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PITUMBIN, a NOVEL KOLAVENE ACYLAL from *Casearia pitumba* Pleumer

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ABSTRACT: The structure of pitumbin 1, a kolavene diterpene possessing a new oxygenation pattern, was determined by the extensive use of one- and two-dimensional NMR techniques.

In addition to a certain number of new triterpenoids[2], several biogenetically interesting compounds - including cyclopentenylglycine[3], which acts as a precursor of equally unusual cyclic fatty acids[4] - have been isolated from plants of the Flacourtiaceae family.

In the present communication we report on the isolation of pitumbin 1, a kolevane acylal from the seeds of *Casearia pitumba* Pleumer. Its structure has been elucidated mainly by a complete set of two-dimensional NMR experiments including the INADEQUATE[5] experiment.

The new compound - a colourless oil - has a molecular formula of $C_{34}H_{48}O_8$ as recognized from the high resolution molecular ion peak. Its IR spectrum presented characteristic bands of saturated ester (1750 cm^{-1}), unsaturated esters (1710 cm^{-1}) and hydroxyl (3400 cm^{-1}) functions.

The ^{1}H-NMR spectrum(CD_3COCD_3) revealed the occurrence of five methyl groups, namely two acetates at 2.15 and 1.96 *ppm*, a further singlet at 1.16 *ppm* , one doublet (J_{HH}=7 Hz) at 1.10 *ppm* and one triplet (J_{HH}=7 Hz) at 1.03 *ppm*. In the low field part of the spectrum the signals of a conjugated diene, a monosubstituted double bond as well as a trisubstituted double bond were recognized. The ^{13}C-NMR spectrum confirmed the above features and indicated that 1 contains also a conjugated carbonyl function (165.99 *ppm*) and five carbon-carbon double bonds (Table 1). Pitumbin 1 is thus a tricyclic compound.

Attempts to chemically modify the basic structure under a variety of mild conditions inevitably led to an extensive degradation of the natural product. For instance, upon basic treatment (*Z,E*)2,4 -decadienoic acid (designated by the abbreviation d in the following Figures and Tables) was recovered as the only reaction product as identified by its ^{1}H and ^{13}C-NMR spectra[6].

We therefore initiated a detailed NMR study using the full potentialities of two-dimensional experiments.

First a homonuclear multiple quanta filtered COSY[7] experiment allows to identify the scalarly coupled proton resonances. A one bond ^{1}H-^{13}C heteronuclear COSY experiment[8,9] (Figure 1) discriminates the methylene protons from the methine protons and provides the protonated substructures which are indicated in Figure 2 along with a tertiary methyl group, the two acetates and two isolated singlets at 6.66 *ppm* and 6.56 *ppm* paired to two carbons atoms at 99.15 and 96.21 *ppm*, whose chemical shifts indicate a probable polysubstitution by heteroatoms.

The positions of the double bonds and oxygen atoms on fragments A-F in Figure 2 were all tentatively deduced from chemical shifts considerations (^1H and ^{13}C, see Table 1).

Among the salient features one should also note the presence of a terminal disubstituted double bond as indicated by the observation of the two singlets at 5.17 and 5.15 *ppm* coupled to a single olefinic carbon (115.71 *ppm*) and the confirmation of the monosubstituted double bond (as evidenced by the observation of an isolated system of three coupled protons at 5.18, 5.44 and 6.61 *ppm*, the former two being coupled to a single carbon atom at 113.08 *ppm*, the latter to a carbon atom at 141.44 *ppm*.

