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ORIGINAL PAPER

Bacteria-induced static batch fungal fermentation of the diterpenoid cyathin A_3, a small-molecule inducer of nerve growth factor

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Abstract Cyathin A_3, produced by the fungus *Cyathus helenae*, is a member of the cyathane family of diterpene natural products. While many of the cyathanes display antibacterial/antimicrobial activity or have cytotoxic activity against human cancer cell lines, their most exciting therapeutic potential is derived from their ability to induce nerve growth factor (NGF) release from glial cells, making the cyathanes attractive lead molecules for the development of neuroprotective therapeutics to prevent/treat Alzheimer's disease. To investigate if cyathin A_3 has NGF-inducing activity, we set out to obtain it using published *C. helenae* bench-scale fungal fermentations. However, to overcome nonproducing fermentations, we developed an alternative, bacteria-induced static batch fermentation approach to the production of cyathin A_3, as described in this report. HPLC, UV absorption spectra, and mass spectrometry identify cyathin A_3 in fungal fermentations induced by the timely addition of *Escherichia coli* K12 or *Bacillus megabacterium*. Pre-filtration of the bacterial culture abolishes cyathin A_3 induction, suggesting that bacteria-associated media changes or physical interaction between the fungus and bacteria underlie the induction mechanism. Through alteration of incubation conditions, including agitation, the timing of induction, and media composition, we optimized the fermentation to yield nearly 1 mg cyathin A_3/ml media, a sixfold increase over previously described yields. Additionally, by comparison of fermentation profiles, we reveal that cyathin A_3 biosynthesis is regulated by carbon catabolite repression. We have used an enzyme-linked immunosorbent assay to illustrate that cyathin A_3 induces NGF release from cultured glial cells, and therefore cyathin A_3 warrants further examination in the development of neuroprotective therapeutics.

Keywords Coincubation · Cyathin A_3 · Cyathane · Bacteria-induced · Silent genes

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Introduction

With more than 55,000 members isolated to date, terpenoids are a large and structurally varied class of natural products, many exhibiting great industrial and medicinal value. Despite their structural diversity, all terpenoids are derived from the five-carbon isoprene units dimethylallyl diphosphate and isopentenyl diphosphate (Fig. 1), formed via the mevalonic acid or methylerythritol phosphate biosynthetic pathways. The terpenoid class of molecules can be subdivided into groups based upon the number of isoprene units used for biosynthesis (Fig. 1). Diterpenoids, comprised of four isoprene units, include the cyathane family of natural products (Fig. 2). Members of the cyathane family are each related by a characteristic 5-6-7 tricarbocyclic fused-core structure, derived from the cyclization of geranylgeranyl diphosphate [3]. While many of the cyathanes display antibacterial/antimicrobial activity or have cytotoxic activity against human cancer cell lines

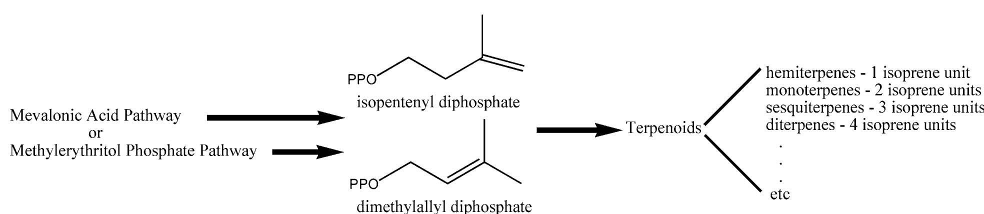

Fig. 1 Biosynthesis of terpenoids. Isopentenyl diphosphate and dimethylallyl diphosphate, produced by the mevalonic acid or methylerythritol phosphate pathways, are the isoprene building blocks of the terpenoid class of natural products. Terpenes are classified by their number of isoprene units

