New Azaphilone Pigments from *Monascus anka*

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Abstract

*Two new azaphilones, monankacin A (**1**) and monankacin B (**2**), were isolated from Monascus anka IFO 30878. Their structure have been elucidated by NMR experiments and other spectral analysis.*

The fungi of the genus *Monascus* were found as contaminants of cereals, starch, silage and other materials and was named by van Tieghem.[1] The color of red-yeast-rice is produced by the growth of *Monascus* species on rice. In Japan, it is known as beni-koji or akakoji, in China and Singapore, it is widely available under the brand name XueZhiKang, and Hypocol™, respectively. Pigments produced by the fungi have been used as natural food colorant for fish, bean, curd and wine in folk medicine in China and East-South Asian countries for hundreds of years. In traditional medicine of China and Japan, it has been used in dyspepsia, diarrhea, excessive lactation, and metrorrhagia mycopathica.[2] Several azaphilone pigments were isolated from *M. anka*. Fungal metabolites having pyrano-quinone structure

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known as azaphilone, because of the affinity of these types of compounds for ammonia, yielding vinylogou γ–pyridones. Monascin, one of the major azaphilonoid pigments of *Monascus anka* (Fam. Monascaceae), has been reported to have inhibitory activity in both peroxynitrile (PN) and ultraviolet light B (UVB) – induced mouse carcinogenic effect[3], and 12-O-tetradecanoylphorbol-13-actate (TPA) - induced inflammation.[4] Monascin also showed immunosuppressive activity on mouse T-splenocytes, but no cytotoxic activity toward rats hepatocytes.[5] Ankaflavin, another azaphilonoid of *M. anka*, was found to be selectively toxic to human cancer cell lines Hep G2 and A549 with a IC_{50} value of 15 μg/mL, while it posed no significant toxicity to normal MRC-5 and WI-38 cells at the same concentration.[6] In our continuing search for monoamine oxidase inhibitors (MAOI) from fungi,[7-9] an azaphilonoid pigment from *Talaromyces luteus*, luteusins A (**8**), has been found to have MAO inhibitory activity.[10-12] As *M. anka* is well known for azapilonoid pigments, we investigated its metabolites for MAOI activity. We obtained a new unique series of coumarin derivatives having MAOI activity,[13,14] and two new azaphilones, monankacin A (**1**) and monankacin B (**2**). This paper deals the structure elucidation of these two new azaphilones.

The strain *Monascus anka* IFO 30873 was cultured in modified Nishikawa's medium at 28°C for 28 days. Harvested dried mycelia was extracted with *n*-hexane, $CHCl_3$ and MeOH successively. From the crude extracts, **1**, **2**, monascin (**3**), ankaflavin (**4**), co-enyme Q (**7**), monankarin A ~ E[13] have been purified by using diffident chromatographic techniques and crystallization. Presence of monascorubrin (**5**) and rubropactating (**6**) have been confirmed by TLC comparison, but not isolated due to low yield. Monascorubrin and rubropactating were purified from a mixtures of the pigments which was obtained as a gift from Nihon University.

Monankacin A (**1**) was obtained as a yellow amorphous powder. The molecular formula was determined to be $C_{22}H_{32}O_4$ by High-Resolution Fast Atom Bombardment mass spectrometry (HR-FABMS) (obs. m/z 399.1913). The UV spectrum of **1** was found to be very similar to

that of monascin. The absorption in the IR spectrum of **1** at 1705 cm^{-1} and 1650 cm^{-1} suggested the presence of an isolated and a conjugated carbonyl groups, respectively.

