EUPENIFELDIN, A NOVEL CYTOTOXIC BISTROPOLONE FROM *Eupenicillium brefeldianum*

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Eupenifeldin was isolated from cultures of *Eupenicillium brefeldianum* ATCC 74184 by extraction and crystallization. The compound was identified as a pentacyclic bistropolone on the basis of spectral data and its complete structure was established by single-crystal X-ray analysis. The compound is cytotoxic against the HCT-116 cell line and has *in vivo* antitumor activity in the P388 leukemia model.

Ongoing efforts to find new natural products with antitumor activity led to isolation of a novel cytotoxic bistropolone from a culture broth produced by *Eupenicillium brefeldianum* ATCC 74184. The active principle of this culture was purified to homogeneity and named eupenifeldin. Its spectral data showed that the molecule had partial symmetry due to the presence of two identically substituted tropolone units each fused in the same manner to dihydropyran rings. The complete structure and relative stereochemical relationships were determined by single crystal X-ray analysis. Only one naturally occuring bistropolone with cytotoxic activity, fusariocin C, has been described previously in the literature.[1,2] It was isolated from the culture filtrate of *Fusarium moniliforme* and had cytotoxic and antitumor activities. Bistropolones made by chemical synthesis have also been reported to possess antitumor activity.[3]

This report describes isolation, physico-chemical characterization, biological properties and the structure of eupenifeldin.

Materials and Methods

Spectral Data

Mass spectra in the FAB mode were obtained on a Kratos MS25 using *m*-nitrobenzylalcohol as matrix. EI spectra were recorded on a Finnigan TSQ70. A Shimadzu UV 2100U spectrophotometer was used for UV spectra and a Perkin-Elmer FTIR Model 1800 spectrometer for IR spectra. All NMR spectra were recorded in $CDCl_3$ with a drop of DMSO-d_6 on a Bruker Model AM-500 spectrometer. Proton-proton coupling constants were obtained by selective irradiations, and carbon-proton one-bond coupling constants were measured in the proton coupled carbon spectrum.

EI mass spectrum: major peaks at m/z 548, 384, 366, 175, 137. IR-spectrum (KBr): 3440, 3260, 2950, 2870, 1630, 1595, 1530, 1445, 1425, 1395, 1280, 1178, 1150, 1082, 889 cm^{-1}.

Fermentation Conditions

To prepare an inoculum for the production phase, 4 ml of the frozen vegetative stock were transferred to a 500-ml Erlenmeyer flask containing 100 ml of vegetative medium consisting of soluble starch 0.5%, glucose 0.5%, fishmeat extract 0.1%, yeast extract 0.1%, NZ-case 0.2%, sodium chloride 0.2% and calcium

carbonate 0.1%. The vegetative culture was incubated at 28°C for 72 hours on a rotary shaker set at 250 rpm. Four ml of seed culture were transferred to a 500-ml Erlenmeyer flask containing 100 ml of production medium. The production medium consisted of soluble starch 2%, Bacto-peptone 0.5%, yeast extract 0.5%, Cerelose 1% and calcium carbonate 0.1%. The production cultures were incubated at 28°C and 250 rpm on the same shaker for 5 days. Maximum production of eupenifeldin was obtained at about 5 days according to the cytotoxicity assay.

Determination of Biological Activities

Cytotoxicity was assessed in HCT-116 and HCT-VM46 human colon carcinoma cells by XTT-assay.[4] Cells were plated at 4,000 cells/well in 96-well microtiter plates and 24 hours later drugs were added and serially diluted. The cells were incubated at 37°C for 72 hours at which time the tetrazolium dye, XTT, was added. The results are expressed as an IC_{50} which is the drug concentration required to inhibit cell proliferation (absorbance at 450 nm) to 50% of that of untreated control cells.

In vivo activity against P388 leukemia in mice is expressed as T/C (%). Samples are given intraperitoneally beginning one day post leukemia implant (10^6 cells ip) and once daily for five consecutive days.