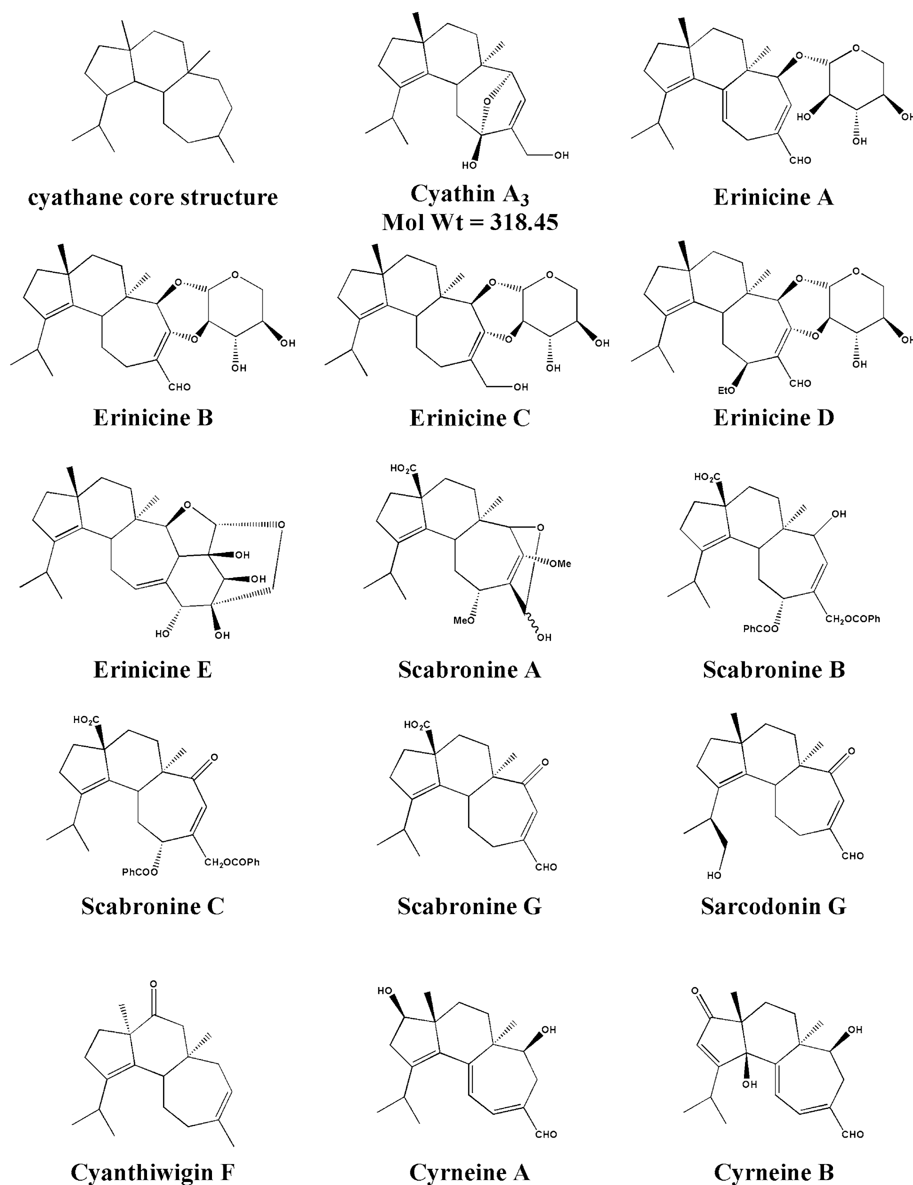

Fig. 2 The cyathane family of diterpenoids. Members of the cyathane family share a 5-6-7 tricarbocyclic cyathane core structure. Cyathin A_3, the founding member of the family, is produced by the bird's nest fungus *Cyathus helenae* [1, 4, 12]. The erinacines [13, 14] are isolated from the Lion's Mane mushroom *Hericium erinaceum*. The sarcodonins [27] and the scabronines [15, 21] are produced by the bitter hedgehog mushroom *Sarcodon scabrosus*. The cyrneines [17] are produced by *Sarcodon cyrneus*. The cyanthiwigins [9, 22–24] are products of the marine sponges *Epipolasis reiswigi* and *Myrmekioderma styx*

[1, 2, 22–24], their most exciting therapeutic potential is derived from their ability to induce nerve growth factor release from glial cells [13–15, 17, 19, 20].

Neurodegeneration is a hallmark of Alzheimer's disease (AD). A neuroprotective therapeutic approach to the treatment/prevention of AD involves the use of molecular agents to defend the neurons from death. Of the most promising neuroprotective agents are the neurotrophins, a family of proteins produced by glial cells in the brain. Nerve growth factor (NGF) is a specific neurotrophin that

binds to basal forebrain cholinergic neurons (the neurons associated with AD) and promotes their survival. Recent preclinical and clinical AD studies have revealed a significant reduction of neuronal loss and a reduced rate of cognitive decline with NGF therapy [6, 30]. However, a critical drawback to NGF is its inability to penetrate the blood–brain barrier. Thus, invasive methods are needed to introduce it into the brain (e.g., surgical implantation of cells engineered to overexpress NGF [30]). Although the outcomes have been positive and offer strong support for the clinical use of NGF for the treatment of AD, the elevated risk and high costs associated with such invasive approaches makes them impractical to be extended to the large number of people suffering from AD. An orally bioavailable drug able to cross the blood–brain barrier and induce the secretion of endogenous NGF would certainly be preferred.

Both enzyme-linked immunosorbent assays (ELISAs) specific for NGF and morphological assays with PC-12 neuronal tumor cells (which differentiate when exposed to NGF) have identified several members of the cyathane family as small-molecule inducers of NGF [13–15, 17–20]. Animal models illustrating cyathane-induced increases in NGF further underscore the potential for this family of molecules as therapeutics for the treatment/prevention of AD [18, 28]. To further the development of the cyathanes as neuroprotective agents, and since it was unknown if cyathin A_3 is a small-molecule inducer of NGF, we set out to obtain cyathin A_3 (Fig. 2) using *Cyathus helenae* bench-scale fungal fermentations. Because our initial approach, based on published conditions, failed to produce detectable levels of cyathin A_3, we describe in this paper the development of a novel approach to the fermentation involving the timely addition of a bacterial culture to the fermentation flask. While the molecular mechanism remains largely unknown, co-cultivation of fungi and bacteria is an emerging strategy for activating silent gene clusters in fungi (reviewed in [7]). The described bacteria-induced static batch fermentation produces cyathin A_3 in yields that are greater than any previously reported. Using purified cyathin A_3 and an NGF-specific ELISA, we illustrate that cyathin A_3 induces NGF from cultured glial cells.

Materials and methods

Mammalian, bacterial, and fungal strains

Human T98G glial cells (ATCC CRL-1690), *Cyathus helenae* strain 1500 (ATCC 28392), and *Escherichia coli* K12 (ATCC 29425) were obtained from American Type Culture Collection (Manassas, VA, USA). *Bacillus megaterium* (ATCC 25300) was a gift from Dr. Clint Smith, Army Corps of Engineers.

Culturing

All culturing procedures were performed aseptically in a laminar flow cabinet. *E. coli* and *B. megaterium* were cultured at 37°C using Luria–Bertani (LB) liquid or solid (1.5% w/v agar) media. Overnight bacterial liquid cultures were incubated in Erlenmeyer flasks (culture volumes were 10% of flask capacity) at 250 rpm using a New Brunswick Scientific I26R shaker (Edison, NJ, USA). *C. helenae* was cultured on 799 Brodie or Potato Dextrose agar (2% w/v agar) in a 28°C water jacketed incubator (Thermo Scientific, Waltham, MA, USA). Media components were purchased from Sigma-Aldrich (St. Louis, MO, USA). Table 1S (Supplementary data) lists the composition of all fungal media used in this investigation. T98G cells were cultured at 37°C, 5% CO_2 in 75-cm^2 tissue culture flasks containing Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12; Invitrogen, Carlsbad, CA, USA).