Analysis of ^{1}H- and ^{13}C-NMR spectra in benzene-d_6 together with 2D-

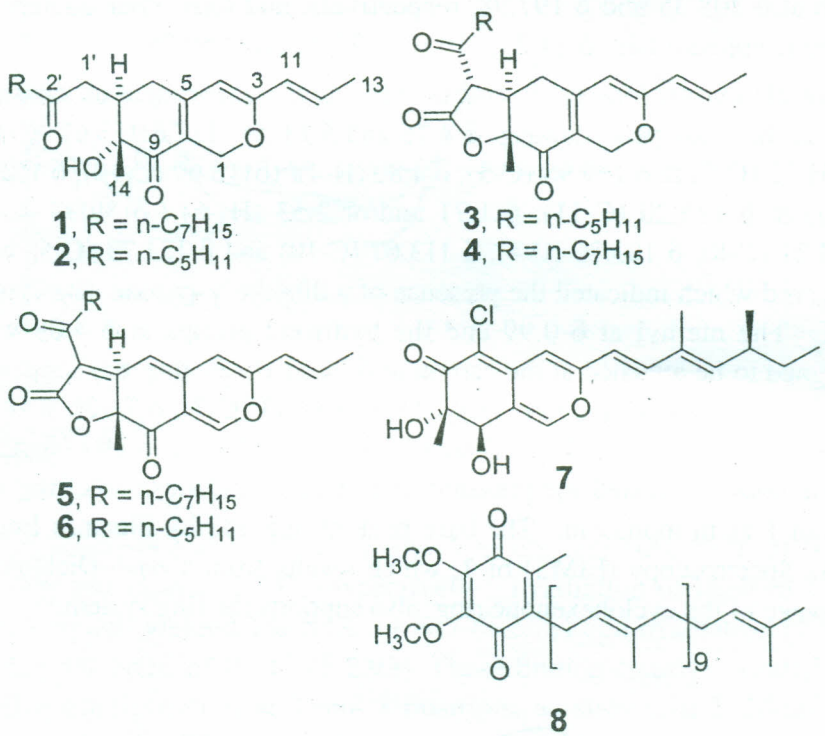
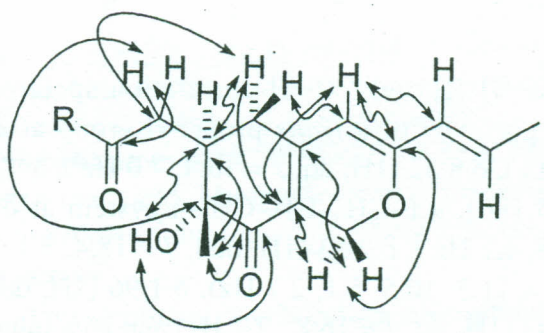


Fig-1

NMR such as ^{1}H-^{1}H and ^{13}C-^{1}H correlation spectroscopy (COSY) revealed the presence of a *trans* propenyl group at δ 1.46 (3H, dd, J = 7.0, 1.0 Hz.), δ 6.32 (1H, dq, J = 15.4, 7.0 Hz.), and δ 5.58 (1H, dq, J = 15.4, 1.5 Hz.), a ($-CH_2-CH-CH_2-$) system at δ 1.71 (1H, ddt, J = 18.5, 11.5, 1.7 Hz.), δ 2.53 (1H, ddd, J = 18.4, 5.1, 1.2 Hz.), δ 2.71 (1H, dddd, J = 11.5, 10.5, 5.1, 2.7 Hz), δ 1.96 (1H, dd, **J** = 16.8, 10.5 Hz) and δ 2.93 (1H, dd, J = 16.8, 2.7 Hz) and an aliphatic side chain at δ 0.90 (3H, t, **J** = 7.1 Hz.), δ 1.16~1.30 (8H, m), δ 1.50~1.57 (2H, m), δ 2.01 ~ 2.05 (2H, m). An isolated olefinic proton at δ 4.08 (1H, s),

