Carbohydrate Research, 245 (1993) 259–270
Elsevier Science Publishers B.V., Amsterdam

On the structure of calonyctin A, a plant growth regulator

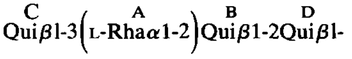
Yiwei Fang, Wengang Chai, Suming Chen, Yuzhen He, Lu Zhao, Jianhe Peng, Haiwang Huang and Bin Xin

Institute of Chemistry, Academia Sinica, Beijing 100080 (China)

(Received July 8th, 1991; accepted in revised form November 9th, 1992)

ABSTRACT

The plant growth regulator calonyctin A, isolated from the dried leaves of *Calonyction aculeatum* L. House (Yue-Guang-Hua), was separated into two pure components by high performance liquid chromatography. By use of mass spectrometry based on various ionization techniques, one- and two-dimensional NMR spectroscopy, IR spectrometry, and chemical methods the molecular structures of the two homologous glycosides were determined. Each molecule contains two hydroxy fatty acid residues and four 6-deoxyhexose units. The fatty acids are 3-hydroxy-2-methylbutanoic acid and 11-hydroxytetradecanoic acid or 11-hydroxyhexadecanoic acid. The 6-deoxyhexose residues (three of quinovose and one of rhamnose) comprise a tetrasaccharide having the following structure:



$$\overset{C}{\text{Qui}}\beta 1\text{-}3\left(\text{L-}\overset{A}{\text{Rha}}\alpha 1\text{-}2\right)\overset{B}{\text{Qui}}\beta 1\text{-}2\overset{D}{\text{Qui}}\beta 1\text{-}$$

The long-chain hydroxy acid is linked glycosidically through its O-11 to Qui D and esterified to O-2 of Qui C, forming a macrocyclic lactone. The 3-hydroxy-2-methylbutanoic acid is ester-linked to O-3 of Qui C.

INTRODUCTION

Yue-Guang-Hua (moonlight flower, *Calonyction aculeatum* L. House) is a plant of the family Convolvulaceae. Extensive field experiments on farms and agricultural experiment stations in the Fujian, Jilin, and Liaoning areas demonstrated the promoting effect of extracts from Yue-Guang-Hua leaves on the growth and yield of various crops, such as yams, peanuts, beans, and wheat[1,2].

In the late 1970s, in collaboration with the phytohormone research group of Amoy University, we undertook a preliminary study of the structure of calonyctin A (designated as DC-2), a white crystalline substance showing the strongest physiological activity among several components isolated from the Yue-Guang-Hua leaves. The preliminary results indicated that DC-2 is a glycoside[3]. The substance was then hydrolyzed in ethanolic sulfuric acid, yielding an aglyconic fragment that was shown to be ethyl 11-hydroxyhexadecanoate[4]. Huang et al.[5] and Hu et al.[6] published further results on the structure of DC-2, and we recently carried out a detailed study which showed that DC-2 is not a single substance. We now report the separation of DC-2 into two components, designated DC-2b and DC-2d, and the complete elucidation of their structures.

EXPERIMENTAL

Extraction.—Yue-Guang-Hua leaves were collected in Tong-An, Fujian Province, China. Dried leaves (200 g) were ground and extracted with 95% EtOH under reflux, essentially as described by the phytohormone research group of Amoy University[2]. The extract was decolorized by a passage through a charcoal column which was previously treated with acetic acid. The glycosides were eluted with 95% EtOH, and the EtOH was evaporated off. The residue was taken up in water, and the solution was extracted with EtOAc several times. Evaporation of the solvent gave a brown solid in a yield of 4% based on crude leaf powder.

Column separation.—The sample was applied to an Al_2O_3 (100–200 mesh, 160 g) column which was previously washed with EtOAc and then eluted sequentially with 350 mL portions each of 1 : 9, 2 : 8, 3 : 7, and 4 : 6 MeOH–EtOAc. The last two eluates, designated as C and D respectively, showed high physiological activity[2]. The combined D and C fractions were further chromatographed on a silica gel column (60–100 mesh, 70 g) that had been washed with $CHCl_3$, using 200 mL portions of 1 : 9, 2 : 8, and 3 : 7 abs EtOH–$CHCl_3$ as successive eluents. The second fraction (eluate 2 : 8) showed the highest physiological activity[2]. Evaporation of the solvent gave a yellowish, brittle solid, designated as DC-2, yield 0.4% based on crude leaf powder.

