for the isolation of CC-00281 is summarized below. Column: Gemini C_{18} with guard column, 250 x 10 mm, 5 µm (p/n 00G-4435-N0); Column Temp: 25 °C; Mobile Phase A: H_2O; Mobile Phase B: Acetonitrile; Flow Rate: 5.0 mL/min; Injection volume: 300 uL at 10 mg/mL of degradation mixture CC-00276 Lot VSPC-2973-6B prepared in water-acetonitrile (75:25). Detection was by UV (210 nm).

Gradient:

Time (min)	%A	%B
0.0	75	25
20.0	69	31
20.5	50	50
25.0	40	60
25.1	75	25
30.0	75	25

3.3 MS and MS/MS. MS and MS/MS data were generated with a Waters Premier QTof mass spectrometer equipped with an electrospray ionization source. Samples were diluted with H_2O:acetonitrile (1:1) containing 0.1% formic acid and introduced via infusion using the onboard syringe pump. The samples were diluted to yield good s/n which occurred at an approximate concentration of 0.01 mg/mL.

3.4 NMR. The sample was prepared in pyridine-d_5 and NMR data were acquired on a Bruker Avance 500 MHz instrument with a 5 mm inverse detection probe. The spectrum was referenced to the residual solvent signal (δ_H 8.71, δ_C 149.9 for pyridine-d_5).

3.5 Degradation of CC-00276. A 0.1 M phosphoric acid solution was made and adjusted to pH 2.0 with concentrated ammonium hydroxide. Ten mg of CC-00276 (Lot VSPC-2973-6B) was added to 10 mL of the phosphoric acid solution. The solution was placed on a heat block at 80 °C for 24 hours. A sample of the degradation mixture was analyzed using the LC-MS method described in Section 3.2.

4.0 Results and Discussion

4.1 Isolation and Purification. Isolation of CC-00281 was performed using the CC-00276 degradation mixture that was prepared as described in Section 3.6. This material was analyzed by LC-MS using the LC-MS method (Section 3.2) and the results are given in Figure 1. The CC-00276 peak was observed at 11.1 min in the UV (210 nm) chromatogram. The mass spectrum for the CC-00276 peak provided the expected $[M-H]^-$ ion at *m/z* 1290.5. The CC-00281 peak was

observed to elute at 24.1 min in the UV chromatogram and showed an $[M-H]^-$ ion at *m/z* 803.9. Relative to CC-00276 this indicated a net loss of 486 Daltons. HPLC purification was performed using HPLC Method 1 and the peak eluting at 23.98 min was collected over several injections and dried by rotary evaporation under reduced pressure (Figure 2).

4.2 Mass Spectrometry. The results of an LC-MS analysis of the isolated peak are shown in Figure 3 and confirmed that it corresponded to CC-00281. A single peak was observed in the TIC, UV and ELS chromatograms. The mass spectrum of the isolate of CC-00281 showed an $[M-H]^-$ ion at *m/z* 803.8 suggesting a nominal mass of 804 Daltons.

The ESI+ TOF mass spectrum acquired by infusing a sample of CC-00281 showed $[M+H]^+$ and $[M+Na]^+$ ions at *m/z* 805.3890 and 827.3707, respectively (Figure 4). The mass of the $[M+H]^+$ ion was in good agreement with the molecular formula $C_{38}H_{60}O_{18}$ (calcd for $C_{38}H_{61}O_{18}$: 805.3858, error: 4.0 ppm) for CC-00281 (Figure 5). The ESI- mass spectrum provided $[M-H]^-$ and $[M+HCOOH-H]^-$ ions at *m/z* 803.3691 and 849.3774, respectively (Figure 6). As above, the mass of the $[M-H]^-$ ion was in good agreement with the molecular formula $C_{38}H_{60}O_{18}$ (calcd for $C_{38}H_{59}O_{18}$: 803.3701, error: -1.4 ppm) for CC-00281 (Figure 7). The +ESI and -ESI data indicated that CC-00281 has a nominal mass of 804 Daltons with the molecular formula, $C_{38}H_{60}O_{18}$. The molecular formula of CC-00281 differs from that of CC-00276 by the net loss of $C_{18}H_{30}O_{15}$ which corresponds to three units of glucose.

The MS/MS spectrum of CC-00281, selecting the $[M+H]^+$ ion at *m/z* 805 for fragmentation indicated the sequential loss of 3 glucose moieties at *m/z* 643.3341, 481.2809, and 319.2302 (Figure 8). A fragment ion was also observed at *m/z* 487.1670 corresponding to 3 glucose moieties and this ion underwent loss of glucose to yield a fragment ion at *m/z* 325.1151.

The -ESI Tof MS/MS spectrum of CC-00281, fragmenting on the $[M-H]^-$ ion at *m/z* 803 showed an ion at *m/z* 317.2130 corresponding to the loss of three glucose residues (Figure 9). Fragment ions were also observed at *m/z* 641.3160 and 479.2651 corresponding to the loss of one or two glucose residues, respectively.

4.3 NMR Spectrometry. A series of NMR experiments including ^1H NMR (Figure 10), ^1H-^1H COSY (Figure 11), HSQC (Figure 12), HMBC (Figure 13) were performed to allow the assignment of CC-00281. A preliminary inspection of the NMR data indicated that the olefinic protons observed for CC-00276 were absent. Together with the MS data this suggested that a rearrangement of the aglycone had occurred with concomitant loss of three sugar residues.

An HMBC correlation from the methyl protons at δ_H 1.42 ppm to the carbonyl at δ_C 175.8 allowed assignment of one of the tertiary methyl groups (C-18) as well as C-19 and provided a starting point for assignment of the rest of the aglycone.

Additional HMBC correlations from the methyl protons (H-18) to carbons at δ_C 37.3, 44.1, and 57.1 allowed assignment of C3 to C5 in comparison with the data for CC-00276 (Ref 6.2). The ^1H chemical shifts for C-3 (δ_H 1.07 and 2.77) and C-5 (δ_H 1.06) were assigned using the HSQC data. A COSY correlation between one of the H-3 protons (δ_H 1.07) and protons at δ_H 2.02 allowed assignment of the H-2 protons which in turn showed a correlation with a proton at δ_H 0.77 which was assigned to C-1. The remaining ^1H and ^{13}C chemical shifts for C-1 and C-2 were then assigned on the basis of additional COSY and HSQC correlations and are summarized in Table 1.

Two additional tertiary methyl singlets were observed at δ_H 0.81 and 0.98 in the ^1H NMR spectrum and showed HSQC correlations to carbons at δ_C 13.8 and 19.9, respectively. One of these overlapped singlets (δ_H 0.81, δ_C 13.8) showed HMBC correlations to C-1 and C-5 and was assigned as C-20 in comparison with the data for CC-00276. The methyl protons showed an additional HMBC correlation to a methine (δ_H 1.04, δ_C 54.4) which was assigned as C-9. COSY correlations between H-5 (δ_H 1.06) and protons at δ_H 1.38 and 2.04 then allowed assignment of the H-6 protons which in turn showed correlations to protons at δ_H 1.34 and 1.61 which were assigned to C-7. The ^{13}C chemical shifts for C-6 (δ_C 19.4) and C-7 (δ_C 41.3) were then determined from the HSQC data.

COSY correlations between H-9 (δ_H 1.04) and protons at δ_H 1.04 and 1.46 allowed assignment of the H-11 protons which in turn showed COSY correlations to protons at δ_H 1.24 and 1.51 which were assigned as the H-12 protons. The HSQC data was then used to assign C-11 (δ_C 20.1) and C-12 (δ_C 37.1). As noted above signals for the olefinic H-17 protons were not observed suggesting a change in this region of the aglycone. The remaining methyl singlet (δ_H 0.98, δ_C 19.9) showed an HMBC correlation to C-12 indicating that it must be attached at C-13. The methyl protons showed additional HMBC correlations to carbons at δ_C 48.4, 54.2, and 220.7. The HSQC data indicated that the carbon at δ_C 48.4 is a methylene group (δ_H 1.83 and 2.62) which was assigned as C-14. The ketone (δ_C 220.7) was assigned to C-16 indicating that the aglycone had undergone rearrangement to isosteviol (Ref 6.3). The quaternary carbon at δ_C 54.2 was assigned as C-13. The HSQC data indicated the presence of one additional methylene group (δ_H 1.27 and 1.36, δ_C 53.8) which was assigned to C-15.

