A New Ene-triyne Antibiotic from the Fungus *Baeospora myosura*

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The isolation and structure elucidation of **1** from the Basidomycete fungus *Baeospora myosura* is described. This new ene-triyne antibiotic was most potent against Gram-positive bacteria, while it was less active against Gram-negative bacteria and a yeast. MICs against several strains of *Staphylococcus aureus* were as low as 0.001 *μ*g/mL. Analogues of **1** that did not contain the ene-triyne moiety were inactive against all microorganisms tested. The isolation of this new natural product was complicated by the highly reactive nature of the conjugated terminal polyacetylene.

While screening for novel antibiotics from microbial sources, a new ene-triyne secondary metabolite was isolated and characterized from the Basidomycete fungus *Baeospora myosura* (Fr.) Singer. The active component (**1**) was determined to be an amide of the C-9 ene-triyne carboxylic acid **2** with *p*-aminobenzoic acid. This natural product demonstrated selective antibacterial activity. It was potent against Gram-positive bacteria, while it was less active against Gram-negative bacteria and the yeast *Candida albicans*.

Polyacetylenic natural products are produced by a number of higher fungi and plants.[1–5] Ene-triyne-containing natural products have been identified from a variety of Basidomycete fungal species. The C-9 ene-triyne carboxylic acid **2** (2*E*-non-2-ene-4,6,8-triynoic acid), containing a terminal acetylene, has been isolated from the Basidomycetes *Psilocybe sarcocephala*,[6] *Poria sinuosa* Fr.,[7] and *Lepista* species.[8,9] A number of structural analogues of this polyunsaturated unit also have been described.[8–10] The only previous example in which the terminal ene-triyne carboxylate was isolated as an amide was the case of the valine analogue.[7]

The isolation of **1** was complicated by the substantial instability of this class of natural products. Concentration of the active component, particularly in a purified state, led to rapid and complete decomposition to an uncharacterized black polymeric material. Generally, terminal conjugated polyacetylenes are much more reactive than internal polyacetylenes.[2] In this case, standard loading and elution of larger samples of active material during chromatography led to partial sample decomposition, which depended on the local concentration of the natural product on the column. Several modifications to standard isolation protocols were required in order to execute the isolation and structure elucidation of this highly reactive compound.

While screening for novel antibiotics, an acetone extract from *Baeospora myosura* was identified as providing potent activity against *Staphylococcus aureus*. This activity was used to guide the isolation of the active component. The antibacterial activity was correlated with one major component present in the whole broth extract. A larger extract (0.7 L) of a solid fermentation of *B. myosura* was prepared with an equal volume of 2:1 acetone–water. An EtOAc extraction at acidic pH, followed by the partitioning of the sample between hexane and acetonitrile, provided a crude extract that retained the activity of the original extract. Approximately 800 mg of solid material was obtained after this initial extractive process.

Further processing of this partially purified extract was accomplished on CHP20P resin. To minimize decomposition of the active component, batch loading of the resin was employed. After addition of the resin, water was added until >95% of the active component was bound. After washing of the resin, the active component was eluted with a linear gradient of aqueous MeOH. Analytical reversed-phase (C$_8$) HPLC was used to follow the adsorption and elution of the desired component. Final purification of the natural product was accomplished by preparative reversed-phase HPLC. Inorganic salts were removed from the purified sample by EtOAc extraction at acidic pH.