Purification.—DC-2 was subjected to HPLC on an ODS column (25 cm × 8 mm i.d.). Aqueous MeOH (13%) was used as the eluent at a flow rate of 2 mL/min, with monitoring by a refractive index detector. A sample chromatogram is shown in Fig. 1. The two major components, DC-2b and DC-2d, were collected for further study.

Both DC-2b and DC-2d form white hygroscopic crystals, easily soluble in MeOH, EtOH, acetone, ethyl ether, EtOAc, $CHCl_3$, acetic acid, DMF, and pyridine. They do not dissolve in petroleum ether and are sparingly soluble in water.

DC-2b had mp 165–166°C; $[\alpha]_D^{20} = -65°$ (c 1.0, abs EtOH); and DC-2d had mp 153–154°C; $[\alpha]_D^{20} = -55°$ (c 1.0, abs EtOH). Neither compound showed UV absorption in the range 200–400 nm.

Preparation of partially methylated alditol acetates and GLC–MS analysis.—Permethylation was carried out essentially by the method of Ciucanu and Kerek[7]. The permethylated glycoside (10 mg) was hydrolyzed with 2 N CF_3COOH (1 mL) for 2 h at 100°C. The hydrolyzate was dried and reduced with $NaBH_4$ (5 mg) in 0.5 N NaOH (3 mL) for 8 h at 25°C, anhyd acetic acid was added drop by drop until bubbling ceased, then borate was removed by four successive additions and evaporations of abs MeOH (1 mL). The reduced product was heated with Ac_2O (0.5 mL) for 3 h at 100°C, then cooled, and the reagent was removed by two successive additions and evaporations of toluene (1 mL). GLC–MS analysis was

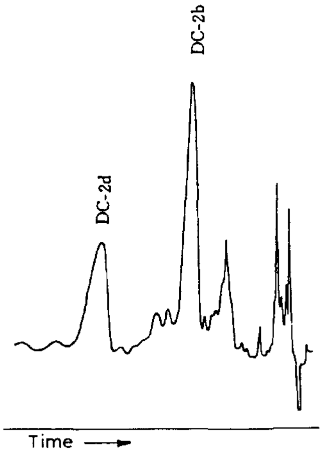


Fig. 1. HPLC resolution of fraction DC-2.

performed on a Finnigan 4500 instrument. The capillary column used was OV 225 (28 m × 0.25 mm i.d.) with He as the carrier gas. The column temperature was initially 180°C programmed to 260°C at 5°C/min. Mass spectra were recorded at 70 eV at a source temperature of 250°C.

Acidic hydrolysis of DC-2b and DC-2d.—Solutions of DC-2b and DC-2d (5 mg) in 10% methanolic HCl (0.2 mL) were heated at 100°C for 3 h in sealed vials filled with N_2. Water (0.2 mL) was added to the residues and the mixtures were extracted with ether several times. The organic phases on drying yielded the nonsugar hydrolysis products.

Instrument measurements.—Liquid secondary ion mass spectrometry (LSIMS), at low resolution in both negative- and positive-ion modes, and at high resolution for accurate mass measurement, was performed on a VG ZAB-2E mass spectrometer. The instrument was fitted with a Cs ion gun operating at 25 keV for negative-ion detection and 35 keV for positive-ion detection, with an emission current of 0.5 μA. Data were collected with a VG Analytical 11-250J data system operating in the continuous acquisition mode, matrix glycerol with 2% PEG 1000 as an internal standard, resolution for exact mass measurements 6500. Field desorption (FD) mass spectra were recorded on a MAT 711 instrument, emission current 1 mA; electron ionization (EI) mass spectra were obtained on an AEI MS 50 at an ionization voltage of 70 eV and an accelerating voltage of 8 kV.

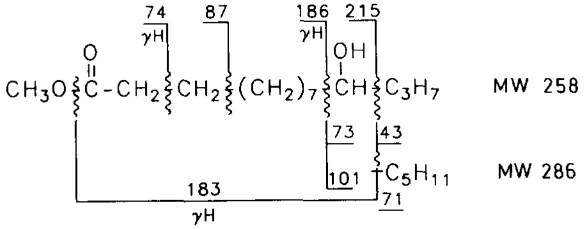
^{13}C DEPT, 2D ^{1}H–^{1}H COSY, 2D ^{13}C–^{1}H COSY, 2D NOESY, and selective INEPT spectra were taken on a Jeol GX 400 spectrometer, and 2D *J*-resolved, 2D TOCSY, and gated decoupled spectra were recorded with a Bruker AM 500 instrument, both operating at 25°C. Pyridine-d_5 was used as the solvent and Me_4Si as the internal standard. Infrared spectra were recorded on a Bruker IFS-113V spectrometer with the samples in KBr pellets. Optical rotations were measured on a Perkin–Elmer 241 polarimeter.