Bacteria-induced batch fermentation of cyathin A_3

Erlenmeyer flasks (125 ml) were used as culture vessels. Fermentation media was added to each flask (50 ml media per flask), the flask capped with a foam plug, then autoclaved for 20 min, 121°C, 15 psi (103 kPa) prior to use. Flasks were inoculated with a 5-mm^3 agar slice of mycelium aseptically taken at the actively growing hyphal tips of a 6 to 10-day-old *C. helenae* colony. The fermentation flasks were incubated statically (Percival Intellus Environmental Controller, Percival Scientific Inc, Perry, IA, USA) or with shaking (New Brunswick Scientific I26R, Edison, NJ, USA) at the temperature indicated. To produce a fermentation profile, at defined time points (daily from 11 through 30 days post-inoculation) 1 ml of an overnight bacterial culture ($OD_{600} = 4.5–5.0$) was added to the fermentation flask and the fermentation was allowed to continue for an additional 7 days. Each time point was evaluated using duplicate flasks. Upon completion of the fermentation, the media was isolated by filtration and cyathin A_3 was purified as described below. Mycelial mass was determined by drying (100°C, 24 h) then weighing. To evaluate if coincubation was necessary for cyathin A_3 production, the overnight bacterial culture was filtered through a 0.2-μm syringe filter and the filtrate was added to the fermentation flask. An evaluation of bacterial titer during coincubation was determined by plating daily aliquots of fermentation media onto LB-agar, incubating the plates overnight at 37°C, and examining the plate for bacterial growth.

Extraction of cyathin A_3

Media filtrate was transferred to a separatory funnel then extracted three times with an equal volume of ethyl acetate. The organic extracts were combined, dried with anhydrous magnesium sulfate, and concentrated under vacuum at 40°C (Rotovapor R210, Buchi Corporation, New Castle, DE, USA). The extract was stored at 4°C until analyzed.

Authentication of cyathin A_3

Extracts were resuspended in acetonitrile and analyzed using a Beckman System Gold HPLC equipped with a diode array detector and/or a Waters 2695 Separations Module equipped with a diode array and Micromass ZQ MS detectors (Waters Corporation, Milford, MA, USA). A Luna 5μ 150 × 4.6 mm C18(2) column was used for HPLC (Phenomenex, Torrance, CA, USA), whereas an Atlantis T3 3μ 150 × 2.1 mm C18 column (Waters) was used for LC-MS. HPLC conditions were as follows: 50% solvent A (0.1% TFA in water), 50% solvent B (0.1% TFA in acetonitrile) for 5 min, linear gradient to 95% B over 5 min, 95% B for 5 min, 50% B over 2 min, then 50% B for 3 min, at a flow rate of 1 ml per min. LC-MS conditions were as follows: 50% solvent A (0.1% acetic acid in water), 50% solvent B (0.1% acetic acid in acetonitrile) for 5 min, linear gradient to 95% B over 15 min, 95% B for 5 min, 50% B over 2 min, then 50% B for 3 min, flow rate of 0.2 ml per min, ESI negative mode with cone voltage set to 27 V. An authentic cyathin A_3 standard [31, 32] (a gift from Dr. Dennis Wright, University of Connecticut, Storrs, CT, USA) was used to identify cyathin A_3 in the extracts.

Purification of cyathin A_3

Cyathin A_3 was purified from media extracts using tandem silica and C18 flash chromatography with ethyl acetate as the mobile phase. The first 200 ml of eluate from the silica flash column (2.5 × 50 cm; silica gel premium rf, 60 Å porosity, 40–75 μm particle size, Sorbent Technologies, Atlanta, GA, USA) was concentrated under vacuum, resuspended in 5 ml ethyl acetate, then passed through a C18 flash column (2.5 × 50 cm; C18 silica gel, end capped, 60 Å porosity, 40–75 μm particle size, Sorbent Technologies). Fractions containing cyathin A_3 (35–60 ml inclusive) were collected, dried under vacuum, then resuspended in acetonitrile or methanol.

NGF ELISA

T98G cells were removed from tissue culture flasks using a trypsin–EDTA solution devoid of calcium and magnesium (ATCC) and plated at confluent density into six-well plates containing DMEM/F12. After 24 h, the media was changed to Optimem I (reduced serum media; Invitrogen) and cyathin A_3 or phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) were added. After 24 h, the conditioned media was harvested, snap frozen in liquid nitrogen, and stored at −80°C until analyzed. NGF titers were determined using a Human β-NGF ELISA Development Kit (Leinco Technologies, Inc, St. Louis, MO USA), per the manufacturer's instructions. Purified recombinant human β-NGF (Leinco Technologies) was used to create a standard curve (A_{450} vs. NGF concentration).

Results and discussion

Originally reported in the 1970s, cyathin A_3 was discovered as a component of an antibiotic complex obtained from static batch fermentations of *C. helenae* [1, 4, 5, 11, 12]. Since cyathin A_3 is not commercially available, we set out to obtain it using bench-scale fermentations performed as described in those previous reports. However, despite repeated attempts, we were unable to obtain detectable levels of cyathin A_3. Our subsequent fermentations performed with alternative media (Table 1S) also failed to produce the metabolite of interest. Since *C. helenae* exhibits strain-dependent variability in cyathin A_3 production [11, 12], we suspect the fungal strain to be the cause of the fermentation failures. Rather than acquire an alternative strain, however, we chose to evaluate a more radical change to our fermentation approach.

