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Isolation and Structure Determination of Pycnidione, a Novel Bistropolone Stromelysin Inhibitor from a *Phoma* sp.

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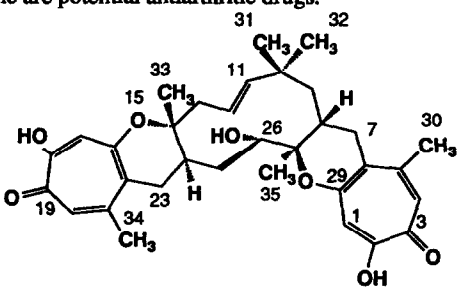
Abstract: A novel bistropolone, pycnidione (1), was isolated by bioassay guided fractionation from fermentations of a *Phoma* sp The structure was determined by spectroscopic methods and single crystal X-ray diffraction

A screening program[1] to identify novel structural types of stromelysin inhibitors from fungal cultures resulted in the isolation and characterization of the novel bistropolone pycnidione (1). Stromelysin is a zinc containing metalloendoproteinase found in elevated levels in the synovial fluid of patients with rheumatoid arthritis. Proteolysis by stromelysin is postulated to cause the cartilage degradation and impaired joint function and thus inhibitors of this enzyme are potential antiarthritic drugs.[2]



1

RESULTS AND DISCUSSION

The producing fungus, strain MF 5726, was isolated from soil collected on Korase Island, Federated States of Micronesia and was identified as a *Phoma* sp Fermentations of the culture produced large quantities of pycnidione which crystallized as hexagonal prisms upon concentration of a methyl ethyl ketone extract of a 21 day fermentation Recrystallization from CH_2Cl_2 MeOH mixtures, EtOH or CH_3CN provided pure **1** HREIMS (M^+ 548 2744) and carbon-13 nmr data (33 unique carbons) determined the molecular formula to be $C_{33}H_{40}O_7$ Silylation of **1** resulted in the formation of a tri-TMS derivative, as indicated by an ion at 749 m/z in the EI mass spectrum of the derivatized sample corresponding to $[(1+TMS_3) - CH_3]^+$, suggesting the presence of three exchangable hydrogens

Crystals suitable for X-ray diffraction were obtained by recrystallization from CH_3CN with traces of H_2O The initial solution of the structure was obtained by statistical methods, without any assumptions concerning the chemical structure of the molecule All atoms were initially assigned as carbon atoms During

the course of the refinement the positions and identities of the oxygen atoms were derived from the temperature factors, bond distances and angles. These considerations also identified the positions of the keto and hydroxyl substituents as well as the C-11 = C-12, C-6 = C-29 and C-16 = C-22 double bonds Both seven membered rings exhibited a high degree of planarity, indicating additional unsaturation No definitive location of the unsaturation could be made on the basis of the observed dimensions of the rings The crystal structure contains an unknown amount of solvation per mole of **1** in a highly disordered arrangement (modelled as 4 half-occupancy water molecules), limiting the accuracy of the coordinates in this structure determination (Figure 1)

Complete ^{1}H and ^{13}C nmr assignments for **1** are shown in Table 1. Both of the spectra exhibited considerable symmetry because of the two identically substituted tropolone / pyran rings COSY and HETCOR experiments were used to identify the three isolated spin systems of the 11-membered ring. Long range ^{1}H-^{13}C correlations from these spin systems to the gem dimethyl quaternary carbon C-10 and the two oxygenated quaternary carbons, C-14 and 27, were observed with a long range HETCOR optimized for a multiple bond J_{CH} of 10 Hz and completed the assignment of this portion of the molecule The tropolone assignments were established with long range HETCOR ^{1}H-^{13}C correlations from H-1,3 and 30 or H-17,20 and 34 to their corresponding quaternary tropolone ring carbons. Correlations from either H-7 or H-23 methylene protons into their respective tropolone ring carbons were not observed in the long range HETCOR and thus the two tropolone spins systems could not be differentiated. An HMBC[3] experiment at 500 MHz (^{1}H dimension) optimized for a ^{1}H-^{13}C multiple bond coupling of 10 Hz provided correlations of H-7a with C-5, C-27, and C-29 and the corresponding H-23a with C-14, C-16 and C-21. The important two and three bond HMBC correlations obtained are shown in Figure 2.