Samples for structure elucidation were prepared without concentration to dryness. Initial manipulations of purified material led to rapid polymerization of **1** upon removal of solvent. Reports have indicated the reactive, and even explosive, nature of this family of natural products, particularly when present in the solid form.[10] DMSO was added to EtOAc solutions of **1** prior to concentration of samples for spectroscopic and biological studies. For NMR measurements, deuterated DMSO was added to the EtOAc solution of **1** prior to concentration. Due to the instability of **1**, sample concentration was determined by preparing a dilution of the DMSO stock solution in MeOH and calculating the concentration based on previously reported extinction coefficients for the ene-triyne chromophore at 325 nm ($\epsilon = 2 \times 10^4$ M^{-1} cm^{-1}).[6] The absorbance spectrum of **1** was characteristic of polyacetylenes with maxima observed at 347, 325, 261, and 248 nm. The UV absorbance of the *p*-aminobenzoic acid residue was masked by DMSO in these samples. The titer of the original culture was estimated as 25 mg/L, and approximately 6 mg of **1** was isolated and purified as a solution in DMSO.

The molecular formula of the active component was C$_{16}$H$_9$NO$_3$ based on HRFTMS ([M + H]$^+$ *m/z* obsd 264.0654; calcd 264.0661). ^{1}H and COSY NMR clearly identified two structural units, a 1,4-disubstituted benzene ring and a *trans*-alkene. Further functionality was revealed in the ^{13}C NMR spectrum, which contained resonances for six alkynic carbons and two carbonyls. The complex absorbance spectrum was consistent with extended conjugation of the acetylenic groups. The connectivity of the structural units
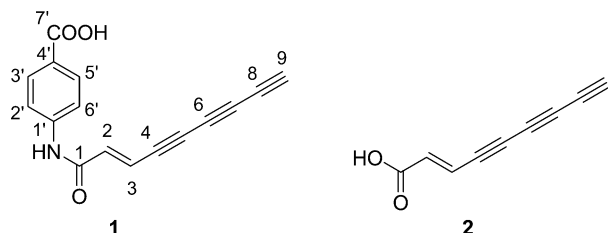
Figure 1. Isolated active component **1** and ene-triyne carboxylic acid **2**.
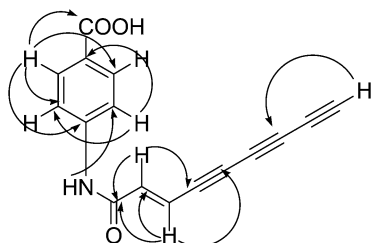

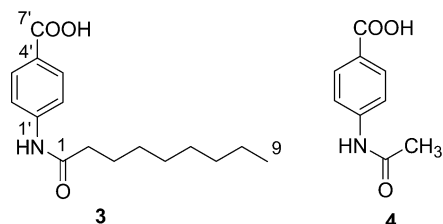**Figure 2.** Selected HMBC correlations observed for **1** in d_6-DMSO.

Figure 3. Reduced analogue **3** and 4-acetimidobenzoic acid (**4**).

Table 1. NMR Assignments of Ene-triyne **1** in d_6-DMSO (500 MHz)

	δ_C	mult	δ_H (mult, J in Hz)	HMBC
1	161.7	qC		H-2, 3
2	118.4	CH	6.80, d, 15.5	H-3
3	139.7	CH	6.91, d, 15.5	H-2
4	79.3	qC		H-2 (w)
5	74.2	qC		H-3
6	58.7	qC		
7	68.8	qC		H-9
8	67.3	qC		
9	76.2	CH	4.19, s, J_{CH}=260	
1'	142.1	qC		H-3', 5'
2', 6'	118.8	CH	7.73, d, 9.0	H-6', 2', NH
3', 5'	130.4	CH	7.89, d, 9.0	H-5', 3'
4'	126.9	qC		H-2', 6'
7'	167.0	qC		H-3', 5'
NH			10.70, s	

of **1** was determined through analysis of HMBC experiments (Figure 2), leading to the assignments in Table 1. The terminal acetylene (^{1}H δ 4.19, s, 1H) was also observed as a QC doublet in the HMBC spectrum with a coupling constant of $^1J_{CH} = 260$ Hz.