Analysis of the NMR data for the aglycone, together with MS data, indicated that a rearrangement of the aglycone to isosteviol with concomitant loss of the glycoside at C-13. An analogous degradant (CC-00212) was isolated from a degradation preparation of Rebaudioside A (CC-00201) in a previous study (Ref. 6.4). A summary of the ^1H and ^{13}C chemical shifts for the aglycone are found in Table 1 and a summary of the key HMBC and COSY correlations used to assign the aglycone region are provided in Figure 14.

An analysis of the HSQC data for CC-00281 indicated the presence of three anomeric positions rather than the six found in CC-00276. All three of the anomeric protons were well resolved at δ_H 6.19 (δ_C 93.0), 5.79 (δ_C 103.4), and

5.35 (δ_C 104.5) in the ^{1}H NMR spectrum. The anomeric proton observed at δ_H 6.19 showed an HMBC correlation to C-19 which indicated that it corresponds to the anomeric proton of Glc_I. This was in agreement with the NMR data described above for the aglycone which indicated that the glycoside at C-13 was absent while the glycoside at C-19 was retained in the degradant.

The Glc_I anomeric proton (δ_H 6.19) showed a COSY correlation to a proton at δ_H 4.51 which was assigned as Glc_I H-2 and in turn showed a COSY correlation to a proton at δ_H 4.27 (Glc_I H-3) which showed a correlation with a proton at δ_H 4.17 (Glc_I H-4). Assignment of the ^{13}C chemical shifts for Glc_I C-2 (δ_C 76.8), C-3 (δ_C 88.4), and C-4 (δ_C 69.1) was made using the HSQC data. The assignments at Glc_I C-5 and C-6 were made using the ^{1}H and HSQC data in comparison with the data for CC-00276.

The two remaining unassigned glucose moieties were assigned as substituents at C-2 and C-3 of Glc_I in comparison with the data for CC-00276. The anomeric proton observed at δ_H 5.79 was assigned as the anomeric proton of Glc_V in comparison with the data for CC-00276. Similarly, the anomeric proton observed at δ_H 5.35 was assigned as the anomeric proton of Glc_{VI} in comparison with CC-00276. The assignments for C-2 through C-6 of Glc_V and Glc_{VI} were made using the ^{1}H, COSY and HSQC data in comparison with the assignment of CC-00276.

A summary of the ^{1}H and ^{13}C chemical shifts for the glycoside at C-19 are found in Table 2 and a summary of the key HMBC and COSY correlations used to assign the C-19 glycoside region are provided in Figure 15. The structure of CC-00276 is shown in Figure 16.

4.4 Chromatography. When analyzed under the conditions of the LC/MS method described above, CC-00281 had a retention time of 24.1 min.

5.0 Conclusions

NMR and MS analyses of CC-00281 allowed a full assignment of its structure. The chemical name of CC-00281 is 13-methyl-16-oxo-17-norkauran-19-oic acid-[(2-*O* -β-D-glucopyranosyl -3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester].

6.0 References

6.1 AMRI-Bothell Research Center Notebook # 833 pp. 1-3, 45, 56-64.

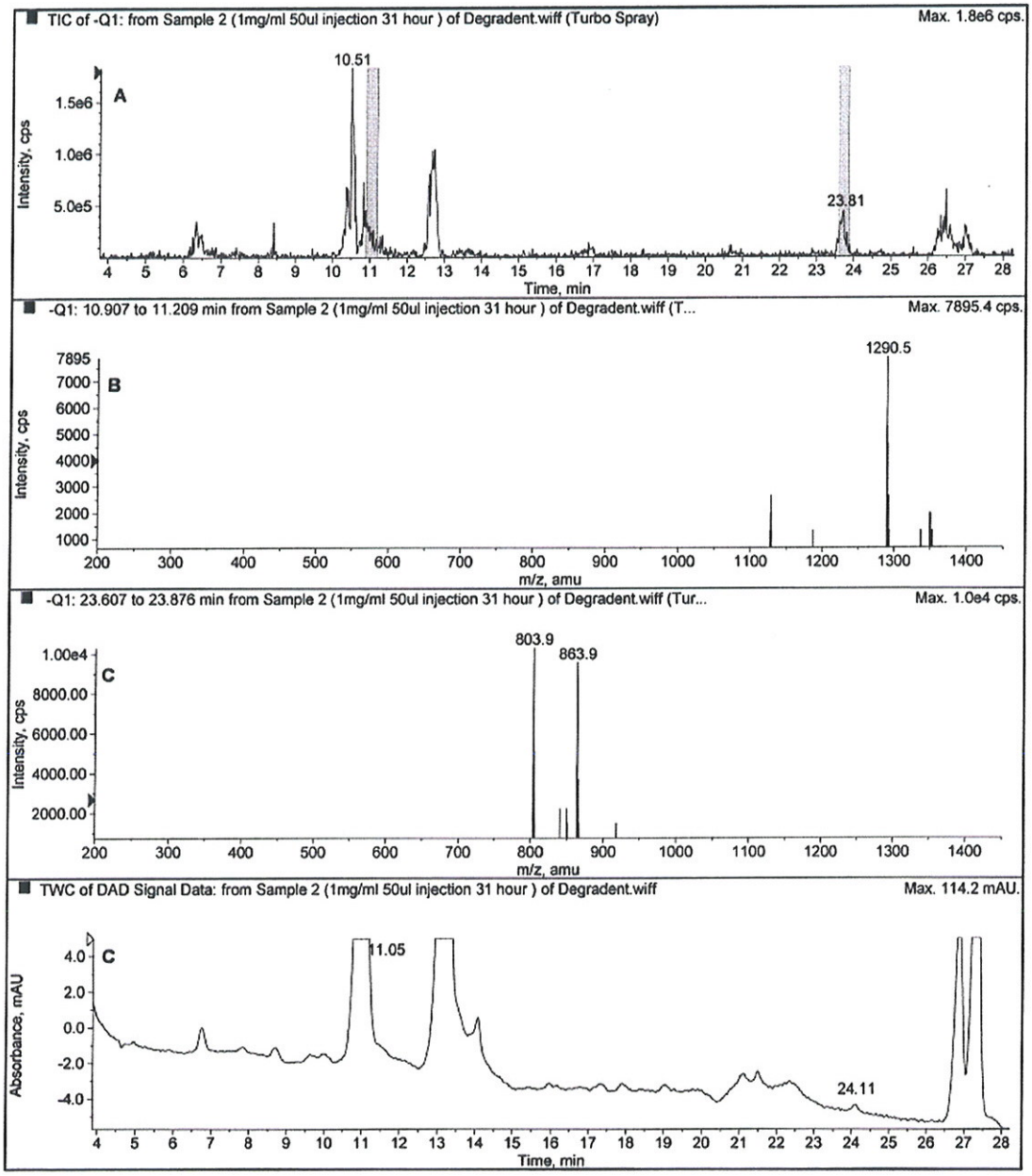
6.2 RC-032 "Structural Characterization of CC-00276".