RESULTS AND DISCUSSION

The negative-ion LSIMS spectra for DC-2b and DC-2d are shown in Fig. 2. From these molecular weights of DC-2b and DC-2d were taken to be 910 and 938, respectively. The FD spectra showed quasimolecular ions (MH^+) at *m/z* 911 and 939 (when K^+ was present, MK^+ appeared in addition to MH^+), which confirmed the proposed molecular weights. The accurate masses of the MH^+ ions obtained from high resolution, positive-ion LSIMS measurements are 911.4863 and 939.5176, respectively, which correspond to the elemental composition $C_{43}H_{75}O_{20}$ for DC-2b and $C_{45}H_{79}O_{20}$ for DC-2d. Deviations in each case are less than 1.2 ppm. From their differences in molecular weight the two compounds must differ by a C_2H_4 unit (28 amu). The LSIMS spectra showed very similar fragmentation patterns in both the positive- and negative-ion modes. The difference between the masses of the corresponding ions from each compound is 28 amu. Fragment ions resulting from sequential losses of 146 amu appeared in all LSIMS spectra, namely *m/z* 911 → 765 → 619 → 473 in the positive-ion spectrum and *m/z* 809 → 663 → 517 → 371 → 225 (or *m/z* 681 → 535 → 389 → 243) in the negative-ion spectrum of DC-2b; *m/z* 939 → 793 → 647 → 501 in the positive-ion spectrum and 837 → 691 → 545 → 399 → 253 (or *m/z* 709 → 563 → 417 → 271) in the negative-ion spectrum of DC-2d, suggesting the pressence of three or four deoxyhexose residues.

DC-2b and DC-2d showed very similar ^1H and ^{13}C NMR spectra. The ^{13}C DEPT data (Table I) obtained from DC-2b showed seven CH_3, eleven CH_2, and twenty-two CH groups, and two quaternary carbon atoms. The four signals appearing at $\sim \delta_C$ 100 and the four signals at δ_C 17–18 are characteristic for C-1 and C-6, respectively, of 6-deoxyhexoses. From the ^{13}C–^1H correlation spectrum (Fig. 3) and the above mass spectral data the presence of four 6-deoxyaldohexoses can be concluded.

The structures of the aglycons.—After the methanolysis of glycosides DC-2b and DC-2d derivatives of the nonsugar fragments were isolated. The EI mass spectra of these derivatives are shown in Fig. 4. Very similar fragment ions were obtained for both compounds, such as those appearing at *m/z* 74, 87, 101, 115, 129, 143, 157, 183, and 215, which are characteristic for the methyl esters of long-chain hydroxy fatty acids (see fragmentation pattern). The position of the hydroxyl in the aglycons is revealed by the *m/z* value of the base peak[8], 183. This indicates a chain of nine methylene groups between the carbonyl group and the carbinol group. FD mass spectra gave protonated molecules (MH^+) at *m/z* 259 and 287, indicating molecular weights of 258 and 286, respectively, for the aglycons. The difference in molecular weights is 28 amu. On the basis of this analysis, the methanolysis products could be assigned the structures of methyl 11-hydroxytetradecanoate and 11-hydroxyhexadecanoate for DC-2b and DC-2d, respectively. The latter structure is in accord with the previous report[4], in which DC-2 was treated with 5% ethanolic sulfuric acid and an ethyl ester obtained. From these results, the aglycons can be identified as 11-hydroxytetradecanoic acid (MW 244) and its structural homolog 11-hydroxyhexadecanoic acid (MW 272). The masses are in good agreement with the prominent ions appearing at *m/z* 243 and 271 in the negative ion LSIMS spectra of DC-2b and DC-2d, respectively.



The structure of the oligosaccharide moiety.—With the aid of ^1H–^1H COSY (Fig. 5), ^{13}C–^1H COSY (Fig. 3), TOCSY (HOHAHA) (Fig. 6), and 2D NOESY (Fig. 7) NMR spectra, all the signals of the four hexose residues could be readily assigned, and the linkage positions could be established. The ^{13}C signal at δ 71.96 was found to represent two carbon atoms, and thus the total number of carbons is 43 for DC-2b, consistent with the high resolution LSIMS data. The chemical shifts of the carbon and hydrogen atoms of the four hexose units A, B, C, and D are listed in Table II, with indication of the linkage positions: A-(1 → 2)-B, B-(1 → 2)-D, C-(1 → 3)-B, D-(1 → aglycon).

