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Production of brefeldin-A

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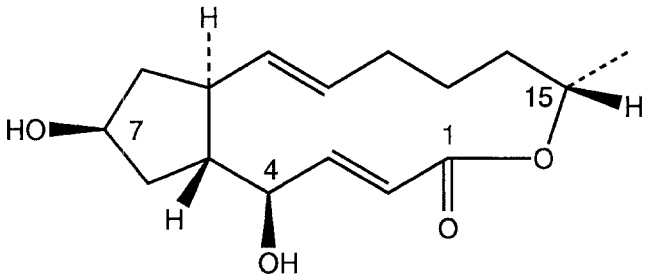
Fermentation conditions are described for the production of the antitumor antibiotic 7-(*S*)-brefeldin-A (brefeldin-A) in liquid culture by *Eupenicillium brefeldianum*, (B. Dodge) Stolk and Scott, ATCC 58665. An analytical hplc method was developed which allowed rapid quantitation of the compound during fermentation. A kilogram of brefeldin-A was isolated from a fermentation at the 6800-liter scale.

Keywords: brefeldin-A; *Eupenicillium brefeldianum*; antitumor antibiotic

Introduction

Brefeldin-A, (synonyms: ascotoxin, cyanein, decumbin, lunatin) a 13-carbon macrolide lactone antibiotic first isolated by Singleton in 1958 from *Penicillium decumbens* [25], has been subsequently identified as a metabolite from *P. brefeldianum* [10,27], *P. cyaneum* [4], and other sources [1,26,30] (Figure 1). The structure was determined by Sigg [24,32]. The 7-(*R*) epimer has been reported from a different microbial source [8]. Biosynthesis of brefeldin-A has been investigated by Hutchinson and others [6,12,13,29,31]. Several total syntheses have been reported [2,5,9,11,14,18]. Because of unexceptional antibiotic properties, interest in the compound was restricted to biomedical research laboratories until recently. The discovery of a previously unknown mechanism of action on animal cells, the disruption of intracellular protein transport by dissociation of endoplasmic reticulum following treatment with brefeldin-A [21,22], and selectivity for certain cell types of the 60 human tumor cell lines which constitute the anticancer screen used by the National Cancer Institute (NCI) to detect potential new chemotherapeutic agents [20] has revived interest in the compound. Because of these findings brefeldin-A was selected by the Developmental Therapeutics Program (DTP), NCI, for scaled-up production to provide sufficient quantities for further *in vitro* and *in vivo* testing. Other biological effects of brefeldin-A have recently been reviewed [3,16,23].



7-(S)-Brefeldin-A

Figure 1 Structure of 7-(*S*)-brefeldin-A

Materials and methods

Two strains of *Eupenicillium brefeldianum* (ATCC 10417 and 58665) were obtained from the American Type Culture Collection. Both were grown in shake flasks at 28° C for 7 days on four media: Czapek-Dox, potato-dextrose, sorbitol-fumarate, and malt extract (MEM). MEM is composed of glucose (2%), malt extract (0.2%), yeast extract (0.25%), peptone (0.2%), KH_2PO_4 (0.2%), $MgSO{\cdot}7H_2O$ (0.2%), and $CaCO_3$ (0.4%), pH 7.0.

Brefeldin-A was detected in the ethyl acetate extract of broth from *E. brefeldianum* ATCC 58665 grown on MEM. Studies were then undertaken to improve fermentation conditions on MEM for the production of brefeldin-A. Stirring rate, aeration, growth rate by centrifugal solids measurement, and glucose utilization were monitored. Time-course monitoring for brefeldin-A titre was done by hplc. Dow Corning type A (DCA) and PEG 1000 antifoams were compared.