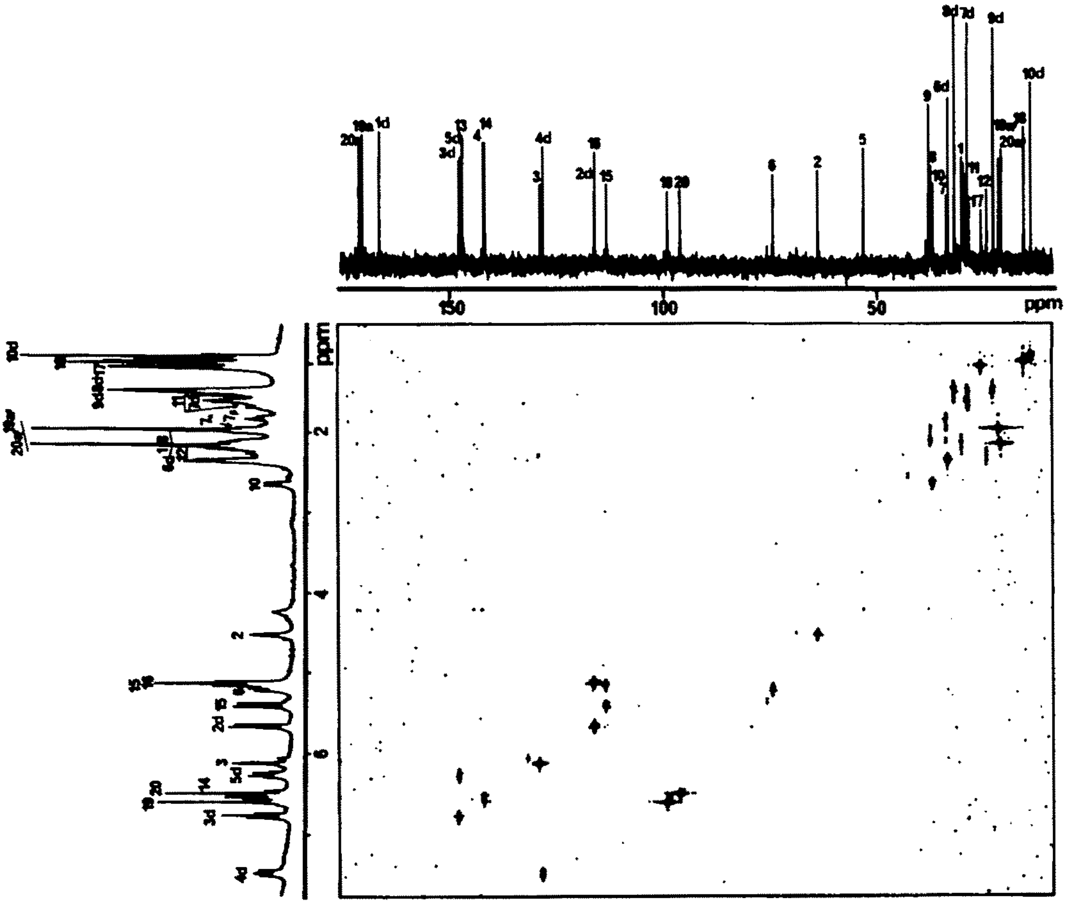


Figure 1 : One-bond ^1H-^{13}C correlation map of pitumbin 1

Additional small couplings could be observed between a signal at 6.56 *ppm* and the signals at 4.55 and 6.18 *ppm* in the COSY experiment.One of them is clearly an allylic coupling. The second corresponds to a homoallylic coupling and will be discussed below.

The interconnections between the various protonated fragments were deduced from long range ^1H-^{13}C experiments using various transfer and refocussing times, as the one represented in Figure 3 (the solid circles correspond to other connectivities revealed by a separate experiment).

In order to avoid the many experiments needed for obtaining all the necessary long range connectivities , we developed a phased, J-selection free experiment that proved to be an interesting alternative to the usual methodology but whose detailed description is beyond the scope of the present paper[10]. The new pulse sequence is shown in Figure 4; the basic idea was simply to replace all the fixed delays of the usual 2D ^1H-^{13}C-correlation sequence by incremented delays, transforming the associative transfer coefficients into an additionnal J-modulation. The phase cycle was elaborated using the general screw-pulse[11] concept and can be choosen as to lead to an amplitude modulation of the signals and consequently to a phase sensitive experiment.