According to the literature[9], a signal from hexose C-6 at δ_C 17–18 is attributable to rhamnose. Further details of the oligosaccharide structure could be obtained through 2D *J*-resolved (homonuclear) spectra. The *J* values for all residues are listed in Table III. The $J_{C1\text{-}H1}$ values for residues A, B, C, and D, obtained from gated decoupling, are 170.9, 158.3, 164.3, and 156.8 Hz, respectively. From this it could be concluded that A is α-rhamnopyranose[10]. The coupling

TABLE I

^{13}C Chemical shifts for compound DC-2b

Stuctural element [a]	Chemical shifts (δ)						
$-CH_3$	20.88	18.78	18.45	18.40	17.67	14.78	13.19
$-CH_2-$	38.84	35.68	33.43	31.43	30.80	30.21	30.13
	28.90	25.46	25.21	18.83			
$>$CH	103.12	101.96	101.58	100.38	84.81	80.79	79.25
	79.17	78.91	77.17	75.98	74.50	74.18	73.91
	73.15	72.84	72.31	72.17	71.96	69.56	69.05
	48.46						
$-\overset{\vert}{\underset{\vert}{C}}-$	173.94	175.02					

[a] From the DEPT spectrum.

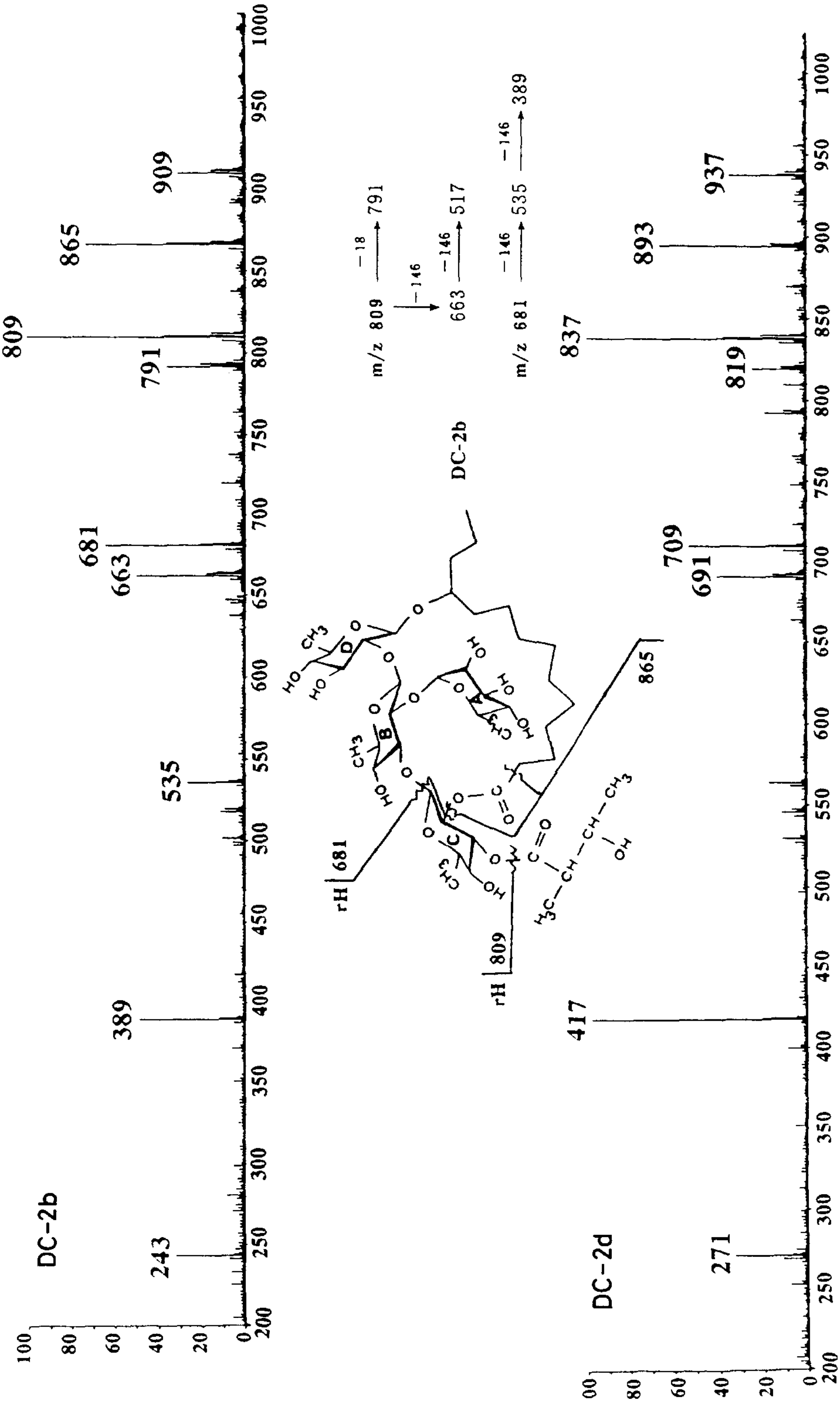


Fig. 2. The negative-ion LSIMS spectra of DC-2b and DC-2d.

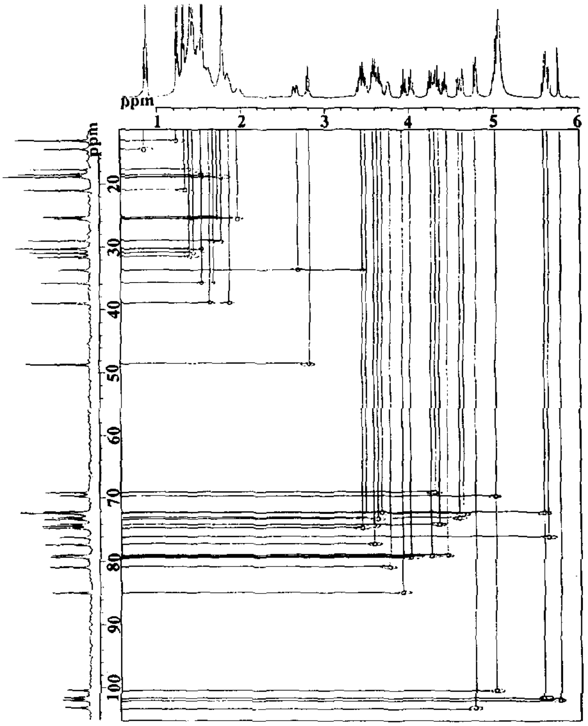


Fig. 3. 400 MHz 2D ^{13}C–^{1}H COSY spectrum of DC-2b transformation involved 256×2048 points; F_1, 4092 Hz; F_2, 20833. 3 Hz.

constants of the protons on adjacent carbon atoms are all relatively large (7–10 Hz), except for $J_{1,2}$ and $J_{2,3}$ of residue A. This indicates that all the ring protons of residues B, C, and D are axially disposed, and hence that these residues must be 6-deoxy-β-glucopyranose, i.e., β-quinovose. The presence of L-rhamnose was es-

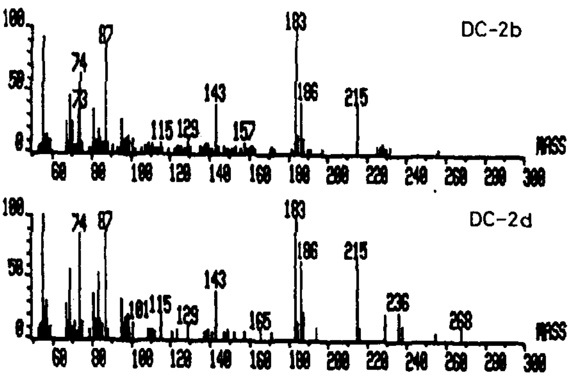


Fig. 4. EI mass spectra of methyl esters of the aglycons DC-2b and DC-2d.