Table 1 ^{1}H and ^{13}C nmr Assignments for **1**

No	^{13}C (δ)	^{1}H (δ), *m*, *J* (Hz)	No	^{13}C (δ)	^{1}H (δ), *m*, *J* (Hz)
1	114 26	7 03, s	19	171.47	-
2	164.44	-	20	124 57	7.08, s
3	172 27	-	21	150 87	-
4	124 20	7 06, s	22	124 31	-
5	151 18	-	23 a	34 23	3 06, dd, 5, 17
			b		2.33, m
6	122.39	-	24	39 99	1 85, m
7 a	34 52	2 67, m	25 a	33 30	2 13, d, 15
b		2 40, m	b		1.22, m
8	31 79	1.66, m	26	76 22	3 58, d, 10
9 a	46 24	1 62, d, 14 5	27	84 40	-
b		1 12, dd, 4, 14.5			
10	37 10	-	29	161.68	-
11	144 29	5 40, m	30	27.20	2 38, s
12	122 27	5 40, m	31*	31 32	1 01, s
13 a	49 26	2 70, m	32*	21 86	1 06, s
b		2 33, m			
14	82 84	-	33	18 70	1.21, s
16	161 85	-	34	27 20	2 42, s
17	114 87	6.92, s	35	15.76	1 19, s
18	165 09	-			

* interchangeable

Figure 1 ORTEP Drawing of **1**

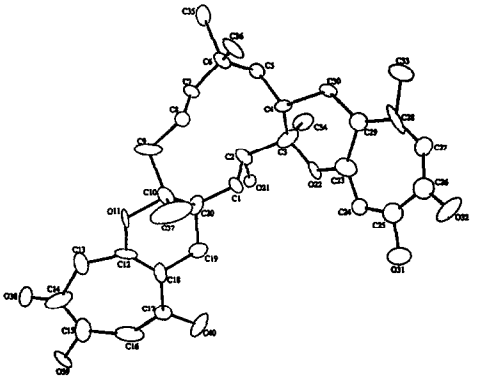
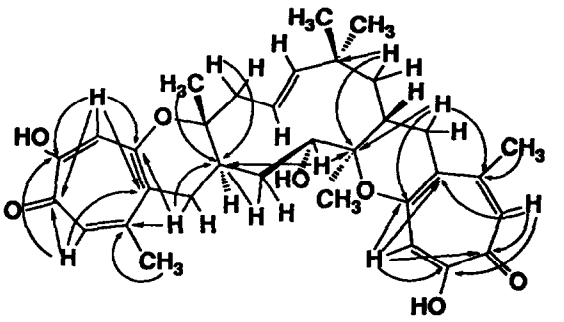
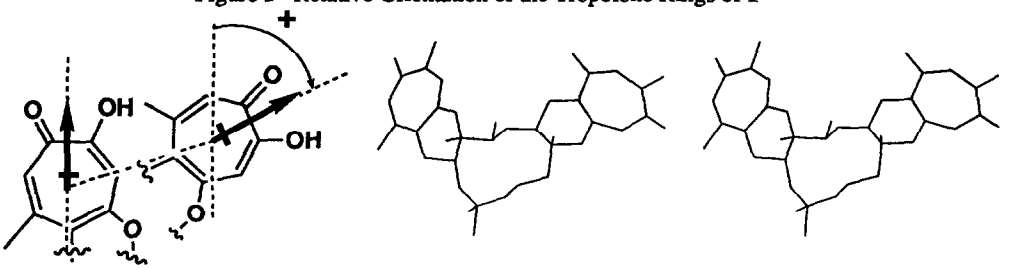


