DYNEMICINS†, NEW ANTIBIOTICS WITH THE 1,5-DIYN-3-ENE AND ANTHRAQUINONE SUBUNIT

I. PRODUCTION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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Dynemicin A, a novel antibiotic containing the bicyclo[7.3.1]-1,5-diyn-3-ene and 1,4,6-trihydroxyanthraquinone functionalities, was isolated from the culture broth of *Micromonospora chersina* sp. nov. M956-1. The antibiotic exhibited potent *in vitro* antibacterial and cytotoxic activity, and in *in vivo*, it cured mice from lethal *Staphylococcus aureus* infection and prolonged survival time of mice inoculated with murine tumors. Three satellite components, dynemicins L, M and N, were also isolated from the culture broth and chemically characterized.

In the first paper of this series, we reported discovery of a novel violet-colored antibiotic dynemicin A produced by *Micromonospora chersina* sp. nov. M956-1[1,2]. Dynemicin A showed potent inhibitory activity against a wide range of bacteria and various tumor cell lines. In *in vivo* test, it protected mice from lethal infection of *Staphylococcus aureus* Smith and prolonged the survival time of mice implanted with the experimental tumors. A unique structure, a hybrid of bicyclo[7.3.1]-1,5-diyn-3-ene and 1,4,6-trihydroxyanthraquinone was subsequently assigned to the antibiotic by the X-ray and spectrometric analyses[3].

In the purification of dynemicin A, three satellite components named dynemicins L, M and N were isolated as blue crystalline solid. The spectral studies disclosed that they were structurally similar to dynemicin A but the 1,5-diyn-3-ene moiety of latter was cyclized to a phenyl ring in all of the new components. Dynemicins L, M and N exhibited antibacterial and cytotoxic activity with much lower potency than that of dynemicin A.

Deoxydynemicin A was recently discovered together with dynemicin A by SHIOMI *et al.*[4] in the broth of *Micromonospora globosa* MG331-hF6. The antibiotic lacks the hydroxyl group on C-15 in our numbering system and shows strong antibacterial activity comparable to that of dynemicin A.

Herein, we present the details of fermentation, isolation and physico-chemical properties of dynemicins. The taxonomy of the producing organism and biological activity of dynemicin components are covered in the companion papers[5,6].

Fermentation

A well grown agar slant of *M. chersina*, strain No. M956-1 (ATCC 53710), was used to inoculate to a 500-ml Erlenmeyer flask containing 100 ml of the seed medium consisting of lactose 1%, soluble starch (Nichiden Kagaku) 3%, fish meal (Hokuyo Suisan) 1%, $CaSO_4 \cdot 2H_2O$ 0.6% and $CaCO_3$ 0.5%, the pH being adjusted to 7.0 before sterilization. The flasks were shaken at 32°C for 7 days on a rotary shaker

† Dynemicin was originally called as Bu-3420T.

(200 rpm) and a 500-ml aliquot of the culture from the flasks was inoculated into a 20-liter stir-jar fermenter containing 12 liters of the second seed medium composed of soluble starch 1.5%, glucose 0.5%, beet molasses (Nihon Tensai Seito) 1%, fish meal 1% and $CaCO_3$ 0.5% (pH 7.0). The fermentation was carried out at 28°C for 92 hours with agitation at 250 rpm and aeration of 12 liters per minute. Two liters of the second seed was transferred to a 200-liter tank fermenter containing 120 liters of production medium having the same composition as the second seed medium described above. Fermentation was carried out at 28°C for 73 hours under agitation at 250 rpm and aeration rate of 120 liters per minute. In order to follow the antibiotic production, aliquots of the fermentation broth were extracted with butanol and the extracts monitored by the paper-disc agar diffusion method using *Bacillus subtilis* PCI 219 (pH 8.0) as the indicator organism. The antibiotic production was extremely low and the maximum potency less than 1 μg/ml was obtained after 3 days.