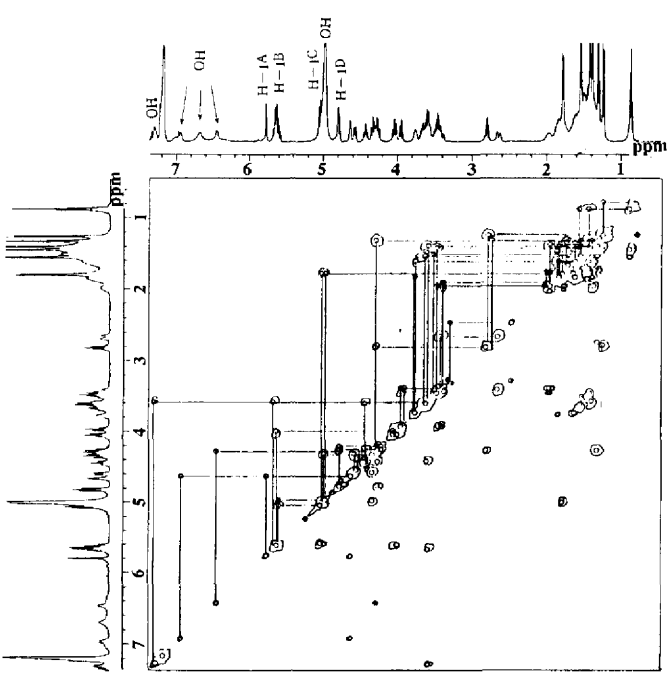


Fig. 5. 400-MHz 2D $^1H-^1H$ COSY spectrum of DC-2b. The 2D transformation involved 512×1024 points; $F_1 = F_2 = 4790.2$ Hz; delay between aquistions, 1.0 s.

tablished by paper chromatography of the component sugars[3] of DC-2. We could not obtain an authentic sample of quinovose, but the quinovose of natural glycosides usually exists in the D-configuration, so the quinovose residues B, C, and D are provisionally indicated as belonging to the D series.

DC-2b and DC-2d were permethylated and hydrolyzed, and the products were reduced and acetylated. GLC–MS analysis of the mixture indicated the presence of 1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methylhexitol (C-1 as the linkage position), 1,2,5-tri-*O*-acetyl-6-deoxy-3,4-di-*O*-methylhexitol (C-1 and C-2 as the linkage positions) and 1,2,3,5-tetra-*O*-acetyl-6-deoxy-4-*O*-methylhexitol (C-1, C-2, and C-3 as the linkage positions), on the basis of comparison with reference spectra[11]. This result was in good agreement with the NMR data. In addition, spectra of the derivatized aglycons were also obtained from the GLC–MS experiment.

The structure of the acyl substituent.—The 2D $^1H-^1H$ COSY spectrum showed the following correlated signals: 1.25 (CH_3, δ_C 13.19) ↔ 2.8 (CH, δ_C 48.46) ↔ 4.28 (CH, δ_C 69.05) ↔ 1.23 (CH_3, δ_C 20.88). Taking this information into account, together with the elemental composition, number of carboxyl groups, and the δ_H and δ_C values, it can be deduced that the first CH is linked to the carboxyl group and the second CH to a hydroxyl group. Subtraction of the masses of the aglycon and the four 6-deoxyaldohexose residues from the molecular masses of DC-2b and DC-2d, respectively, suggested that there is an additional nonsugar moiety and

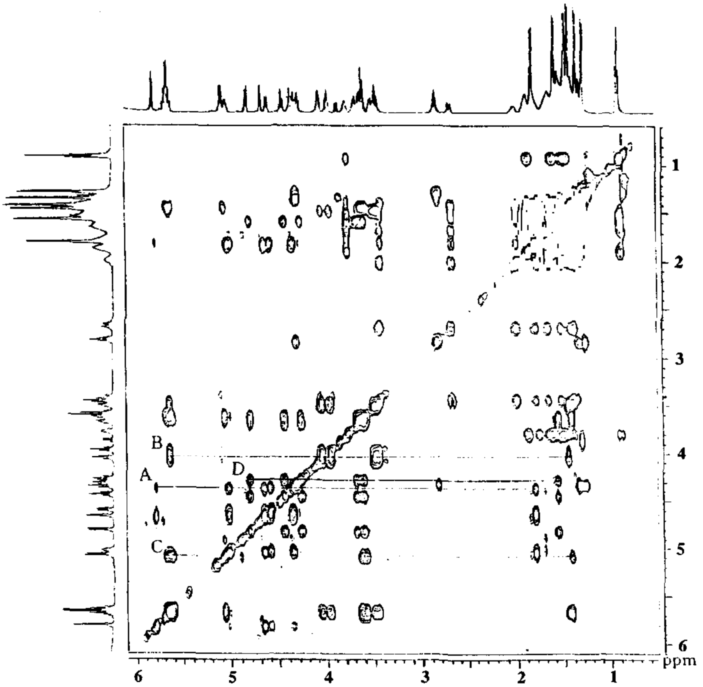
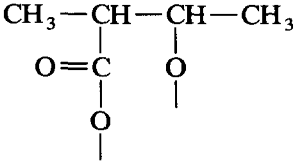


Fig. 6. 500-MHz 2D TOCSY spectrum. The 2D transformation involved 512×1024 points.

that its molecular mass is 118 Da. The following structural element was thus established:



$$\begin{array}{ccccccc} CH_3 & - & CH & - & CH & - & CH_3 \\ & & | & & | & & \\ & O= & C & & O & & \\ & & | & & | & & \\ & & O & & & & \\ & & | & & & & \end{array}$$

It can be concluded from further evidence based on methylation, hydrolysis, and GLC–MS analysis of DC-2d that this structural element is an acyl substituent, and that its parent acid is 3-hydroxy-2-methylbutanoic acid.