Figure 2 Selected HMBC Correlations for **1**



The presence of two tropolone chromophores in pycnidione suggested that the absolute stereochemistry might be obtained by application of the exciton chirality method.[4] The CD spectrum of **1** exhibited a positive first Cotton effect at 260 nm and a negative second Cotton effect at 238 nm for the $\pi \rightarrow \pi^*$ transition observed in the UV spectrum at 255 nm The first (270 - 400 nm) and second (210 - 270 nm) bands of tropolone and tropone are known to each be composed of two electronic transitions with parallel (bands II and IV, charge transfer from the ring to the carbonyl) and perpendicular (bands I and III) polarizations with respect to the long axis of the molecule [5,6] Thus the second (255 nm) adsorption maximum of **1** should consist of two sub-bands, one polarized parallel (corresponding to band IV of tropolone) to the carbonyl and the other perpendicular (band III of tropolone). The Cotton effect observed can be explained by exciton coupling of band IV of the two tropolone rings The positive sign of the first of the two Cotton effects implies that the intramolecular charge transfer electric transition moments of the tropolones have a positive chirality relative to each other, oriented in the sense of right-handed screw (Figure 3) This result suggests that **1** has the absolute stereochemistry shown.

Figure 3 Relative Orientation of the Tropolone Rings of **1**



Pycnidione inhibited the cleavage of ^{3}H-β casein by stromelysin[7] with an IC_{50} = 31 μM but did not inhibit a panel of other proteases including angiotensin converting enzyme (metalloprotease), human leucocyte elastase (serine protease), papain (thiol protease), and HIV-1protease (aspartic acid protease)

The occurrence of the tropolone moiety in natural products is rare and only two other bistropolones have been reported, utahin from *Juniperus utahensis* [8] and fusariocin C from *Fusarium moniliforme* [9] Lucidene[10], a sesquiterpene isolated recently from the higher plant *Uvaria lucida ssp lucida*, possesses a similar 11-membered

ring system with bis-benzopyranyl substitution As for lucidene, pycnidione appears to be of mixed biosynthetic origin It can be viewed as the condensation of a sesquiterpene such as humulene and two tropolones, formed from acetate / malonate and methionine as described for stipitatic acid.[11] Lucidene crystallized as enantiomeric pairs and was optically inactive which led to the proposal of a non-enzymatic biosynthesis. In contrast, pycnidione is optically active and formed in large quantities suggesting that it is formed enzymatically

EXPERIMENTAL

General Methods - Proton nmr spectra were recorded either at 300 MHz on a Varian XL-300 spectrometer or at 500 MHz on a Varian Unity 500 spectrometer. ^{1}H Chemical shifts are reported in ppm downfield from TMS at 0 ppm and spectra are referenced to $\underline{C}HDCl_2 = 5.32$ ppm Carbon spectra were recorded at either 75 MHz or 125 MHz on the above spectrometers ^{13}C Chemical shifts are reported downfield from TMS at 0 ppm and are referenced to $\underline{C}D_2Cl_2 = 53.8$ ppm. All nmr spectra were recorded in a mixture of CD_2Cl_2 CD_3OD (2·1). Ultraviolet spectra were recorded on a Beckman DU70 spectrophotometer. The CD spectra were obtained on a AVIV Model 62DS circular dichroism spectrophotometer. High resolution electron ionization mass spectrometry (HR EIMS, 90 eV) data were obtained on a MAT 212 double-focusing mass spectrometer Perfluorokerosene was used as the internal reference compound. Optical rotation was recorded on a Perkin Elmer 241 polarimeter at 25°C. The infrared spectrum was recorded as a thin film on a ZnSe multiple internal reflectance crystal on a Perkin Elmer 1750 FTIR spectrophotometer. Trimethylsilyl derivatives were prepared with a 1·1 mixture of bis(trimethylsilyl) trifluoroacetamide - pyridine at room temperature.