6.3 Nanayakkara et al., *Journal of Natural Products*, **1987**, *50*: 434-441.

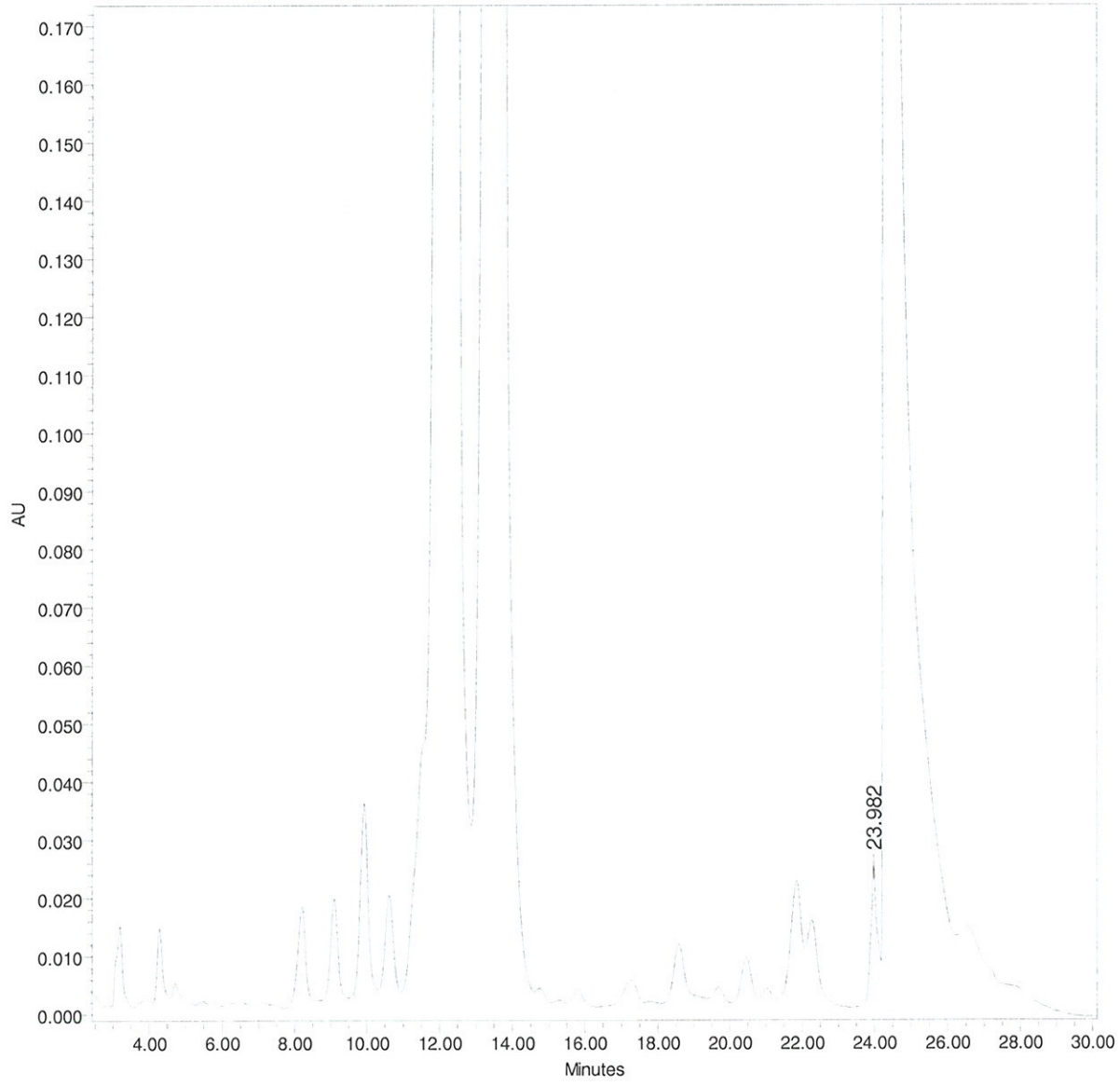
6.4 RC-006 "Isolation and Identification of CC-00212 (DAQ 5)".

7.0 Appendices and Attachments

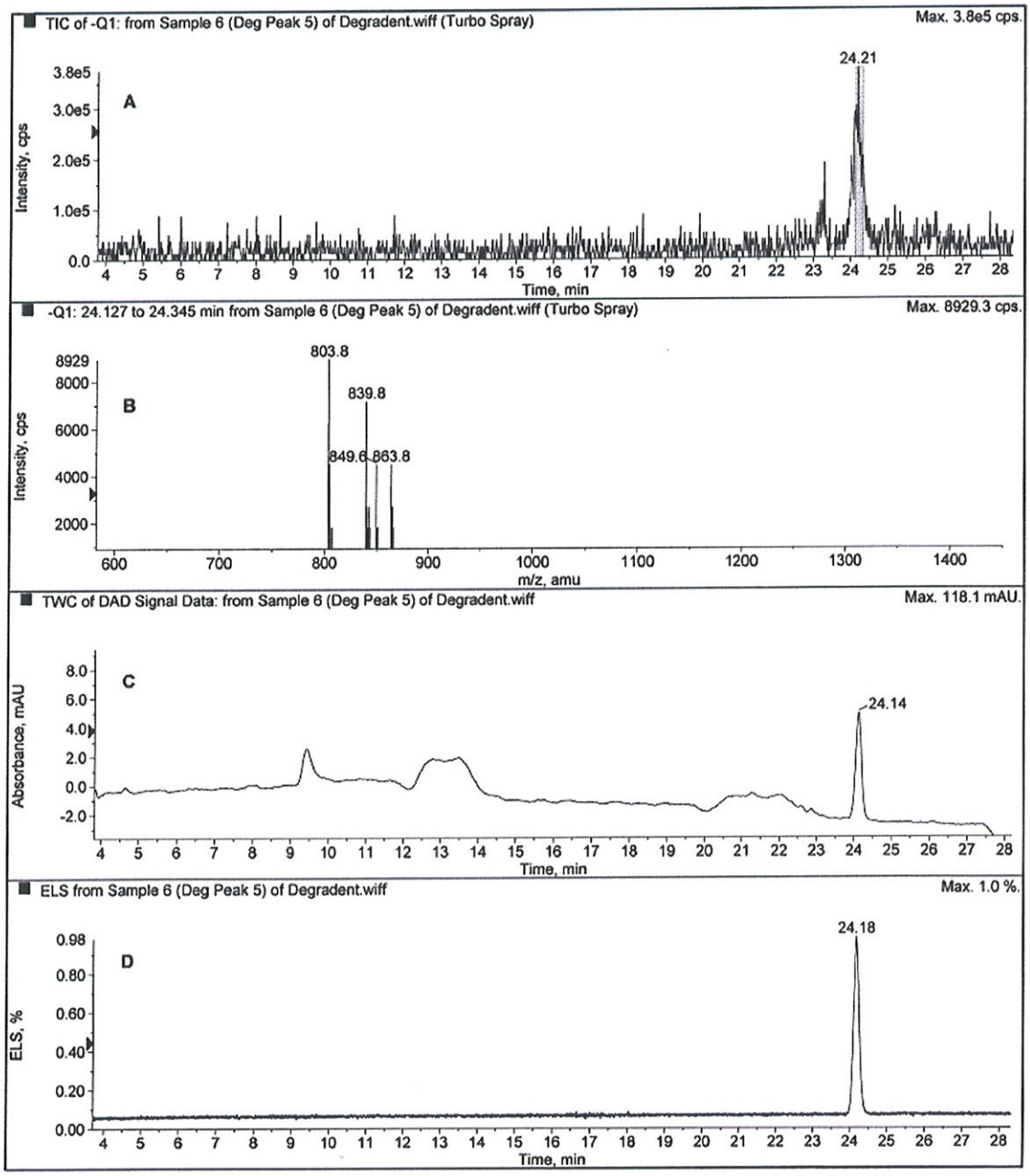
7.1 Figure 1. LC-MS analysis of CC-00276 Lot VSPC-2973-6B degradation preparation showing TIC (**A**), mass spectrum of the CC-00276 peak at 11.1 min (**B**), mass spectrum of the CC-00281 peak at 23.8 min (**C**) and UV (210 nm) chromatogram (**D**).



