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Improved processes for the production and isolation of dynemicin A and large-scale fermentation in a 10000-liter fermentor

Kin Sing Lam[a], Jeffrey A. Titus[b], Thomas T. Dabrah[a], Dennis L. Kimball[b], Jacqueline M. Veitch[a], Donald R. Gustavson[a], Bruce J. Compton[b], James A. Matson[a], Salvatore Forenza[a], John Ross[c], David Miller[c], John Roach[c] and John Beutler[c]

[a] *Bristol-Myers Squibb Company, Pharmaceutical Research Institute, Wallingford, CT, USA,* [b] *Bristol-Myers Squibb Company, Industrial Division, Syracuse, NY, USA and* [c] *Frederick Cancer Research and Development Center, Frederick, MD, USA*

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SUMMARY

Supplementing the culture of *Micromonospora chersina* sp. nov. No. M956-1 with NaI (0.5 mg/l) enhanced the production of dynemicin A by 35-fold in shake flask culture. Homogeneous dynemicin A was obtained from the whole broth extract by Dicalite chromatography, Sephadex LH-20 chromatography and vacuum liquid chromatography. Gram quantities of dynemicin A were obtained from the fermentation of *M. chersina* sp. nov. No. M956-1 in a 10000-liter fermentor.

INTRODUCTION

A novel antitumor antibiotic, dynemicin A, was discovered in the fermentation broth of *Micromonospora chersina* sp. nov. No. M956-1 (ATCC 53710) [2]. Strain ATCC 53710 was isolated from a soil sample collected in Gujarat State, India. Structural studies [4] revealed that dynemicin A is a unique hybrid of an anthraquinone and a 1,5-diyn-3-ene system. The anthraquinone moiety of dynemicin A is identical to those of the anthracycline anticancer drugs daunorubicin and adriamycin [12]. The 1,5-diyn-3-ene system of dynemicin A is similar to those of the esperamicin [1,3] and calicheamicin [6] class of extremely potent antitumor antibiotics. Sugiura et al. [10] demonstrated that dynemicin A possessed potent DNA cleavage activity in the presence of NADPH and dithiothreitol. The mechanism of action of dynemicin A may be similar to that of esperamicin-calicheamicin which involves a bioreductively activated, highly efficient, DNA strand scission [7,11,13,14].

Dynemicin A, a violet-colored antibiotic, exhibits very potent antibacterial activity, especially against Gram-positive bacteria [2]. It also prolongs the life span of mice inoculated with P388 leukemia and B16 melanoma [2]. The production of dynemicin A in the original medium described by Konishi et al. [2] was very low, about 0.1 μg/ml. Further development of dynemicin A as an anticancer drug would have been difficult due to such a low titer. Therefore, extensive medium development was needed to improve the production of dynemicin A. Also, large-scale fermentation was required to obtain gram quantities of dynemicin A for further studies on this new class of antibiotics. This paper describes the development of new improved media for the production of dynemicin A, a rapid HPLC assay for monitoring dynemicin A production in different media, a new improved purification scheme and large-scale fermentation process for obtaining gram quantities of dynemicin A at the National Cancer Institute's Frederick Cancer Research and Development Center (FCRDC).

MATERIALS AND METHODS

Microorganism

The dynemicin A producing microorganism was *Micromonospora chersina* sp. nov. No. M956-1 (ATCC 53710). Frozen vegetative preparations were maintained in 10% glycerol/5% sucrose solution stored at −80 °C for use as working stocks.

Correspondence to: K.S. Lam, Department 114, Bristol-Myers Squibb Company, P.O. Box 5100, Wallingford, CT 06492, USA.