Results and Discussion

Extraction and Crystallization

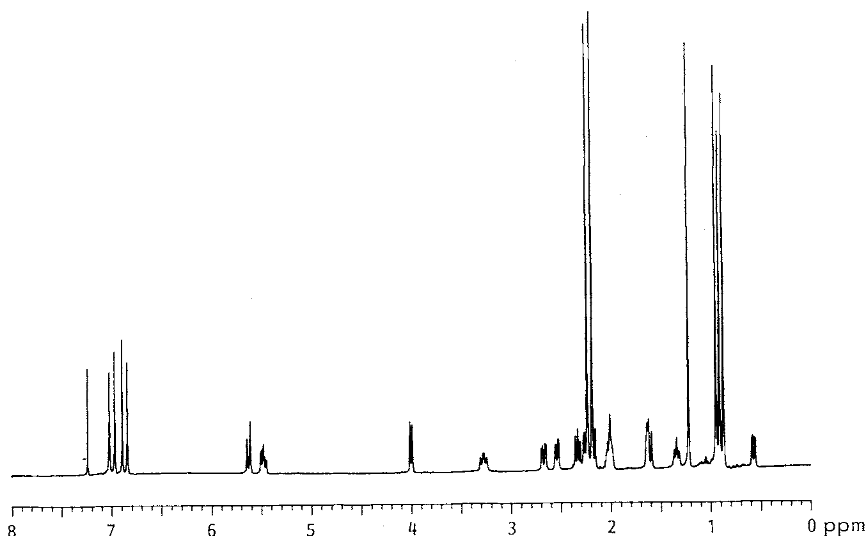
Culture broth from shake flask cultures was pooled for whole broth extraction with ethyl acetate. Extraction studies have shown that most eupenifeldin is mycelium bound and extraction of broth supernatant alone leads to significantly lower yields. Extraction of ten liters of whole broth with ethyl acetate provided an oily yellow residue upon concentration of the extract. Trituration of the residue with cold methanol triggers formation of a solid which after further washing with cold methanol yielded 474 mg eupenifeldin. Crystals for X-ray analysis were prepared by recrystallization from $CHCl_3$ - EtOH.

Physico-chemical Properties and Structure

Molecular weight determination of eupenifeldin by FAB-MS gave rise to a strong M+H ion at *m/z* 549, in addition to low level cluster ions of M+Na (*m/z* 571) and M+K (*m/z* 587). The exact mass of the protonated molecular ion was determined as *m/z* 549.2840 with the most likely molecular composition being $C_{33}H_{40}O_7$. The molecular weight of 548 was confirmed by spectra derived from other ionization methods. DCI with methane produces again a prominent M+H at *m/z* 549 accompanied by a small $M+C_2H_5$ at *m/z* 577, while electron impact ionization generated a molecular ion at *m/z* 548. The EI spectrum is characterized by an intense *m/z* 384 whose exact mass corresponded to a neutral loss of $C_9H_8O_3$. The FAB spectrum of a microscale acetylation experiment showed predominant incorporation of two acetyl groups (*m/z* 633), together with peaks of monoacetate (*m/z* 591) and a triacetate (*m/z* 675), indicating the presence of three HO-groups.

A UV spectrum of the compound in methanol showed maxima at 256, 324 (sh), 360 (sh), and 366 nm and was similar in appearance to the one reported for 3,7-dihydroxytropolone.[5] In the IR spectrum, an absorption band at 1595 cm^{-1} pointed to a highly conjugated or hydrogen bonded carbonyl group. The position of this signal is very close to the reported 1590 cm^{-1} of the carbonyl group in 3,7-dihydroxytropolone[5] and together with the UV spectrum suggested the presence of a tropolone moiety. In fact, from the observation that most carbonyl and olefinic signals in the carbon spectrum appeared in sets of two, we assumed that two substituted tropolone rings were part of the structure. Of the 14 unsaturations in the entire molecule, 10 would be accounted for by two tropolone rings.

Fig. 1. 500 MHz ^1H NMR spectrum of eupenifeldin in $CDCl_3$ - DMSO-d_6.



