STUDIES ON A NEW ANTIBIOTIC M-92 PRODUCED BY *MICROMONOSPORA*

II. ISOLATION AND PHYSICOCHEMICAL PROPERTIES OF M-92 AND ITS COMPONENTS

KATO TANI and TETSUO TAKAISHI

Microbiological Research Laboratory, Tanabe Seiyaku Co., Ltd.,
Toda, Saitama, Japan

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A new antibiotic complex, M-92 was isolated from the whole fermentation broth of *Micromonospora verruculosa* MCRL 0404. The whole broth was mixed with talc and filtered. The filter cake thus obtained was extracted with acidic methanol to give a crude powder of M-92 complex, which was then separated into A (acidic) and N (neutral or weakly acidic) groups. The A group components are soluble in alkaline water (pH 8.5), while the N group components are not. These components were further separated into six major components designated VA-2, BA-4, BA-5, BN-1, BN-2 and BN-3 by silica gel column chromatography. Components with the letter "V" are reddish violet and those with "B" are blue.

The IR and UV spectra of these components suggest that their chromophores may be the same or very closely related to one another. The molecular formula of BN-3 was determined to be $C_{29}H_{23}NO_9$ by mass spectrometry. The results of various spectroscopies on BN-3 suggest that M-92 components consisted of chromophores in which juglone (5-hydroxynaphthoquinone) is conjugated with naphthazarin (5,8-dihydroxynaphthoquinone).

In screening for new antibiotics produced by *Micromonospora*, we found that a new strain for which the name *Micromonospora verruculosa* sp. nov.[1] was proposed, produced a new antibiotic complex named M-92 which was exceedingly active against some Gram-positive and Gram-negative bacteria. M-92 complex was extracted with acidic methanol from the mycelial cake. Crude complex was then separated into six major components designated VA-2, BA-4, BA-5, BN-1, BN-2 and BN-3 by silica gel H column chromatography. They were acidic or neutral in nature and blue or violet in color. This paper deals with the isolation and purification of the M-92 components and their physicochemical properties. Biological properties of these components will be reported in the succeeding paper[2]. The structural elucidation of these components is now under way.

Isolation and Purification

Isolation of a crude powder of the M-92 complex was achieved as shown in Chart 1. Thus, 24 g of a crude powder of the M-92 complex was obtained from 1,000 liters of fermentation broth.

According to the procedures shown in Chart 2, the chloroform solution of the M-92 complex was separated into A group (acidic substances) which is soluble in alkaline water (Na_2CO_3, pH 8.5) and N group (weakly acidic or neutral substances) which is insoluble. The A group components in the chloroform layer were washed with water and then evaporated to dryness *in vacuo*. Thus, 6.5 g of a crude powder of the A group components and 10.4 g of a crude powder of the N group components were obtained from 20 g of crude M-92 complex.

The isolation and purification of the A group components were carried out by silica gel H [Merck, impregnated with 0.5 M McIlvain buffer (pH 5)] column chromatography as shown in Chart 3. Then,

Chart 1. Extraction of M-92 complex.

Fermentation whole broth (1,000 liters)
- adjusted to pH 2 with 6 N HCl
- added talc (1 kg) and Celite 545 (2 kg)
- stirred for 30 minutes, filtered

Mycelial cake
- extracted with 100 liters (×2) of acidic methanol (adjusted to pH 2 with 6 N HCl)

Methanol extract (160 liters)
- adjusted to pH 10 with 6 N NaOH
- added Celite 545 (1.6 kg) filtered

Cake
- suspended in 14 liters of water
- adjusted to pH 2 with 6 N HCl
- extracted with 7 liters (×2) of chloroform

Chloroform layer (13 liters)
- washed with 5 liters of water and evaporated

Crude oil (130 g)
- added 1.5 liters of *n*-hexane

Precipitate
- dried *in vacuo*

Crude powder of M-92 complex (24 g)

the A group components were separated into the VA group components which are acidic violet substances, and BA-4 and BA-5 components which are blue and acidic. Further, the VA group components were separated to VA-1, VA-2 and VA-4. Thus, 2 mg of VA-1, 250 mg of VA-2, 1 mg of VA-4, 2.45 g of BA-4 and 2.54 g of BA-5 were obtained from 6 g of crude powder of the A group components.