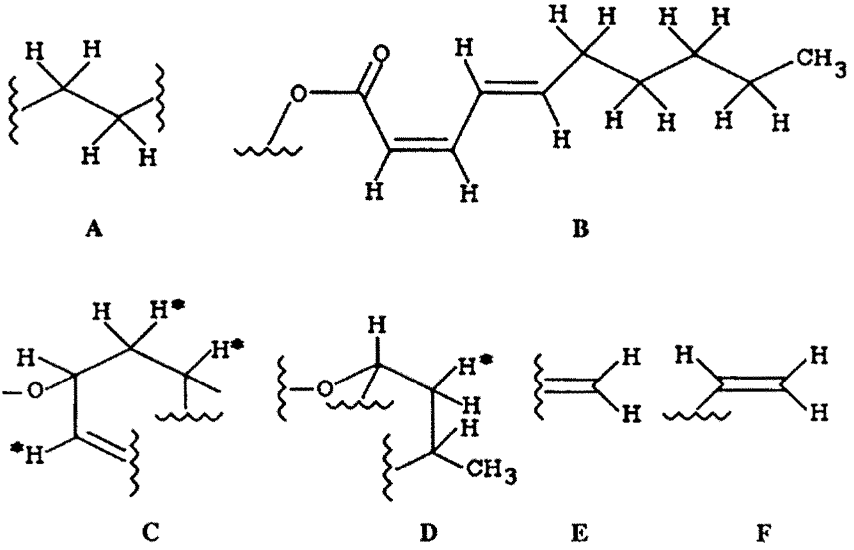


Figure 2 : Protonated fragments of pitumbin 1 as deduced from the MQT COSY and the one-bond ^{1}H-^{13}C correlation map.

Its potentialities are exemplified in Figure 4 corresponding to the vertical cross-section of a quaternary carbon atom at 53.48 *ppm* and showing the various proton connectivities to that carbon atom. Based on the reasonable hypothesis[12] that the sizeble long range couplings most probably correspond to couplings through two to four bonds, one can easily see that accommodation

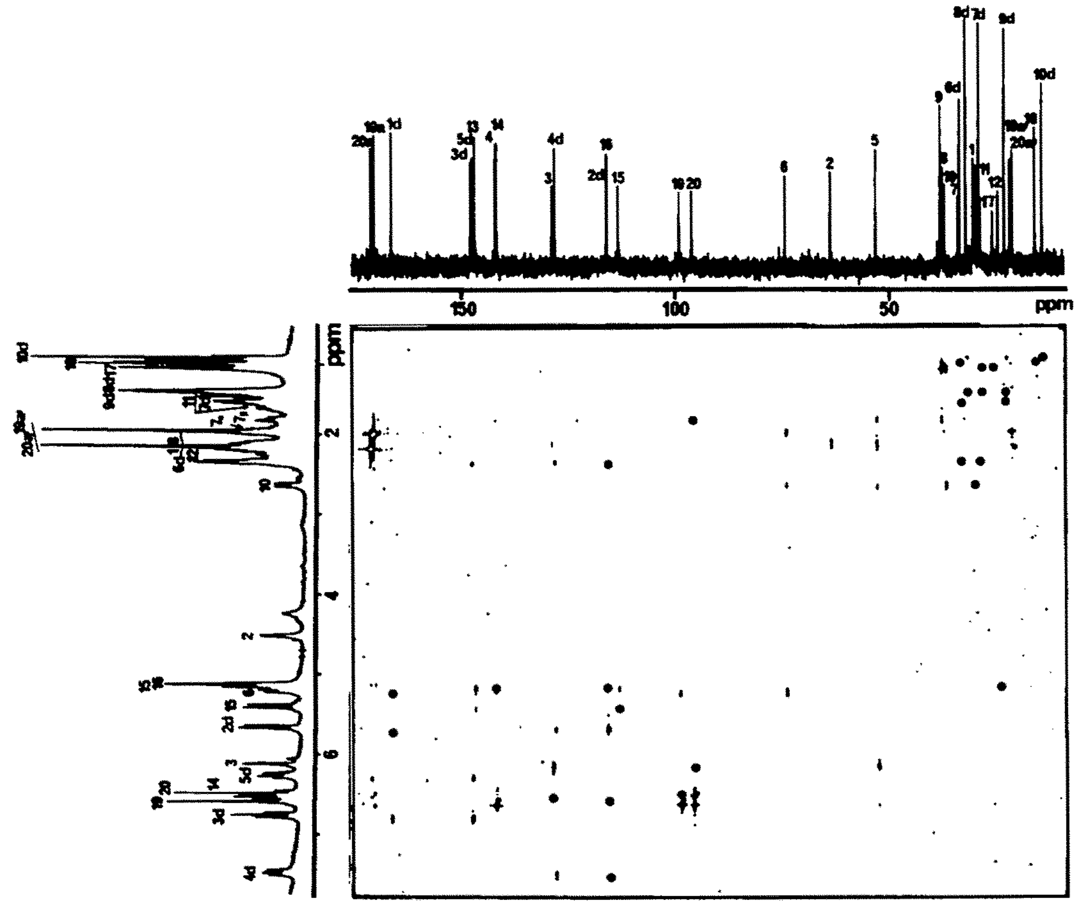


Figure 3 : Long range ^{1}H-^{13}C correlation map of pitumbin 1

of the observed couplings of that carbon atom to the various protons of the molecules (starred in Figure 2) simply leads to the closure of a six membered ring and allows to link the two protonated fragments C and D.