Inverse gated decoupling showed signals for 33 carbons confirming the molecular formula. Information from the proton spectrum shown in Fig. 1 with data from DEPT, COSY and HETCOR experiments provided the complete set of carbon building blocks for eupenifeldin. Molecular connectivity among the pieces was derived from long-range COSY, HMBC and COLOC. Table 1 summarizes correlations from the COSY spectrum and Table 2 shows HETCOR results and combined long range couplings from HMBC and COLOC.

In the proton spectrum, four singlet signals in the downfield region were observed. Two of the protons correlated to five quaternary carbons through long-range couplings. Of those five quaternary carbons, the one at 170.5 ppm is characteristic of a carbonyl carbon, and two carbons at 160.7 ppm and 162.6 ppm were assigned to oxygen-attached sp^2 carbons. The remaining two downfield protons showed analogous long-range couplings with the respective carbon shifts being 172.3, 159.7, and 162.2. Chemical shifts and long-range carbon-proton correlations seemed to indicate the presence of tropolones A and B in the structure of eupenifeldin (Fig. 2), in agreement with IR and UV spectra. Chemical shifts and coupling constants of tropolones A and B were compared and

Table 1. COSY data of eupenifeldin ($CDCl_3$ - DMSO-d_6).

H position	$\delta\,^1H$, ppm	Multiplicity	H-H correlations (Hz)
H-7	7.02	s	
H-7′	6.97	s	
H-3′	6.87	s	
H-3	6.84	s	
H-11″	5.64	d	5.48 (15.9)
H-12″	5.48	m	5.64 (15.9), 2.53 (4.0), 2.33 (11.0)
H-4″	4.00	d	1.33 (11.3)
H-1″	3.27	dd	2.26 (18.4), 2.00 (13.5)
H-7″	2.67	dd	2.16 (17.3), 1.62 (4.8)
H-13″	2.53	dd	2.33 (13.3), 5.48 (4.0)
H-13″	2.33	dd	2.53 (13.3), 5.48 (11.0)
H-1″	2.26	dd	3.27 (18.4), 2.00 (3.1)
H-8	2.23	s	
H-8′	2.18	s	
H-7″	2.16	d	2.67 (17.3)
H-3″	2.03	m	1.33 (13.8)
H-2″	2.00	m	3.27 (13.5), 2.26 (3.1)
H-8″	1.62	m	2.67 (4.8), 0.56 (4.1)
H-9″	1.61	m	0.56 (14.4)
H-3″	1.33	dd	2.03 (13.8), 4.00 (11.3)
H-19″	1.22	s	
H-16″	0.94	s	
H-17″	0.91	s	
H-18″	0.87	s	
H-9″	0.56	dd	1.61 (14.4), 1.62 (4.1)

Table 2. HETCOR data and long-range couplings of eupenifeldin ($CDCl_3$ - DMSO-d_6).

Carbon position	$\delta^{13}C$, ppm	Multi-plicity	$\delta^{1}H$, ppm	Long-range ^{1}H-^{13}C
Tropolone A				
1′	172.3	s		6.87, 6.97
2′	162.2	s		6.97, 6.87
3′	112.9	d	6.87	
4′	159.7	s		
5′	118.7	s		6.87, 2.67, 2.18, 6.97, 1.62
6′	151.2	s		2.18
7′	124.9	d	6.97	2.18
8′	26.9	q	2.18	6.97
Tropolone B				
1	170.5	s		6.84, 7.02
2	162.6	s		7.02, 6.84
3	114.1	d	6.84	
4	160.7	s		6.84
5	123.8	s		3.27, 2.23, 6.84, 7.02
6	150.7	s		2.23
7	124.3	d	7.02	2.23
8	26.9	q	2.23	7.02
Aliphatic rings				
1″	32.5	t	3.27, 2.26	2.00
2″	40.9	d	2.00	4.00, 3.27, 2.53, 2.33, 2.03, 1.22
3″	29.6	t	2.03, 1.33	4.00
4″	69.9	d	4.00	2.03, 1.33, 0.94
5″	81.5	s		4.00, 2.16, 0.94, 1.61
7″	33.8	t	2.67, 2.16	1.62
8″	31.3	d	1.62	1.61, 2.67, 0.56, 0.94
9″	45.9	t	1.61, 0.56	2.67, 0.91, 0.87
10″	34.4	s		1.61, 5.48, 5.64, 0.56, 0.87, 0.91
11″	143.7	d	5.64	2.53, 2.33, 5.48, 0.56, 0.87, 0.91
12″	125.0	d	5.48	5.64, 2.53, 2.33
13″	45.6	t	2.53, 2.33	5.64, 1.22
14″	80.6	s		2.33, 1.33, 1.22, 2.53
16″	15.5	q	0.94	4.00
17″	26.6	q	0.91	5.64, 1.61, 0.87
18″	29.1	q	0.87	5.64, 1.61, 0.91
19″	18.8	q	1.22	2.33