Bacterial cocultivation was recently shown to induce silent fungal secondary metabolic genes in an *Aspergillus nidulans* polyketide fermentation [26]. Since the cyathin complex exhibits antibacterial activity, we hypothesized that the addition of bacteria to the *C. helenae* fermentation would trigger the production of cyathin A_3. To test our hypothesis, actively growing hyphal tips of *C. helenae* were excised from agar plates and then used to inoculate Erlenmeyer flasks containing 799 Brodie Media, as detailed in "Materials and methods". The flasks were statically incubated until the mycelial mat was well established on the surface of the media (20 days at 28°C) and then 1 ml of an overnight *E. coli K12* or *B. megaterium* culture was added directly to the media (the bacterial culture was added beneath the mycelial mat). After addition of the bacteria, the flasks were returned to the incubator for an additional week (this duration was chosen for convenience). Upon completion of the fermentation, the media was isolated via filtration, extracted with ethyl acetate, and the extract analyzed for cyathin A_3. As shown in Fig. 3, both bacteria-induced static batch fermentations produced cyathin A_3, as predicted by our hypothesis. Compared to an authentic cyathin A_3 standard,

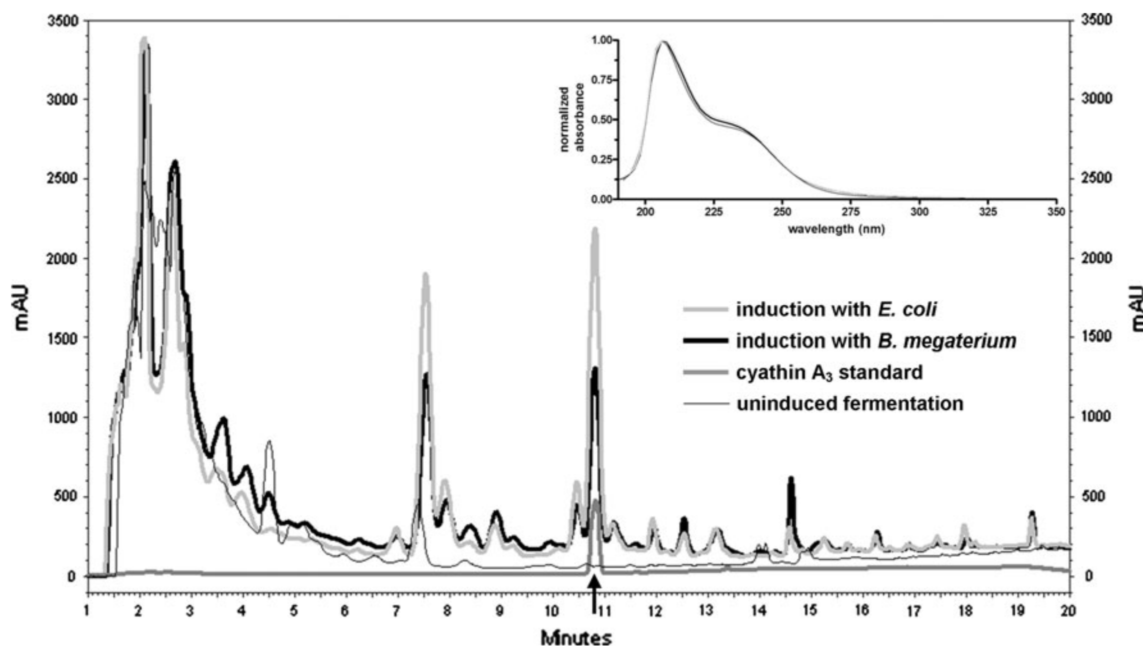

Fig. 3 Identification of cyathin A_3 in the media extract. Compared to an authentic cyathin A_3 standard, identical retention time (*arrow*), UV absorption spectra (*inset*), and *m/z* ratio ($[M\text{-}H]^- = 317.4$ amu) identify cyathin A_3 in media extracts obtained from *C. helenae* fermentations induced with either *E. coli* or *B. megaterium*

a chromatographic peak with an identical retention time (10.8 min) is observed in each of the extracts. The UV absorption spectra ($\lambda_{max} = 207$ nm, shoulder peak at 233 nm) and molecular ion mass (predicted $m/z = 317.4$ ($[M\text{-}H]^-$); actual $m/z = 317.4$) are also identical to the authentic standard, confirming the identity of the peak as cyathin A_3. Thus, the addition of either Gram-positive or Gram-negative bacteria to the *C. helenae* fermentation triggers cyathin A_3 production, however, the yield is significantly greater when *C. helenae* is cocultivated with *E. coli* (Fig. 4). Filtration of the *E. coli* overnight culture before addition to the fungal fermentation flask completely eliminates the induction of cyathin A_3 (Fig. 4), confirming that the bacteria must coincubate with the fungus for stimulation of cyathin A_3 production to occur. This is consistent with the observation that a physical bacteria–fungal interaction is required for bacteria-induced polyketide production by *Aspergillus nidulans* [26], but may also reflect media changes associated with bacterial growth. Bacterial titering of the fermentation media indicates that both *E. coli* and *B. megaterium* remain present throughout the fermentation period (the bacterial cell density remains consistent throughout (data not shown)). While the underlying mechanism remains unclear, the success of the bacteria-induced static batch fermentation prompted us to create fermentation profiles to evaluate the timing of induction as well as alternative conditions for improving the yield of cyathin A_3.

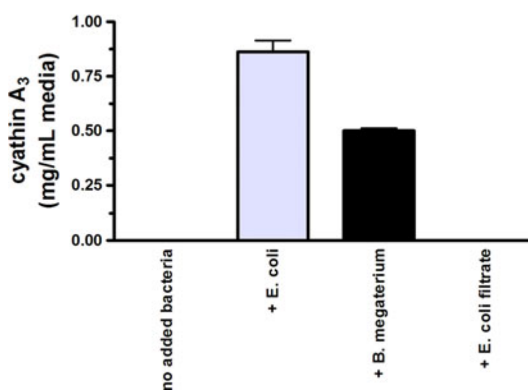

Fig. 4 Bacteria-induced production of cyathin A_3. Twenty-day-old *C. helenae* static flask cultures were induced by the addition of an overnight *E. coli* culture, an overnight *B. megaterium* culture, or filtrate obtained from an overnight *E. coli* culture. After an additional week of incubation, the media was isolated and analyzed for cyathin A_3. Cocultivation with bacteria leads to cyathin A_3 production, with the greatest titers obtained with *E. coli*. Each condition was performed in duplicate

Static versus shaken cultures and timing of induction

In early reports, static flask *C. helenae* fermentations were shown to produce a maximum of ~120–165 μg cyathin A_3 per ml media (after ~29 days of incubation), fivefold greater than maximal yields obtained from shaken flasks [1, 11]. To evaluate how incubation condition and timing of