Producing Organism - The producing strain was recovered from soil using a modified soil-washing protocol.[12] Colony morphology is based on growth at 25° C under continuous fluorescent light Conidiomata formation is best observed on oatmeal agar under a combination of near-ultraviolet light and fluorescent light with a 12 hr photoperiod

Colonies on oatmeal agar (Difco) after 14 days > 90 mm, plane at margin, with irregular stromatic growths radiating from inoculation point, with cottony, white, aerial mycelium on surface of stroma, but aerial mycelium sparse and floccose or appressed toward margin, pale olive gray to gray, Light Olive - Gray[13], Olive - Gray, Olive -Buff, Dark Olive - Gray, with a few drops of clear exudate. After 21 days conidomata formed over inner third of colony. Conidomata less than 0 3 mm in diameter, small dark grayish areas emedded in amoung stromatic growths, exuding pale grayish yellow conidial masses.

Conidomata are pycnidia, 250-400 µm in diameter, subglobose, translucent to dark greenish gray, densely gregarious to confluent, exuding pale grayish yellow conidial masses, ostiolate, covered with white mycelium when young, glabrous in age, with walls composed of psedoparenchymatous tissue, 2-4 cell layer thick, with internal cavity lined with conidiogenous cells Conidiogenous cells enteroblastic, phialidic, 4-6 µm in diameter, pyriform, subglobose to doliform, with 2-4 conidiogenous loci. Conidia aseptate, hyaline, smooth-walled, 1.5-2.5 x 3-4 µm

The producing strain was assigned to form-genus *Phoma* (Deuteromycotina, Coelomycetes) based on the criteria established by Sutton.[14] The isolate has been accessioned in the Merck Microbial Resources Culture Collection as *Phoma* MF5726 Dried culture mats have been deposited at U S.D A the National Fungus Collection (BPI 1112552)

Fermentation - Culture MF5726 was grown in cotton-plugged, 250 ml Erlenmeyer flasks containing either 54 ml of the seed medium described previously[15] or 45 ml of a production medium consisting of yellow cornmeal 5 0%, glucose 4 0% and yeast extract 0 1%. Media were steam sterilized at 15 psi for 20 minutes Cultures were incubated at 28° C for 3 days with shaking at 220 rpm (seed medium) or at 25° C for 21 days with shaking at 220 rpm (production medium) A standardized inoculum was prepared by growing the fungus in seed medium, mixing the resulting culture with an equal volume of 20% glycerol and storing 2 ml portions of the mixture at -80° C The standard inoculum was diluted 28-fold into seed medium to prepare an inoculum for the production medium Production of **1** was accomplished by adding 2 ml of this seed culture to each production flask

Isolation of 1 - Fermentations (16 flasks) as described above were extracted with 48 ml methyl ethyl ketone MEK) per flask by stirring 1 hr at room temperature. The MEK layer was removed and concentrated to dryness *in vacuo* . The extract was dissolved in CH_2Cl_2 MeOH (1.1, 25 ml), and after standing 30 min. at room temperature, **1** crystallized as hexagonal prisms and was collected by filtration The collected **1** (90 mg) was recrystallized from CH_3CN (45 ml) to obtain crystals suitable for X-ray crystallography

Pycnidione (1): ^1H and ^{13}C nmr (CH_2Cl_2 : MeOH, 2 : 1) seeTable 1; UV λ_{max} 255 nm (ε 67,600), 362 (24,300), 366 (24,400); CD (EtOH) λ_{max} 260 nm ($\Delta\varepsilon$ = +63), 238 nm ($\Delta\varepsilon$ = -15), IR (film) 3400, 3215, 2934, 1631, 1594, 1445, 1280, 1180, 1152 cm^{-1}; $[\alpha]^{25}_D$ = +278° (*c* 0 23 in CH_2Cl_2 CH_3OH, 2 1), HREIMS obsd for $C_{33}H_{40}O_7$ 548 2744, calcd 548.2774, mp 216 - 219°C (decomp)