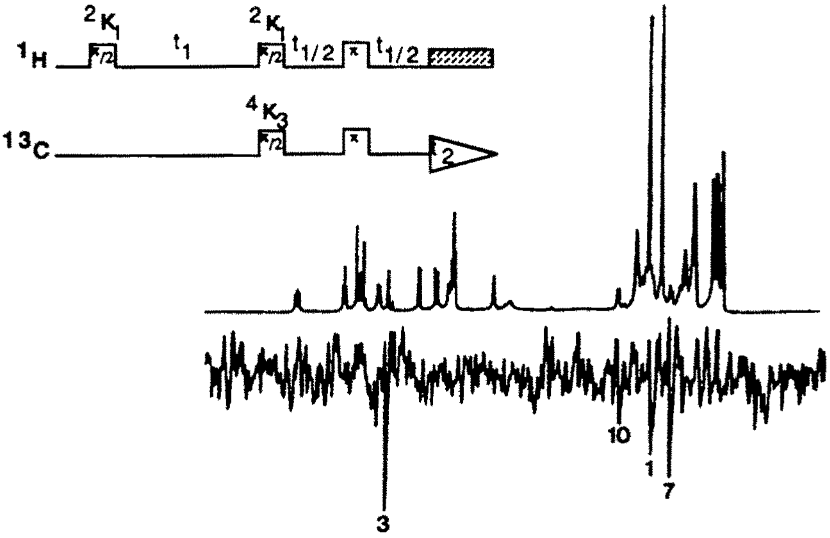
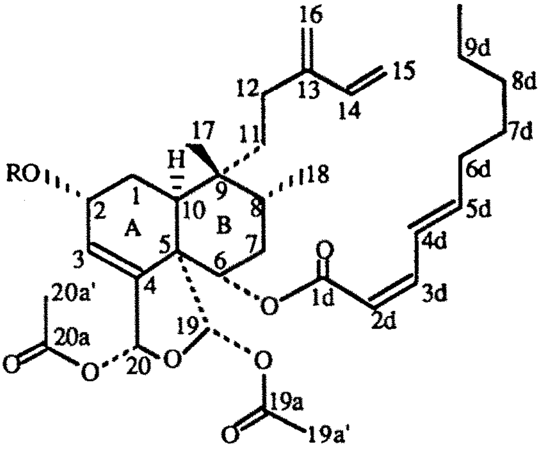


Figure 4 : Cross section of carbon 5 in the phased, selection free ^1H-^{13}C correlation map related to the ^1H 1D-spectrum and the corresponding pulse sequence.

The same strategy established the positions of the three ester functions, taking advantage of the ^3J couplings that relate the carbonyl carbon atoms to the protons on the oxygenated carbon atoms. It also demonstrated the presence of a conjugated diene side chain in position 9 which is the only fragment compatible with the long range couplings seen in the low field region of the ^1H and ^{13}C-NMR spectra.

Finally, using all the available information, the most probable structure appeared to be 1.



1 R = H
2 $R = \underset{\underset{O}{\|}}{C}\text{-}CH_3$

However, the structure proposal resulting from this first set of experiments remained somewhat puzzling, mainly because of the coexistence of a very oxidized "southern" part and a rather fragile "northern" part. In biogenetically related natural products the carbon atoms corresponding to the side chain in position 9 are indeed often strongly oxidized and are found in the form of furans, difurans or lactones.

We searched therefore for additional evidence and took advantage of the relatively large amount of natural product available and turned to the INADEQUATE experiment. This experiment is one of the most powerful tools for the NMR spectroscopist, in that it reveals all the carbon-carbon covalent bonds and consequently the carbon skeleton of the molecule.