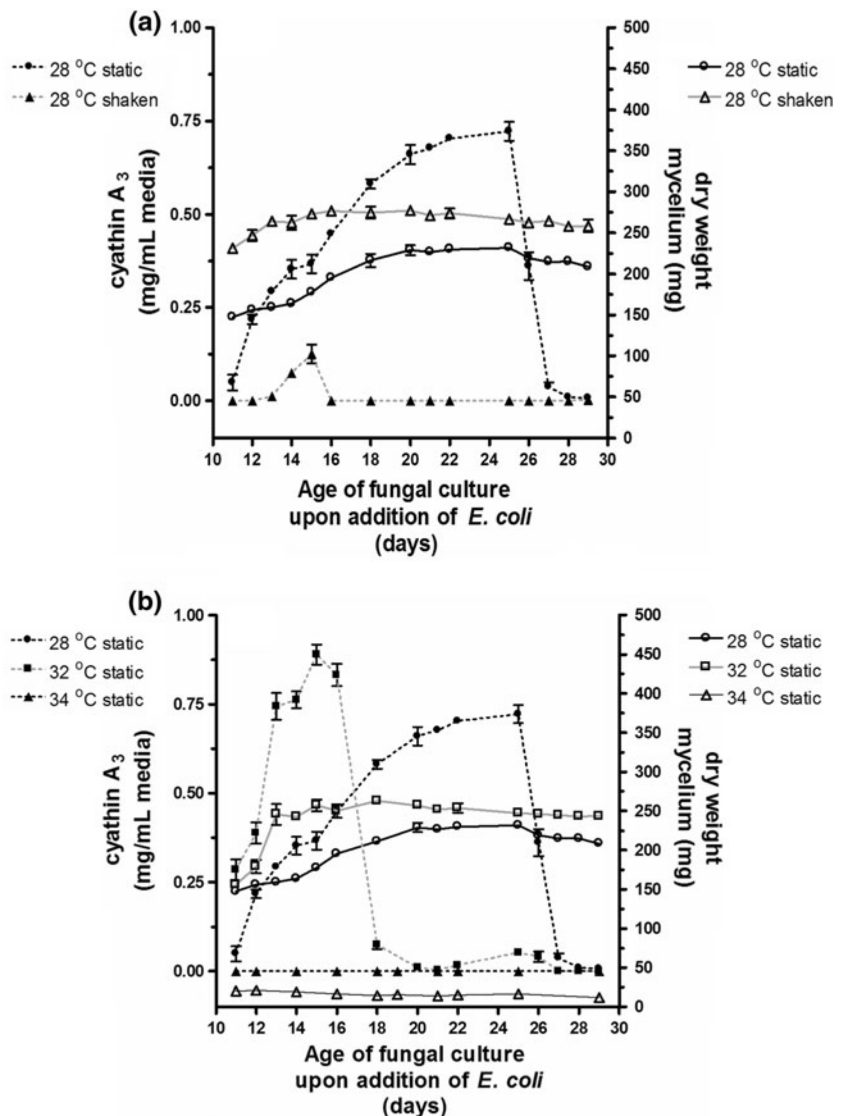

Fig. 5 The effect of incubation condition and timing of induction on the cyathin A_3 fermentation. **a** Fermentation profiles obtained from static and shaken (250 rpm) flasks reveal that significantly greater yields are obtained from static flasks (maximal production = 747 µg/ml media). **b** Fermentation profiles from static flasks incubated at either 28, 32, or 34°C. Incubation at 32°C increases the cyathin A_3 yield to 918 µg/ml media and shifts maximal production to earlier in the profile. Each condition was performed in duplicate

induction affect the bacteria-induced cyathin A_3 fermentation, we created both static and shaken fermentation profiles by monitoring mycelial mass and cyathin A_3 concentration as a function of the age of the fungal culture upon addition of *E. coli* (Fig. 5). Erlenmeyer flasks containing 799 Brodie Media were inoculated with actively growing *C. helenae* hyphal tips and then incubated (28°C) either statically or with shaking (250 rpm). Beginning 11 days post-inoculation, flasks were sequentially induced by the addition of *E. coli* (1 ml overnight culture), then after 1 week cocultivation, the fungal mycelium and fermentation media were isolated from each flask and analyzed. As illustrated in Fig. 5a, reciprocal shaking enhances *C. helenae* growth (relative to static flasks), and the bacteria-induced shaken batch fermentations produce cyathin A_3 in titers that are comparable to the early reports (125 µg/ml maximal production when induced at day 15). In contrast, while mycelial growth in static flasks is less robust, the bacteria-induced static batch fermentation produces substantially greater amounts of cyathin A_3 (Fig. 5a). Maximal cyathin A_3 yield (747 µg/ml; fivefold improvement relative to the titers described in the early reports) is obtained when 25-day-old static flasks are induced with *E. coli*. Thus, both the timing of induction and incubation condition have a profound effect on the yield obtained from bacteria-induced batch fermentation of cyathin A_3. While we speculate that static fermentations may favor the formation of intimate bacterial-fungal contacts, thereby leading to enhanced cyathin A_3 production, the early reports also described improved cyathin A_3 titers with static flasks, suggesting that an additional factor may also contribute to production (such as the dissolved oxygen level). While the static fermentation is clearly preferred, a shorter production run was desirable, so we next sought to alter the fermentation

Table 1 Carbohydrate content of select media used in this study (per l)

799 Brodie Media	799 Brodie Media + glucose	799 Brodie Media + ammonium chloride
2.0 g glucose	10.0 g glucose	2.0 g glucose
5.0 g maltose	5.0 g maltose	5.0 g maltose
Sweet potato media[a]	Potato media[a]	Potato dextrose media[a]
1.9 g glucose	0.8 g glucose	15.8 g glucose
5.0 g sucrose	0.6 g sucrose	0.6 g sucrose
1.4 g fructose	0.6 g fructose	0.6 g fructose
25.3 g starch	26.7 g starch	26.7 g starch

[a] The carbohydrate content of sweet potato and potato are obtained from the USDA National Nutrient Database for Standard Reference, release 22

conditions to shift the appearance of maximal titers to earlier in the fermentation profile.

