cis-2-AMINO-1-HYDROXYCYCLOBUTANE-1-ACETIC ACID, A HERBICIDAL ANTIMETABOLITE PRODUCED BY
Streptomyces rochei A13018

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Microorganisms represent a well-documented source of herbicidal compounds with the potential to provide agricultural researchers with new structural classes for herbicide synthesis efforts1*. In the continuation of a screening program to discover novel natural product herbicides from microbial sources2), Streptomyces rochei A13018 was found to produce cis-2-amino-1-hydroxycyclobutane-1-acetic acid (CBAA: 1), which causes L-cysteine- or L-methionine-reversible chlorosis in Arabidopsis thaliana. CBAA is a constituent of the naturally occurring antibacterial dipeptide (l\(\land\),2S)-1-hydroxy-2-[((S)-valylamino)cyclobutane-1-acetic acid (2)3), and has been reported synthetically by Baldwin et al.4). However, no previous reports cite the isolation of CBAA as the free amino acid or describe its herbicidal properties. This note briefly describes the producing organism, fermentation, isolation, structure identification, and biological activities of CBAA.

Strain A13018 was isolated from a soil sample collected in Madera Canyon, Santa Rita Mountains, Arizona, U.S.A. The aerial mycelium bore chains of spores forming spirals (S), as described by Shirling and Gottlieb5), bearing up to five turns. When examined by scanning electron microscopy, the spores were observed as short rods (0.6 by 1.0~1.3 \(\mu\)m) bearing a hairy surface. The color of the aerial mycelium was white to gray. Substrate mycelium was not fragmented and no spores were observed. The strain grew at 10°C but no growth was observed at 45°C. The optimum growth temperature was 27°C. On International Streptomyces Project (ISP) media 1, 2, 3, and 5, the strain produced yellow diffusible pigment and the reverse side color of the colonies was yellow. Melanin production was not observed on either tyrosine or peptone iron agar. The following tests were positive: starch hydrolysis; degradation of allantoin, lecithin, pectin, xanthine, and hypoxanthine; resistance to rifampicin (50 \(\mu\)g/ml); tolerance to sodium azide (0.01% w/v); utilization of D-glucose, D-raffinose, \(\alpha\)-lactose, L-arabinose, D-galactose, L-valine, salicin, \(\alpha\)-(+) melibiose, D-fructose, D-mannitol, (+)-mannose, L-sorbose, D-ribose, (+)-rhamnose, (+)-maltose, sucrose, (+)-xylose, myo-inositol, and (+)-cellobiose; antibiotic to Streptomyces marlensis ATCC 19788 and Bacillus subtilis ATCC 6633. Negative responses were: \(H_2\)S production; nitrate reduction; degradation of arbutin; tolerance to NaCl (7% w/v) and phenol (0.1% w/v); resistance to neomycin (50 \(\mu\)g/ml); antibiotic to Aspergillus niger ATCC 36233; utilization of D-phenylalanine, L-tyrosine, hydroxy-L-proline, L-threonine, L-serine, L-histidine, L-cystine, DL-\(\alpha\)-amino-\(\beta\) -butyric acid, L-glutamic acid, adonitol, xylitol, dulcitol, and inulin. The strain utilized DL-\(\alpha\)-amino-\(\beta\) -butyric acid and hydroxy-L-proline as nitrogen sources. The whole cell hydrolysate contained L-diaminopimelic acid, and the whole cell sugar pattern was not characteristic, indicating that the cell wall of strain A13018 was type 1. Based on the above results, strain A13018 closely resembled Streptomyces rochei, as defined in Bergey's Manual of Systematic Bacteriology6).

CBAA (1) was produced by Streptomyces rochei A13018 in shake flask fermentations. A 250-ml Erlenmeyer flask containing 50 ml of a medium consisting of Tryptone 0.5% and yeast extract 0.3% was inoculated and fermented at 30°C for 3 days on a rotary shaker at 250 rpm. The inoculum broth was transferred to a 2-liter Erlenmeyer flask containing 1 liter of production medium consisting of dextrin 1.0%, glucose 0.1%, soybean flour 0.5%, yeast extract 0.15%, and CaCO\(_3\) 0.15% (pH 6.5), to which

\[ \begin{align*}
1 & \quad \text{H}_2\text{N} \quad \text{OH} \quad \text{COOH} \\
2 & \quad \text{NH}_2 \quad \text{O} \quad \text{NH} \quad \text{OH} \quad \text{COOH}
\end{align*} \]
was added 0.1% antifoam (Dow Corning Type A). The fermentation was carried out on a rotary shaker at 30°C and 120 rpm, with 1 liter/minute aeration, for 4 days. The herbicidal activity of the resulting broth was assayed by an agar diffusion method using Arabidopsis thaliana as the test organism.

The culture broth (1-liter) was filter sterilized and the sterile filtrate (350ml) was passed through a column of Bio Rad AG50W-X8 (H+form, 50ml) which was washed with water (500ml) and eluted with 3% NH₄OH (500ml). The 3% NH₄OH eluate was evaporated under reduced pressure and the resulting crude active material (0.34g) was fractionated by reversed phase C18 chromatography eluting with water. The active fractions were combined and concentrated in vacuo to give a yellow solid (0.07g), which was further purified on Sephadex LH-20 eluting with water-methanol (3:1). Active fractions (0.07g) were combined, concentrated, and chromatographed on QAE Sephadex A-25 (acetate form). Elution with a linear NH₄OAc gradient (0.05M, pH 9 to 0.3M, pH 7) afforded, after removal of NH₄OAc via passage through a column of Bio Rad AG50W-X8 (H+ form, 10ml), elution with 3% NH₄OH, and evaporation under reduced pressure, a total of 12mg of CBAA (1).