Isolation and Purification

The fermentation broth (230 liters) was stirred for 1 hour with butanol (96 liters) and the mixture was filtered. The separated butanol layer was concentrated *in vacuo* azeotropically by occasional addition of water to 3 liters of solution which was added to ethyl acetate (18 liters) under stirring. After removal of the biologically inactive precipitate by filtration, the clear filtrate was concentrated to dryness *in vacuo* to an oily residue (124 g). It was dissolved in 70% aqueous methanol (1 liter) and the solution was charged onto a Diaion HP-20 column (1.8 liters). After washing thoroughly with 80% aqueous methanol (12 liters), the activity was eluted from the column with 80% aqueous acetone. The eluate was examined by the bioassay with *B. subtilis* PCI 219 and HPLC (column: YMC gel A301-3, 4.6 i.d. × 100 mm, solvent: MeOH - 0.15% KH_2PO_4, pH 3.5 (75 : 25)). The early eluted fractions containing dynemicins L, M and N were concentrated to yield a brown solid (3.24 g). The following most bio-active fractions were pooled and concentrated *in vacuo* to give a brown solid (15.23 g) containing dynemicin A. This solid was chromatographed on a column of Sephadex LH-20 (4.0 i.d. × 65 cm) developing with methanol. The eluate was examined by the HPLC system described above and TLC (xylene - methyl ethyl ketone - MeOH, 5 : 5 : 1). Evaporation of the faster eluted fractions gave a blue solid containing dynemicins L, M and N (351 mg). The following fractions containing dynemicin A were pooled and concentrated to yield violet solid (79 mg). Further purification of dynemicin A was effected by Sephadex LH-20 column chromatography (4.0 i.d. × 65 cm) with methanol elution. Upon examination with the HPLC, the appropriate eluates were concentrated to dryness yielding an violet solid of homogeneous dynemicin A (18 mg).

The solids containing dynemicins L, M and N were pooled (3.59 g) and applied onto a column of reversed phase silica gel (ODS-60, Yamamura Chem. Lab. 1.0 i.d. × 128 cm) prewashed with 50% aqueous methanol. Elution was carried out first with 55% aqueous methanol and then with 65% aqueous methanol. Dynemicins N, L and M were eluted with 65% aqueous methanol in the order. The HPLC-directed fractionation and evaporation of the relevant fractions resulted in the isolation of the crude solids of dynemicins N (18 mg), L (15 mg) and M (70 mg). The solid of dynemicin L was then subjected to column chromatography on silica gel (2.0 i.d. × 60 cm) using a mixture of methylene chloride - methanol (4 : 1). The fractions containing the homogeneous compound were concentrated and further chromatographed on Sephadex LH-20 (4.0 i.d. × 56 cm) with methanol elution. Evaporation of the heart-cuts of the column afforded pure dynemicin L (1.6 mg). The solid of dynemicin M was also purified by silica gel (2.0 i.d. × 35 cm, methylene chloride - methanol, 10 : 1) and Sephadex LH-20 (4.0 i.d. × 65 cm, methanol) to yield a blue solid

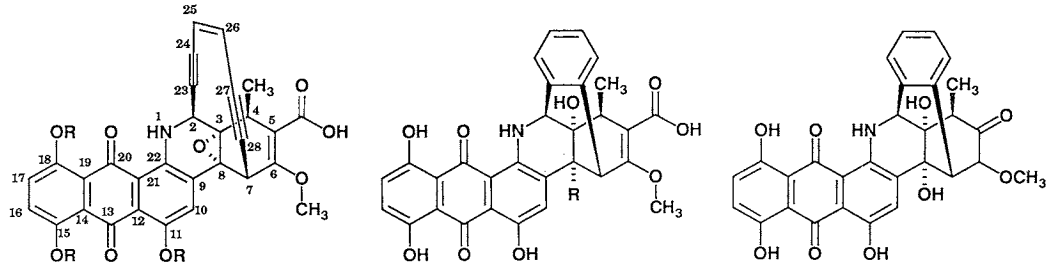
Table 1. Physico-chemical properties of dynemicins A, A-triacetate, L, M and N.