Increased incubation temperature

Elevated temperature was recently shown to enhance the fermentation of the antifungal agro-antibiotic validamycin A, resulting in an increased yield at an earlier onset of production [16]. To evaluate how increased incubation temperature would affect the fermentation of cyathin A_3, we generated bacteria-induced static batch fermentation profiles by monitoring mycelial growth and cyathin A_3 titers in flasks incubated at either 28, 32, or 34°C. To create the fermentation profiles, flasks containing 799 Brodie Media were inoculated and sequentially induced with *E. coli* in the manner described above. After 1 week of cocultivation, the fungal mycelium and fermentation media were isolated and analyzed. As illustrated in Fig. 5b, incubation at 32°C results in increased cyathin A_3 yields, appearing 10 days earlier in the fermentation profile. In contrast, incubation at 34°C completely inhibits fungal growth and concomitantly abolishes the production of cyathin A_3. Thus, of the incubation temperatures evaluated, 32°C is preferred, producing a maximum titer of 918 μg cyathin A_3/ml media, a sixfold improvement over yields described in the previous reports [1, 11], and a twofold improvement over the fermentation performed at 28°C (61.2 vs. 29.9 μg/ml/day of induction).

Evaluation of alternative fermentation media

The onset of fungal secondary metabolism is generally under the control of global and pathway-specific transcription factors that respond to environmental cues such as nutrient availability [8, 10, 29]. To evaluate the influence of media composition on the production of cyathin A_3, we generated bacteria-induced static batch fermentation profiles by monitoring cyathin A_3 titers in various fermentation media (Table 1S and Fig. 6). To create the fermentation profiles, flasks were inoculated with *C. helenae*, statically

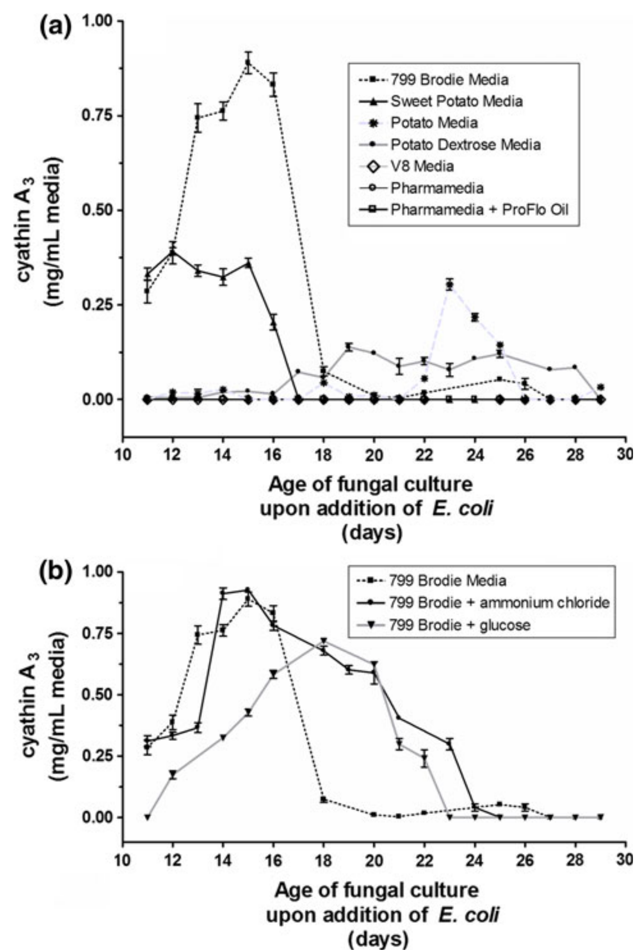

Fig. 6 An evaluation of alternative fermentation media. **a** Bacteria-induced static batch fermentation of cyathin A_3 in various fermentation media. Maximal yield is obtained with 799 Brodie Media. See text for further discussion. **b** Bacteria-induced static batch fermentation in altered 799 Brodie Media. The addition of 4 g/l ammonium chloride permits the continued induction of cyathin A_3 in 19- to 23-day-old fungal cultures, suggesting that nitrogen depletion is responsible for the rapid decline in cyathin A_3 titers beginning with 18-day-old cultures in 799 Brodie Media. The addition of 8 g/l glucose to 799 Brodie Media suppresses and delays the appearance of maximal titers, indicative of carbon catabolite repression. All fermentations were performed in duplicate at 32°C

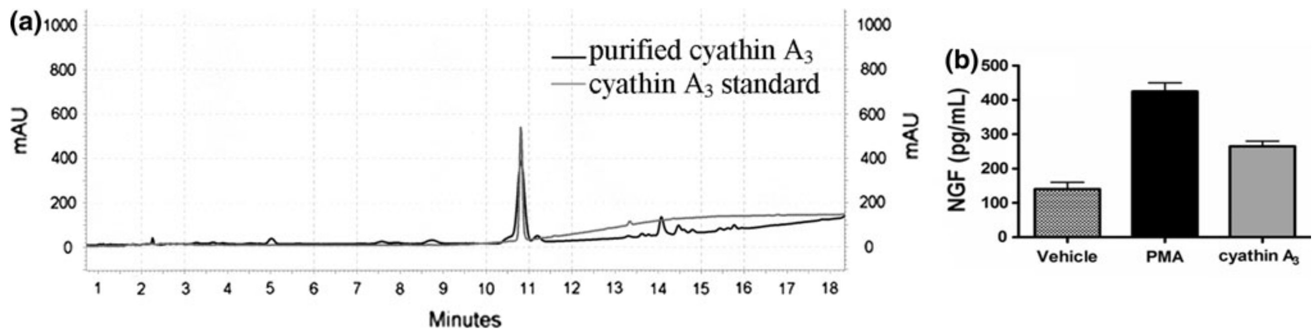

Fig. 7 Cyathin A_3 induced NGF. **a** Silica and C18 flash chromatography were used to purify cyathin A_3 from bacteria-induced static batch fermentation media extracts. Shown are overlayed HPLC chromatograms of purified cyathin A_3 and an authentic cyathin A_3 standard. **b** An NGF-specific ELISA was used to titer the NGF concentration in media obtained from glial cells exposed to vehicle, 10 nM PMA (*positive control*), or 100 μM cyathin A_3. Cyathin A_3 induces the glial cells to release NGF. Assays were performed in duplicate