agreed well with those observed for the tropolone ring in colchicine.[6)] Table 3 summarizes the data for tropolone B.

The prominent neutral loss of $C_9H_8O_3$ in the electron impact mass spectrum constitutes additional evidence for tropolone rings at both ends of the molecule. Determining structural features of the aliphatic portion started with three independent spin systems that were determined by their proton-proton couplings from COSY and long-range COSY spectra. Together with corresponding carbon chemical shifts from the HETCOR spectrum, three

Table 3. ^{13}C chemical shifts and $^{n}J_{C,H}$ of tropolone B in eupenifeldin and of colchicine.

Position	Tropolone B of eupenifeldin		Colchicine	
	$\delta^{13}C$ (ppm)	$^{n}J_{C,H}$ (Hz)	$\delta^{13}C$ (ppm)	$^{n}J_{C,H}$ (Hz)
1	170.5		179.6	
2	162.6	$^{2}J_{C2,H3}=6.3$	164.1	$^{2}J_{C2,H3}=6.0$
3	114.1	$^{1}J_{C3,H3}=155.4$	113.2	$^{1}J_{C3,H3}=151.7$
4	160.7		135.8	
5	123.8		137.2	
6	150.7		152.9	
7	124.3	$^{3}J_{C2,H7}=7.5$	130.5	$^{3}J_{C2,H7}=5.8$

Fig. 2. Structure of eupenifeldin as determined by single crystal X-ray analysis.

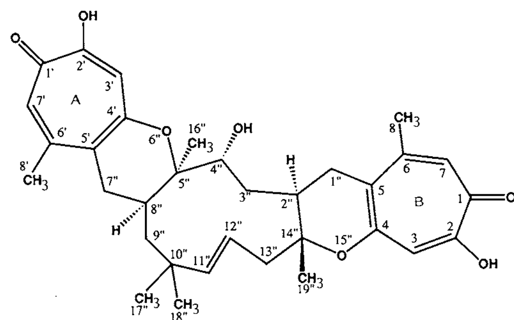


Table 4. *In vivo* activity of eupenifeldin in treating P388 leukemia in mice.

Dose (mg/kg/day)	Median survival time (days)	% T/C	Body weight change on day 4 (grams)
Olivomycin A			
0.8	15.5	141	−2.2
0.4	16.0	145	−1.5
Eupenifeldin			
2	9.0	82	−3.0
1	15.0	136	−3.5
0.3	13.5	123	−1.6
Control	11.0	100	−0.7

structural fragments comprising carbons C(7″) to C(9″), C(11″) to C(13″) and C(4″) to C(1″) were defined. Connectivities of these fragments to the three quaternary centers established the structure of the 11-membered ring at the center of eupenifeldin. Protons on carbons C(9″), C(11″) and C(12″) coupled to quaternary carbon C(10″), while protons on carbons C(7″) and C(4″) coupled to C(5″). Couplings of the third quaternary carbon C(14″) to protons on carbons C(3″) and C(13″) placed the last two bonds of the central ring structure. Attachment of tropolones A and B through carbons C(1″) and C(7″) was evident from three bond couplings between C(1″) and C(4), and between C(7″) and C(4′). To complete the structure and account for two remaining degrees of unsaturation, the location of two ring forming ether linkages from the tropolones to the central macrocycle and the position of one hydroxy group had to be determined. Assuming that C(4″) carried the hydroxy group, several possible structures could be drawn: C(5″) connecting to O(15″) and C(14″) connecting to O(6″); or C(5″) connecting to O(6″) and C(14″) connecting to O(15″); or C(5″) connecting to O(15″) and C(4) connecting to O(6″). However, since C(5″) and C(14″) are alternative carriers of the one remaining hydroxy group, even more permutations would have to be considered.