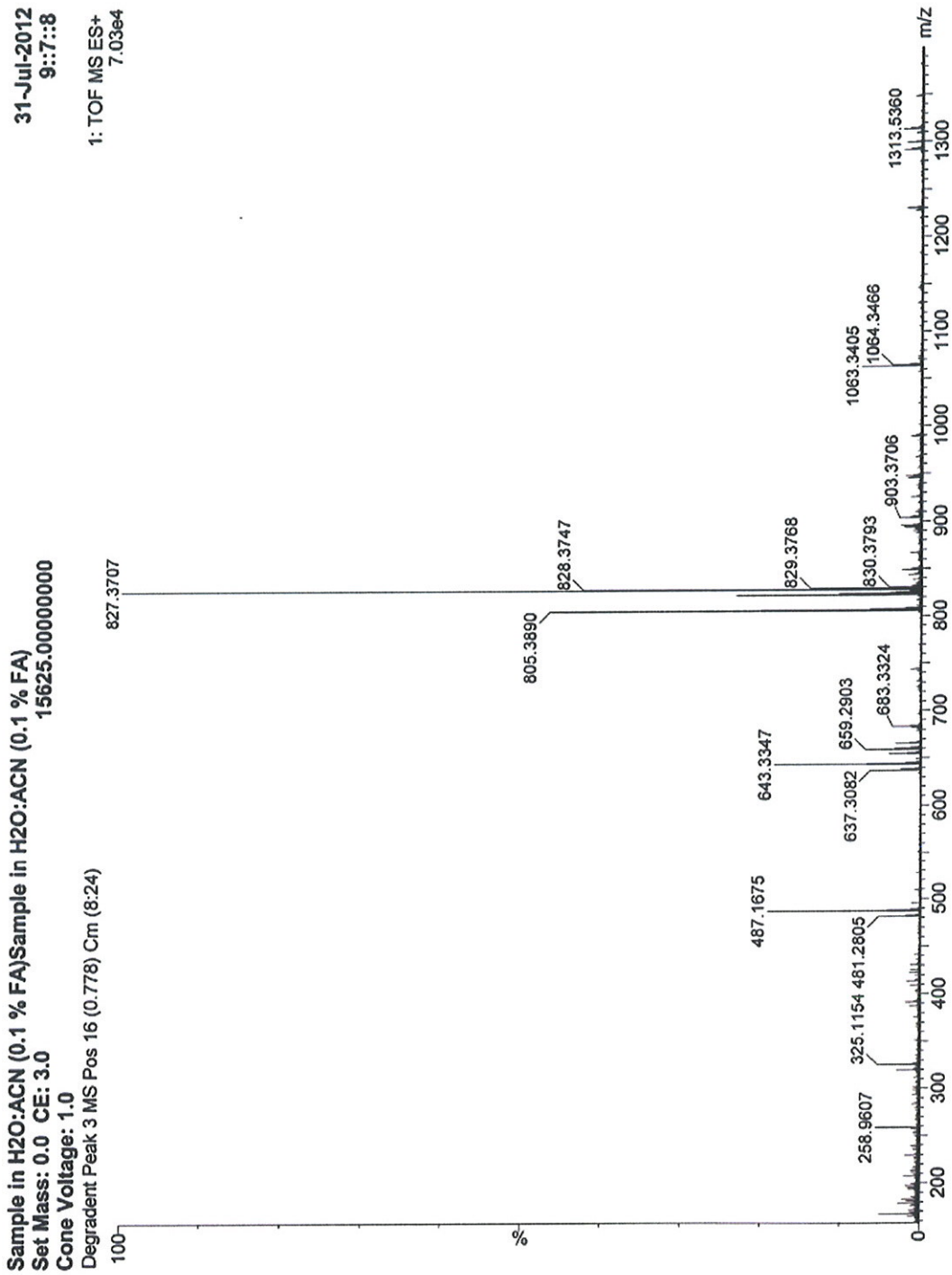
7.2 Figure 2. Representative HPLC UV (210 nm) chromatogram for CC-00276 Lot VSPC-2973-6B degradation preparation.



7.3 Figure 3. LC-MS analysis of isolated sample of CC-00281 showing TIC (**A**), mass spectrum of the CC-00281 peak at 24.2 min (**B**), UV (210 nm) chromatogram (**C**) and ELS chromatogram (**D**).



7.4 Figure 4. ESI+ TOF mass spectrum of CC-00281.

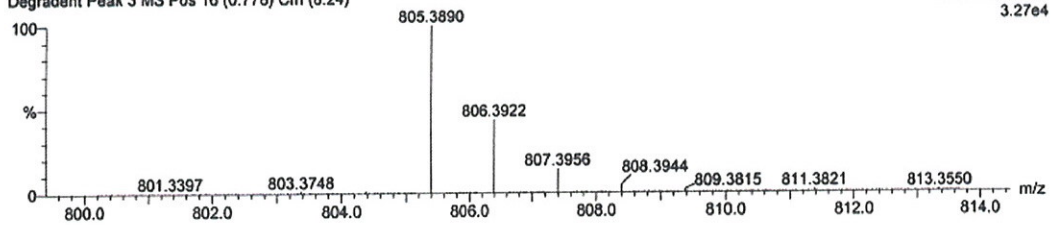


7.5 Figure 5. Accurate mass analysis of the $[M+H]^+$ ion of CC-00281.

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Selected filters: None

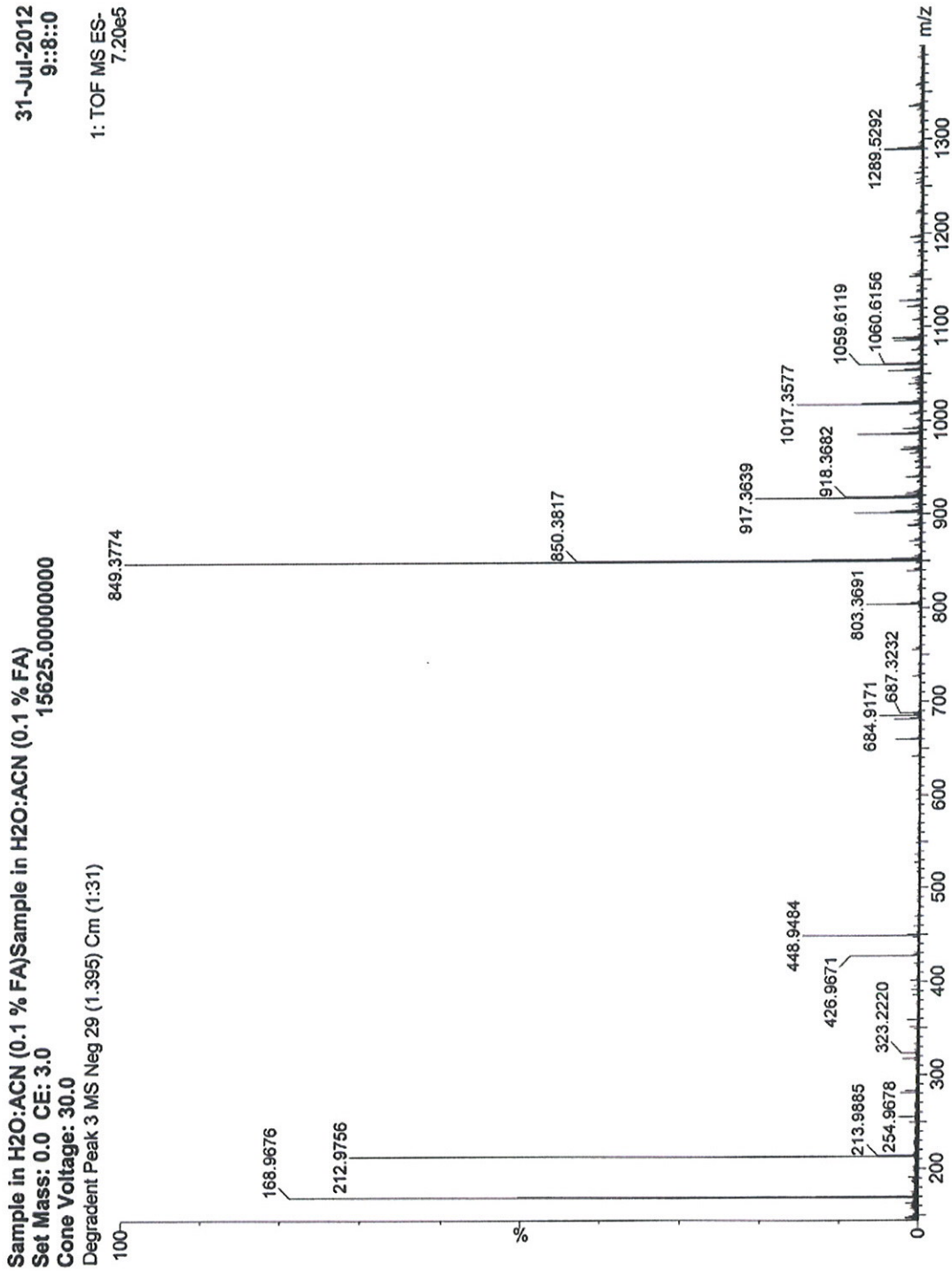
Monoisotopic Mass, Even Electron Ions
61 formula(e) evaluated with 2 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-40 H: 0-70 O: 0-25

Sample in H2O:ACN (0.1 % FA)Sample in H2O:ACN (0.1 % FA)
Set Mass: 0.0 CE: 3.0
Cone Voltage: 1.0
Degradent Peak 3 MS Pos 16 (0.778) Cm (8:24)
15625.00000000
31-Jul-2012
9::7::8
1: TOF MS ES+
3.27e4