Crystallography - Single crystals, grown from acetonitrile at room temperature, were hexagonal in space group $P6_5$ with a=22.023(8), c=15 638(7) Å for Z=6 and a calculated density of 0.887 g cm^{-1}. An automatic four circle diffractometer equipped with Cu Kα radiation (λ=1 5418 Å) was used to measure 3708 potential diffraction peaks of which 1686 were observed ($I \geq 3\sigma I$). Application of a multi-solution tangent formula approach to the phase solution solved the structure which was subsequently refined with least squares and Fourier methods Anisotropic temperature factors were refined for most of the non-hydrogen atoms (some would not refine and stay positive definite), while isotropic temperature factors were applied to the hydrogens but not refined There was some disordered solvent present in the crystal structure, which was represented in the final calculations as 4 oxygen atoms with partial occupancy factors, estimated from the final electron density difference maps The function $\Sigma\omega(|F_o|-|F_c|)^2$ with $w = 4F_o^2/\sigma^2(F_o^2)$ was minimized with full matrix least squares calculations to give an unweighted residual of 14 6%.

The following library of crystallographic programs was used SHELXS-86, G M Sheldrick, University of Gottingen, West Germany (1986); ORTEP II, C K Johnson, Oak Ridge National Laboratory (1965), a version of SDPV 3, Enraff-Nonius, Delft, The Netherlands (1985) locally modified for a Sun Microsystems computer The author (K H) has deposited atomic coordinates for this structure with Cambridge Crystallographic Data Centre The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK.

Assay of Protease Activity - Assay of stromelysin, angiotensin converting enzyme, human leucocyte elastase, papain, and HIV-protease inhibition was performed as described previously [7]

ACKNOWLEDGEMENTS

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REFERENCES

1. Lingham, R B , Harris, G H ,, Dougherty, H , McCauley, E , Dey, T., Lark, M , Koch, G.; Stevens, S., Huang, L., Raghoobar, S , Silverman, K C *Annual Meeting of the Society for Industrial Microbiology*, August 9 - 14, 1992
2 MacNaul, K.L , Chartrain, N , Lark, M , Tocci, M J ; Hutchinson, N I. *J Biol Chem* **1990**, *28*, 17238-17245
3 Bax, A.; Summers, M F *J Am Chem. Soc* **1986**, *108*, 2093
4. Harada, H., Nakanishi, K *Circular Dichroic Spectroscopy - Exciton Coupling in Organic Stereochemistry*; University Science Books· Mill Valley, California, 1983
5 Hoshi, T , Tanizaki, Y Z *Physik Chem. NF* **1970**, *71*, 230-246.
6 Yamaguchi, H , Ikeda, N , Hirayama, F.; Uekama, *K Chem Phys. Lett* , **1978**, *55*, 75-76
7. Lingham, R B , Hsu, A ; Silverman, K.C., Bills, G F , Dombrowski, A , Goldman, M., Darke, P.L ; Huang, L ; Koch, G ; Ondeyka, J.G , Goetz, M.A. *J Antibiotics* **1992**, *45*, 686-691
8 a Runeberg, *J Acta Chem Scand* **1960**, *14*, 797, b Baggaley, K H ; Norin, T *J Chem Soc Chem Commun* **1968**, 233-234
9 a Ito, T *Agric Biol Chem* **1979**, *43*, 1237-1242; b Ito, T , Arai, T ; Ohashi, Y , Sasada, Y *Agric Biol Chem* **1981**, *45*, 1689-1692
10 Weegen, H ; Nkunya, M H H , Adul El-Fadl, A.; Harkema, S , Zwanenburg, B *J Org Chem* **1990**, *55*, 5107-5109.
11 Bryant, R.W , Light, R J *Biochemistry*, **1974**, *13*, 1516
12 Williams, S T , Parkinson, D , Burges, *N.A Plant and Soil*, **1965**, *22*, 167-186
13 Ridgway, R *Color Standards and Nomenclature*, **1912**, Published by the author, Washington, D C
14 Sutton, B C *The Coelomycetes*, **1980**, Commonwealth Mycological Institute Kew, U K
15 Schwartz, R E ,Giacobbe, R A ; Bland, J A , Monaghan, R L *J Antibiotics* **1989**, *42*, 163-167