	Dynemicin A	Dynemicin A triacetate	Dynemicin L	Dynemicin M	Dynemicin N
Nature	Violet amorphous powder	Orange rods	Blue amorphous powder	Blue amorphous powder	Blue amorphous powder
MP (dec) (°C)	208~210	228~231	222~225	238~240	253~256
Optical rotation	$[\alpha]_D^{24}$ +270° (*c* 0.01, DMF)	$[\alpha]_D^{24}$ +1,300° (*c* 0.05, MeOH)	$[\alpha]_D^{27}$ −820° (*c* 0.01, MeOH)	$[\alpha]_D^{27}$ −2,460° (*c* 0.01, MeOH)	$[\alpha]_D^{27}$ −200° (*c* 0.01, MeOH)
UV λ_{max}^{MeOH} nm (ε)	239 (24,900), 282 (sh), 569 (10,800), 599 (10,100)	244 (40,900), 313 (7,900), 482 (8,100)	241 (48,100), 454 (2,400), 594 (18,000), 639 (17,900)	241 (41,700), 453 (1,500), 589 (17,000), 633 (17,200)	241 (53,100), 452 (4,300), 592 (24,500), 639 (25,200)
$\lambda_{max}^{0.01\,N\,HCl\text{-}MeOH}$ nm (ε)	239 (27,100), 282 (sh), 568 (11,400), 597 (10,800)	246 (37,100), 318 (7,500), 494 (7,200)	241 (47,900), 453 (1,800), 594 (17,700), 637 (17,500)	241 (41,300), 453 (1,400), 589 (16,900), 632 (17,000)	241 (52,700), 452 (3,900), 592 (24,100), 638 (25,000)
$\lambda_{max}^{0.01\,N\,NaOH\text{-}MeOH}$ nm (ε)	246 (27,900), 276 (7,400), 598 (13,300), 642 (13,800)	215 (37,600), 245 (42,700), 272 (sh), 592 (15,900), 641 (15,600)	242 (47,000), 450 (sh), 612 (18,900), 654 (20,500)	243 (44,200), 450 (sh), 607 (19,200), 655 (22,400)	243 (56,900), 450 (sh), 605 (18,300), 651 (28,300)
Molecular formula	$C_{30}H_{19}NO_9$	$C_{36}H_{25}NO_{12}$	$C_{30}H_{22}NO_9Cl$	$C_{29}H_{23}NO_9$	$C_{30}H_{23}NO_{10}$
Microanalysis		$C_{36}H_{25}NO_{12}\cdot H_2O$	$C_{30}H_{22}NO_9Cl\cdot 2H_2O$		
		Calcd: Found:	Calcd: Found:		
C:		63.43 63.20	58.68 58.86		
H:		3.99 3.75	4.27 4.00		
N:		2.06 2.16	2.28 1.93		
Cl:			5.78 5.56		
HRFAB-MS ($(M+H)^+$)	Calcd: 538.1138 Found: 538.1132				
SI-MS (*m/z*)	538 $(M+H)^+$	664 $(M+H)^+$	576 $(M+H)^-$	532 $(M+3H)^+$	558 $(M+H)^-$
TLC, SiO_2 (Rf)[a]	0.40	0.33	0.15	0.63	0.08
HPLC (Rt: minutes)[b]	10.9		9.4	8.3	7.2

[a] Merck: Xylene - methyl ethyl ketone - MeOH (5 : 5 : 1).

[b] Column: A 301-3S-3-120A ODS (4.6 × 100 mm, YMC); mobile phase: MeOH - 0.15% KH_2PO_4, pH 3.5 (75 : 25); detection: UV 254 nm; flow rate: 1 ml/minute.

Fig. 1. The structures of dynemicins A, L, M and N and triacetyldynemicin A.



Dynemicin A R=H
Triacetyldynemicin A R=$COCH_3$

Dynemicin L R=Cl
Dynemicin N R=OH

Dynemicin M

sample of pure dynemicin M (16 mg). Purification of the dynemicin N sample was effected by preparative TLC (Kieselgel $60F_{254}$, xylene-methyl ethyl ketone-methanol, 5:5:2). The appropriate band (Rf 0.18) was scraped and dynemicin N was eluted out with a mixture of methylene chloride-methanol (4:1). Evaporation of the eluate yielded a homogeneous sample of dynemicin N (2.3 mg).

