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

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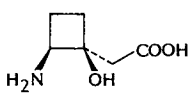
Microorganisms represent a well-documented source of herbicidal compounds with the potential to provide agricultural researchers with new structural classes for herbicide synthesis efforts[1]. In the continuation of a screening program to discover novel natural product herbicides from microbial sources[2], *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 occuring antibacterial dipeptide (1*S*,2*S*)-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 GOTTLIEB[5], 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 μm) 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 μg/ml); tolerance to sodium azide (0.01% w/v); utilization of D-glucose, D-raffinose, α-lactose, L-arabinose, D-galactose, L-valine, salicin, α-(+)-melibiose, D-fructose, D-mannitol, (+)-mannose, L-sorbose, D-ribose, (+)-rhamnose, (+)-maltose, sucrose, (+)-xylose, *myo*-inositol, and (+)-cellobiose; antibiosis to *Streptomyces murinus* ATCC 19788 and *Bacillus subtilis* ATCC 6633. Negative responses were: H_2S production; nitrate reduction; degradation of arbutin; tolerance to NaCl (7% w/v) and phenol (0.1% w/v); resistance to neomycin (50 μg/ml); antibiosis to *Aspergillus niger* ATCC 36233; utilization of L-phenylalanine, L-tyrosine, hydroxy-L-proline, L-threonine, L-serine, L-histidine, L-cystine, DL-α-amino-*n*-butyric acid, L-glutamic acid, adonitol, xylitol, dulcitol, and inulin. The strain utilized DL-α-amino-*n*-butyric acid and hydroxy-L-proline as nitrogen sources. The whole cell hydrolysate contained LL-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 Bacteriology[6].

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



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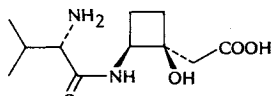

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was added 0.1% antifoam (Dow Corning Type A). The fermentation was carried out on a rotary shaker at 30°C and 120 rpm, with 1 liter/minute aeration, for 4 days. The herbicidal activity of the resulting broth was assayed by an agar diffusion method using *Arabidopsis thaliana* as the test organism.

The culture broth (1-liter) was filter sterilized and the sterile filtrate (350 ml) was passed through a column of Bio Rad AG50W-X8 (H^+ form, 50 ml) which was washed with water (500 ml) and eluted with 3% NH_4OH (500 ml). The 3% NH_4OH eluate was evaporated under reduced pressure and the resulting crude active material (0.34 g) was fractionated by reversed phase C18 flash chromatography eluting with water. The active fractions were combined and concentrated *in vacuo* to give a yellow solid (0.076 g), which was further purified on Sephadex LH-20 eluting with water-methanol (3:1). Active fractions (0.070 g) were combined, concentrated, and chromatographed on QAE Sephadex A-25 (acetate form). Elution with a linear NH_4OAc gradient (0.05 M, pH 9 to 0.3 M, pH 7) afforded, after removal of NH_4OAc *via* passage through a column of Bio Rad AG50W-X8 (H^+ form, 10 ml), elution with 3% NH_4OH, and evaporation under reduced pressure, a total of 12 mg of CBAA (**1**).

The physico-chemical properties of CBAA were as follows: $[\alpha]_D^{22}$ +3.8° (*c* 0.025, H_2O); CD (*c* 0.067 mg/ml, H_2O): $[\theta]$ (193) −1,094, (217) +42; IR ν_{max} (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 **1** showed an M+H ion peak at *m/z* 146.0818, indicating the molecular formula of the protonated species to be $C_6H_{12}NO_3$ (Calcd 146.0817); ^1H NMR (300 MHz, D_2O) δ 1.90~2.10 (3H, m), 2.10~2.21 (1H, m), 2.42 and 2.49 (2H, J_{AB}=16 Hz), 3.63 (1H, m); ^{13}C NMR (75 MHz, D_2O) δ 23.67 (t, $^1J_{CH}$=139 Hz), 32.77 (t, $^1J_{CH}$=138 Hz), 48.60 (t, $^1J_{CH}$=127 Hz), 54.13 (d, $^1J_{CH}$=152 Hz), 76.50 (s), 181.69 (s); TLC SiO_2 (Merck) Rf 0.46 (BuOH-AcOH-H_2O, 2:1:1), Rf 0.45 (CH_3CN-AcOH-H_2O, 4:1:1), ninhydrin positive.

^1H and ^{13}C NMR studies of **1** revealed one sp^3 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, $C_6H_{11}NO_3$, required one site of unsaturation in addition to the carboxylic acid functionality. In the absence of further sp^2 hybridized carbon atoms, as indicated by ^{13}C NMR, the molecule had to contain one ring. The presence of four exchangeable protons required that the ring of compound **1** 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 ($^2J_{CH}$=6.0 Hz), indicating the presence of a CH_2COOH unit. The presence of a cyclobutane ring as opposed to a cyclopropane ring was suggested by ^1H and ^{13}C 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, $^1J_{CH}$=138 Hz), and δ 23.67 (t, $^1J_{CH}$=139 Hz)). Thus, in addition to the CH_2COOH partial structure, compound **1** contained a cyclobutane ring substituted with hydroxyl and primary amine functionalities.

A comparison of spectroscopic data with that for synthetically derived stereoisomers of CBAA[4] suggested the assignment of the *Streptomyces rochei* A13018 metabolite as **1**, in which the relative stereochemistry with respect to the OH and NH_2 groups is *cis*. This assignment is consistent with the relative stereochemistry of the known dipeptide **2**. The absolute stereochemistry of **1**, as indicated by the optical rotation (+3.8°), is identical with synthetic (1*S*,2*S*)-1-hydroxy-2-aminocyclobutane-1-acetic acid.

Confirmation of this structural assignment was provided by a comparison of ^1H and ^{13}C NMR data with that for racemic *cis*-CBAA (**1**) prepared as per BALDWIN *et al.*[4]: (^1H NMR (300 MHz, D_2O) δ 1.92~2.09 (3H, m), 2.11~2.20 (1H, m), 2.42 and 2.49 (2H, J_{AB}=16 Hz), 3.63 (1H, m); ^{13}C NMR (75 MHz, D_2O) (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[3,4], compound **1** 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, OCH_3, $O(CH_2)_3CH_3$ esters and $NHCH(CH_3)_2$, NEt_2, NBn_2 amides), exocyclic methylene ($C(CH_3)_2$), hydroxyl (OCH_3, $OCH_2CH=CH_2$), and amino (NBn_2) functionalities. One analog, containing NBn_2 and $CONBn_2$ 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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