The physico-chemical properties of CBAA were as follows: [α]2 25 +3.8° (c 0.025, H₂O); CD (c 0.067mg/ml, H₂O): [θ] (193) -1,094, (217) +42; IR νmax (neat) cm⁻¹ 2940 (br), 1630, 1610, 1565, 1520, 1380; FAB-MS (glycerol) negative mode m/z 144 (M-H)⁻, positive mode m/z 146 (M+H)⁺. The HRFAB-MS of I showed an M+H ion peak at m/z 146.0818, indicating the molecular formula of the protonated species to be C₆H₁₂NO₃ (Calcd 146.0817); ¹H NMR (300MHz, D₂O) δ 1.90-2.10 (3H, m), 2.10-2.21 (1H, m), 2.42 and 2.49 (2H, AB=16Hz), 3.63 (1H, m); ¹³C NMR (75MHz, D₂O) δ 23.67 (t, JCH=139Hz), 32.77 (t, JCH=138Hz), 48.60 (t, JCH=127Hz), 54.13 (d, JCH=152Hz), 76.50 (s), 181.69 (s); TLC SiO₂ (Merck) Rf 0.46 (BuOH-ACOH-H₂O, 2:1:1), Rf 0.45 (CH₃CN-ACOH-H₂O, 4:1:1), ninhydrin positive.

¹H and ¹³C NMR studies of I revealed one sp³ hybridized quaternary carbon, one methine, three methylenes, and a signal at δ 181.69 assigned to a carboxylate salt on the basis of IR absorptions at 1565 and 1380 cm⁻¹. The four protons which were not observed in the ¹H NMR spectrum were attributed to exchangeable protons of carboxylic acid, hydroxyl, and primary amine groups. The molecular formula, C₆H₁₁N₂O₃, required one site of unsaturation in addition to the carboxylic acid functionality. In the absence of further sp² hybridized carbon atoms, as indicated by ¹³C NMR, the molecule had to contain one ring. The presence of four exchangeable protons required that the ring of compound I be carbocyclic.

A ¹H-coupled carbon experiment showed that the carboxylic acid carbonyl at δ 181.69 was coupled to two protons on an adjacent carbon (JCH=6.0Hz), indicating the presence of a CH₂COOH unit. The presence of a cyclobutane ring as opposed to a cyclopropane ring was suggested by ¹H and ¹³C NMR chemical shifts and was supported by the magnitude of the one bond proton-carbon coupling constants for two of the ring methylene groups (δ 32.77 (t, JCH=138Hz), and δ 23.67 (t, JCH=139Hz)). Thus, in addition to the CH₂COOH partial structure, compound I contained a cyclobutane ring substituted with hydroxyl and primary amine functionalities.

A comparison of spectroscopic data with that for synthetically derived stereoisomers of CBAA suggested the assignment of the Streptomyces rochei A13018 metabolite as I, in which the relative stereochemistry with respect to the OH and NH₂ groups is cis. This assignment is consistent with the relative stereochemistry of the known dipeptide 2. The absolute stereochemistry of I, as indicated by the optical rotation (+3.8°), is identical with synthetic (1S,2S)-1-hydroxy-2-aminocyclobutane-1-acetic acid.

Confirmation of this structural assignment was provided by a comparison of ¹H and ¹³C NMR data with that for racemic cis-CBAA (1) prepared as per Baldwin et al. ¹H NMR (300MHz, D₂O) δ 1.92 - 2.09 (3H, m), 2.11 - 2.20 (1H, m), 2.42 and 2.49 (2H, JAB=16Hz), 3.63 (1H, m); ¹³C NMR (75MHz, D₂O) (referenced to the δ 23.67 carbon of the natural product) δ 23.67, 32.75, 48.61, 54.10, 76.50, 181.71.

Utilizing an agar-based titration assay, treatment of Arabidopsis thaliana with optically active CBAA resulted in moderate chlorosis at 10 µg/ml. Severe bleaching and a slight reduction in growth were observed at 50 µg/ml. Racemic CBAA applied post-emergence at 11.2 kg/ha caused significant chlorosis on morning glory and Indian mustard. In agreement with previous reports of its antibacterial activity, compound I inhibited the growth of Bacillus subtilis on a minimal salts medium. This activity was partially reversible by l-cysteine or L-methionine, and more completely reversible by a combined application. Similarly, the addition of
L-cysteine or L-methionine to CBAA-treated Arabidopsis thaliana seeds showed partial or complete reversal, respectively, of the observed chlorosis.

In an attempt to improve upon the herbicidal activity of racemic CBAA, twenty-five analogs were synthesized. These compounds included substitutions at the carboxylic acid (O-tert-butyl, OCH₃, O(CH₂)₃CH₃ esters and NHCH(CH₃)₂, NEt₂, NBn₂ amides), exocyclic methylene (C(CH₃)₂), hydroxyl (OCH₃, OCH₂CH=CH₂), and amino (NBn₂) functionalities. One analog, containing NBn₂ and CONBn₂ substitutions, showed enhanced activity, but unlike 1, this compound showed plant stunting and necrosis.

As part of an ongoing program to discover natural product herbicides produced by microorganisms, cis-2-amino-1-hydroxycyclobutane-1-acetic acid (1) was identified as the chlorosis-inducing metabolite produced by Streptomyces rochei A13018. When tested against Arabidopsis thaliana, CBAA apparently interfered with the biosynthesis or utilization of sulfur-containing amino acids as suggested by the reversal of chlorosis upon addition of L-cysteine or L-methionine. Although CBAA exhibited relatively weak post-emergence phytotoxicity, the isolation of this compound provides further demonstration of the utility of microorganisms as a resource for new areas of synthetic herbicide chemistry.

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References