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ACICULATIN, A NOVEL FLAVONE-C-GLYCOSIDE WITH DNA BINDING ACTIVITY FROM *CHRYSOPOGON ACICULATIS*

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Abstract A novel flavone-C-glycoside, aciculatin (**1**), has been isolated from the methylene chloride extract of *Chrysopogon aciculatis* (Poaceae) collected in the Philippines. The structure of **1** was determined by analysis of spectral data Aciculatin exhibits cytotoxicity towards KB cells that is reduced by an order of magnitude in the presence of exogenous DNA indicating that **1** binds to DNA DNA binding assays indicated an apparent K_d of 15 - 50 μM for binding of **1** to calf thymus DNA

Compounds bind to DNA by one or more of several recognized mechanisms [1-4] Frequently, the consequence of this binding is an alteration in DNA structure, which in turn represents the basis for the biological activity of a number of antimicrobial and antineoplastic agents [5-9] Regardless of the eventual biochemical consequences of DNA binding, one aspect of the interaction common to such agents is the initial step of binding We have undertaken a program to identify novel DNA binding molecules from natural sources with the goal of discovering novel classes of compounds with potential antitumor activity Natural products have been a traditional source of DNA interactive agents,[10-16] and many natural products with *in vitro* antitumor activity have been discovered.[17-20] Moreover, a number of antineoplastic drugs derived from natural products have been successful clinically, including bleomycins, vinca alkaloids, actinomycin, etc [21] To discover novel DNA-interactive compounds, we examined a collection of plant extracts to identify those with cytotoxic activity against the transformed human cell line, KB, that could be inhibited by evaluating the test substance in the presence of excess exogenous DNA The rationale for this protocol was that chemicals which bound to DNA would be prevented from entering the cells and exerting their cytopathic effect The assay was validated using a wide variety of structurally dissimilar agents [22]

The crude CH_2Cl_2 extract of the roots and rhizomes of *Chrysopogon aciculatis* exhibited cytotoxicity towards KB cells that was reduced on the addition of exogenous calf thymus DNA (50% reduction at 25 μg/mL) Using this activity to guide isolation, the crude extract of *C aciculatis* was chromatographed on silica gel to give an active fraction that was was further purified by crystallization to give aciculatin (**1**) as yellow plates, mp 257-260° C The molecular formula of **1** was established by high resolution FABMS as $C_{22}H_{22}O_8$ which implies the presence of 12 double bond equivalents H/D exchange by FABMS indicated the presence of four exchangeable

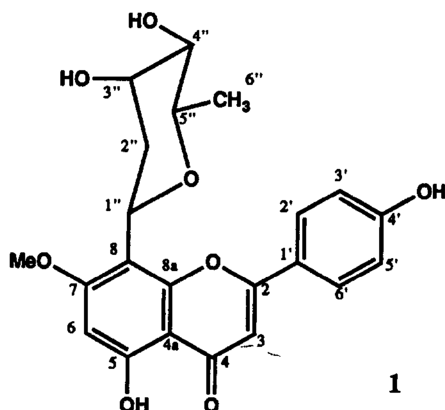

Table 1 [1]H (360 MHz) and ^{13}C (90 MHz) NMR Data for Aciculatin (1) in 1 1 $CDCl_3/CD_3OD$

Atom #	δH (multiplicity, integration, *J*)	δC (multiplicity)
2	-	164 9 (s)
3	6 58 (s, 1 H)	102 2 (d)
4	-	182 7 (s)
4a	-	104 7 (s)
5	-	161 2 (s)
6	6 43 (s, 1 H)	94 7 (d)
7	-	162 5 (s)
8	-	106 9 (s)
8a	-	155.2 (s)
1'	-	121 9 (s)
2'	7.96 (d, 2 H, *J* = 8.7 Hz)	128 3 (d)
3'	6 96 (d, 2 H, *J* = 8 7 Hz)	115 2 (d)
4'	-	160.9 (s)
5'	6 96 (d, 2 H, *J* = 8 7 Hz)	115.2 (d)
6'	7 96 (d, 2 H, *J* = 8 7 Hz)	128.3 (d)
1''	5 64 (dd, 1 H, *J* = 12 0, 1 8 Hz)	65 3 (d)
2ax''	2 60 (m, 1 H)	35 8 (t)
2eq''	1 86 (m, 1 H)	35 8 (t)
3''	4 16 (m, 1 H)	67 2 (d)
4''	3 49 (dd, 1 H, *J* = 9 6, 2 9 Hz)	73 0 (d)
5''	3.95 (dd, 1 H, *J* = 9 6, 6.1 Hz)	72 4 (d)
6''	1 39 (d, 3 H, *J* = 6 1 Hz)	17 8 (q)
-OMe	3 93 (s, 3 H)	55 3 (q)