Minimum:				-1.5		
Maximum:		3.0	5.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
805.3890	805.3858	3.2	4.0	8.5	12.3	C38 H61 O18
	805.3917	-2.7	-3.4	-0.5	202.1	C31 H65 O23

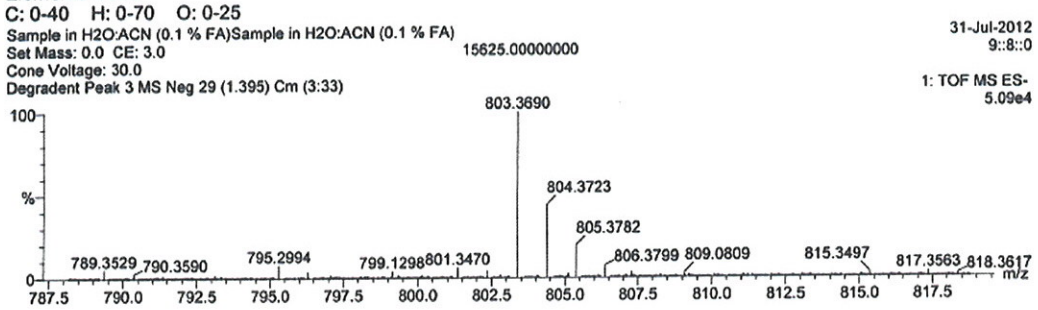
7.6 Figure 6. ESI- TOF mass spectrum of CC-00281.



7.7 Figure 7. Accurate mass analysis of the $[M-H]^-$ ion of CC-00281.

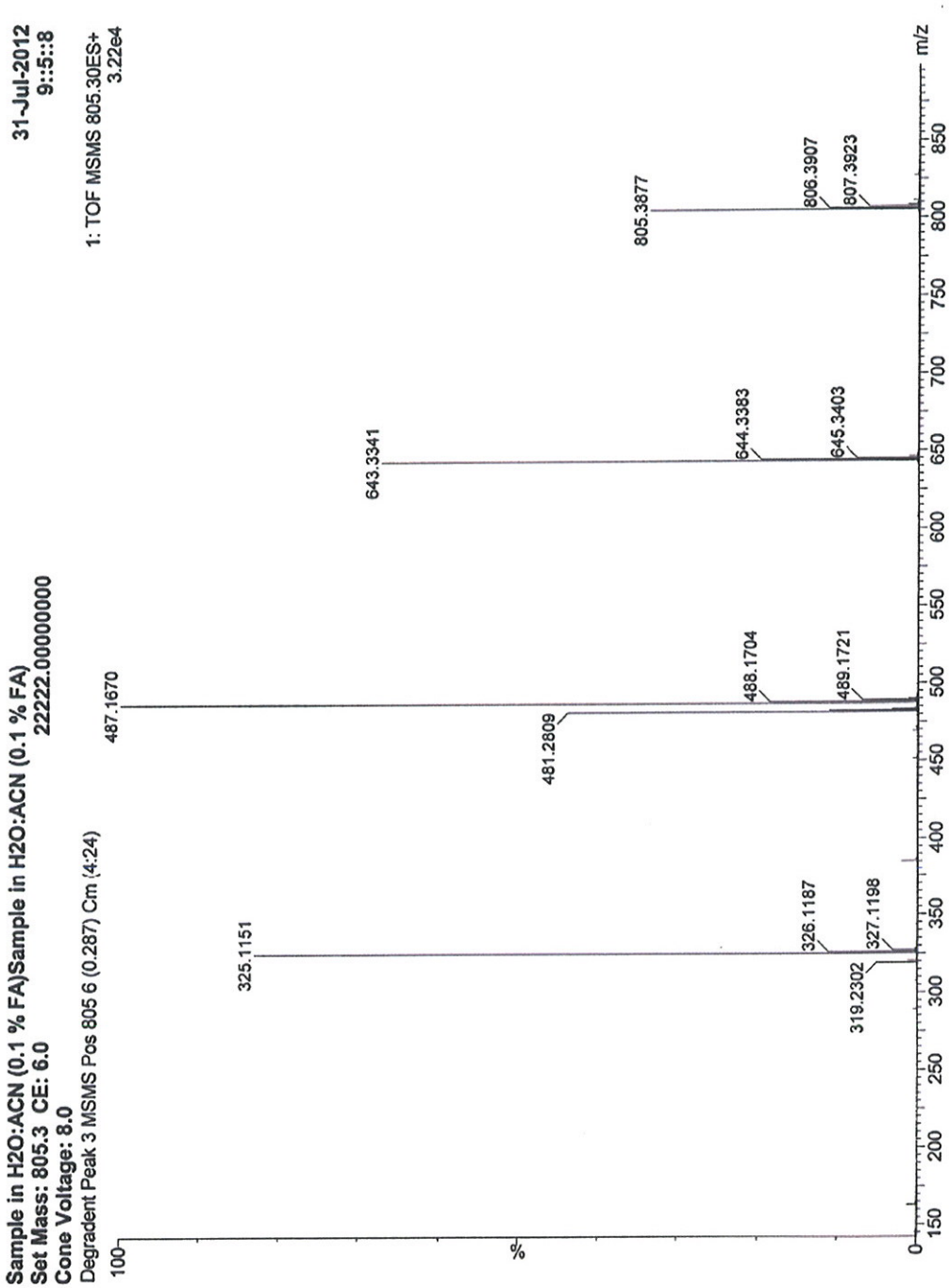
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Selected filters: None

Monoisotopic Mass, Even Electron Ions
62 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-40 H: 0-70 O: 0-25