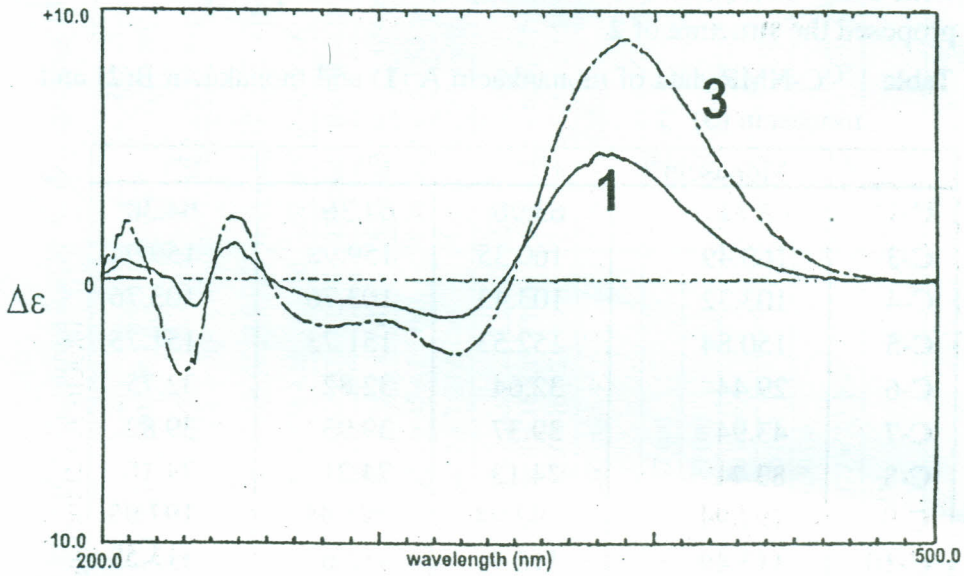
two AB type of protons at δ 4.75 (1H, dt, J = 12.4, 1.5 Hz.), δ 5.13 (1H, dd, J = 12.5, 1.5 Hz), a methyl group at δ 0.99 (3H, s) and an aliphatic -OH proton at δ 4.08 (1H; s) were also observed in the ^{1}H-NMR. ^{13}C-NMR indicated an isolated and a conjugated carbonyl carbons at δ 208.35 and δ 197.98, respectively, and four other quaternary carbons appeared at δ 74.21, 113.67, 151.73 and 159.98.

In correlation via long range coupling (COLOC) experiment as shown in Fig. 2, the cross peaks among δ 4.75 and 5.13 (H_2-1) / δ 113.67 (C-10), δ 151.73 (C-5) & δ 159.9S (C-3); δ 4.80 (H-4)/ (δ113.69 (C-10), δ 159.98 (C-3) & δ 125.20 (C-11); δ 1.71 and/or 2.53 (H_2-6) / δ 39.93 (C-7), δ 74.21 (C-8), δ 103.76 (C-4), δ 113.67 (C-10) and δ 151.73 (C-5) were observed which indicated the presence of a dihydro γ–pyrone ring system in **1**. The methyl at δ 0.99 and the hydroxyl groups at δ 4.08 were assigned to be attached at the carbon at δ 74.21 (C-8), that was supported by the cross peaks of δ 0.99 (CH_3-14) / δ 39.93 (C-7), δ 74.21 (C-8) & δ 197.98 (C-9), and δ 4.08 (OH-8) / δ 39.93 (C-7) and δ 197.98 (C-9). These results revealed the presence of a pyrano-cyclohexenone ring system in **1** as in monascin. The base peak at m/z 162 by Electron Impact Mass Spectroscopy (EIMS) of **1**, which results from a *retro*-Diels-Alder cleavage of the cyclohexenone ring, also supports the ring system.[15]



Fig–2 Cross peaks in COLOC experiment

Relative configuration of C-7 and C-8 were assigned by the differential nuclear Overhauser (NOEDF) experiments. Irradiation of the



Fig–4 Comparison CD spectra of **1** and **3**

methyl resonance at δ 0.99 (C-14) yielded 6% and 10% NOE of H_a-1´ (δ 1.96) and H_a-6 (δ 1.71), respectively. Irradiation of H_b-6 (δ 2.53) proton signal yielded 1% NOE of H-7 (δ 2.71) and irradiation of H-7 yielded 5% NOE of H_b-1´ (δ 2.93). These finding clearly revealed the relative configuration at 7 and 8-positions as shown in **1**. Measurement of Circular Dichroic (*CD*) spectrum of **1** showed close similarity to that of **3** (Fig. 3). This finding revealed that **1** and **3** both have the same absolute configuration. Thus the absolute stereochemistry of both of C-7 and C-8 were determined to be **R**.