The overall structures of DC-2b and DC-2d.—The 2D NOESY spectrum of the aglycon suggested that the oxygen of the hydroxyl group (hydroxylated -CH–, δ_C 80.79, δ_H 3.78) is linked to C-1 (δ_H 4.79) of hexose D. The inequality of the chemical shifts (δ_H 2.65 and 3.44) of the two protons of the α-CH_2 group of the aglycon excluded an adjacent free carboxyl function. Furthermore, the lack of a characteristic peak for a methyl or ethyl ester in the ^{13}C NMR spectrum suggested a lactone in which the carboxylic acid is esterified with a hydroxyl group of a sugar.

As shown in Table II both H-2 and H-3 of hexose C exhibited lowfield shifts (δ_H 5.64 and 5.65, respectively). Therefore, it can then be deduced that C-2 and C-3 of hexose C are connected with the carboxyl groups of the two acids.

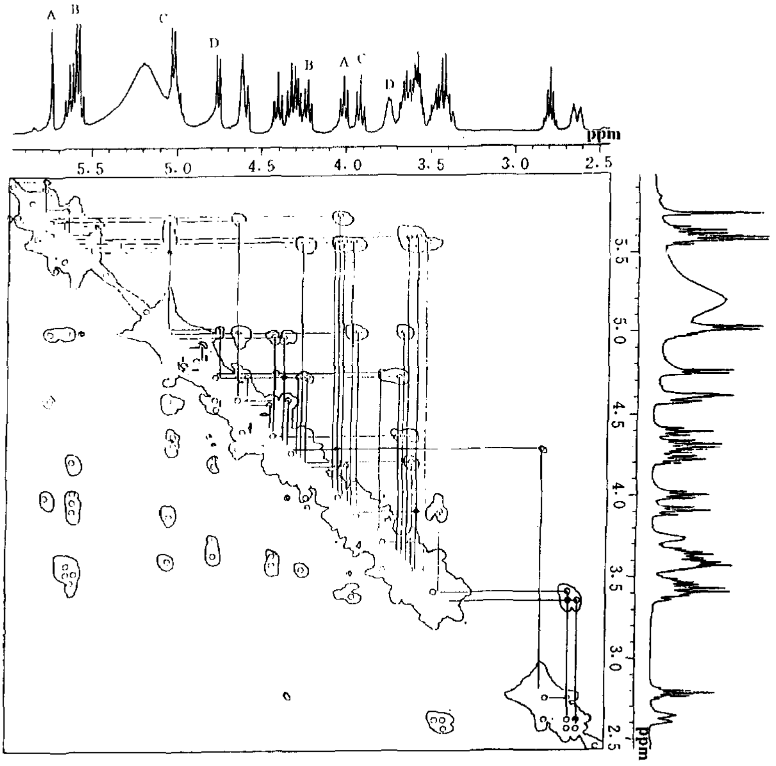


Fig. 7. Symmetry contour plot of 400-MHz 2D NOESY spectrum of DC-2b. The 2D transformation involved 512×1025 points; $F_1 = F_2 = 4970.2$ Hz; delay between acquisitions, 1.0 s; mixing time, 500 ms.

TABLE II

The ^{13}C and ^{1}H chemical shifts for the sugar residues of DC-2b and their linkage positions

Sugar residue	Chemical shifts (δ_C, first line; δ_{H_1} second line)					
	C-1	C-2	C-3	C-4	C-5	C-6
A	101.96 5.78 ↓ C-2B	72.31 4.64	72.84 4.58	73.91 4.33	69.55 4.98	18.78 1.79
B	101.58 5.64 ↓ C-2D	79.25 4.04 ↓ C-1A	84.81 3.95 ↓ C-1C	74.50 3.44	71.96 3.49	18.40 1.43
C	100.38 5.05 ↓ C-3B	72.17 5.64	75.98 5.65	77.18 3.59	73.15 3.62	17.67 1.40
D	103.12 4.79	79.17 4.26 ↓ C-1B	78.91 4.43	74.18 3.59	71.96 3.67	18.45 1.55