Media

The seed media used were medium 53 (lactose (Sigma) 1%, dextrin (Sigma) 3%, fishmeal (Menhaden) 1%, $CaSO_4$ (Sigma) 0.6% and $CaCO_3$ (Pfizer) 0.5%) as the first seed and medium 118F (soluble starch (A.E. Staley) 1.5%, cerelose (Corn Products) 0.5%, fish emulsion (Alaska Fish Fertilizer) 1%, beet molasses (Pacific Molasses) 1% and $CaCO_3$ 0.5%) as the second stage seed. The production media used were 118D-M1 (soluble starch 1.5%, cerelose 0.5%, fishmeal 1%, beet molasses 1%, $CaCO_3$ 0.5% and NaI (Fisher) 0.5 mg/l), H881 (soluble starch 1%, Pharmamedia (Traders Protein) 0.5%, $CaCO_3$ 0.1%, $CuSO_4 \cdot 5H_2O$ (Fisher) 0.005% and NaI 0.5 mg/l) and LED (sucrose (Sigma) 2%, Bacto peptone (Difco) 0.2%, cane molasses (Pacific Molasses) 0.5%, $FeSO_4 \cdot 7H_2O$ (Fisher) 0.01%, $MgSO_4$ (Fisher) 0.02%, $CaCO_3$ 0.5% and KI (Fisher) 0.01%).

Culturing conditions

To prepare an inoculum for the production phase, 5 ml of the frozen vegetative stock were transferred to a 500-ml Erlenmeyer flask containing 100 ml of medium 53. This seed culture was incubated at 28 °C for 7 days on a rotary shaker set at 250 rpm. Five ml of seed culture were transferred to a 500-ml Erlenmeyer flask containing 100 ml of production medium 118D-M1, H881 or LED. The production cultures were incubated at 28 °C and 250 rpm on the same shaker for 4–5 days. For production in laboratory fermentors (50 liter nominal volume), 20 ml of the seed culture were transferred to a 2-liter Erlenmeyer flask containing 400 ml of medium 118D. This second seed culture was incubated at 28 °C and 250 rpm for 4 days. Four second seed cultures were combined and transferred to the fermentor containing 30 liter production medium (118D-M1, H881 or LED). The incubation temperature was 28 °C, agitation rate was 250 rpm and the air flow was 0.7 vol. per min. The back pressure of the fermentor was set at 0.35 kg/cm^2.

Assays of dynemicin A

The production of dynemicin A in the fermentation was quantitated by HPLC using a C-18 Shandom ODS-hypersil column (5 μm, 4.6 × 100 mm). The solvent system was CH_3CN/0.1% H_3PO_4 (65:35) and detector wavelength set at 570 nm. The flow rate was 2 ml/min. Dynemicin A was eluted at about 2.7 min. The fermentation extract was processed by extracting the culture broth with an equal volume of ethyl acetate. The extract was concentrated to 10-fold and 10–50 μl of this concentrate were used for HPLC analysis.

The production of dynemicin A in the fermentation can be qualitatively monitored by TLC using Analtech GHLF plate and $CHCl_3/CH_3OH$ (9:1) as the mobile phase. The R_F value of dynemicin A was 0.35.

Isolation and purification of dynemicin A

The isolation and purification of dynemicin A from a 30-liter fermentor culture are summarized in Fig. 1.

RESULTS

Media formulation in shake flask culture

The structure of dynemicin A was elucidated by X-ray crystallography [4] and shown to be a fusion product of an anthraquinone and a 1,5-diyn-3-ene system (Fig. 2). The rational used for the development of media formula-

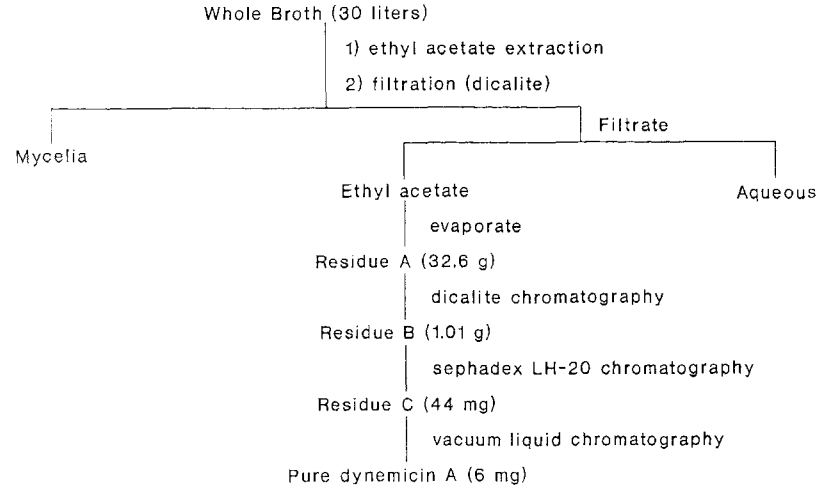

Fig. 1. Producedure for isolation of dynemicin A.

