FUNICULOSIN, A NEW ANTIBIOTIC

II. STRUCTURE ELUCIDATION AND ANTIFUNGAL ACTIVITY

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Structure elucidation and some biological properties of an antiviral and antifungal antibiotic, funiculosin, are summarized. Funiculosin is a derivative of N-methyl-4-hydroxy-3, 5-disubstituted-2-pyridone containing a novel substituent, cyclopentanetetrol moiety, that is reported here for the first time in a natural product. The antibiotic protects guinea pigs efficiently against experimental trichophytosis. Toxicity of funiculosin is unique and highly selective for animal species.

In 1969, we reported the fermentation, isolation and biological properties of funiculosin[1]. In a screening program for antiviral and antitumor antibiotics[2], an acetone extract of *Penicillium funiculosum* THOM mycelium showed activity against some animal viruses in the agar-diffusion plaque-inhibition test[3]. This fungal species was already known to produce an antiviral principle, helenine[4]; the later proved to consist of virus-like particles with interferon-inducing activity[5]. An antiviral substance, $C_{27}H_{41}NO_7$, was isolated in crystalline form from the acetone extract of *P. funiculosum* mycelium and named funiculosin.

In addition to its antiviral activity, the antibiotic showed antifungal and marginal antitumor activity against EHRLICH ascites tumor.

This paper deals with further advances in the study of funiculosin, with special emphasis on structure elucidation.

Structure Elucidation

Funiculosin readily crystallizes into fine needles on repeated purification through silica gel column chromatography. Crystals are so fine that they are unsuitable for X-ray crystallographic analysis. The molecular formula, $C_{27}H_{41}NO_7$, was determined from a molecular ion peak at *m/e* 491 and elementary analysis. The molecular formula is suggestive of a steroid, but funiculosin is negative in the LIEBERMAN-BURCHARD test. The antibiotic forms a pentaacetate, $C_{37}H_{51}NO_{12}$, *m/e* 701, and can be converted to dihydro-, $C_{27}H_{43}NO_7$, *m/e* 493, and tetrahydrofuniculosin, $C_{27}H_{41}NO_7$, *m/e* 495, on catalytic hydrogenation.

The presence of N-methylpyridone was deduced from its UV, IR and pmr spectra which had been demonstrated in a previous paper[1]. Funiculosin possesses an aromatic proton signal but the absence of absorption bands at 1600 and 1500 cm^{-1} in IR indicates that the ring is not a phenyl moiety. The strong bands at 1656 and 1566 cm^{-1} are very similar to those of N-methyl-2-pyridone and N-methyl-3-methyl-2-pyridone. Comparison of the UV spectrum with those of pyridones revealed that the absorption

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band and the molar absorptivity are approximately the same as those of N-methyl-3-ethyl-4-hydroxy-5-methylpyridone. Therefore, funiculosin must be a derivative of N-methyl-4-hydroxy-3, 5-disubstituted-2-pyridone.

The base peak is *m*/*e* 360 in mass spectrum of funiculosin corresponding to $[M-131]^+$. A fragment of $C_5H_7O_4$ (m=131) is suggestive of cyclopentanetetrol moiety, the presence of which is supported by positive periodate-benzidine test and formation of mono- and diisopropylidene derivatives. The locus of this moiety should be at C–5 of the pyridone, since a proton signal attached to the ring shifted to 0.4 and 0.25 ppm higher field in the pmr spectra of the pentaacetate and diisopropylidene derivative.

The structure of another side chain, $C_{16}H_{27}O$, was determined by spin-spin decoupling technique in pmr spectrometry[7]. The presence of 5 methyl groups, in which 3 are attached to aliphatic groups and 2 to double bonds, and 2 olefinic bonds is evident from the pmr spectrum. On irradiation at δ 2.25, a broad signal at δ 5.75 (1H) and a triplet at δ 4.93 (1H, J=6.7 Hz) collapsed correspondingly into two sharp singlets. This fact indicates the following partial structure

In pmr spectrum of dihydrofuniculosin, a triplet at δ 4.93 changed to a doublet doublet (J=9.0 and and J=4.6 Hz) and an original broad singlet at δ 4.60 shifted to δ 3.52 (doublet, J=8.7), indicating that this part forms a 6-membered ring, shown as follows:

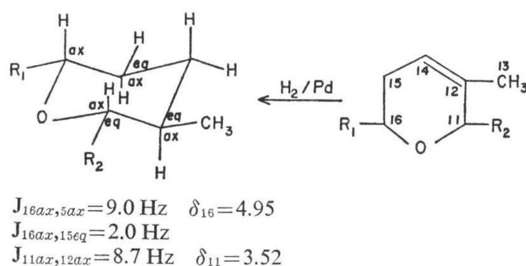
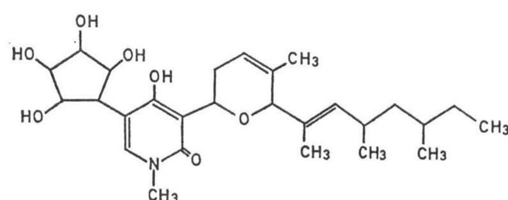


Fig. 1. Structure of funiculosin.