TABLE III

^1H–^1H Coupling constants for the sugar residues of DC-2b

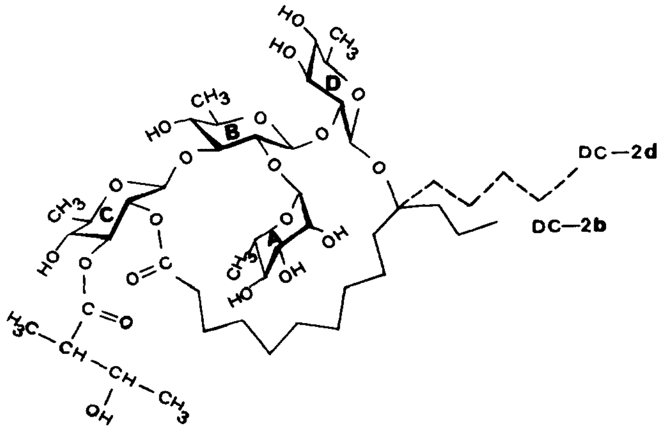
Sugar residue	Coupling constants (Hz)				
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$
A	2.1	3.5	9.3	9.5	6.4
B	7.7	9.2	9.0	9.2	6.4
C	7.7	9.2	9.2	9.2	6.0
D	7.7	9.1	9.1	9.5	6.0

The FT-IR spectra of DC-2b and DC-2d were essentially the same. In both the carbonyl group of an ester exhibits a prominent peak at 1735 cm^{-1}, with a shoulder on the high frequency side.

A selective INEPT (NMR) experiment (400 MHz, in deuterated DMF to increase the shift difference between H-2 and H-3 of hexose C) further indicated that the carboxyl group of the aglycon is linked to O-2 of hexose C, and that the second acyl substituent is linked to O-3 of the same hexose. Moreover, the assignment of the signals of the two carboxyl carbons can also be readily made. The chemical shift of the carboxyl C of the aglycon is 173.94, and that of the second acyl substituent is 175.02 ppm.

In the negative-ion SIMS spectra of both DC-2b and 2d prominent ions at m/z 865 or 893 $(M-44)^-$ could be attributed to the products of bond cleavage between the ester oxygen of the aglycon and C-2 of hexose C followed by loss of CO_2. The ions at m/z 809 or 837 $(M-100)^-$ result from the cleavage of the 3-hydroxy-2-methylbutanoyl group from the sugar with rearrangement of a hydrogen atom. The ions at m/z 791 or 819 $(M-118)^-$ are the products of the elimination of 3-hydroxy-2-methylbutanoic acid followed by a hydrogen rearrangement, and the ions at m/z 681 or 709 $(M-228)^-$ result from the loss of the acid and hexose C, each followed by a hydrogen rearrangement.

In conclusion, the overall structures of DC-2b and DC-2d, which are homologs, are as follows:



ACKNOWLEDGMENTS

Support of this work by the National Natural Science Foundation of China is gratefully acknowledged. We thank the Phytohormone Research Group of Amoy University, in particular Professors Guanghui Wang, Zeliang Bian, Yongquan Lu, and Zhiquan Kang, for their valuable assistance.

REFERENCES

1 J.-L. Ding, *Acta Nong Ye*, 3 (1952) 17–24.
2 Phytohormone Research Group of Amoy University, *Acta Amoy University*, Issue 1 (1974) 40–50.
3 Q.-Z. Guo, T.-S. Wang, Y.-W. Fang, and Z.-L. Bian, *Acta Amoy University*, Issue 1 (1980) 83–91.
4 Y.-W. Fang, Z.-L. Bian, T.-C. Chiang, Q.-Z. Guo, *Kexue Tongbao (Chin. Ed.)*, 26 (1981) 468–470; Engl. Ed., 26 (1981) 806–809.
5 H.-S. Huang, Y.-C. Hu, R. Pastov, G. Serratrice, *Acta Amoy University*, 25 (1986) 588–593.
6 Y.-C. Hu, Q.Z. Guo, R. Pastov, G. Serratrice, A. Cambon, C. Bosso, *Youji Huaxue*, 9 (1989) 146–150.
7 I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
8 F.W. McLafferty, *Mass Spectrometry of Organic Ions*, Academic, NY, 1963, p 430.
9 K. Bock, C. Pedersen, and H. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 193–224.
10 R. Kasai, M. Okihara, J. Asakawa, K. Mitzutani, and O. Tanaka, *Tetrahedron*, 35 (1979) 1427–1432.
11 B. Founet, G. Strecker, Y. Leroy, and J. Montreuil, *Anal. Biochem.*, 116 (1981) 489–502.