incubated at 32°C, sequentially induced with *E. coli*, and after 1 week of cocultivation, the fermentation media was isolated and analyzed, in the manner described above. As shown in Fig. 6a, of the fermentation media evaluated, 799 Brodie Media is clearly preferred for the production of cyathin A_3, as the maximal yield is over twofold greater than those obtained from the alternative media. Although fungal growth was comparable in each (not shown), fermentations performed with V8 Media, Pharmamedia (cottonseed flour), and Pharmamedia with added Proflo Oil (cottonseed oil) failed to produce detectable levels of cyathin A_3, while Sweet Potato Media, Potato Media, and Potato Dextrose Media all support the production of cyathin A_3. Interestingly, the fermentation profile in Sweet Potato Media parallels that observed with 799 Brodie Media (but with a lower maximal titer), suggesting that maltose is preferred for cyathin A_3 biosynthesis, while sucrose, fructose, and/or starch are not (Table 1). Additionally, differences observed in Potato Media relative to Potato Dextrose Media suggests that carbon catabolite repression may regulate cyathin A_3 production (Fig. 6a and Table 1), as the maximal cyathin A_3 titer is suppressed in the presence of the added glucose. Carbon catabolite repression and nitrogen derepression are well-known regulators of secondary metabolism, and glucose and NH_4^+ in particular are known to inhibit many diverse fermentations [25]. Interestingly, while the addition of ammonium chloride to 799 Brodie Media does not significantly alter the maximal yield or onset of cyathin A_3 production (Fig. 6b), the time window in which cyathin A_3 can be induced by addition of *E. coli* becomes extended (i.e., 11- through 23-day-old cultures when supplemented with ammonium chloride vs. 11- through 18-day-old cultures in media without supplementation), suggesting that the rapid decline in cyathin A_3 titers observed on day 18 in the 799 Brodie Media may be caused by nitrogen depletion in the media. As anticipated, the addition of glucose to 799 Brodie Media suppresses and delays cyathin A_3 production (Fig. 6b), further implicating carbon catabolite repression as a regulator of cyathin A_3 biosynthesis. Overall, evaluation of alternative fermentation media identifies 799 Brodie Media supplemented with ammonium chloride as the best tested for production of cyathin A_3 (maximum titer = 936 μg/ml on day 15).

Cyathin A_3 induction of NGF

To evaluate the effect of cyathin A_3 on NGF release from cultured glial cells, we used an NGF-specific ELISA and culture media from cells exposed to cyathin A_3, PMA (a potent inducer of NGF), or solvent alone (vehicle). Tandem silica and C18 flash chromatography were used to purify cyathin A_3 from bacteria-induced static batch fermentation media extracts (Fig. 7a) then 100 μM cyathin A_3 was added to the glial cells. After 24 h of exposure, the culture media was isolated and NGF titers were determined. As shown in Fig. 7b, cyathin A_3 induces NGF from cultured glial cells with titers (280 pg/ml) that are comparable to those reported for the cyathane scabronine G (100 μM scabronine G induces 320 pg NGF/ml) [20]. Thus, cyathin A_3 can be used to facilitate the development of neuroprotective agents for the treatment/prevention of AD.

Conclusions

In summary, we have developed a novel approach to the fermentation of cyathin A_3 involving the timely addition of a bacterial culture to the fermentation media. We have shown that static fermentations generate far greater yields than do shaken fermentations, and incubation at 32°C shifts the onset of production to earlier in the fermentation profile relative to incubation at 28°C, and is thus preferred. We propose that cyathin A_3 biosynthesis is regulated by carbon

catabolite repression and identify 799 Brodie Media supplemented with ammonium chloride as the optimal production media evaluated. We have also shown that cyathin A_3 induces NGF from cultured glial cells, with activity comparable to that of scabronine G. Efforts are now underway to further characterize cyathin A_3-induced production of NGF by glial cells.