Monankacin B (**2**) was obtained as a yellow amorphous powder. In EIMS, **2** showed molecular ion peak at m/z 332 which was two methylenes (28 mass unit) less than that of **1**, although it had the same base peak, m/z 162. The ^{13}C-NMR of **2** was very similar to **1**, except for the absence of two secondary carbons at δ 29.52 and δ 29.56. The ^{1}H-NMR (Table 1) was also similar to that of **2**, the only difference

being the absence of four methylenes at δ 1.16–1.30. Taken together with these evidence, the similarity of the CD spectra in **1** and **2** proposed the structure of **2**.

Table 1 ^{13}C-NMR data of monankacin A (**1**) and monakacin B(**2**) and monascin (**3**)

	Monascin[a)]	1[a)]	1[b)]	2[b)]
C-1	63.81	63.96	64.36	64.30
C-3	160.49	160.35	159.98	159.94
C-4	103.32	103.43	103.76	103.76
C-5	150.84	152.53	151.73	151.75
C-6	29.44	32.64	32.82	32.75
C-7	43.94	39.37	39.93	39.82
C-8	83.21	74.13	74.21	74.16
C-9	197.94	197.94	197.98	197.95
C-10	113.49	112.94	113.67	113.58
C-11	124.41	124.70	125.20	125.13
C-12	135.43	134.67	133.78	133.79
C13	18.51	18.45	18.16	18.15
C-14	17.75	19.93	20.01	19.98
C=O	169.58			
C-1'	54.89	42.02	42.11	42.04
C-2'	202.51	210.08	208.35	208.33
C-3'	43.94	43.26	43.05	42.93
C-4'	22.78	23.87	24.11	23.71
C-5'	31.16	29.06[c)]	29.52[d)]	31.67
C-6'	22.42	29.19[c)]	29.56[d)]	22.81
C-7	13.90	31.67	32.10	14.12
C-8'		22.61	23.02	
C-9'		14.07	14.29	

a) in $CDCl_3$, b) in C_6D_6, c) & d) interchangeable

Table:2 ^{1}H-NMR assignment and comparison of monankacin A(**1**), monankacin A (**2**) monascin (**3**).

	3[a]	1[a]	1[b]	2[b]
H_2-1	4.72 (d,*J* = 12 Hz) 5.06 (dd, J= 2,12 Hz)	4.75 (d, *J* = 12 Hz) 5.02 (dd, J= 2,12 Hz)	4.75 (td,J = 2,12 Hz) 5.13 (dd,2,13)	4.75 (dt, *J* = 2, 12 Hz) 5.13 (dd, *J* = 2, 13 Hz)
H-4	5.27 (s)	5.22 (s)	4.08 (s)	4.80 (s)
H_a-6	2.44 (dd, *J* = 17.6, 17 Hz)	2.10 (ddt,*J*=19.4, 12.4, 1.5 Hz)	1.71(tdd, *J* = 18.5,11.5,1.7 Hz)	1.71 (tdd, *J* = 2,12,18 Hz)
H_b-6	2.67 (dd, *J* = 4,17 Hz)	2.57-2.64 (m)	2.53(ddd, *J* = 18.4,15.1,1.2 Hz)	2.53 (ddd, *J* =18.4,4.8, 1.2 Hz)
H-7	3.23(1H,ddd, *J* = 11,13,14 Hz)	2.57-2.64 (m)	2.71(dddd, J =11.4,10.7,4.8, 2.7 Hz)	2.71 (dddd, J =11.4,10.7,4.8, 2.7 Hz)
H-11	5.91 (dd, *J* = 15.1, 1.6 Hz)	5.89 (dd, *J* = 15.3, 1.5 Hz)	5.58 (dq, *J* = 15.1, 1.5 Hz)	5.58 (dq, *J* = 15.1, 1.56 Hz)
H-12	6.51 (qd, *J* = 15.4,7.0 Hz)	6.47 (qd, *J* = 15.4, 7.0 Hz)	6.32 (qd, *J* = 15.4,7.0 Hz)	6.30 (qd, *J* = 15.4,7.0 Hz)
H_3-13	1.87 (dd, *J* = 7.1,1.6 Hz)	1.86 (dd, *J* = 7.1,1.5 Hz)	1.46 (dd, *J* = 7.0,1.0 Hz)	1.46 (dd, *J* = 7.0,1.6 Hz)
H_3-14	1.45 (s)	1.16 (s)	0.99 (s)	0.99 (s)
Ha-1′ H_b-1′	3.65 (d, *J* = 13 Hz,)	2.36 (dd, *J* = 16.8,10.3 Hz) 2.97 (dd, *J* = 16.8,2.6 Hz)	1.96((dd, *J* = 16.8,10.7 Hz) 2.93 (dd, *J* = 16.8,2.7 Hz)	1.96 (dd, J = 16.8,10.7 Hz) 2.92 (dd, J = 16.8,2.7 Hz)
H_a-3′ H_b-3′	2.61 (dt, *J* = 18.1,7.4 Hz) 3.01 (dt, *J* = 18.1,7.4 Hz)	2.33-2.50 (m)	2.02-2.05 (m)	1.99-2.03 (2H,m)
H_2-4′	1.60-1.66 (m)	1.56-1.61 (m)	1.50-1.57 (m)	1.49-1.55 (m)
H_2-5′	1.26 -1.36 (m)	1.18-1.29 (m)	1.16-1.30 (m)	1.10-1.27 (m)
H_2-6′H2-	1.26 -1.36 (m)	1.18-1.29 (m)	1.16-1.30 (m)	
H-7′		1.18-1.29 (m)	1.16-1.30 (m)	
H2-8′		1.18-1.29 (m)	1.16-1.30 (m)	1.10-1.27 (m)
H_3-9′	0.90 (t, *J* = 7.0 Hz)	0.87 (t, *J* = 7.0 Hz)	0.90 (t, *J* = 7.1 Hz)	0.86 (t-like, J = 7.2 Hz)
HO-9		3.79 (s)	4.08 (s)	4.08 (s)