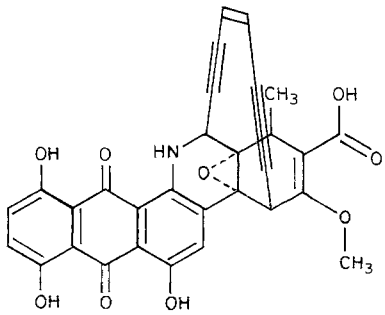

Fig. 2. Structure of dynemicin A.

tions with improved dynemicin A production was to examine media that supported good production of other anthraquinone-containing antibiotics (daunorubicin and adriamycin) [12] and 1,5-diyn-3-ene containing antibiotics (esperamicins and calicheamicins) [5,8]. It was found that the media that supported good production of daunorubicin and adriamycin did not improve the production of dynemicin A by strain ATCC 53710. However, media that supported good production of other 1,5-diyn-3-ene antibiotics significantly improved the production of dynemicin A (Table 1). Three media in particular, H881, LED and 118D-M1, showed significant improvement. Media LED [8] and H881 (unpublished results) were fermentation media developed for the production of calicheamicins. The production titer of dynemicin A in media LED and H881 was 3.2 and 3.5 μg/ml, respectively. This corresponded to a 32–35-fold increase in titer over the original production medium 118D (0.1 μg/ml). Media LED and H881 contained NaI or KI since calicheamicins are iodinated antibiotics. However, we have been shown that NaI enhanced the production of esperamicins [5] which are not halogenated. The effect of NaI on the production of dynemicin A was examined by adding NaI (0.5 mg/l) to medium 118D. This new medium, designated medium 118D-M1, also increased the production of dynemicin A to 3.6 μg/ml. A 36-fold improvement over the original production medium. Omission of NaI from medium H881 and LED significantly lowered the production of dynemicin A.

Evaluation of dynemicin A production in different media

The HPLC assay used for examining multiple fermentation samples generated during medium development was rapid, specific and sensitive. It adequately separated the other related minor dynemicin analogs in the extract from dynemicin A (Fig. 3). About 4 min were required for each sample. With this HPLC system, fermentation samples generated from medium formulation was quickly evaluated.

Production of dynemicin A in laboratory fermentors

Initial scale-up production of dynemicin A was carried out in 50-liter fermentors containing 30 liters of production media 118D-M1, H881 and LED. The titers of dynemicin A in the fermentor containing media 118D-M1, H881 and LED were 3.8, 1.1, and 0.26 μg/ml, respectively (Table 2). The pH of the fermentation in the three different media was about the same. However, the centrifuged solid values were significantly different for these media in the fermentor cultures but were very similar in the shake flask cultures (Table 1). The drop in the production of dynemicin A in going from shake flask cultures to 50-liter fermentor cultures when H881 and LED were used as the production media may be correlated to the drop in the growth of the organism as indicated from the % centrifuged solid data.

Isolation of dynemicin A from fermentor culture (30 liters)

Fermentation broth (30 liters) from medium 118D-M1 was mixed with an equal volume of ethyl acetate and stirred for 1 h. The suspension was mixed with approx. 2 kg of Dicalite and the resulting mixture filtered. The

TABLE 1

Production of dynemicin A in shake flask culture

Media	Dynemicin A (μg/ml)	pH	Centrifuged solid (%)[a]
118D	0.1	7.9	10
H881	3.5	7.9	9
LED	3.2	7.6	8
118D-M1	3.6	7.8	9

[a] Centrifuged solid (%) was obtained by centrifuging 10 ml of the broth at 3000 rpm for 15 min. The volume (ml) of the sediment was divided by 10 ml and multiplying by 100 to yield the value of centrifuged solid (%). Centrifuged solid (%) was used to measure the growth of the organism.