Confirmation of the structural assignments was obtained by the hydrogenation of the ene-triyne system of **1**. After hydrogenation, the molecular weight increased by 14, indicating that seven units of unsaturation had been reduced. The aromatic moiety of **1** was still intact by NMR analysis. Further characterization of the reduced product identified it as the saturated *n*-nonanoic acid analogue **3** containing a terminal methyl group (^{1}H δ 0.85, t, 3H, $J = 7.0$ Hz). The structure of the hydrogenated analogue was fully consistent with the assigned structure of the natural product.

The antibiotic activity of **1** was determined against a panel of microorganisms. Ene-triyne **1** showed extremely potent activity against the Gram-positive bacteria *Staphy-*

Table 2. Antimicrobial Activity of **1**[a]

organism	MIC (μg/mL)
Gram-positive	
Staphylococcus aureus (Meth^S) (1)	0.0005
Staphylococcus aureus (Meth^S) (+50% serum) (1)	16
Staphylococcus aureus (Meth^R) (2)	0.001
coagulase negative Staph. (Meth^R) (1)	0.0005
Enterococcus faecalis (Van^S) (1)	0.12
Enterococcus faecium (Van^R) (1)	0.015
Streptococcus pneumoniae (3)	8
Streptococcus pneumoniae (Pen^R) (1)	16
Streptococcus pyogenes (1)	0.008
Bacillus megaterium (1)	4
Gram-negative	
Haemophilus influenzae (1)	0.5
Escherichia coli (2)	32 to >32
Pseudomonas aeruginosa (1)	>32
Klebsiella pneumoniae (1)	>32
Yeast	
Candida albicans (1)	>32

[a] Number of organisms tested in parentheses. Meth^S = methicillin sensitive; Meth^R = methicillin resistant; Pen^R = penicillin resistant; Van^R = vancomycin resistant; Van^S = vancomycin sensitive.

lococcus aureus (MIC $\leq$ 0.001 μg/mL) and *Streptococcus pyogenes* (0.008 μg/mL). The reduced analogue **3** and 4-acetimidobenzoic acid (**4**), both lacking the reactive ene-triyne chromophore, were not active against *S. aureus*. The activity of **1** against *S. aureus* was significantly reversed in the presence of 50% serum, with the MIC increasing from 0.0005 to 16 μg/mL. There was less activity against *Bacillus megaterium* and *Streptococcus pneumoniae*, with MICs of 4 and 8−16 μg/mL, respectively. For Gram-negative bacteria, while there was no measured activity against *Escherichia coli*, *Klebsiella pneumoniae*, or *Pseudomonas aeruginosa*, inhibition was observed for *Haemophilus influenzae* with an MIC of 0.5 μg/mL. Compound **1** was inactive against *C. albicans* at 32 μg/mL. A preliminary toxicity study in which **1** was administered subcutaneously to mice at 25 mg/kg resulted in the overnight death of two of three treated animals.

Thus, the isolation and biological activity of polyacetylene **1** from a Basidomycete fungus have been described. The potent antibacterial activity of this substance is attributed to its highly reactive and toxic properties, but some selectivity against a panel of microorganisms was observed. To facilitate the purification of **1**, isolation protocols were adapted due to the extreme reactivity of the conjugated ene-triyne system.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Beckman DU-70 spectrophotometer. IR spectra were obtained with a Perkin-Elmer Spectrum One spectrophotometer. All NMR spectra were recorded on Varian Inova 500 MHz instruments, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. High-resolution mass spectral data were obtained with a Thermo Quest FTMS using electrospray ionization. Analytical HPLC was performed on a Hewlett-Packard 1100 HPLC system. Preparative HPLC was performed on a Waters Delta Prep 4000 preparative system. CHP20P resin was purchased from MCI.

Fungal Materials. The strain of *Baeospora myosura* used for this investigation has been deposited into the Merck Culture Collection (MF7010). Fermentation conditions for the production of **1** from this microorganism in F1 medium are described below.