To prepare inoculum for the production phase, potato dextrose agar slants were inoculated with 1.0 ml of glycerol vegetative stock which had been stored at −70° C. The slants were incubated at 25° C for up to 96 h or until confluent growth was obtained. These were washed with 5 ml of sterile MEM and the slant washings were used to inoculate the first stage seed flask (100 ml culture medium/500-ml baffled Erlenmeyer flask). The flasks were incubated at 25° C for 48 h at 250 rpm on a 1″ stroke shaker. After examining the culture for purity under a microscope, the second stage seed flask (1 L medium/4-L baffled Erlenmeyer flask) was inoculated with a 5% volume. Again, the flasks were incubated for 48 h at 25° C, 250 rpm.

After checking each flask for purity, the contents of several 4-L flasks were pooled. The organism tended to grow in 'pellets' and it was determined that disruption of the

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pellets by homogenization in a sterilized stainless steel Waring commercial blender (Waring Products Division, Dynamics Corporation of America, New Hartford, CT, USA), model CB-6, at high speed for 2 min, resulted in a more rapid growth rate following transfer. Sufficient homogenate to give 5% v/v of inoculum was transferred to a 100-L fermentor containing 68 L of MEM. Dow Corning type A antifoam (Dow Corning Corporation, Midland, MI, USA) added at a level of 0.1% in the media in fermentors of 100 L and larger was effective in controlling the frothing problem.

The incubation temperature was 25° C ± 0.5, agitation rate 250 rpm giving a tip speed of 327.0 ft min^{-1} and air flow was 0.73 vvm. After 15–24 h (mid-log phase), the contents of the 100-L fermentor were checked for culture purity, then transferred into a 1100-L fermentor containing 680 L of MEM. Fermentation was continued for about 24 h under the same conditions, at which point the entire microbial culture was again checked for purity, then transferred into a 11000-L fermentor containing 6800 L of MEM. Operating conditions were essentially unchanged for about 50 h. The sequence of events from initial vial through harvest, including a time line and volume at each stage, is depicted in Figure 2.

At harvest diatomaceous earth was added and the broth was filtered through a pad of the same filter aid in a filter press. The clarified broth was extracted once by vigorous stirring with a half volume of ethyl acetate. Following concentration of the ethyl acetate extract to one-tenth initial volume in a wiped-film evaporator and holding overnight at room temperature without agitation, a fine, off-yellowish precipitate formed that was removed by filtration through a coarse filter paper. The remaining liquors were evaporated to dryness.

An analytical hplc method was developed which permitted near-real-time monitoring of brefeldin-A production during the course of fermentation (Figure 3). Whole fermentation broth was extracted with an equal volume of ethyl acetate, the organic solvent was removed, dried under nitrogen, resolubilized in methanol to a concentration of 1 mg ml^{-1}, filtered through a 0.2-μm pore-size nylon filter (Rainin 38-109, Rainin Instrument Co, Inc, Woburn, MA, USA) and an appropriate volume (usually 20 μl) injected onto a 4.6 × 150 mm, 3-μm C-18 phase bonded silica column, (Rainin Microsorb 'Short One'), fitted with a 3-μm C-18, 4.6 × 15 mm guard column. All solvents were Burdick & Jackson hplc grade (Burdick and Jackson Division, Baxter Diagnostics, Inc, Muskegon, MI, USA). Isocratic elution with 57% methanol/43% water at 1 ml min^{-1} gave a retention time of 5.8 min. A Waters 996 photodiode array detector (Waters Corporation, Milford, MA, USA) with the Waters Millennium 2010 software package was used to acquire and analyze data. Standard curves were constructed using absorbance at both 230 and 254 nm with authentic brefeldin-A obtained from the DTP repository. The standard was dissolved in methanol. Routine analysis was performed at 230 nm. Linearity was found between 0.3 and 250 μg ml^{-1} at both wavelengths. A quantification of brefeldin-A in crude extracts is calculated by the chromatographic software from the values used to construct the stan-