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References

1. Allbutt AD, Ayer WA, Brodie HJ, Johri BN, Taube H (1971) Cyathin, a new antibiotic complex produced by *Cyathus helenae*. Can J Microbiol 17(11):1401–1407
2. Ayer WA, Lee SP (1979) Metabolites of birds nest fungi.11. Diterpenoid metabolites of Cyathus-Earlei Lloyd. Can J Chem 57(24):3332–3337
3. Ayer WA, Lee SP, Nakashima TT (1979) Metabolites of birds nest fungi.12. Studies on the biosynthesis of the cyathins. Can J Chem 57(24):3338–3343
4. Ayer WA, Taube H (1972) Metabolites of *Cyathus helenae*. Cyathin A3 and allocyathin B3, members of a new group of diterpenoids. Tetrahedron Lett 19:1917–1920
5. Ayer WA, Taube H (1973) Metabolites of *Cyathus helenae*. A new class of diterpenoids. Can J Chem 51:3842–3854
6. Blesch A, Grill RJ, Tuszynski MH (1998) Neurotrophin gene therapy in CNS models of trauma and degeneration. Prog Brain Res 117:473–484
7. Brakhage AA, Schroeckh V (2010) Fungal secondary metabolites—strategies to activate silent gene clusters. Fungal Genet Biol. doi:10.1016/j.fgb.2010.04.004
8. Fox EM, Howlett BJ (2008) Secondary metabolism: regulation and role in fungal biology. Curr Opin Microbiol 11(6):481–487
9. Green D, Goldberg I, Stein Z, Ilan M, Kashman Y (1992) Cyanthiwigin A-D, novel cytotoxic diterpenes from the sponge *Epipolasis reiswigi*. Nat Prod Res 1(3):193–199
10. Hoffmeister D, Keller NP (2007) Natural products of filamentous fungi: enzymes, genes, and their regulation. Nat Prod Rep 24(2):393–416
11. Johri BN, Brodie HJ (1971) The physiology of production of the antibiotic cyathin by *Cyathus helenae*. Can J Microbiol 17(9):1243–1245
12. Johri BN, Brodie HJ, Allbutt AD, Ayer WA, Taube H (1971) A previously unknown antibiotic complex from the fungus *Cyathus helenae*. Experientia 27(7):853
13. Kawagishi H, Shimada A, Shirai R, Okamoto K, Ojima F, Sakamoto H, Ishiguro Y, Furukawa S (1994) Erinacine-A, erinacine-B and erinacine-C, strong stimulators of nerve growth-factor (NGF)-synthesis, from the mycelia of *Hericium erinaceum*. Tetrahedron Lett 35(10):1569–1572
14. Kawagishi HSA, Hosokawa S, Mori H, Sakamoto H, Ishiguro Y, Sakemi S, Bordner J, Kojima N, Furukawa S (1996) Erinacines E, F, and G, stimulators of nerve growth factor (NGF)-synthesis, from the mycelia of *Hericium erinaceum*. Tetrahedron Lett 37:7399–7402
15. Kita T, Takaya Y, Oshima Y, Ohta T, Aizawa K, Hirano T, Inakuma T (1998) Scabronines B, C, D, E and F, novel diterpenoids showing stimulating activity of nerve growth factor-synthesis, from the mushroom *Sarcodon scabrosus*. Tetrahedron 54(39):11877–11886
16. Liao Y, Wei ZH, Bai L, Deng Z, Zhong JJ (2009) Effect of fermentation temperature on validamycin A production by *Streptomyces hygroscopicus* 5008. J Biotechnol 142(3–4):271–274
17. Marcotullio MC, Pagiotti R, Maltese F, Mwankie GNOM, Hoshino T, Obara Y, Nakahata N (2007) Cyathane diterpenes from *Sarcodon cyrneus* and evaluation of their activities of neuritegenesis and nerve growth factor production. Bioorg Med Chem 15(8):2878–2882
18. Mori K, Obara Y, Hirota M, Azumi Y, Kinugasa S, Inatomi S, Nakahata N (2008) Nerve growth factor-inducing activity of *Hericium erinaceus* in 1321N1 human astrocytoma cells. Biol Pharm Bull 31(9):1727–1732
19. Obara Y, Hoshino T, Marcotullio MC, Pagiotti R, Nakahata N (2007) A novel cyathane diterpene, cyrneine A, induces neurite outgrowth in a Rac1-dependent mechanism in PC12 cells. Life Sci 80(18):1669–1677
20. Obara Y, Kobayashi H, Ohta T, Ohizumi Y, Nakahata N (2001) Scabronine G-methylester enhances secretion of neurotrophic factors mediated by an activation of protein kinase C-zeta. Mol Pharmacol 59(5):1287–1297
21. Ohta T, Kita T, Kobayashi N, Obara Y, Nakahata N, Ohizumi Y, Takaya Y, Oshima Y (1998) Scabronine A, a novel diterpenoid having potent inductive activity of the nerve growth factor synthesis, isolated from the mushroom, *Sarcodon scabrosus*. Tetrahedron Lett 39(34):6229–6232
22. Peng J, Kasanah N, Stanley CE, Chadwick J, Fronczek FR, Hamann MT (2006) Microbial metabolism studies of cyanthiwigin B and synergetic antibiotic effects. J Nat Prod 69(5):727–730
23. Peng JN, Avery MA, Hamann MT (2003) Cyanthiwigin AC and AD, two novel diterpene skeletons from the Jamaican sponge *Myrmekioderma styx*. Org Lett 5(24):4575–4578
24. Peng JN, Walsh K, Weedman V, Bergthold JD, Lynch J, Lieu KL, Braude IA, Kelly M, Hamann MT (2002) The new bioactive diterpenes cyanthiwigins E-AA from the Jamaican sponge *Myrmekioderma styx*. Tetrahedron 58(39):7809–7819
25. Sanchez S, Demain AL (2002) Metabolic regulation of fermentation processes. Enzyme Microb Technol 31(7):895–906
26. Schroeckh V, Scherlach K, Nutzmann HW, Shelest E, Schmidt-Heck W, Schuemann J, Martin K, Hertweck C, Brakhage AA (2009) Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. Proc Natl Acad Sci USA 106(34):14558–14563
27. Shibata H, Tokunaga T, Karasawa D, Hirota A, Nakayama M, Nozaki H, Tada T (1989) Isolation and characterization of new bitter Diterpenoids from the fungus *Sarcodon scabrosus*. Agric Biol Chem 53(12):3373–3375
28. Shimbo M, Kawagishi H, Yokogoshi H (2005) Erinacine A increases catecholamine and nerve growth factor content in the central nervous system of rats. Nutr Res 25(6):617–623
29. Shwab EK, Keller NP (2008) Regulation of secondary metabolite production in filamentous ascomycetes. Mycol Res 112:225–230
30. Tuszynski MH, Thal L, Pay M, Salmon DP, Sang UH, Bakay R, Patel P, Blesch A, Vahlsing HL, Ho G, Tong G, Potkin SG, Fallon J, Hansen L, Mufson EJ, Kordower JH, Gall C, Conner J (2005) A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. Nat Med 11(5):551–555
31. Wright DL, Whitehead CR (2000) Recent progress on the synthesis of cyathane type diterpenes. A review. Org Prep Proced Int 32(4):307–330
32. Wright DL, Whitehead CR, Sessions EH, Ghiviriga I, Frey DA (1999) Studies on inducers of nerve growth factor: synthesis of the cyathin core. Org Lett 1(10):1535–1538