Chemical shift of a proton attached to C_{16} is unaltered when hydrogenated. Therefore, C_{16} should be adjacent to the pyridone ring. Upon further hydrogenation of dihydrofuniculosin, a methine proton at C_{11} transformed to a doublet doublet ($J_{11,12}$=7.0 Hz, $J_{11,9}$=2.0 Hz) and shifted to 0.37 ppm higher field. Thus, C_{11} should be adjacent to a double bond with a methyl group,

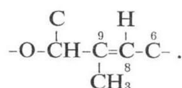
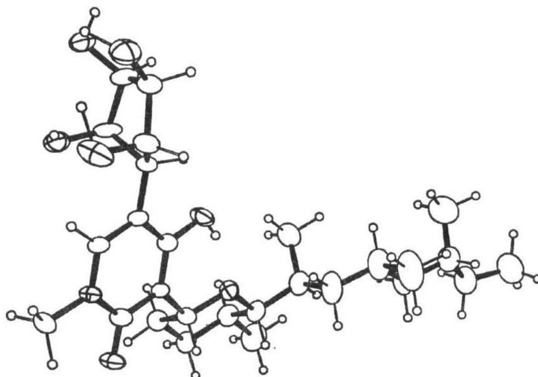
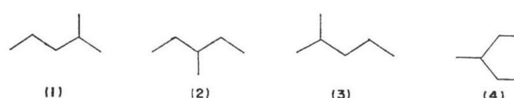


Fig. 2. Absolute configuration of tetrahydrofuniculosin.



On irradiation of a multiplet at δ 2.53, a doublet at δ 5.22 (1 H, J=9Hz) and a methyl proton at δ 0.9 collapsed to a singlet. Therefore, a methyl is attached to C–6. The double bond between C–9 and –8 was determined to be *trans*

by NOE. Two methyl groups are present in the rest of the molecule, C_5H_{11}. According to LINDEMAN-ADAMS equation[8], the most plausible structure (2) was selected from 4 possibilities.



The structure proposed by the chemical study is demonstrated in Fig. 1.

Conclusive evidence of the structural validity was obtained by X-ray crystallography using tetrahydrofuniculosin. The absolute configuration determined by the multi-solution method is shown in Fig. 2. The cyclopentanetetrol moiety is reported for the first time in natural products[9].

Toxicity

Toxicity of funiculosin is unique and highly specific; the acute toxicity varies with animal species. Funiculosin is highly toxic to mice and rats (LD_{50} 5~7 mg/kg through all routes), whereas it is virtually non-toxic to guinea pigs and rabbits. The antibiotic is well absorbed through the gastrointestinal tract so that oral LD_{50} is approximately the same as intraperitoneal LD_{50} for rats and mice. Unexpectedly, the antibiotic is not toxic to guinea pigs and rabbits; these animals are able to tolerate even 2 g/kg of the oral dose. Moreover, guinea pigs showed no sign of toxicity when administered an intraperitoneal injection of 500 mg/kg. The dermal toxicity is also depend on the animal species; when the ointment containing funiculosin was repeatedly applied to naked skin of rats or mice, some animals died. However, guinea pigs were normal even under repeated application of the ointment containing 10% funiculosin. At autopsy, congestion of lungs was noted in rats and mice, but not in guinea pigs and rabbits. As far as we know, such species difference in the acute toxicity of an antibiotic has never been reported. The reason for this selective toxicity is unknown.

Antifungal Activity

Funiculosin showed activity against a wide variety of pathogenic fungi. As shown in Table 1, the minimum effective concentrations, that is, the concentrations that inhibit growth for 3 days, are comparable to those of polyene macrolide antibiotics. When incubation was prolonged to 7 days, most of

Table 1. Antifungal activity of funiculosin.