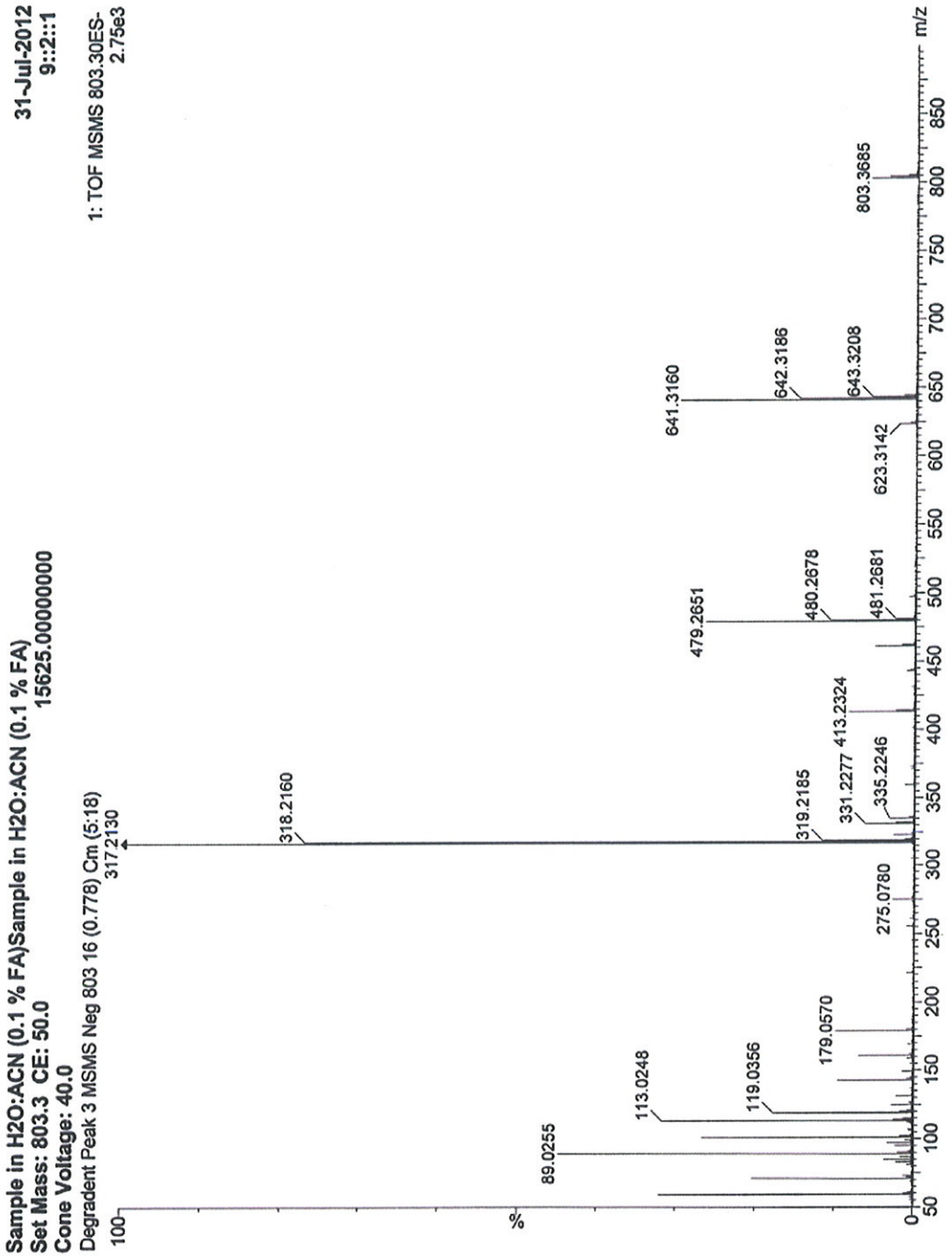


Minimum:				-1.5		
Maximum:		3.0	5.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
803.3690	803.3701	-1.1	-1.4	9.5	485.7	C38 H59 O18

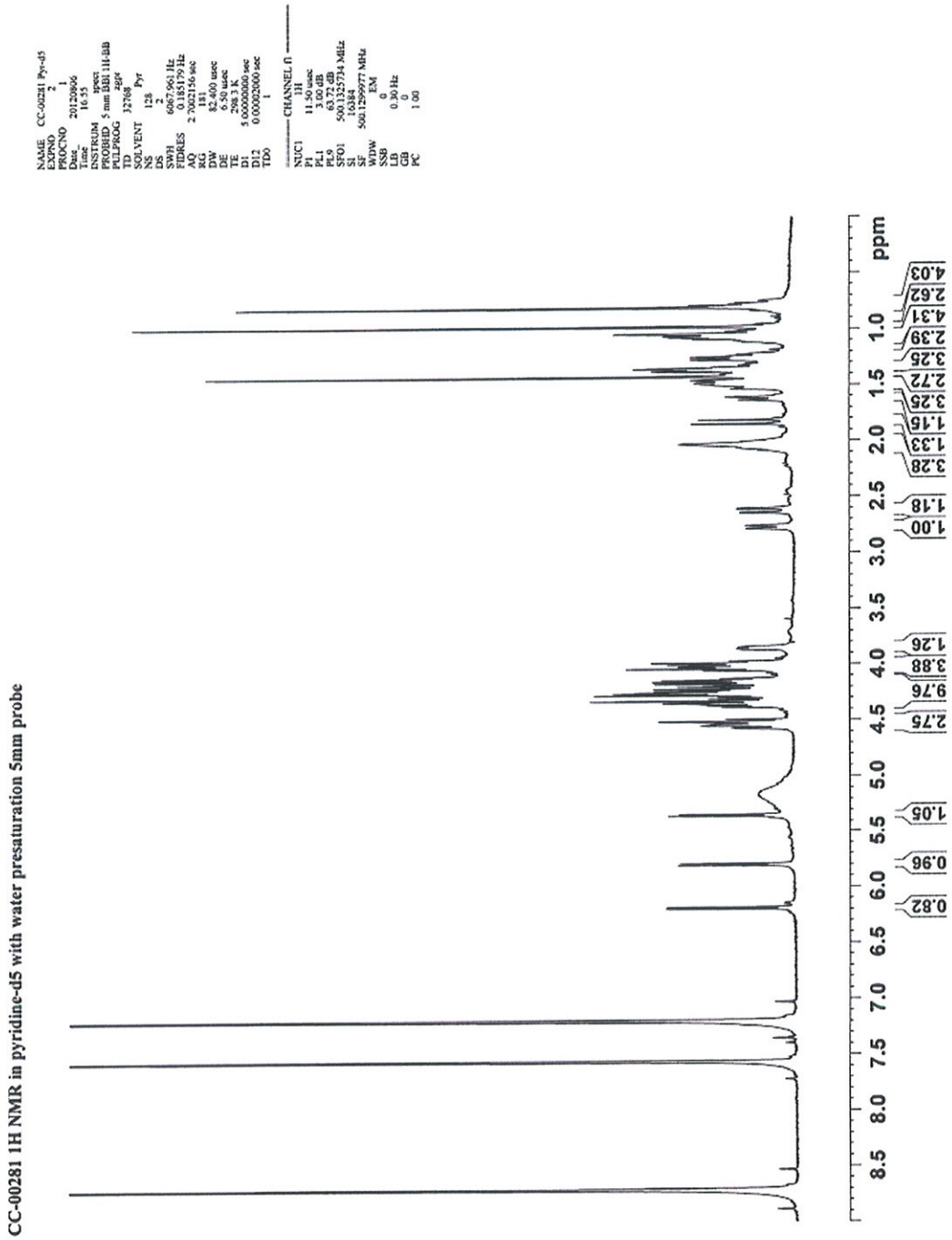
7.8 Figure 8. ESI+ TOF MS/MS analysis of CC-00281 selecting the $[M+H]^+$ ion at *m/z* 805 for fragmentation.



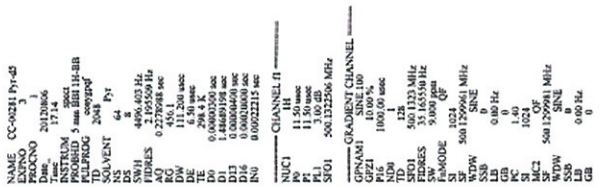
7.9 Figure 9. ESI- TOF MS/MS analysis of CC-00281 selecting the $[M-H]^-$ ion at *m/z* 803 for fragmentation.