Despite its fantastic potentialities in the structural elucidation problematics, this experiment has found until now only rare applications, probably because it suffers from many drawbacks. Particularly, the inherent low sensitivity remains a major difficulty. A first run with the natural product was not successful, probably because association of the molecules due to the presence of a hydroxyl group, led to a faster transverse relaxation and thus aggravated the sensitivity problem. However, after acetylation of the hydroxyl group at position 2, the concentrated solutions needed for the INADEQUATE experiment proved much more fluid and the experiment finally was successful as seen from Figure 5.

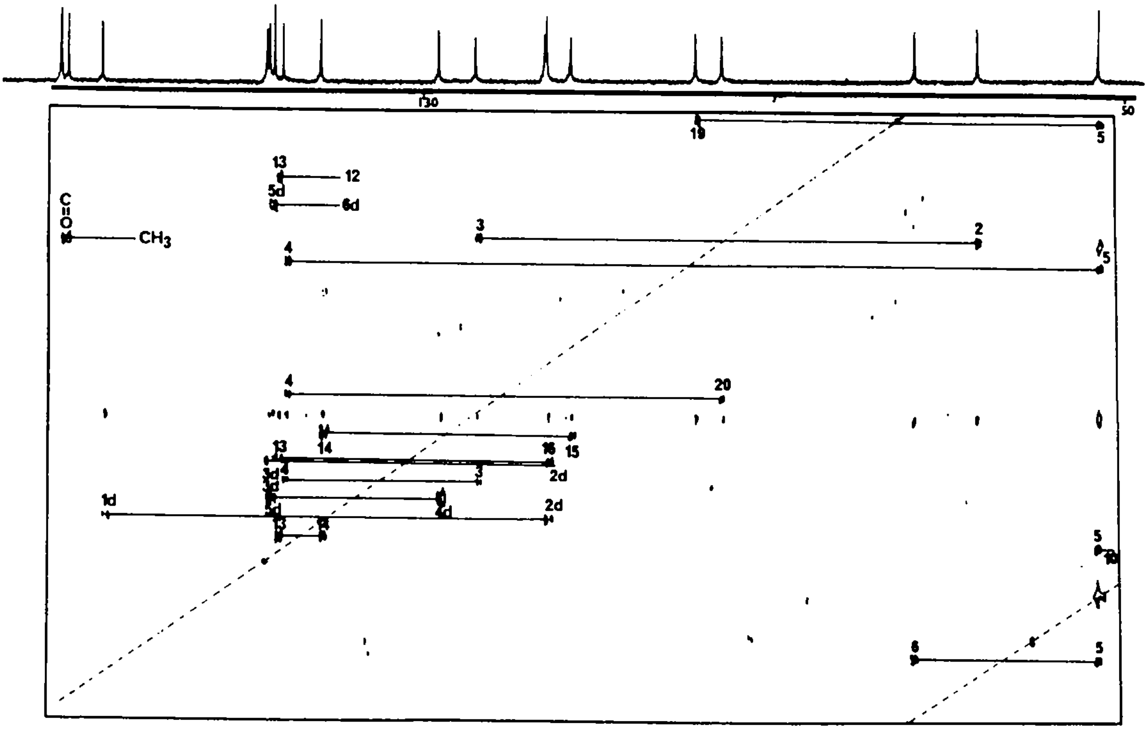


Figure 5 : Expansion of the INADEQUATE experiment of pitumbin acetate 2 showing the connections in the region from δ 50 to 175 *ppm*.

Several spectacular aspects of this experiment deserve explicit citation : the filiations within the olefinic carbons : an *exo*-methylene carbon to a quaternary carbon to a monosubstituted carbon to an other *exo*-methylene carbon :

CH_2 (116.04 *ppm*) —— C (146.78 *ppm*) —— CH (141.62 *ppm*) —— CH_2(113.33*ppm*)

confirm the presence of a terminal diene entity.

Furthermore, the link of C-13 (146.78 *ppm*) to the saturated carbon C-12 (24.80 *ppm*) is clearly observable and connects this entity to the —CH_2—CH_2— part of the side chain. The six membered ring as deduced from the long-range ^1H-^{13}C experiment is also firmly established through the observation of the C-3—C-4, C-4—C-5 and C-5—C-10 connectivities. The C-20—C-4 and C-19—C-5 connectivities firmly establish the positions of the acetates in the molecule.