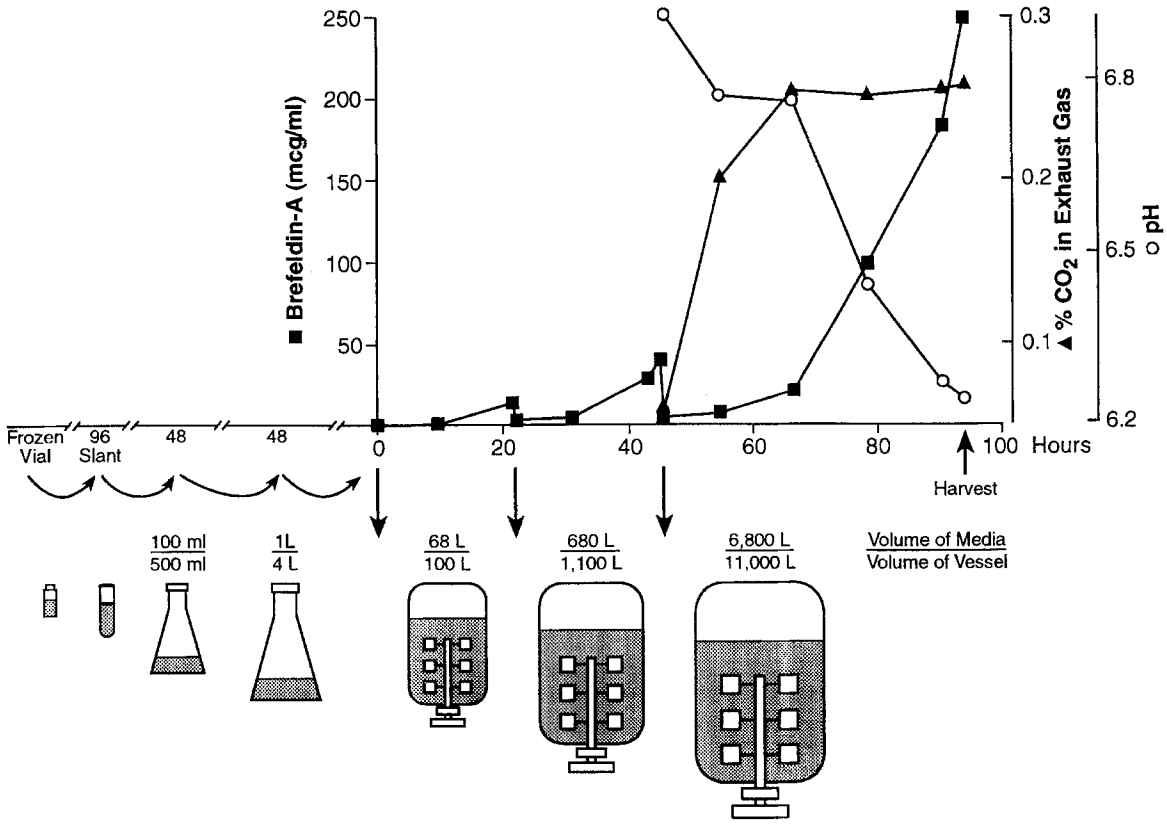

Figure 2 Scheme for scale-up production of brefeldin-A to an 11000-L fermentor providing a time line and volume at each stage