Fungal strains	Funiculosin		Griseofulvin	
	MIC	MEC	MIC	MEC
Trichophyton asteroides	63	0.97	15	15
T. mentagrophytes	15	0.97	15	15
T. rubrum	3.9	0.12	15	15
T. schoenleinii	>500	7.8	>500	>500
T. ferrugineum	125	0.97	31	31
Microsporum canis	125	0.48	7.8	3.9
M. gypseum	<0.12	<0.12	<0.12	<0.12
Epidermophyton floccosum	500	31	>500	250
Blastomyces dermatitidis	<0.12	<0.12	<0.12	<0.12
Cladosporium sp.	>500	>500	>500	>500
Hormodendrum pedrosoi	15	15	>500	>500
Sporotrichum schenckii	>500	>500	>500	>500
Aspergillus fumigatus	>500	>500	>500	>500
A. flavus	>500	>500	>500	>500

(to be continued)

Table 1. (continued)

Fungal strains	Funiculosin		Griseofulvin	
	MIC	MEC	MIC	MEC
A. niger	>500	>500	>500	>500
A. oryzae	>500	>500	>500	>500
Penicillium notatum	>500	>500	>500	>500
P. chrysogenum	>500	>500	>500	>500
Mucor pusillus	125	125	>500	>500
Rhizopus nigricans	>500	>500	>500	>500
Candida albicans ouke No 2	>1,000	62	>1,000	>1,000
C. albicans yu-1200	>1,000	62	>1,000	>1,000
C. albicans yu-1202	>1,000	31	>1,000	>1,000
C. albicans yu-1201	>1,000	125	>1,000	>1,000
C. tropicalis No 1	>1,000	125	>1,000	>1,000
C. tropicalis No 2	>1,000	125	>1,000	>1,000
C. pseudotropicalis No 1	>1,000	7.8	>1,000	>1,000
C. pseudotropicalis No 2	>1,000	31	>1,000	>1,000
C. krusei No 1	>1,000	250	>1,000	>1,000
C. krusei No 2	>1,000	25	>1,000	>1,000
C. parakrusei No 1	>1,000	125	>1,000	>1,000
C. parakrusei No 2	>1,000	62	>1,000	>1,000
C. guilliermondii No 1	>1,000	125	>1,000	>1,000
C. guilliermondii No 2	>1,000	31	>1,000	>1,000
Cryptococcus neoformans No 1	>1,000	125	>1,000	>1,000
Cryp. neoformans No 2	>1,000	62	>1,000	>1,000
Cryp. neoformans No 3	>1,000	62	>1,000	>1,000
Saccharomyces delbrueckii	>1,000	125	>1,000	>1,000
S. exiguus	>1,000	62	>1,000	>1,000
S. cerevisiae	>1,000	31	>1,000	>1,000
S. rouxii	>1,000	62	>1,000	>1,000
Pichia membranefaciens	>1,000	125	>1,000	>1,000

MIC and MEC: minimum inhibitory concentration and minimum effective concentration (μg/ml). SABOURAUD dextrose agar was used.

the fungi tested can propagate even under relatively high concentrations of funiculosin. It is noteworthy that the antibiotic inhibits the growth of Dermatophytes, such as *Trichophyton asteroides*, *T. mentagrophytes* and *T. rubrum* at low concentrations. Yeasts and bacteria were resistant to the antibiotic action, though growth of the former was slightly affected by high concentrations. As shown in Table 2, the antifungal activity of funiculosin, in contrast to that of griseofulvin, was reduced in the presence of bovine serum. However, the reduction rate was lower for pentachlorophenol which is virtually inactivated by serum.

Table 2. Effect of bovine serum on the antifungal activity of funiculosin.

Bovine serum added (%)	Minimum inhibitory concentration (μg/ml)		
	Funiculosin	Griseofulvin	Pentachlorophenol
0	7.8	7.8	1.9
20	62	15	250
50	62	15	500

Trichophyton mentagrophytes strain T-1 was used. The minimum inhibitory concentrations were determined after 7 days of incubation using SABOURAUD's dextrose agar plate.

Effects on Experimental Trichophytosis in Guinea Pigs

Guinea pigs were infected by the inoculation of scratched skin with a mycelial suspension of *T.*

mentagrophytes T-1[10]. Treatment was initiated 48 hours after infection by applying the funiculosin-containing ointment once daily for 6 successive days. At an early stage of this investigation, we used a tincture or a vaseline ointment as a carrier, but the curative rates were poor (20~50%). Later, it was observed that the curative rate depends on the vehicle to a great extent; a hydrophylic ointment was found to be highly effective. A 0.5% ointment yielded a high curative rate, 97% in 10 days. Other antifungal agents, now commercialized in Japan, were used as positive controls in this study. Griseofulvin showed the best curative rate, although its application was accompanied with skin stimuli. Naphthiomate was comparable to funiculosin in activity and superior to any others, since it showed no skin-stimuli to guinea pigs. The others were much less effective than the three agents described above (Table 3).

Table 3. Effect of funiculosin on experimental trichophytosis in guinea pigs.