The culture was maintained on agar plugs in vials containing sterile glycerol (10%) and stored at −80 °C until ready for

use. The seed culture was inoculated by aseptically transferring four agar plugs into a 250 mL Erlenmeyer flask containing 60 mL of seed medium of the following composition (in g/L): corn steep powder, 2.5; tomato paste, 40.0; oat flour, 10.0; glucose, 10.0; agar, 4; and trace elements solution, 10 mL/L. The trace elements solution consisted of the following components: $FeSO_4 \cdot 7H_2O$, 1.0 g/L; $MnSO_4 \cdot 4H_2O$, 1.0 g/L; $CuCl_2 \cdot 2H_2O$, 0.025 g/L; $CaCl_2 \cdot 2H_2O$, 0.1 g/L; H_3BO_3, 0.056 g/L; $(NH_4)_6MoO_{24} \cdot 4H_2O$, 0.019 g/L; and $ZnSO_4 \cdot 7H_2O$, 0.2 g/L.

Seed medium was prepared with distilled water. The pH was adjusted to 6.8 by adding NaOH, and the medium was dispensed into 250 mL Erlenmeyer flasks and capped with cellulose plugs before being autoclaved at 121 °C for 20 min.

The seed culture was incubated at 22 °C on a gyratory shaker (220 rpm) for 5−8 days prior to the inoculation of fermentation flasks. The F1 production medium was formulated (per 250 mL flask) as follows: cracked corn (10 g) and base liquid (25 mL). Base liquid stock solution contained the following (per 2.5 L): ardamine pH (0.2 g); KH_2PO_4 (0.1 g); $MgSO_4 \cdot 7H_2O$ (0.1 g); sodium tartrate (0.1 g); $FeSO_4 \cdot 7H_2O$ (0.01 g); $ZnSO_4 \cdot 7H_2O$ (0.01 g); no pH adjustment. The production medium was prepared with distilled water, and no adjustment was made to the pH. It was dispensed into 250 Erlenmeyer flasks and capped with cellulose plugs before being autoclaved at 121 °C for 20 min. Fermentation flasks were inoculated with 2 mL of vegetative seed growth and were incubated at 22 °C, 70% humidity for 28 days. This solid fermentation broth was extracted as described below.

Extraction and Isolation. A solid fermentation of *B. myosura* (0.7 L) was extracted with an equal volume of 2:1 acetone−water and filtered to remove the mycelium. The acetone was removed in vacuo, and the volume was brought to 0.5 L with water, adjusted to pH 2.5 with 2 N HCl, and extracted twice with an equal volume of EtOAc. The combined organic layers were partially concentrated and then, to further remove lipid material, partitioned between acetonitrile and hexane (0.5 L each). The desired component was present only in the acetonitrile layer. Analytical HPLC was used to monitor the isolation and identify samples containing the active component (Waters Symmetry C_8, 4.6 × 50 mm, 3.5 μm, 2 mL/min, 45:55 acetonitrile−pH 2.5 10 mM potassium phosphate, t_R ∼1.8 min).

To a stirred solution of the crude extract in MeOH−acetonitrile (30 mL) was added CHP20P (50 mL) in water. Additional water was added until less than 5% of the active component remained in solution, as analyzed by HPLC. At that point, the solution was approximately 65% aqueous. The resin was washed with 25% MeOH and eluted with a linear gradient of aqueous MeOH (25−90% MeOH), with elution of **1** beginning at 55% MeOH. Final purification of the active component was achieved by preparative HPLC (Waters Symmetry C_8, 7.8 × 300 mm, 7 μm, 8 mL/min, 45:55 acetonitrile−pH 2.5, 10 mM potassium phosphate, t_R ∼35 min). Compound **1**: UV (MeOH) λ_{max} 347, 325, 261, 248 nm; ^{1}H NMR (d_6-DMSO, 500 MHz) δ 10.70 (1H, s, amide), 7.89 (2H, d, J = 9.0 Hz, H-3′, 5′), 7.73 (2H, d, J = 9.0 Hz, H-2′, 6′), 6.91 (1H, d, J = 15.5 Hz, H-2), 6.80 (1H, d, J = 15.5 Hz, H-3), 4.19 (1H, s, H-9); ^{13}C NMR (d_6-DMSO, 125 MHz) δ 167.0 (amide CO, C-7′), 161.7 (acid CO, C-1), 142.1 (qC, C-1′), 139.7 (CH, H-2), 130.4 (CH, C-3′, 5′), 126.9 (qC, C-4′), 118.8 (CH, C-2′, 6′), 118.4 (CH, C-2), 79.3 (qC, C-4), 76.2 (CH, C-9), 74.2 (qC, C-5), 68.8 (qC, C-7), 67.3 (qC, C-8), 58.7 (qC, C-6); HRFTMS m/z 264.0654 (calcd for $C_{16}H_9NO_3$ + H, 264.0661).

