Communications to the Editor

ISOLATION AND STRUCTURAL ELUCIDATION OF PYRIDOXATIN, A FREE RADICAL SCAVENGER OF MICROBIAL ORIGIN

Sir:

Oxygen-derived free radicals have been known to play a role in a variety of diseases such as autoimmune disease, cardiovascular diseases, diabetes, inflammation, rheumatism and cancer-initiation[1,2]. Thus, it could be expected that free radical scavengers may contribute to the control of these diseases.

In the course of our screening for free radical scavengers of microbial origin, we have isolated new active substances, naphterpin[3], antiostatins[4] and benthocyanin A[5]. Further screening resulted in the isolation of a new free radical scavenger, designated pyridoxatin, from a fungus culture identified as *Acremonium* sp. BX86. It inhibited lipid peroxidation induced by free radicals in rat liver microsomes free from vitamin E[6]. In this paper, we report the isolation and structure elucidation of pyridoxatin.

The producing organism was cultivated in 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of starch 2.5%, soybean meal 1.5%, dry yeast 0.2% and $CaCO_3$ 0.4% (pH 6.2, before sterilization) at 27°C for 4 days on a rotary shaker.

The mycelial cake collected by centrifugation from the fermentation broth (2 liters) was extracted with acetone. The extract was concentrated *in vacuo* to a small volume and then partitioned between EtOAc and water. The separated organic layer was evaporated to dryness and applied to a silica gel column, which was eluted with $CHCl_3$-MeOH-conc NH_4OH (40:8:1). The pooled active fraction was concentrated and rechromatographed on a silica gel column with $CHCl_3$-MeOH (10:1). After evaporation of the active eluate, the dried residue

Table 1. Physico-chemical properties of pyridoxatin.

Appearance	Colorless needle
MP	195~197°C (dec)
$[\alpha]_D^{23}$ (*c* 0.09, MeOH)	−23°
Molecular formula	$C_{15}H_{21}NO_3$
HREI-MS (*m/z*)	Calcd: 263.1522
	Found: 263.1472 (M^+)
Analysis	
Calcd for $C_{15}H_{21}NO_3$:	C 68.42, H 8.03, N 5.32
Found:	C 68.20, H 8.05, N 5.56
UV λ_{max}^{MeOH} nm (ε)	218 (11,600), 288 (4,700)
$\lambda_{max}^{MeOH+HCl}$ nm (ε)	216 (10,200), 246 (3,900), 267 (3,600)
$\lambda_{max}^{MeOH+NaOH}$ nm (ε)	230 (15,900), 262 (4,700), 295 (3,600)
IR (KBr) cm^{-1}	3300, 1630, 1560, 1550, 1530,1470, 1450, 1250

Table 2. ^{13}C and ^1H NMR chemical shifts of pyridoxatin.

No.	Rotamer A (major)		Rotamer B (minor)	
	^{13}C	^1H	^{13}C	^1H
2	160.4		162.7	
3	115.0		115.3	
4	163.9		163.2	
5	99.0	5.98 (d, 8)	99.9	5.98 (d, 8)
6	132.7	7.58 (d, 8)	132.7	7.56 (d, 8)
7	47.5	2.51 (dd, 11, 11)	48.1	2.68 (dd, 11, 11)
8	44.0	3.06 (dddd, 12, 11, 9, 3)	45.2	2.89 (dddd, 12, 11, 9, 3)
9	43.7	0.92 (ddd, 12, 12, 12), 1.74 (ddd, 12, 3, 2)	43.7	0.97 (ddd, 12, 12, 12), 1.74 (ddd, 12, 3, 2)
10	33.0	1.64 (m)	33.0	1.64 (m)
11	45.9	0.76 (ddd, 12, 12, 12), 1.77 (ddd, 12, 3, 2)	45.9	0.80 (ddd, 12, 12, 12), 1.77 (ddd, 12, 3, 2)
12	33.0	2.43 (m)	34.1	2.28 (m)
13	144.7	5.55 (ddd, 17, 10, 9)	144.7	5.59 (ddd, 17, 10, 9)
14	113.0	4.66 (dd, 10, 2), 4.82 (d, 17, 2)	113.0	4.66 (dd, 10, 2), 4.79 (dd, 17, 2)
15	23.1	0.97 (d, 7)	23.1	0.97 (d, 7)
16	21.0	0.73 (d, 7)	21.0	0.76 (d, 7)

ppm from internal TMS in CD_3OD. Values in parentheses show ^1H-^1H coupling constants in J=Hz.

(75 mg) was further purified by Toyopearl HW-40F column chromatography with MeOH to give a pure material. Finally, pyridoxatin was obtained as colorless needles (14 mg) by crystallization from acetone - ethyl acetate.

The physico-chemical properties of pyridoxatin are summarized in Table 1. Its molecular formula was determined as $C_{15}H_{21}NO_3$ by HREI-MS and elemental analysis.

The ^{13}C and ^1H spectral data for pyridoxatin are summarized in Table 2. These NMR spectra indicated that pyridoxatin existed as a mixture of two rotamers A and B. Their ratio was estimated to be approximately 1 : 1 in methanol-d_4 and 2 : 1 in acetone-d_6 by ^1H NMR spectral analysis. For clarity, the NMR spectral analysis is explained with rotamer A.