protons The IR of 1 had absorptions at 3300 - 2800 and 1654 cm^{-1} corresponding to hydroxyl and hydrogen bonded unsaturated carbonyl groups. The UV spectrum of 1 exhibited bands at 222, 270 and 328 nm, the latter gave a bathochromic shift of 60 nm on the addition of base. This information along with a cursory examination of the NMR data (Table 1) indicated that 1 was a flavone glycoside.

The 360 MHz ^1H NMR spectrum of 1 revealed a high field methyl doublet, a methoxyl group, two high field aromatic singlets, an AX pair of aromatic doublets (4H) characteristic of a para-disubstituted aromatic ring, and six well-resolved signals of the sugar portion of 1 in the low-field aliphatic region. The carbon NMR spectrum of 1 was obtained and fully assigned with aid of ^1H-^{13}C correlation and COLOC spectra The chemical shift values of the carbons associated with the para-disubstituted aromatic residue and those of the associated protons lead to the conclusion that the para-substituents were an electron-releasing group (e g OH or OMe) and an electron-withdrawing group with some shielding anisotropy (e g carbonyl)

The identity of the sugar moeity was established from analysis of the ^1H-^1H COSY NMR spectrum. The anomeric proton signal at δ5 64 ppm was coupled to the methylene protons at 2 60 and 1 86 ppm with coupling constants of 12.0 and 1 8 Hz respectively This suggested that the sugar was a 2-deoxy sugar and that the anomeric proton was axially disposed. The chemical shift of the anomeric methine carbon (δ 65 3) indicated that the sugar was attached to the aglycone as a C-glycoside Each of the methylene proton signals was coupled to H-3" with small coupling constants (2 9 and 2 0 Hz) H-3" showed no couplings larger than 2 9 Hz, and therefore was required to be equatorial H-3" was coupled to H-4" (3 49 ppm, J = 2 9 Hz) which was in turn coupled to H-5" ($J_{4,5}$ = 9 6 Hz), the large coupling constant required H-4" and H-5" to be trans-diaxial to each other H-5" is coupled to the methyl doublet implying that the sugar was a 2,6-dideoxy sugar The sugar has thus been identified as digitoxose, which is linked to the flavone aglycone through a β-C-glycosidic bond.

Difference NOE experiments allowed final determination of the complete structure of aciculatin (1) One immediate conclusion was that the methoxyl group was not the electron-releasing group on the paradisubstituted aromatic ring It relaxed the aromatic singlet at δ 6 43 ppm and the anomeric H-1" proton The methoxyl must therefore have been central in space to a cluster of these three resonances Since the IR carbonyl stretch of 1654 cm^{-1} indicated a hydroxyl group at C-5, the methoxyl must be at C-7 and the digitoxose moeity must have been attached at either C-8 or C-6 A weak NOE between the sugar methyl group and H-2',6' places the sugar residue at C-8 Finally the aromatic singlet at δ6 58 ppm also relaxed H-2',6'and could therefore be assigned as H-3

In order to confirm the structure of 1, the sample was permethylated and analysed using electron ionization MS The spectrum indicated the uptake of four methyls with a molecular ion at m/z 470 Typical flavanoid glycoside ions[23] were observed at m/z 132, 135, 338 and 339 Figure 1 suggests structures to account for these ions The mass spectra of O-glycosides of flavones always exhibit an abundant fragment corresponding to the oxonium ion of the carbohydrate moiety Ions corresponding to hexosyl, deoxyhexosyl, or dideoxyhexosyl species were absent, confirming that the compound was a C-glycoside. Final confirmation of the structure of aciculatin (1) was achieved in a single crystal x-ray diffraction study to be presented elsewhere [24]