As expected, the main effect of the acetylation concerns one proton and one carbon :

H-2 (4.55 *ppm* ——> 5.65 *ppm*) and C-2 (63.96 *ppm* ——> 66.99 *ppm*)

confirming the position of the free hydroxyl group.

The various coupling constants were measured directly from the 1D spectra after their gross value had been evaluated from the phased multiple quanta filtered experiment[13] and are reported in Figure 6. They allowed to precise the geometry of the various cycles and -together with additionnal 1D and 2D NOE-experiments- solved the problem of the relative stereochemistry of the different chiral centers.

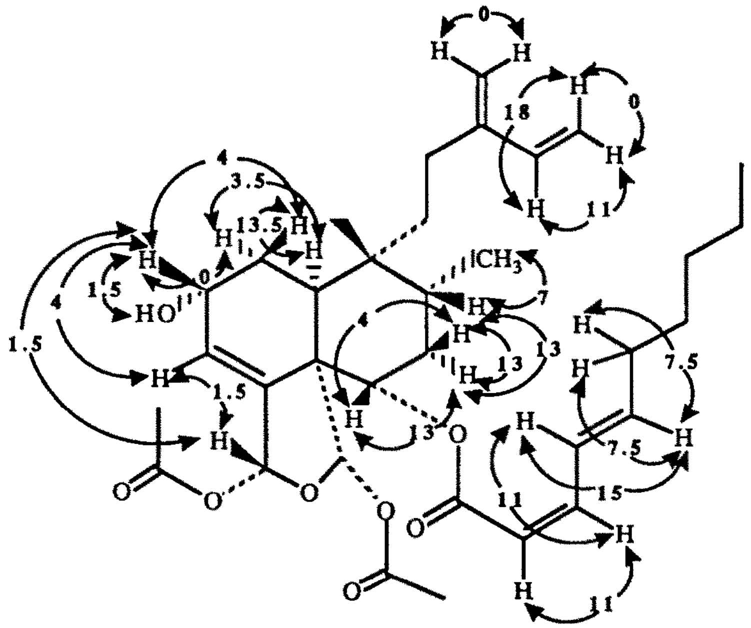


Figure 6 : ^{1}H-^{1}H coupling constants of pitumbin 1 (in Hz).

The observation of the Nuclear Overhauser Effects in Figure 7 for instance demonstrates the stereochemistry at C-9 and C-19 and the *cis* -junction of the cycles.

The proximities and dihedral angles revealed by these two types of experiments demonstrate that the B ring adopts a boat conformation as depicted in Figure 7 .

The only as yet undetermined stereochemistry (C -20) was ascertained by the observation of the above mentioned long range (^5J) coupling between H-20 and H-2. This type of homoallylic coupling necessitates a certain amount of overlap of the π-orbitals of the double bond and the 1s orbitals of the hydrogen atoms and thus demonstrates the β-orientation of the acetalic proton at C-20.

In conclusion, the structure of pitumbin deserves two comments :

The presence of two completely different halves from the oxidation level point of view represents an interesting puzzle as far as its biosynthesis is concerned.

The presence of a protected unsaturated dialdehyde makes pitumbin 1 a potential member of a growing family of biologically important terpenoids having this functionality such as onchidal[14](a fish repellent), ancistrodial[15] (an insecticide) and polygodial[16] (an antifeedant). The physiological significance of pitumbin 1 as well as its possible applications remain however to be investigated.