Determination of MICs. Strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Bacillus megaterium*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* were tested in cation-adjusted Mueller-Hinton Broth (CAMHB), inoculation = 10^5 cfu/mL, incubated at 35 °C for 20 h. *Streptococcus pneumoniae* was tested in CAMHB with 2.5% lysed horse blood, inoculation = 10^5 cfu/mL, incubated at 35 °C for 20 h. *Haemophilus influenzae* was tested in Haemophilus Test medium, inoculation = 10^5 cfu/mL, incubated at 35 °C for 20 h. *Candida albicans* was tested in RPMI 1640, inoculation = 10^3 cfu/mL, incubated at 35 °C for 24 h.

Reduction of Ene-triyne 1 to 3. To a solution of **1** (1 mg, 0.004 mmol) in MeOH was added 10% Pd/C (1 mg). The atmosphere of the flask was evacuated and purged with H_2 gas (1 atm) five times, and the reaction was stirred at room temperature for 90 min. After removing the H_2 gas in vacuo, the reaction was filtered through Celite and concentrated. The reduced product was purified by semipreparative HPLC (Waters Symmetry300 C_{18} 300 Å, 7.8 × 300 mm, 7 μm, 3 mL/min, gradient elution 10−75% acetonitrile (12 min) in pH 2.5 10 mM sodium phosphate) and desalted with a plug of C_{18} resin (YMC), eluting with MeOH. Compound **3**: UV (MeOH) λ_{max} 273 nm; FT-IR ν_{max} 1666 (amide) cm^{-1}; ^{1}H NMR (d_6-DMSO, 500 MHz) δ 10.14 (1H, s, amide-NH), 7.85 (2H, d, J = 9.0 Hz, H-3′, H-5′), 7.68 (2H, d, J = 9.0 Hz, H-2′, H-6′), 2.32 (2H, t, J = 7.3 Hz, H_2-2), 1.57 (2H, m, H_2-3), 1.25 (6H, m, H_2-4, 5, 6), 1.22 (2H, m, H_2-7), 1.24 (2H, m, H_2-8), 0.85 (3H, t, J = 7.0 Hz, H_3-9); ^{13}C NMR (d_6-DMSO, 125 MHz) δ 171.8 (amide CO, C-1), 166.9 (acid CO, C-7′), 143.3 (C, C-1), 130.3 (CH, C-3′, 5′), 124.9 (C, C-4′), 118.2 (CH, C-2′, 6′), 36.5 (CH_2, C-2), 31.2 (CH_2, C-7), 28.7 (CH_2, C-5), 28.6 (CH_2, C-6), 28.6 (CH_2, C-4), 25.0 (CH_2, C-3), 22.0 (CH_2, C-8), 13.9 (CH_3, C-9); HRFTMS m/z 278.1731 (calcd for $C_{16}H_{23}NO_3$ + H, 278.1756).

Supporting Information Available: ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, and UV spectra of **1** are available free of charge via the Internet at http://pubs.acs.org.

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