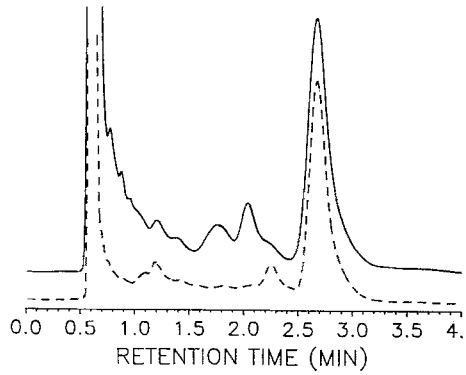

Fig. 3. HPLC of a fermentation extract of strain ATCC 53710 (——) and standard of dynemicin A (– – –).

TABLE 2

Production of dynemicin A in laboratory fermentor (50 liter nominal volume)

Media	Dynemicin A (μg/ml)	pH	Centrifuged solid (%)
H881	1.10	7.8	5
LED	0.26	7.8	3
118D-M1	3.83	7.7	8

organic layer was concentrated in vacuo to give 32.6 g of a dark blue residue A (Fig. 1). Residue A was dissolved in 100 ml of chloroform and preabsorbed onto 40 g of Dicalite. The dried mixture was added to a flash chromatography column (4.1 x 46 cm) packed with 40 g of Dicalite. The column was eluted under pressure (N_2, 3 psi) using the following eluotropic series: 2 liters of hexane, 1 liter of toluene and 500 ml of methanol. The toluene fraction which contained dynemicin A was evaporated to dryness in vacuo to give 1.01 g of residue B (Fig. 1).

Residue B was further purified by Sephadex LH-20 column chromatography (2.5 x 70 cm) using hexane/chloroform/methanol (1:1:1) mixture as the mobile phase. After an initial 400 ml forerun, twenty 100 ml fractions were collected. Aliquots (2–3 μl) from each fraction were analyzed by TLC. Fractions 9–20 were pooled and concentrated in vacuo to give residue C (44 mg, Fig. 1) which was highly enriched in dynemicin A. The residue was then subjected to vacuum liquid chromatography (6 g silica gel 60, Merck) eluting first with $CHCl_3$ (200 ml) and then with 5% CH_3OH in $CHCl_3$ (100 ml), 10% CH_3OH in $CHCl_3$ (100 ml) and 20% CH_3OH in $CHCl_3$ (100 ml). The 5% CH_3OH in $CHCl_3$ fraction was shown by TLC analysis to contain exclusively dynemicin A. The fraction was concentrated in vacuo to yield 6 mg of homogeneous dynemicin A.

Fermentation of dynemicin A in a 10000-liter fermentor

In order to obtain sufficient quantity of dynemicin A for further studies, large-scale fermentation of dynemicin A was carried out in a 10000-liter fermentor in the National Cancer Institute's FCRDC located at Frederick, MD. Due to the bottom drive configuration of the fermentor at FCRDC, fishmeal could not be used as an ingredient of the production medium. Even though medium 118D-M1 was shown to be the best production medium among the three media tested in the 50-liter fermentor (Table 2), it was not the medium of choice because it contained fishmeal. Without any further optimization, medium H881 was used as the production medium for the dynemicin A fermentation in the 10000-liter fermentor to meet the demand of dynemicin A for the expanded biological activities evaluation. Seed cultures of strain ATCC 53710 were built-up in four stages before inoculation into the production tank (10000 liter nominal volume) containing 6800 liters of medium H881 (Fig. 4). Production of dynemicin A