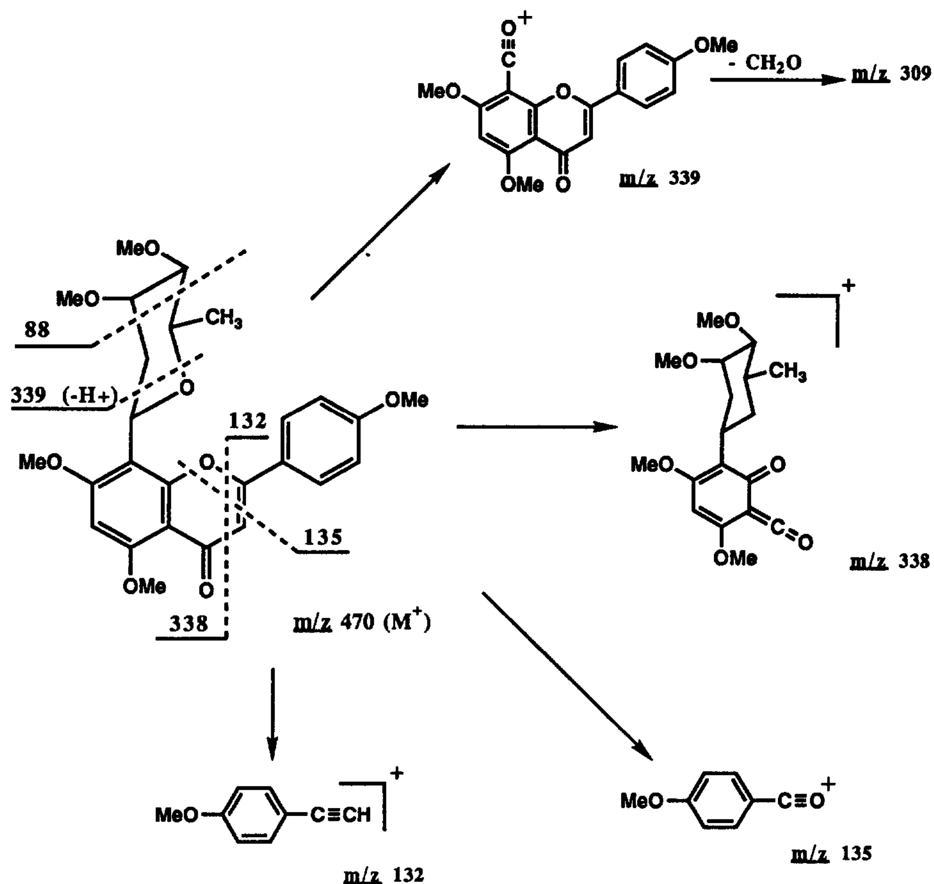

Figure 1 EIMS fragmentation pattern of permethylated aciculatin

The dependence of cytotoxicity on the concentration of **1** is shown in Figure 2 After a 48 hour exposure, the EC_{50} in the absence of exogenous DNA was 7 6 μg/mL In the presence of DNA, this was increased 10-fold to 72 0 μg/mL This decrease in cytotoxicity was due to the compound being bound to DNA and effectively removed from solution, thereby preventing it from entering the cells to exert its cytopathic effect From these data, an apparent dissociation constant was calculated to be 50 μM Analyisis of binding of **1** to purified calf thymus DNA by equilibrium dialysis confirmed this value, yielding an apparent K_d of 15 μM (data not shown) The ability of **1** to interact with DNA stimulated our interest in its ability to prevent tumor cell growth Furthermore, a related compound, flavone 8-acetic acid (NSC 347512) is of particular interest as an antineoplastic agent because it has demonstrated selectivity for solid tumors rather than leukemias and lymphomas [25-26] The antitumor activity of flavone 8-acetic acid also has been ascribed to its ability to cleave DNA [27] The cellular pharmacology of **1** will be reported in a subsequent manuscript [28]