The N group components were isolated and purified in similar ways to the A group components as shown in Chart 4. Then, the N group components were separated into components BN-1, BN-2 and BN-3 which are blue and weakly acidic, and VN-3 which is violet and weakly acidic. In this manner, 0.73 g of BN-1, 1.0 g of BN-2, 4.8 g of BN-3 and 1 mg of VN-3 were

Chart 2. Isolation of A and N group components.

Crude powder of M-92 complex (20 g)
- dissolved in 3 liters of chloroform
- added 1.5 liters of 1% Na_2CO_3 solution
- stirred

Chloroform layer
- washed with 1 liter of water
- evaporated to dryness

Crude powder of N group components (10.4 g)

Aqueous layer
- adjusted to pH 2 with 6 N HCl
- added 1 liter of chloroform

Chloroform layer
- evaporated to dryness

Crude powder of A group components (6.5 g)

Chart 3. Isolation and purification of A group components.

Crude powder of A group components (6 g)
- dissolved in MeOAc

Silica gel H column (1.2 kg, 90×7.5 cm diameter)
- developed with MeOAc saturated with H_2O and collected 100 ml fractions

Fractions 14~19
- evaporated to dryness

VA-group (0.96 g)
- dissolved in EtOAc

Silica gel H* column (500 g, 70×5 cm diameter)
- developed with benzene - EtOAc (5: 1) and collected 20 ml fractions

Fractions 34~35
- evaporated to dryness

VA-1 (2 mg)

Fractions 36~56
- evaporated to dryness

VA-2 (250 mg)

Fractions 63~69
- evaporated to dryness

VA-4 (1 mg)

Fractions 40~49
- evaporated to dryness

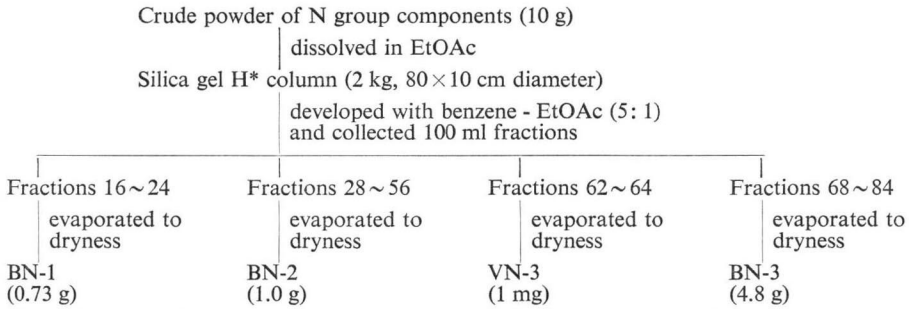
BA-4 (2.45 g)

Fractions 51~59
- evaporated to dryness

BA-5 (2.54 g)

* Silica gel H (Merck) was treated with 0.5 M McIlvain buffer (pH 5) and dried at room temperature.

Chart 4. Isolation and purification of N group components.



* Silica gel H (Merck) was treated with 0.5 M McIlvain buffer (pH 5) and dried at room temperature.

Table 1. Physicochemical properties of M-92 components.