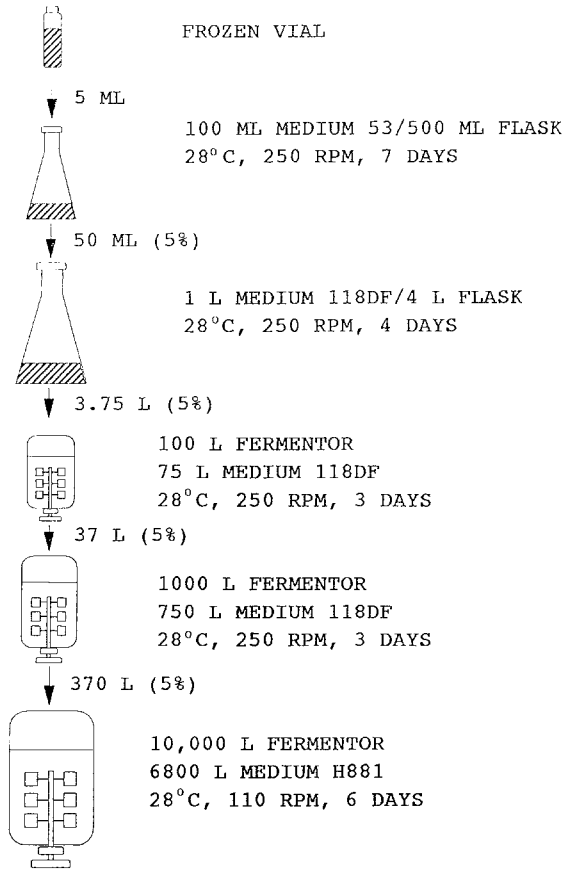

Fig. 4. Scheme for scale-up production of dynemicin A in a 10000-liter fermentor.

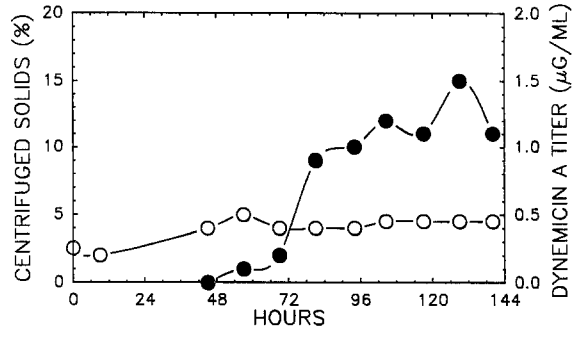

Fig. 5. Time-course of dynemicin A fermentation in a 10000-liter fermentor containing medium H881. Dynemicin A titer (●) and % centrifuged solids (○).

began at about 50 h and leveled off at around 130 h (Fig. 5). The titer of this fermentation was about 1.2 to 1.5 μg/ml and seemed to correlate well with data obtained from the laboratory fermentors. The dissolved oxygen level was maintained at high levels (>90%) by supplying the fermentor culture with the aeration rate of 0.7 vol. per min and very little carbon dioxide was detected in the off gas (<0.08%). After extracting the fermentation broth with ethyl acetate, approx. 7.1 g of dynemicin A was observed by HPLC analysis in the crude solid.

DISCUSSION

After screening about 100 different media, the production of dynemicin A was increased by 35-fold in shake flask cultures. The rapid success of this project was primarily due to the development of a convenient HPLC assay to monitor the production of dynemicin A in different media and elucidation of the structure of dynemicin A; in fact, knowing the structure of dynemicin A helped in designing the medium for the production of this antibiotic. Based upon our experience in the fermentation of esperamicins [5], it was established that sodium iodide also significantly enhanced the production of dynemicin A. Recently, Shiomi et al. [9] reported that inorganic and organic iodine compounds increased the production of dynemicin A and deoxydynemicin A in *M. globosa*. The titer of dynemicin A in the 10000-liter fermentor with medium H881 was about 1.2 to 1.5 μg/ml and correlated well with the data obtained from the laboratory fermentors. Extraction of dynemicin A from the culture broth with ethyl acetate at neutral pH resulted in more efficient recovery than using acidic butanol extraction as described by Konishi et al. [2]. Approx. 7.1 g of dynemicin A was obtained in the crude solids generated by extraction of the culture broth from the 10000-liter fermentor with ethyl acetate. The purification scheme for dynemicin A shown in Fig. 1 is simple, suitable for scale-up and has good recovery yield. Further purification of this crude solid yielded about 2 g of pure dynemicin A (M. Konishi, personal communication). Given the potency of this compound, 2 g of pure material is adequate for initial biological activity testing. The collaborative effort of National Cancer Institute's FCRDC and Bristol-Myers Squibb Company yielded enough dynemicin A for initial testing of this antibiotic. However, higher yields of approx. 15–20 μg/ml in the fermentation will be required for potential commercialization of dynemicin A. Additional experiments in 2-liter fermentors (data not shown) suggested that initial pH value and length of sterilization of the medium were important factors to consider in optimization of the dynemicin A fermentation and may explain the drop in the production of dynemicin A in going from shake flash cultures to fermentor cultures. Further work on optimization of the parameters of the tank fermentation is in progress.

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