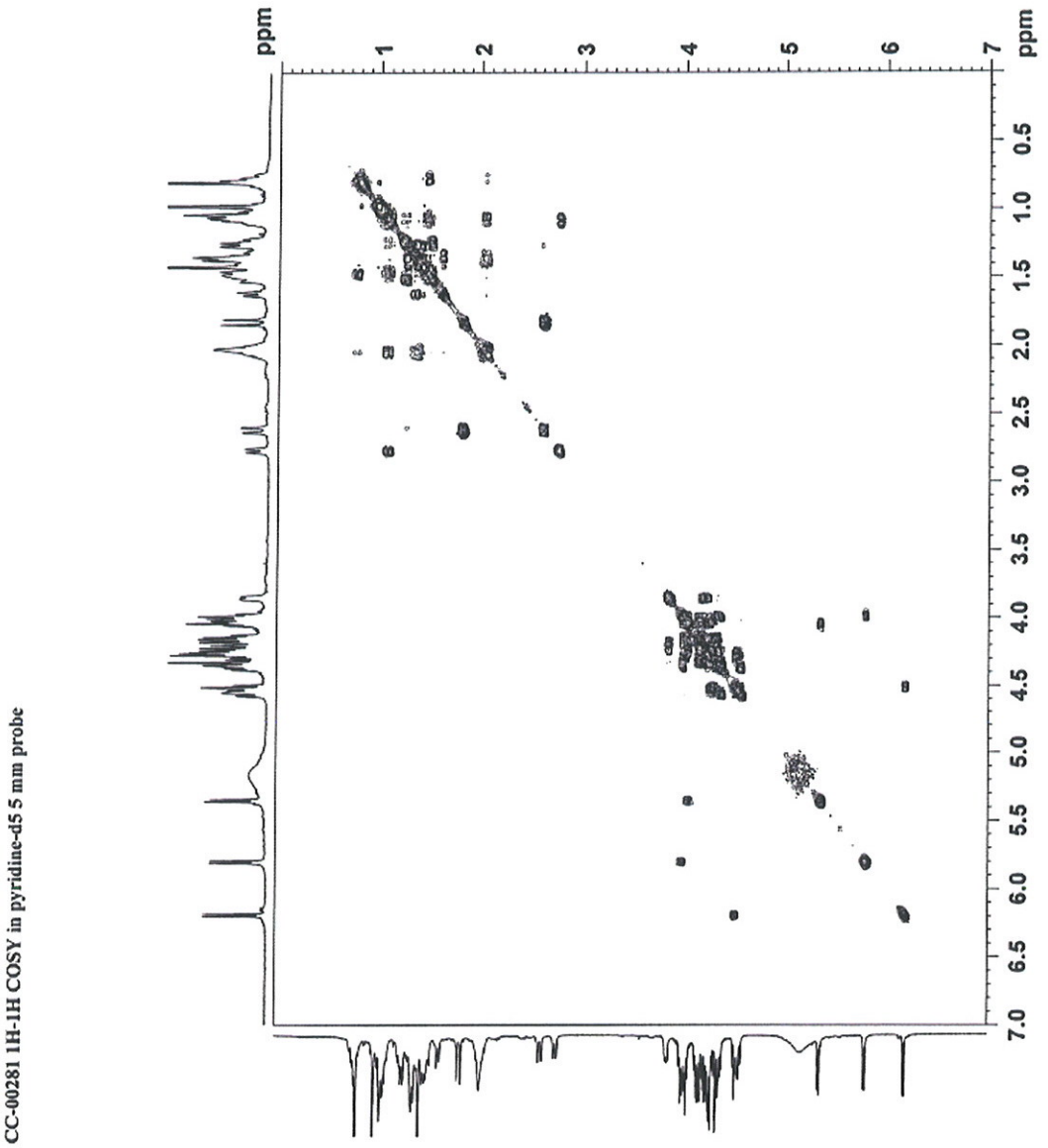


7.10 Figure 10. ^{1}H NMR (500 MHz, pyridine-d_5) of CC-00281.



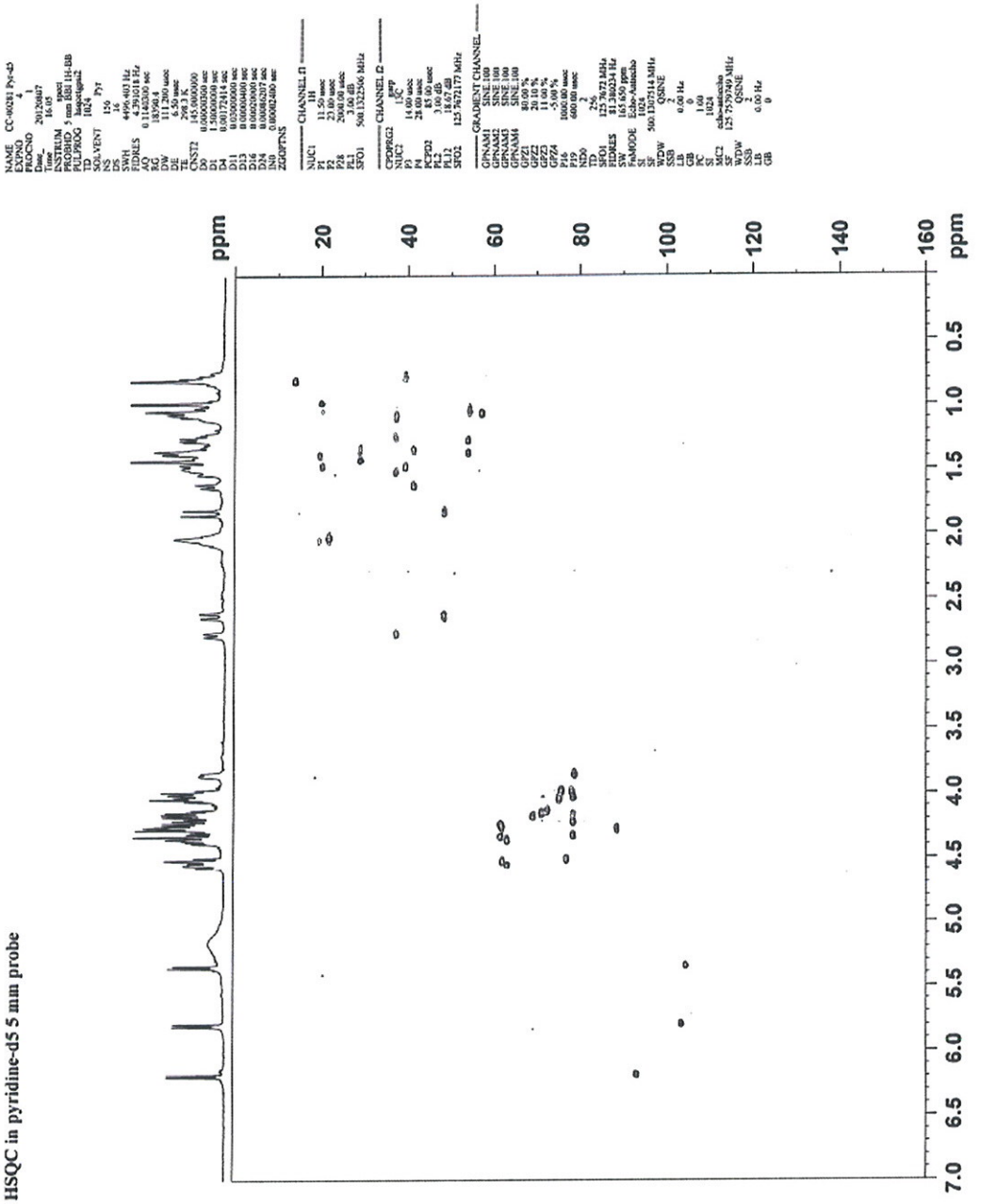
7.11 Figure 11. ^{1}H-^{1}H COSY Spectrum (500 MHz, pyridine-d_5) of CC-00281.