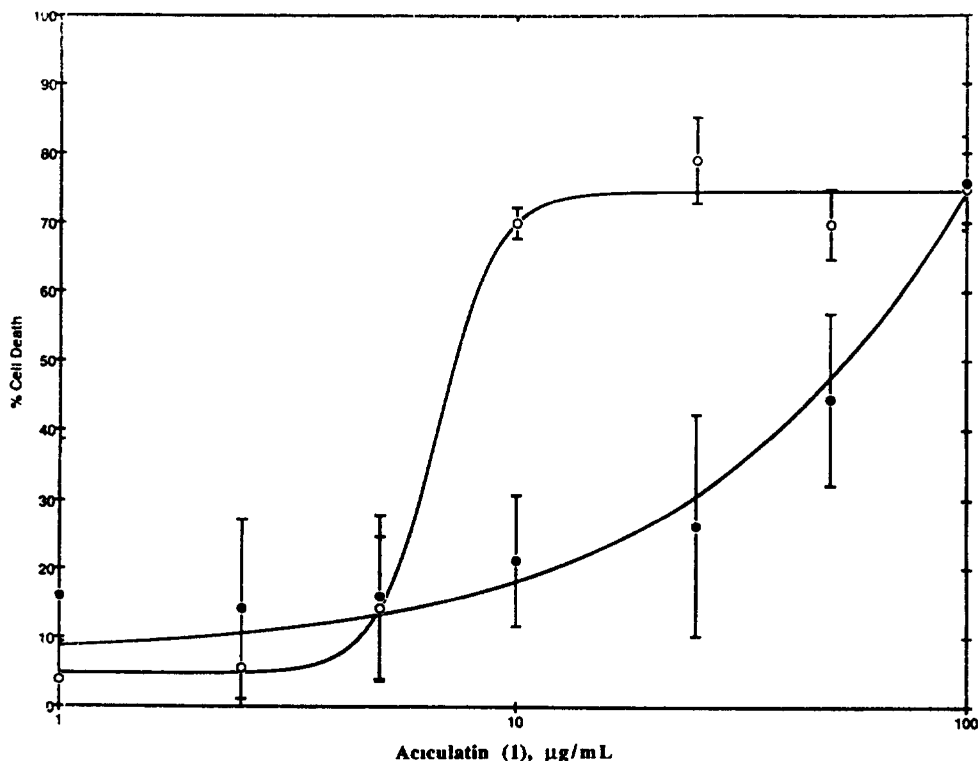

Figure 2 Cytotoxicity of aciculatin (1) and reversal by exogenous DNA. o, drug tested in the absence of DNA, •, drug tested in the presence of exogenous DNA

EXPERIMENTAL

General Methods: Melting points were obtained on a Kofler hot stage microscope apparatus and are uncorrected Optical rotations were performed in MeOH relative to the D line of sodium using a Jasco DIP-360 digital polarimeter equipped with a constant temperature bath held at 20 °C Infrared spectra were recorded on a Nicolet Model 20 DXB FTIR Spectrometer Ultraviolet spectra were recorded on a Beckman DU-7 spectrophotometer ^1H and ^{13}C-NMR spectra were obtained using a Bruker WM360 operating at 360 13 MHz (^1H) and 90 56 MHz (^{13}C) and maintained at ambient temperature (29 °C) using a 1 1 mixture of $CDCl_3$/MeOD NMR experiments included ^1H COSY and $^1H/^{13}C$ correlation 2D NMR measurements, proton decouplings, difference NOE, COLOC and ^{13}C edited spectra All chemical shifts are reported with respect to TMS (δ 0) Fast atom bombardment (FAB) mass spectra were obtained on a VG ZAB-HF mass spectrometer; the sample (ca 10 µg) was dispersed on a stainless steel probe tip in a matrix of α-monothioglycerol or D_3-α-monothioglycerol/MeOD FAB accurate mass measurements were made by peak matching at an instrument resolution of 10,000 (M/ΔM) using polyethylene glycerol as the reference Electron ionization (EI) mass spectra were obtained on a Finnigan MAT 4610 mass spectrometer using ca 1 µg of sample All solvents used were either HPLC or spectrophotometric grade