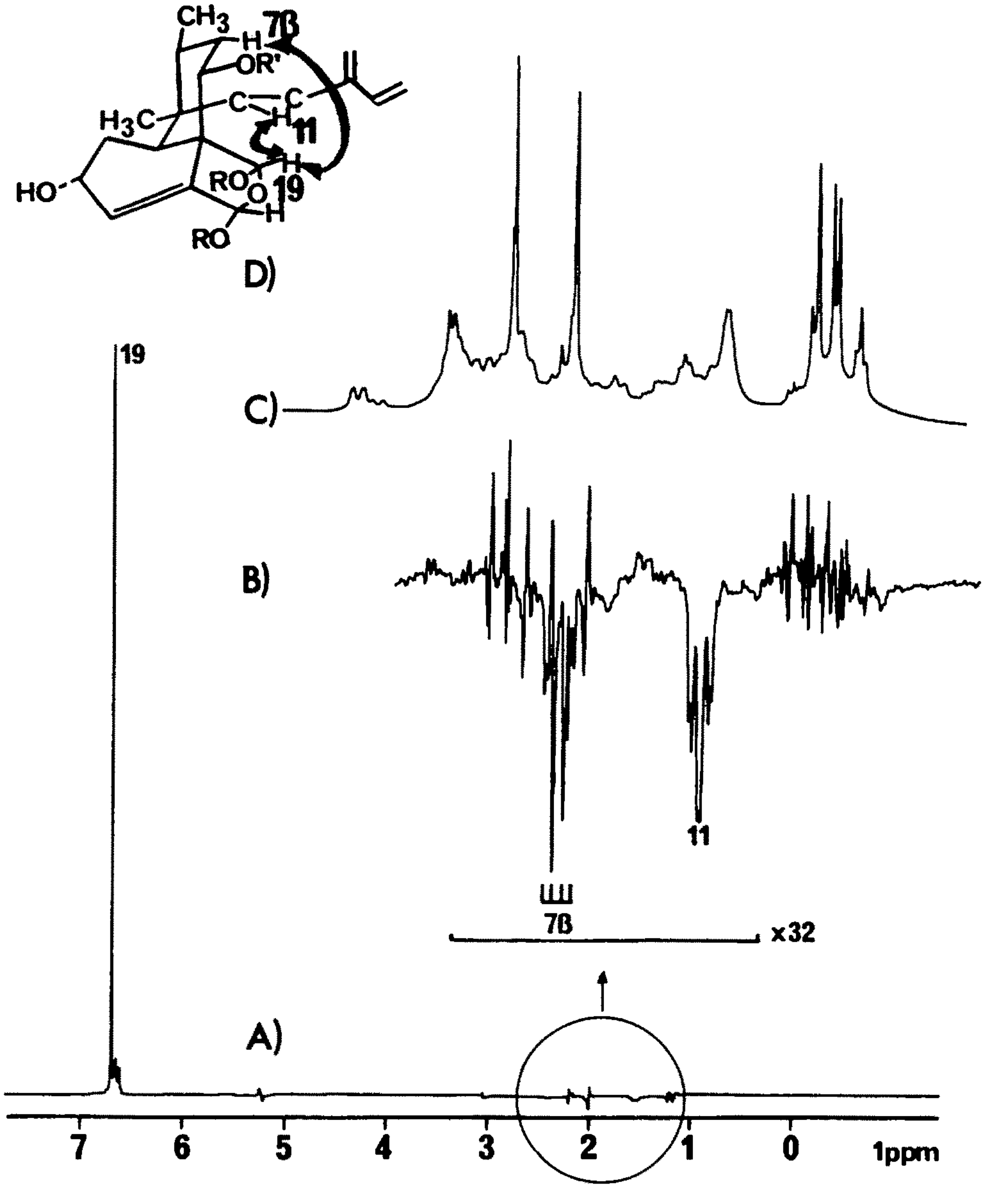


Figure 7 : A) NOE difference spectrum of pitumbin 1 obtained after irradiation of H-19.
B) Enlargement of the encircled part of A. C) The ^1H -NMR spectrum of the same region.
D) The spatial arrangement of the corresponding protons.

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EXPERIMENTAL PART

The NMR spectra were measured on Bruker WM 400 and SY 200 spectrometers. The samples for NOE measurements were treated several times by the freeze -pump - thaw cycle before sealing the tube. The mass spectra were recorded on a Nermag R10-10C instrument in the electron impact and chemical ionisation (NH_3) mode. The IR spectra were measured on a Perkin-Elmer model 397 in $CHCl_3$. UV spectra were recorded on a Unicam SP 1800 . A Zeiss polarimeter was used for optical rotation determinations.

Extraction and isolation of pitumbin 1:

320 g of dry seeds of *Casearia pitumba* PLEUMER were extracted with methylenechloride in a Soxhlet apparatus to yield 108 g of a crude, oily extract after evaporation of the solvent.