The ^1H-^1H COSY experiment revealed the sequence from a vinyl residue (δ 4.66 and δ 4.82, 14-H_2; δ 5.55, 13-H) through four methines (δ 3.06, 8-H; δ 2.51, 7-H; δ 2.43, 12-H; δ 1.64, 10-H) and two methylenes (δ 1.77 and δ 0.76, 11-H_2; δ 1.74 and δ 0.92, 9-H_2) to two secondary methyl groups (δ 0.73, 16-H; δ 0.97, 15-H), thereby indicating the presence of a 4,6-dimethyl-2-vinylcyclohexyl moiety as shown in Fig. 1. The assignments of the methylene protons were made by spin decoupling experiments to overcome the severe overlapping of the protons at 0.7 ~ 1.8 ppm. Likewise, the presence of the same moiety in the other rotamer B was deduced by ^1H-^1H COSY and spin decoupling experiments.

The heterocyclic hydroxamic acid part (see Fig. 2) was analyzed by a heteronuclear multiple-bond correlation (HMBC)[7] experiment. The methine proton (δ 2.51, 7-H) in the cyclohexane ring was coupled to C-2 (δ 160.4), C-3 (δ 115.0) and C-4 (δ 163.9). Based on the ^{13}C chemical shifts, C-2 and C-4 were assignable to phenolic or carbonyl carbons. In addition, 5-H (δ 5.98) was coupled to C-3 and C-6 (δ 132.7), and 6-H (δ 7.58) to C-2 and C-4.

By taking into consideration these relations and the molecular formula, there remained three possible structures for the heterocyclic part as shown in Fig. 2. The structure **III** containing a conjugated ketone function was easily excluded due to the higher chemical shift of C-4 (δ 163.9).

Upon methylation with CH_3I in acetone in the presence of K_2CO_3, pyridoxatin gave a dimethyl ether ($C_{17}H_{25}NO_3$, HREI-MS (*m/z*), Calcd: 291.1834, Found: 291.1808 (M^+)) as a mixture of two rotamers (in the ratio of *ca.* 2 : 1 as determined by ^1H NMR taken in $CDCl_3$). In the HMBC spectrum of this ether derivative taken in $CDCl_3$ (see Fig. 3), only

Fig. 1. Structure for the 4,6-dimethyl-2-vinylcyclohexyl moiety of pyridoxatin.

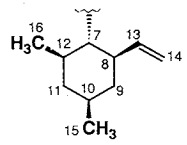


Fig. 2. The possible structures for the heterocyclic hydroxamic acid part of pyridoxatin.

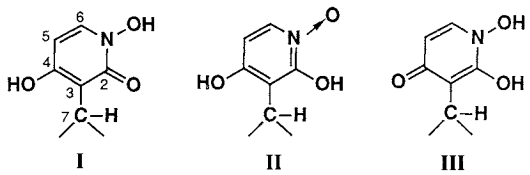


Fig. 3. HMBC analysis for the heterocyclic hydroxamic acid moiety of the dimethyl ether of pyridoxatin.

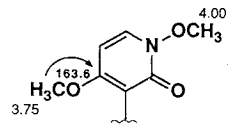
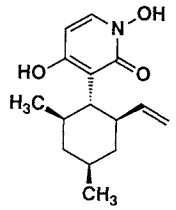


Fig. 4. Structure of pyridoxatin (relative stereochemistry).



one methoxy proton signal at δ 3.75 showed a long-range coupling to the carbon at δ 163.6 in the major rotamer, indicating that the other methoxy group (δ 4.00) must have been introduced to an *N*-hydroxyl group. Therefore, structure **II** was excluded to leave only the 1-hydroxy-2-pyridone moiety **I** for pyridoxatin. A similar phenomenon was observed with the minor rotamer.

In agreement with the conclusion, the direct ^{13}C-^1H coupling constant observed with C-6 (J = 186.8 Hz) was very similar to the corresponding moiety in tenellin[8] (3,4,5-trisubstituted 1-hydroxy-2-pyridone, J = 183.6 Hz). The relative stereochemistry of the cyclohexane ring moiety was assigned as shown in Fig. 1 based on the analysis of the coupling constants summarized in Table 2. Thus, the methine proton (7-H) was coupled to two adjacent methine protons (8-H and 12-H) with large

coupling constants ($J=11$ Hz). Similarly, the methine proton (10-H) has large coupling constants ($J=12$ Hz) with the methylene protons (9-H and 11-H) indicating that these four methine protons of the cyclohexane ring (7-H, 8-H, 10-H and 12-H) are axially oriented. Therefore, the relative stereochemical structure of pyridoxatin is depicted as shown in Fig. 4. The presence of the two rotamers can be ascribed to the hindered rotation along the C-3 ~ C-7 axis.

Pyridoxatin was approximately twenty times (IC_{50} 0.55 μg/ml) as active as vitamin E (IC_{50} 10.8 μg/ml) in the assay system employed[6]. Pyridoxatin inhibited hemolysis of rat erythrocytes catalyzed by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)[9], a free radical initiator (IC_{50} 1.95 μg/ml). So far pyridoxatin inhibited the growth of HeLa cells at the concentration of 1.0 μg/ml (IC_{50}), but it did not show any antimicrobial activity except in tests against *Candida albicans* (MIC 1.64 μg/ml).

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