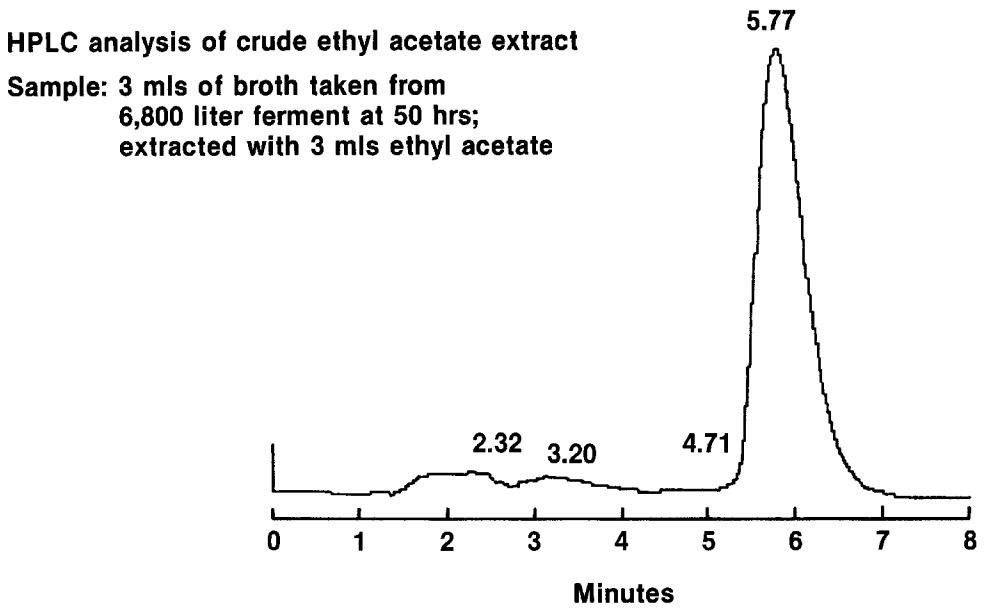

Figure 3 Representative hplc chromatogram showing brefeldin-A

dard curve. Application of this software additionally confirms identity and provides a measure of purity of a compound at a preselected retention time by comparison of the UV spectrum to the spectrum of a standard.

An isocratic normal phase hplc method was developed using a 4.6 × 250 mm, 8-μm silica column (Rainin Dynamax 83-101C) and guard column (Rainin 83-101G) eluted with 95% dichloromethane (DCM)/5% methanol at 1 ml min^{-1}. Under these conditions brefeldin-A has a retention time of 11.2 min. This method was used as an additional confirmation of purity of crystalline material.

The presence of brefeldin-A in broth extracts or column fractions was qualitatively determined by thin layer chromatography using glass-backed 0.25-mm silica 60 plates (EM 5729-6), developed with either DCM : methanol (85 : 15, Rf 0.50), or hexane : acetone (30 : 70, Rf 0.52). Visualization was accomplished by spraying with 5% H_2SO_4 in 95% ethanol and heating briefly with a hot air gun. Brefeldin-A turns a dark reddish color and is detectable at <5 ng.

Large-scale flash chromatography was performed in a 130 mm × 875 mm thick-walled borosilicate glass column (Kontes 584800–4000, Kontes, Vineland, NJ, USA) using Davison grade 22 silica chromatographic media, 60 × 200 mesh (Davison Chemical Company, Baltimore, MD, USA). The silica was dry-packed, then solvated by forcing pure DCM through at <10 psi nitrogen pressure. Liquors remaining after a first crop of crude crystals had been removed were pooled and dried, giving 283 g of a dark brown semi-solid material. The entire pool was dissolved in DCM/methanol (1 : 1), mixed with twice its weight of the same silica, rotary evaporated to dryness and vacuum dried prior to overlayering onto 2000 g of silica. Development was initiated with DCM (10 L), and progressed stepwise through 1% (8 L), 2% (8 L), 3% (8 L), 4% (30 L), and 10% (2 L) methanol in DCM, with flow controlled by the application of nitrogen pressure. The DCA was eluted by DCM, followed by brefeldin-A-enriched material when the solvent composition reached 4% methanol in DCM. Following evaporation to dryness and resolubilization in warm methanol, incubation in a refrigerator overnight yielded crystalline brefeldin-A.

Brefeldin-A is remarkably stable to acid and base but following treatment of extracted broth or equipment used in production in aqueous 0.25% sodium hypochlorite solution for 1 h at ambient temperature, none of the compound could be detected by hplc analysis.

Results

A crystalline substance isolated from the ethyl acetate extract of *E. brefeldianum* ATCC 58665 grown on MEM in a small shake flask was shown to be 7-(*S*)-brefeldin-A by thin layer, hplc, NMR, MS and optical rotation (OR) by comparison to an authentic standard. The literature value given for the OR of 7-(*S*)-brefeldin-A is +82° in ethyl acetate compared with our measurement of +85° in methanol. *E. brefeldianum* strain 10417 did not produce the desired compound, and no effort was made to determine if brefeldin-like compounds [7,28,33] or other bioactive substances [17,19] were present in the extract from either organism.