While efforts were under way to use NOESY spectra to find a definitive answer, we succeeded in growing crystals suitable for X-ray analysis. Single crystal X-ray analysis was performed and provided the complete structure including relative stereochemistry. The structure is shown in Fig. 2. Details of the X-ray analysis will be published separately.

Biological Activity

A variety of natural and synthetic tropolones has been reported to possess antitumor activity.[3,5,7,8] So far, the only natural bistropolone with cytotoxic activity is fusariocin C.[1,2] Eupenifeldin produced by *Eupenicillium brefeldianum* ATCC 74184 is the second example of a naturally occurring cytotoxic bistropolone. Its cytotoxic and antileukemic activities contrast with very weak activity against bacteria (*Bacillus subtilis*, *Micrococcus luteus*) and *Saccharomyces cerevisiae*. While not general for tropolones, weak activity against bacteria and fungi has also been reported for fusariocin C[1] and the fungal metabolite BMY-28438, a 3,7-dihydroxytropolone.[5]

Cytotoxicity of eupenifeldin was determined as described in the Materials and Methods section. Against the HCT-116 cell line, the IC_{50} was 0.005 μg/ml, against HCT-VM46 cells it was 0.002 μg/ml. While no IC_{50} values were reported for fusariocin C, toxic effects of the compound on HeLa cells were

observed at 5 μg/ml, and toxic effects on leukemia L1210 cells ranged from 1 to 20 μg/ml.[1] The IC_{50} for BMY-28438 was reported as 0.04 μg/ml against murine B16-F10 melanoma cells.[5]

In vivo activity of eupenifeldin was tested by treating P388 leukemia in mice. Olivomycin A served as a positive control. As can be seen, eupenifeldin provided moderate prolongation of life span. Table 4 summarizes the data. Fusariocin C was reported weakly active in prolonging survival against Ehrlich carcinoma, but inactive against sarcoma 180.[1] BMY-28438 was inactive against P388 leukemia, but prolonged the life span of mice with B16 melanoma.[5]

Taxonomy

The results of taxonomic studies performed on strain ATCC 74184 indicate that the producing organism is a strain of *Eupenicillium brefeldianum*.[9] Colonies on CZAPEK's Agar attain a diameter of 4.1 cm in 14 days at 28°C. They show a thin basal felt radially furrowed. The center is raised and the surface appears floccose. The center is light yellow with white radiating out. The colony reverse has an orange-yellow center and colorless towards the margin. Exudate is lacking and no soluble pigments are formed. Conidial structures are very few in number. Cleistothecia occur in a layer adjacent to the agar surface obscured by a layer of vegetative hyphae.

Colonies on malt agar reach a diameter of 4.9 cm in 14 days at 28°C. They are floccose with cleistothecia imbedded under the vegetative hyphae. The reverse is pale yellow. No exudate or soluble pigments are formed. Cleistothecia are not hard, measure 150~200 μm and are light tan in color. Ripening seems to start in the center of the colony, asci are produced within 7 days and mature in 15 days. By 20 days, the entire colony consists of ascospores.

Asci are born singly on short branches from ascogenous hyphae.[10] They are globose, measure 7~8 μm in diameter and contain 8 spores. Ascospores are lenticular in shape and measure 7~8 μm in diameter. They are hyaline and finely spinulate with a very faint equatorial furrow.

Conidiophores are smooth-walled and short. Penicilli are monoverticillate with one or two short branches.[11] Phialides measure 7 μm by 3 μm. Conidia are smooth and subglobose measuring 3 μm by 2 μm. Conidial structure was rarely observed to give a statistically significant number of measurements.

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