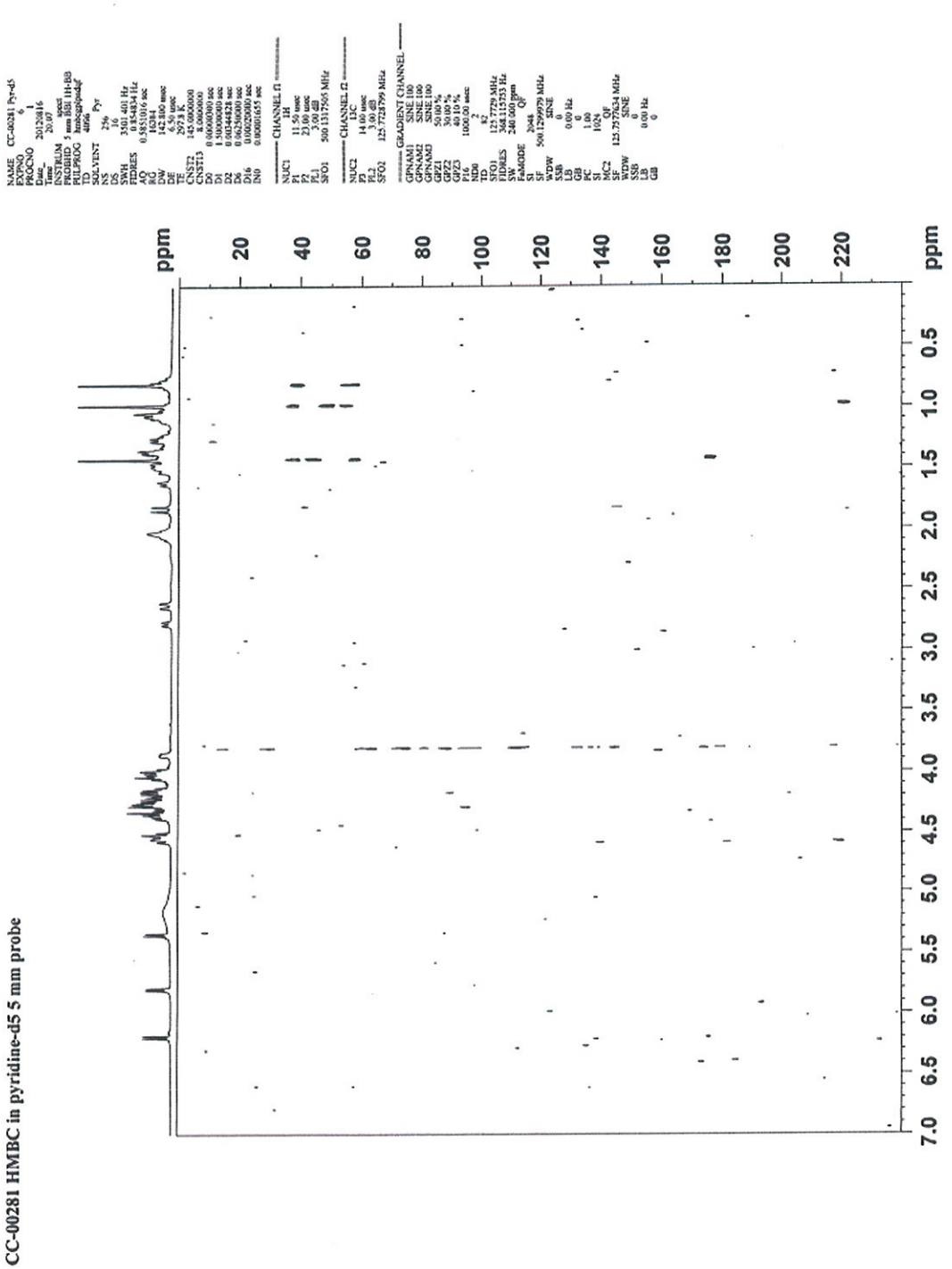


CC-00281 1H-1H COSY in pyridine-d5 5 mm probe

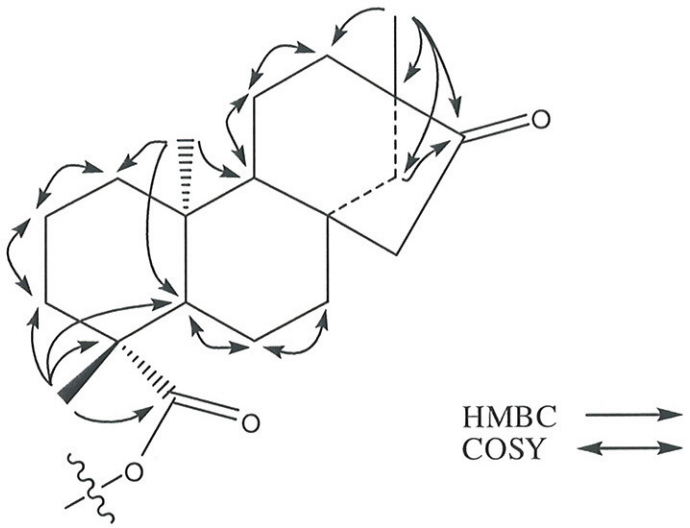
7.12 Figure 12. HSQC Spectrum (500 MHz, pyridine-d_5) of CC-00281.



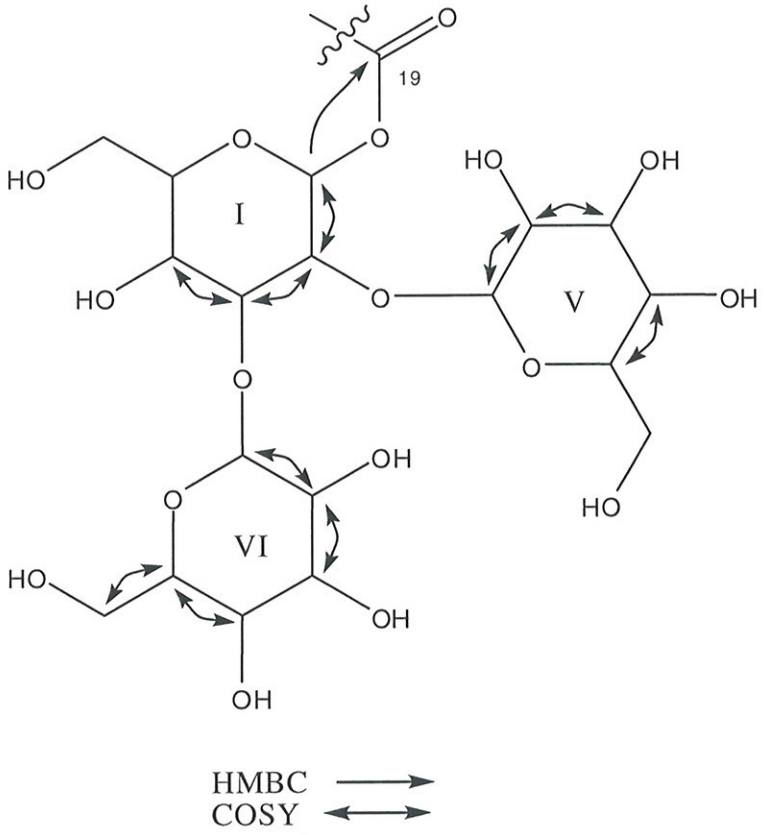
7.13 Figure 13. HMBC Spectrum (500 MHz, pyridine-d_5) of CC-00281.



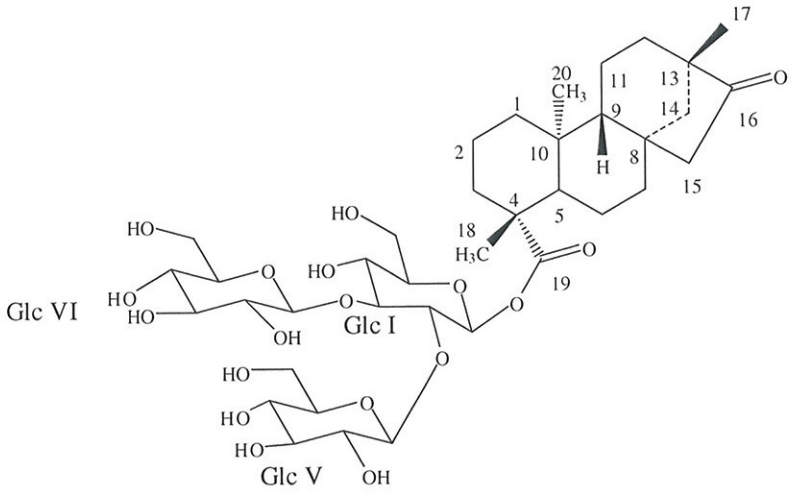
7.14 Figure 14. Summary of Key HMBC and COSY correlations used to assign the aglycone region of CC-00281.



7.15 Figure 15. Summary of Key HMBC and COSY correlations used to assign the C-19 glycoside region of CC-00281.



7.16 Figure 16. Structure of CC-00281.



CC-00281

7.17 Table 1. ^{1}H and ^{13}C NMR (500 and 125 MHz, pyridine-d_5) Assignments of the CC-00281 aglycone.

	CC-00281	
Position	^{13}C	^{1}H
1	39.4	0.77 td (3.8, 13.2) 1.47 m
2	21.6	2.02 m
3	37.3	1.07 m 2.77 d (13.1)
4	44.1	---
5	57.1	1.06 m
6	19.4	1.38 m 2.04 m
7	41.3	1.34 m 1.61 m
8		---
9	54.4	1.04 m
10		---
11	20.1	1.04 m 1.46 m
12	37.1	1.24 m 1.51 m
13	54.2	---
14	48.4	1.83 d (18.5) 2.62 dd (3.1, 18.5)
15	53.8	1.27 m 1.36 m
16	220.7	---
17	19.9	0.98 s
18	28.8	1.42 s
19	175.8	---
20	13.8	0.81s

7.18 Table 2. ^{1}H and ^{13}C NMR (500 and 125 MHz, pyridine-d_5) Assignments of the CC-00280 C-19 glycoside.

	CC-00281	
Position	^{13}C	^{1}H
Glc_I-1	93.0	6.19 d (8.1)
Glc_I-2	76.8	4.51 t (8.9)
Glc_I-3	88.4	4.27 t m
Glc_I-4	69.1	4.17 m
Glc_I-5	78.1	3.98 m
Glc_I-6	62.0	4.24 m 4.52 m
Glc_V-1	103.4	5.79 d (7.9)
Glc_V-2	75.7	3.99 m
Glc_V-3	78.3	4.32 m
Glc_V-4	72.4	4.13 m
Glc_V-5	78.4	4.02 m
Glc_V-6	63.2	4.36 m 4.56 m
Glc_{VI}-1	104.5	5.35 d (7.9)
Glc_{VI}-2	75.2	4.04 t (8.2)
Glc_{VI}-3	78.3	4.21 m
Glc_{VI}-4	71.2	4.14 m
Glc_{VI}-5	78.7	3.85 m
Glc_{VI}-6	61.6	4.24 m 4.33 m