Triacetyldynemicin A

Dynemicin A (15 mg) was dissolved in acetic anhydride (1.5 ml) and pyridine (2 ml) and the mixture was allowed to stand at room temperature for 18 hours. The solution was diluted with ethyl acetate (10 ml) and washed with water. The organic layer was evaporated to dryness *in vacuo*. The crude triacetyldynemicin A was purified by preparative TLC (SiO_2 plate, xylene-methyl ethyl ketone-methanol, 5:5:1) and the homogeneous orange solid (8 mg) obtained was crystallized from aqueous acetonitrile to give orange rods of triacetyldynemicin A (7 mg).

Physico-chemical Properties

Dynemicin A was obtained as a violet amorphous solid, while dynemicins L, M and N as blue amorphous solids. Dynemicins A, L, M and N are soluble in dimethyl sulfoxide, *N*,*N*-dimethylformamide and dioxane, silightly soluble in ethyl acetate, methanol and ethanol but insoluble in water and *n*-hexane. When treated with acetic anhydride in pyridine, dynemicin A yielded the triacetyl derivative with increased solubility. The molecular formula of triacetyldynemicin A was established as $C_{36}H_{25}NO_{12}$ based on HRFAB-MS, microanalysis and ^{13}C NMR data and therefore, that of dynemicin A was as $C_{30}H_{19}NO_9$ by comparative spectral analysis of both compounds. The molecular formulae of dynemicins L, M and N were determined as $C_{30}H_{22}NO_9Cl$, $C_{29}H_{23}NO_9$ and $C_{30}H_{23}NO_{10}$, respectively, by the microanalysis FAB-MS, and ^1H and ^{13}C NMR spectra. The physico-chemical data of dynemicin A and its acetate and dynemicins L, M and N are summarized in Table 1. The UV spectrum of dynemicin A exhibited absorption maxima at 239, 287, 569 and 599 nm in methanol and acidic methanol and 246, 276, 589 and 642 nm in alkaline methanol, while those of dynemicins L, M and N were exhibited the maxima at around 241, 454, 591 and 637 nm in methanol and acidic medium and around 243, 613 and 661 nm in alkaline medium. The structures of dynemicins A, L, M and N have been determined by the X-ray chrystallographic analysis of triacetyldynemicin A and spectral data comparison of the four antibiotics[2] (Fig. 1).

Discussion

Our fermentation screening directed to potent *in vivo* P388 activity resulted in discovery of another

diynene-containing antibiotic, dynemicin A. Two bicyclo[7.3.1]-1,5-diyn-3-ene antibiotics, esperamicins[7] and calicheamicins[8] were recently reported and attracted considerable interest due to their exceptionally potent antitumor activity[9], novel structures and unprecedented action mechanism[10]. Neocarzinostatin chromophore, the active principle of macromolecular antibiotic neocarzinostatin[11], is now recognized to be a member of this family in its chemical structure and mode of action.

Dynemicin A appears to be a new addition to the family of diynene antibiotic, but it is distinctly different from the preceding ones in its structure and activity profile. Dynemicin A has a number of unique structural features; unlike the preceding 1,5-diyn-3-ene containing antibiotics, the bicyclo-1,5-diyn-3-ene moiety of dynemicin A is assembled with 1,4,6-trihydroxyanthraquinone to form a tetracyclo nucleus. The absence of sugar is another structural uniqueness of this antibiotic. The trigger for activation of dynemicin A is believed to be reduction of the anthraquinone to the corresponding hydroquinone, which rearranges to a quinone methide with concomitant opening of the epoxide. Subsequent saturation of the C-8 to sp^3 carbon induces the Bergman cyclization to produce a phenyldiyl radical which cleaves DNA as in the cases of the esperamicin group antibiotics[12]. Isolation of the aromatized compound dynemicin H[12] seems to evidence the reaction. Involvement of ionic species in triggering this reaction is illustrated by the formation of dynemicins L and N upon mild acid treatment of dynemicin A[3]. The anthraquinone chromophore might also play an active role in the interaction of dynemicin A with DNA, which will be the subject of future papers.

The esperamicin/calicheamicin antibiotics are known to be strongly toxic to the mammalian. It is of particular interest that dynemicin A exhibited rather weak acute toxicity in mice[6] compared to its strong cytotoxic effect comparable to that of esperamicins and calicheamicins. It should also be emphasized that dynemicin A cured mice from lethal infection caused by *Staphylococcus aureus* Smith[1].

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