Collection, Extraction and Isolation Procedures *C aciculatus* (Poaceae) was collected in Philippines in September, 1981, a voucher specimen B861962 is preserved in the National Herbarium, Washington, DC Dry pulverized roots and rhizomes from *C aciculatus* (250 g) were exhaustively extracted with 10% IPA in CH_2Cl_2 to give 5 6 g of crude extract which was chromatographed on silica gel 60 (EM Reagents, 230-400 mesh) using an increasing gradient of EtOAc in Et_2O Eluting fractions were analyzed for DNA binding The active fraction, which was eluted with 50% EtOAc, was crystalized twice from CH_2Cl_2/MeOH to give **1** as yellow plates (660 mg, 0 26% dry wt)

Aciculatin (1) mp 257 - 260 °C (1 1 CH_2Cl_2/MeOH), $[\alpha]_D^{25}$ +50 8 (c = 0.5, MeOH), IR (KBr) 3300-2800, 1654, 1604, 1563, 1497, 1447, 1392, 1369, 1336, 1283, 1298, 1207, 1179, 1123, 1110, 1084, 1073, 1020, 1007, 908, 838, 736, 577 cm^{-1}, UV (MeOH) 222 nm (ε 17,800), 270 nm (ε 15,000), 328 nm (ε 15,200), UV MeOH + NaOH) 225 nm (ε 18,000), 270 nm (ε 15,000), 388 nm (ε 20,900), ^1H and ^{13}C NMR - Table 1, high resolution FABMS, observed m/z = 415 1393, $C_{22}H_{22}O_8$ $(M + H)^+$ requires m/z = 415 1393 (Δ = 0 0 mDa)

Methylation of Aciculatin (1) A sample of **1** (250 µg) was dissolved in DMSO and reacted with a solution of sodium t-butoxide in DMSO for 5 min. Methyl iodide was added and reacted for 2 min The sample was dried under N_2 and partitioned first between $CHCl_3$ and 1 N acetic acid and then between $CHCl_3$ and H_2O The organic layer was evaporated and the permethylated sample analyzed by GC and EIMS (See Figure 1)

DNA Binding Assays. KB cells were routinely grown in antibiotic-free minimal essential medium (GIBCO Laboratories, Grand Island, NY) containing 20 mM HEPES and 10% fetal bovine serum (Flow Laboratories, McLean, VA) For cytotoxicity assays, cells were added to 96-well microtiter plates at 5 x 10^4 cells/well and allowed to attach overnight. Test substances (crude extract, isolation fractions, or pure compound) were dissolved at 10 mg/mL in DMSO and mixed with an equal volume of water or 10 mg/mL calf thymus DNA (Sigma Chemical Co , St Louis, MO) dissolved in water. This mixture was incubated at rt for 18 - 20 hr before adding to cells DNA premixed with DMSO only was included as a control and cell cultures were tested with a final DMSO concentration of 1% Cultures were incubated in whole medium at 37 °C for 48 hr, the medium was removed, and the remaining cells were stained with 0 5% crystal violet in 70% methanol The amount of stain (proportional to the number of live cells remaining) was quantified by absorbance using a Dynatech MR 600 microplate reader and expressed as a percentage of controls which were not exposed to the drug

Binding of purified aciculatin was also measured by equilibrium dialysis Calf thymus DNA was repurified by repeated phenol/chloroform extractions and ethanol precipitation It was dissolved in 1X SSC (0 15 M NaCl, 0 015 M sodium citrate, pH 7.4) at 3 mM, and dialyzed against the same buffer using a 12,000 dalton cut-off membrane The same molecular weight cut-off was employed in equilibrium dialysis chambers, and equilibrium was established within 24 hr at rt The UV-visible spectrum of the solution in each dialysis chamber was obtained in a Perkin-Elmer Lamda 4C spectrophotometer, and the amount of flavone bound to DNA was computed by subtracting the DNA background

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