		VA-2	BA-4	BA-5	BN-1	BN-2	BN-3
Elementary analysis	C	66.59	68.69	66.12	62.12	62.92	65.79
	H	3.29	7.02	6.72	5.25	4.54	4.44
	N	2.68	2.10	2.01	2.02	2.60	2.65
MP (°C)		250	251~254	185~190	146~151	249~252	300
$[\alpha]_D^{26}$ (c 0.01, MeOH)		+500° (MeOH+ dimethyl-formamide)	−980°	−1040°	−980°	−1160°	−620°
UV λ_{max}^{MeOH} nm($E_{1cm}^{1\%}$)		242 (465) 290sh (110) 580 (189.5) 610 (182)	243 (635) 290sh (112) 590 (152) 640 (267)	245.5 (367.5) 590 (152) 640 (152)	242 (352.5) 277sh (210) 590 (171) 640 (170)	243.5 (577.5) 289sh (140) 590 (247.5) 640 (244.5)	243 (820) 290sh (130) 590 (195) 640 (193.5)
$\lambda_{max}^{0.1N\ NaOH\text{-}MeOH}$ nm ($E_{1cm}^{1\%}$)		248 (457.5) 290sh (110) 610 (200) 650 (217.5)	245.5 (635) 290sh(132.5) 615 (279) 660 (635)	245.5 (442.5) 290sh(185) 615 (202) 660 (240)	244.5 (395) 330(202.5) 615 (178) 660 (210)	246 (602) 287sh (140) 615 (264) 660 (316.5)	245.5 (821) 290sh (150) 615 (204) 660 (240)
IR ν_{max}^{Nujol} cm^{-1}		3300, 1660, 1630, 1585	3450, 3300, 1730, 1640, 1600, 1580	3500, 3320, 1735, 1645, 1600, 1590	3200, 1675, 1650, 1605, 1585	3450, 1690, 1670, 1600, 1585	3450, 3300, 1730, 1640, 1600, 1580
Solubility	Soluble	Dioxane, dimethyl-formamide, alkaline water	Ethylacetate, dioxane, alkaline water	Similar to BA-4	Acetone, chloroform, ethyl acetate	Similar to BN-1	Similar to BN-1
	Slightly soluble	Acetone, ethyl acetate	Acetone, chloroform, methanol	〃	Methanol, ether	〃	〃
	Insoluble	Ether, hexane	Ether, hexane, water	〃	Hexane, water	〃	〃
Rf value on silica gel* TLC	a**	0.50	0.15	0.09	0.58	0.41	0.26
	b	0.54	0.34	0.18	0.89	0.57	0.76

* Silica gel H plates (Merck) were treated with 0.5 M McIlvain buffer (pH 5) and dried at room temperature and activated for 1 hour at 110°C.

** Solvent system a: Benzene - ethyl acetate (5: 1).
b: Chloroform - methanol (19: 1).

obtained from 10 g of crude powder of the N group components.

Physicochemical Properties

The physicochemical properties and the Rf values on TLC of these six major components are listed

in Table 1. These components, obtained as reddish violet to blue amorphous powder, are acidic or weakly acidic in nature. These antibiotics gave a reddish brown color with conc. H_2SO_4, and reddish orange color with formic acid and zinc powder, but were negative to the ninhydrin test. The IR spectra of M-92 components showed the chelated OH band at around 3300 cm^{-1} and the hydrogen bonded C=O band at 1630~1640 cm^{-1}. The UV spectra exhibited characteristic peaks in common to all components, suggesting that their chromophores may be the same or very closely related to one another.

The acidic (A) group components of M-92 did not give molecular ions on EI-mass spectroscopy, but BN-3 afforded the molecular ion peak at *m/z* 529.1366 ($C_{29}H_{23}NO_9$, Δ −0.4 mmu) and fragment ions at *m/z* 514 (M−CH_3), 501 (M−CO), 498, 497 (M−CH_3O, M−CH_3OH). PMR spectrum of BN-3 ($CDCl_3$) showed three methyl signals at δ 1.27 (d), 1.36 (s) and 3.57 (s) and three chelated phenolic hydroxyl groups as sharp singlets at δ 12.03, 12.38 and 12.78. These singlets vanished after the addition of CD_3OD, so that these hydroxyl groups must be in the *peri*-positions to the quinone carbonyls. The spectrum showed also five aromatic portons but not quinoid protons[3]. These results suggested that M-92 consisted of chromophores built up from conjugated juglone (5-hydroxynaphthoquinone) and naphthazarin (5,8-dihydroxynaphthoquinone) like actinorhodins[4,5]. Moreover, M-92 components are different from any of the known antibiotics in their physicochemical properties. M-92 complex is thus concluded to be new antibiotic.

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