Filtration of the crude extract over 1 kg of silicagel (Merck Kieselgel 60, solvent: methylenechloride with increasing amounts of ethyl acetate) provided essentially 2 fractions of 50 g (fraction 1) and 46 g (fraction 2) respectively. Both fractions contained mainly a mixture of lipids, but TLC analysis of fraction 2 revealed the presence of an additional compound showing an UV absorption at 254 nm. Repeated chromatography of fraction 2 followed by preparative HPLC (Jobin-Yvon Miniprep, solvent: 5% ethyl acetate in methylene chloride) led to the isolation of 13 g of pitumbin **1** as a colourless oil. $[\alpha]_D = -71{,}6°$ (c=1, $CHCl_3$). $C_{34}H_{48}O_8$. Calc. : C 69.83, H 8.27; found : C 70.01, H 8.35. Mass spectrum (EI) : $M^{+\cdot}$ at *m/z* : 584, major fragments at *m/z* : 524 (M-60), *m/z* : 464 (524-60); base peak at *m/z* : 151 ($C_{10}H_{15}O^{+\cdot}$).

Pitumbin acetate **2** : Compound **2** was prepared from **1** by treatment with acetic anhydride / pyridine at room temperature followed by standard work-up procedures in 91% yield as a colourless oil. $[\alpha]_D = +0{,}1°$ (c=1, $CHCl_3$). $C_{36}H_{50}O_9$. Calc. : 69.83, H 8.27; found: C 70.01, H 8.35. Mass spectrum (CI/ NH_3) : M+18 = *m/z* 644.

NMR measurements :

One level of zerofilling was used in the F_1 dimension in all 2D experiments. Except when specified, a shifted ($\pi/2$) sine bell multiplication was applied in both dimensions.

The two dimensional multiple quantum COSY experiment was recorded with quadrature detection in both dimensions, in the phase sensitive mode. It included 512x1K experiments (64 scans each).

Table 1 : ^1H and ^{13}C NMR data of **1** and **2***

	1		2			1		2	
no	^{13}C	^1H	^{13}C	^1H	n°	^{13}C	^1H	^{13}C	^1H
1	30.72	2.17	29.02	-	18	16.10	1.10	16.01	1.13
2	63.96	4.55	66.99	5.65	19	99.15	6.66	99.10	6.70
3	128.69	6.18	124.13	6.26	20	96.21	6.56	96.07	6.58
4	141.87		145.79		1d	165.99		166.19	
5	53.48		53.20		2d	116.01	5.70	116.28	5.70
6	74.51	5.24	74.21	5.25	3d	147.23	6.84	147.65	6.87
7	34.24	1.99(α)	33.86	1.99(α)	4d	128.07	7.56	128.28	7.56
		1.81(β)		1.85(β)	5d	146.98	6.32	147.33	6.36
8	37.78	2.11	37.29	-	6d	33.67	2.35	33.50	6.36
9	38.40	-	38.16	-	7d	29.35	1.59	29.01	-
10	37.52	2.64	35.16	2.54	8d	32.23	1.46	32.02	-
11	29.26	1.67,1.48	27.50	-	9d	23.20	1.46	23.00	-
12	25.07	2.28(x2)	24.80	-	10d	14.36	1.03	14.32	1.04
13	146.91	-	146.78	-	19a'	21.93	2.15	21.77	2.29
14	141.44	6.61	141.62	6.63	20a	21.27	1.96	21.25	2.17
15	113.08	5.44,5.18	113.33	5.90,5.45					
16	115.71	5.15,5.17	116.04	5.80,5.18	20a	170.58	-	170.87	-
17	25.89	1.16	25.58	1.17	19a	169.85	-	170.77	
					C=O			169.98	
					CH_3			21.20	1.99

* The spectra were recorded in acetone-D_6 and are reported in *ppm* relative to the signal of the solvent.

The two dimensional proton-carbon chemical shift-correlation experiments were recorded in the magnitude mode except for the new experiment discussed in the text. They consisted of 256x1K experiments (64 scans each). The first fixed delay of the usual sequence was set at 3.57 ms (one bond correlation), 100ms (Figure 3) and 72ms (solid circles in Figure 3), the refocussing delays were set at half these values.The phase sensitive selection free experiment consisted of 512x1K experiments (64 scans each). Cosine and sine bell filters were employed in the F_2 and the F_1 dimensions respectively. 256x1K experiments (400 scans each) were needed for the INADEQUATE spectrum. The fixed delay was set such as to emphasize the carbon-carbon coupling constants of 40Hz.

The 1D NOE difference experiments were obtained using 1s saturation delays; the NOESY experiment (not shown) was recorded in the phase sensitive mode using a fixed 300ms mixing time and consisted in 512x1K experiments (64 scans each).

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