a = in $CDCl_3$, b = in C_6D_6

MAO inhibitory activities of **1** and **2**, together with the related compounds, monascm, ankaflavin and monascorubrin and rubropanctatin were measured by modified Kraml's methods using crude MAO fraction prepared from mice liver homogenate.[16] None of these azaphilones showed significant activity up to a concentration of 1×10^{-4}M. This result indicates that azaphilone ring system on its own is not responsible for MAO inhibitory activity, rather the presence of other substituents in the ring system might be responsible for the inhibitory activity of **7** (IC_{50} 6.6×10^{-4} M).

Experimental

All melting points were determined on Yanagimoto melting point apparatus and are uncorrected. UV spectra were measured on Hitachi U-3200. IR spectra were measured on Hitachi EPI-G3 and Hitachi 26040. ^{1}H- and ^{13}C-NMR spectra were measured on JEOL GSX-400, GSX-500 and GSX-A500 spectrometers. EIMS was measured on Hitachi M-60, FAB-MS on Finnigan MAT TSQ-70 and JEOL JMX-HX 110A, HREIMS on Hitachi RMU-7M and JMS-HX110A. ORD and CD spectra were recorded on JASCC J-20 and J-500 polarimeters, respectively.

Cultivation and Isolation

Monascus anka IFO 30873 was grown at 31°C for 28 days in stationary culture medium (20 L). The medium consists of 10 g peptone, 100 g sucrose, 2.0 g KNO_3, 2.0 g $(NH_4)_2HPO_4$, 0.5 g $MgSO_4$, 0.5 g $ZnSO_4$, and 0.14g $CaCl_2$ in one litter. After sterilization, the mycelia were collected and dried at 40°C for 48 hours.

Mycelia (508.5 g) were extracted by *n*-hexane, chloroform and methanol, successively, to get *n*-hexane-ext. (29.7 g), chloroform-ext. (50.0 g), methanol-ext. (35.0 g), respectively. The *n*-hexane-ext. gave precipitates (4.7 g) during concentration, which was applied to flash chromatography using silica gel (silica gel C-60). Fractions 1d and 1f

eluted with benzene-acetone 100:1 were crystallized independently from benzene and/or *n*-hexane-acetone to yield ankaflavin (16 mg) and monascin (19 mg), respectively. Separation of chloroform-ext. (31.0 g) by silica gel column chromatography eluted with benzene-ethyl acetate, and then by precipitation from *n*-hexane gave crude monascin (1.50 g), which was further purified by crystallization from ethanol. As previously reported, coumarin-compounds were obtained from the methanol-ext. after combined together with the extract of the next large scale culture.[13]