Agents used	Concentration (%)	Curative rate (%)	Stimuli	Redness	Scale
Funiculosin	0.5	99	±	±	−
	1.0	93	±	±	−
	3.0	97	±	±	−
Griseofulvin	3.0	100	±	±	±
Pyrrolnitrin	3.0	26	±	++	+++
Iodoundecylenic acid	3.0	83	+	±	±
Undecylenic acid	3.0	47	+	+	±
Naphthiomate	3.0	98	−	−	−
None	−	0		+++	+++

All the control agents used in this study were commercial preparations. Stimulus, redness and scale are expressed as follows; − none, ± very slight, + slight, ++ moderate and +++ severe. Curative rate is calculated as follows; (total numbers of skin pieces − numbers of skin pieces with positive fungal growth) ÷ total numbers of skin pieces ×100. *Trichophyton mentagrophytes* T-1 was used for the infection.

In conclusion, funiculosin is interesting for (a) its novel structure, (b) its specificity in acute toxicity, and (c) its potent activity against experimental trichophytosis in guinea pigs.

Experimental

Fermentation and isolation of funiculosin were carried out as previously reported with slight modifications. Since production of funiculosin is inhibited by glucose, lactose was used in fermentation. At an early stage of this study, the productivity was 10~30 mg/liter of the fermented broth, but later, it attained 1 g/liter. However, the recovery rate of crystalline funiculosin from broth was consistently poor because extraction from the mycelium is difficult and the antibiotic is labile. Nevertheless, a large scale fermentation (10 m^3 in 15 m^3 tank) afforded appreciable amounts of crystalline funiculosin. In the usual process[1], crystalline material was readily obtained by evaporating the methanol extract from the mycelium and extracting the residue with ethylacetate; the organic solvent extract was subsequently concentrated *in vacuo* without chromatography. Crystals have the shape of fine colorless needles, mp 165~166°C, anal., calculated for $C_{27}H_{41}NO_7$: C 65.96, H 8.41, N 2.85; found: C 66.01, H 8.41, N 2.87.

Determination of MIC and MEC:

Funiculosin is insoluble in water, therefore, a methanol solution was used for serial dilution. SABOURAUD agar was used for fungi and nutrient agar for bacteria. Suitable amounts of funiculosin dissolved

in 0.1 ml methanol were mixed with 10 ml of melted agar and the mixture, after thorough mixing, was poured into a Petri dish. Inoculation was carried out by stamping suspensions of either spores or cells on the agar surfaces, and the plates were incubated at appropriate temperature. On the third day of incubation, growth was examined and the minimum concentrations inhibiting the growth were expressed as MEC. The incubation was further continued and the growth was finally determined after 7 days. The minimum inhibitory concentrations after 7 days were expressed as MIC. The incubation temperature was 37°C for bacteria and 28°C for fungi. The growth of the following bacterial strains was unaffected even in the presence of 1,000 μg/ml; *Staphylococcus aureus*, strains FDA 209 P and EOP-3, *Staph. epidermidis*, strains S-3, R-9, B-5 and 222, *Micrococcus luteus*, strains 1711 and 1732, *Sarcina lutea* strains A and B, *Bacillus subtilis* HA-3, *B. thuringensis*, *Escherichia coli*, strains NIHJ, No 9 and No 11, *Salmonella enteritidis* 7-1, *Shigella flexneri*, strains 3a and 3a-r, *Pseudomonas aeruginosa*, strains 31, Kansai, No 5, Toranomon and GNB-1 70-pa-II, *Klebsiella pneumoniae*, strains 3k-25 and 3k-37 and *Proteus vulgaris* strains, 19 and 1287.

Protection against Experimental Trichophytosis[10]:

Male guinea pigs, strain Hartley, weighing 250 g, were used in this study. They were fed a commercial pellet diet (Oriental Yeast Co., R-1) and tap water *ad libitum*. The hair of both sides and back was shaved, and the skin scratched with sand paper. The inoculum of *Trichophyton mentagrophytes* T-1 was made by scraping the growth from the agar culture in a Petri dish, and grinding the suspension of mycelium and spore in a mixer. The inoculum suspension was applied to the skin and the treatment was initiated 48 hours after the infection. Either ointments or tinctures of the antifungal agents under study were generously applied once daily for 6 successive days. The guinea pigs were sacrificed 10 days after infection by a blow to the head. Small pieces of the skin at infected loci, three pieces each from the back and both sides, were excised. Each piece was divided into 6 portions which were placed on SABOURAUD agar plates supplemented with penicillin G, 100 u/ml, and streptomycin, 100 μg/ml. Plates were examined for fungal growth after 7 days of incubation at 27°C.

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