Having determined that the desired compound was being produced by *E. brefeldianum* ATCC 58665, trial fermentations on MEM in 10-L stir jars were performed until consistent production of brefeldin-A at a titre greater than 100 mg L^{-1} in <90 h was attained. This was considered satisfactory for further scale-up. *E. brefeldianum* ATCC 58665 grew luxuriantly, with a yellow pigment sometimes observed [15]. Appearance of the yellow pigment did not correlate with brefeldin-A production.

Experience was gained through nine progressively larger-scale fermentations (multiple 10-, 100- and 1100-L fermentors) which led to successful completion of four fermentations of 6800 L each. Data typical of a fermentation at that scale is presented here.

The titre of brefeldin-A began climbing sharply approximately 20 h following inoculation of the 11 000-L tank, and continued to rise to a peak after about 50 h of fermentation (Figure 2). During developmental runs, a slow decrease in brefeldin-A titre occurred in the 12 h following the peak titre if fermentation was allowed to continue. This was coincident with a decrease in pH and in centrifugal solids (packed cell volume), both of which could also be used as alternative indicators for harvest. A consistent decrease in pH was observed during the course of fermentation. A pH below 5.0 was deleterious to the optimum production of brefeldin-A. The presence of 0.4% calcium carbonate in the production medium maintained the pH above 5.0 making upward adjustment by addition of base unnecessary.

Analysis by hplc showed that 92% of the desired compound was removed from the broth by a single extraction with ethyl acetate, and that typically >60% of the mass of this extract was brefeldin-A. The off-yellow precipitate obtained from the ethyl acetate extract following concentration, 1123 g, was recrystallized twice from methanol to yield 1026 g of large, white crystals of brefeldin-A. Analysis by both normal and reverse phase hplc showed these crystals to be >99% of the title compound.

Column chromatographic purification of brefeldin-A from the solids remaining in the pool of liquors following crystallization (283 g) yielded an additional 120 g of the desired compound. Isolated yield in this instance was 1146 g of brefeldin-A from 6800 L of broth, or 169 mg L^{-1}.

Discussion

A practical method for the production of kilogram quantities of the anticancer antibiotic brefeldin-A by liquid fermentation has been reported. The isolated yield of brefeldin-A, 169 mg L^{-1}, was less than that reported (278 mg L^{-1}) for stationary culture on a potato dextrose medium [25], but scale-up of that method to provide a large quantity of the desired compound, though possible, would be considerably more difficult than the method reported here. Liquid fermentation at the 50-L scale on a medium similar to MEM resulted in a yield of <15 mg L^{-1} [10].

The determination of optimum fermentation conditions for the production of brefeldin-A was greatly facilitated by the development of a rapid hplc assay which allowed for quantitation of the desired compound from 3 ml of whole fermentation broth within 2 h of the time a sample was taken. As this project progressed from shake flasks to fermentors of increasing volume, the rapid hplc assay provided important information indicating that drug titre in the broth climbed faster and reached a peak earlier in larger vessels than it did in smaller fermentors. The ease and speed at which a brefeldin-A titre profile could be constructed through use of the hplc assay permitted harvest at very nearly the peak of production, thus yield of the desired product could be maximized.

Extraction of brefeldin-A with a half-volume of ethyl acetate from the clarified broth was highly effective, giving, after concentration, a first crop of crude brefeldin-A solids equal to 89% of the isolated yield. Considering the time, labor and materials expended in chromatography, a careful evaluation of the economics of purifying a rather small additional quantity of product from liquors after crystallization should be made in any future large-scale production of brefeldin-A. If testing of this compound by the National Cancer Institute demonstrates its usefulness as an adjunct in cancer chemotherapy, further scale-up of fermentative production of brefeldin-A should be possible with little modification of the techniques reported here.

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