Large scale culture was proceeded similarly as before in stationary culture medium (40 L). Mycelia (887 g) were extracted with *n*-hexane and methanol, successively, at room temperature. The *n*-hexane ext. (91 g) was chromatographed on silica gel (Wakogel C -200) with *n*-hexane-EtOAc 20:1 to get fr. 1A–1C. Fraction 1B (4.7 g) was separated by Sephadex LH-20 (MeOH) and then by silica gel flash chromatography (YMC gel, *n*-hexane-EtOAc 10:1) to give fr. 3A-3E. Fraction 3B (214 mg) and fr. 3C (247 mg) were further separated by flash chromatography on silica gel (YMC gel) with *n*-hexane-EtOAc 10:1 as an eluent. The former fraction yielded monankacin A (**1**, 113 mg), and the latter gave monankacin A (**1**, 70 mg) and B (**2**, 60 mg) together with both mixture (50 mg). Fr. 1C (2.2 g) showed the presence of a mixture of monascin and ankaflavin on TLC, but they were not isolated.

Monankacin A (1): It obtained as yellow amorphous mass. ; $[\alpha]_D$ +166.2° (c = 0.0351, MeOH). HR-EIMS m/z: 399.1913 (M^+; for $C_{22}H_{32}O_4K$). EIMS m/z (%): 360 (10), 342 (7), 201 (14), 162 (100); UV λ_{max} nm (log ε): 227 (4.19), 288 (3.33), 302 (3.32), 382 (4.16); IR ν_{max} cm^{-1}: 3520, 2960, 2940, 2855, 1705, 1650, 1635, 1560, 1525, 1410, 1375, 1270; CD (c=0.0361, MeOH) $\Delta\varepsilon^{25}$ (nm): +4.6 (384) (positive maximum), -1.4 (329) (negative maximum), -0.8 (286) (negative maximum), +1.2 (249) (positive maximum).

Monankacin B (2): It obtained as yellow amorphous mass; $[\alpha]_D$ +101° (c = 0.0128, MeOH); EIMS m/z (%): 332 (M^+,10), 342 (7), 201 (14), 162 (100).UV λ_{max} nm (log ε): 223, 271, 282, 295, 361; IRν_{max} cm^{-1}: 3520, 2950, 2920, 2855, 1705, 1650, 1635, 1560, 1525, 1410, 1375, 1270; CD (c =6.0273, MeOH) $\Delta\varepsilon^{25}$ (nm) : +2.1 (380) (positive maximum), -0.4 (324) (negative maximum), -0.3 (290) (negative maximum), +0.6 (248) (positive maximum)

Monascin: Yellow fine needles from EtOH, m.p.146–147°C; $[\alpha]_D$ +383° (c = 0.0166, $CHCl_3$); EIMS m/z (%): 386 (M^+, 22), 162 (100), 134 (13), 69 (17), 43 (21); UV λ_{max} nm (log ε): 230 (4.40), 287 (3.71), 388"(4.19), IRν_{max} cm^{-1}: 2958, 2920, 2860, 2310, 1785,1720, 1660, 1642, 1525, 1410, 1394, 1268, 1200, 1100, 960; CD (c =6.0273, MeOH) $\Delta\varepsilon^{25}$ (nm) : +9.0 (398) (positive maximum), +2.4 (330) (negative maximum), -1.0 (269) (negative maximum), 2.6 (positive maximum).

Ankaflavin: It obtained as yellow fine needles from EtOH; m.p. 116–117°C; EIMS m/z (%); 386 (M^+, 23), 162 (100), 134 (13), 69 (17), 43 (21); UV λ_{max} nm (log ε): 213 (4.15), 287 (3.24), 388 (4.19); IR ν_{max} cm^{-1}: 2950, 2920, 2870, 2360, 1790, 1727, 1680, 1680, 1532, 1410, 1395, 1278, 1208, 1100, 965.

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