Short Communication

Isolation of *o*-Acetylbenzeneamidinocarboxylic Acid, a New Metabolite of *Gibberella saubinetii*†

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In the course of our screening program for selective inhibitors against SV40-transformed cells, we have found that a strain of *Gibberella saubinetii* IAM8049 produced a selective cytotoxic substance against SV40-transformed cells (*in vitro*) and which showed a slight antitumor activity against Ehrlich carcinoma (*in vivo*).

This paper reports the isolation of a new selective cytotoxic substance, *o*-acetylbenzeneamidonocarboxylic acid (**I**). The compound **I** was isolated from jar fermentation carried out according to the following procedures: 100 ml of the medium in a 500 ml flask, consisting of 5.0% glucose, 1.0% malt extract, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.1% NH_4Cl, and 0.04% $MgSO_4 \cdot 7H_2O$, was inoculated with *Gibberella saubinetii* IAM8049 and shake-cultured at 28°C for 72 hr. This culture was then transferred into a 30-liter jar fermentor, containing 15 liters of the same medium described above, and cultured at 28°C for 96 hr under an aeration rate of 15 liters per minute and agitation at 250 rpm. The filtrates of cultured broth and mycelium collected by centrifugation were separately extracted with ethyl acetate at room temperature. The combined ethyl acetate extract was dehydrated with anhydrous sodium sulfate and concentrated *in vacuo* to remove the solvent.

TABLE I. ^{13}C-NMR AND ^{1}H-NMR SPECTRA OF *o*-ACETYLBENZENEAMIDINOCARBOXYLIC ACID

Determined on a JEOL FX90-Q NMR spectrometer in DMSO-d_6, relative to internal TMS.

Carbon No.	^{13}C-NMR (22.5 MHz)				^{1}H-NMR (89.55 MHz)	
	Chemical shift (ppm)	OFR	J ^{13}C–H (Hz)	Functionality	Chemical shift (ppm)	J(Hz)
9	196.3	s*	q* 6.7	–C(=O)–		
8	170.2	s	s	–COOH	12.65 (brs)	
7	158.6	s	s	–C(=NH)–	7.76 (brs)	
1	138.0	s	t {7.9, 9.8}	=C(–)–NH	8.30 (brs)	
5	132.2	d	dd 150.9, 7.3	=CH–	7.56 (ddd)	7.6, 7.6, 1.3
3	128.7	d	dd 151.5, 8.0	=CH–	7.84 (dd)	7.9, 1.6
4	123.4	d	dd 164.8, 8.0	=CH–	7.19 (ddd)	7.6, 7.6, 1.5
2	120.7	s	m	=C(–)–		
6	119.8	d	m 164.3	=CH–	8.58 (dd)	7.8, 1.2
10	24.1	q	q 128.7	CH_3–	2.43 (s)	

*, multiplicity; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

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Purification was performed by repeated chromatography on silicic acid (Mallinckrodt) and silica gel (Merk) columns. **I** was eluted with a mixture of benzene and ethyl acetate (6:4). This compound was a more potent growth inhibitor against SV40-transformed cells than against non-transformed cells.[1)]

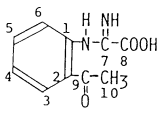

FIG. 1. The structure of **I**.

I forms colorless needle crystals of mp 175~175.5°C. It has a low solubility in hexane, benzene, chloroform and ether, but is readily soluble in pyridine, DMSO and dioxane. The physicochemical properties of **I** are as follows: $C_{10}H_{10}N_2O_3$, CI-MS *m/z*: 207 ($M^+ + H$), p*K*a′ in 50% dioxane: 10.65 and <2, UV λ_{max}^{EtOH} nm: 207 (ε 7800), 242 (sh, 4000), 295 (sh, 2700), 306 (2850), IR ν_{max}^{KBr} cm^{-1}: 3390, 3200, 1720, 1680, 1580, 1510, 1380, 1350, 1240, 745. These spectral data indicate the presence of amine groups and a carbonyl function conjugated with an aromatic ring in the molecule.

The ^{1}H-NMR spectrum of **I** in DMSO-d_6 exhibited signals due to four aromatic protons at 7.19, 7.56, 7.84 and 8.58 ppm, one methyl

TABLE II. CYTOTOXICITY OF *o*-ACETYLBENZENEAMIDINOCARBOXYLIC ACID ON THE N/T ASSAY *in vitro*

Cytotoxicity is expressed as follows: −, no toxic effect; ±, slight toxic effect; +, cells expanded, a small number of detached cells; ++, mixture of expanded and rounded cells, +++, a large number of rounded and detached cells.

o-Acetylbenzeneamidinocarboxylic acid was dissolved in a small amount of DMSO and diluted with Eagle's MEM medium (containing 10% calf serum) for the experiment (concn. of DMSO: <1%).

Concn. (mcg/ml)	C3H-2K Cells			SV40-C3H-2K Cells		
	Days of treatment					
	1	2	3	1	2	3
2000	++	++	+++	+++	+++	+++
1000	+	++	++	+++	+++	+++
500	−	±	±	+++	+++	+++
250	−	−	−	++	+++	+++
125	−	−	−	++	+++	+++
62.5	−	−	−	++	+++	+++
31.3	−	−	−	+	++	++
15.6	−	−	−	−	−	−

TABLE III. ANTITUMOR EFFECT OF *o*-ACETYLBENZENEAMIDINOCARBOXYLIC ACID ON EHRLICH CARCINOMA (SOLID FORM) IN ddy MICE

Compound	*o*-Acetylbenzeneamidino-carboxylic acid	Micophenolic acid	PBS
Daily dose* (mg/kg)	30	120	
Av. tumor wt. (mg)	130	99	206
% Inhibition	32	52	0
Av. body wt. gain **(g)	−0.5	−1.0	−1.6

* Injection intraperitoneally 1, 3, 5 days after tumor inoculation.

** Difference in body weight before and 11 days after inoculation. *o*-Acetylbenzeneamidinocarboxylic acid was dissolved in a small amount of DMSO and diluted with PBS (−) solution for the experiment (concn. of DMSO: <1%).

proton at 2.43 ppm (s) (see Table I), two amino protons at 7.76 (broad s) and 8.3 ppm (broad s) and one carboxylic acid proton at 12.65 ppm (broad s). The last three protons disappeared with the addition of D_2O. Spin decoupling experiments proved the presence of an ortho-substituted benzene ring. The ^{13}C-NMR spectrum of **I** (see Table I) showed signals due to aromatic (138.0, 132.2, 128.7, 123.4, 120.7 and 119.8 ppm) acetyl (24.1, 196.3 ppm), carboxylic acid (170.2 ppm) and sp^2 carbon (158.6 ppm) linked to the amino-nitrogen atom.

Alkaline hydrolysis of **I** (saturated $Ba(OH)_2$, 100°C, 10 hr) gave a basic compound which was isolated by extraction with ethyl acetate followed by HPLC (Waters μPorasil, chloroform–methanol = 95 : 5).

This compound, C_8H_9ON, M^+ (m/z) 135, was identified as *o*-amino acetophenone by comparison with an authentic specimen. Thus, the remaining part of **I** must have been an amidinocarboxylic acid to give the structure of **I** as shown in Fig. 1. The two pKa' values of **I** (<2, 10.65) are in agreement with this conclusion.[2] Amidinocarboxylic acid is very unique among the microbial metabolites and only kasugamycin[2] has been previously reported with this partial structure.

In the bioassay, using SV40-C3H-2K and C3H-2K cells,[1] *o*-acetylbenzeneamidinocarboxylic acid had a selective cytotoxicity from the first day (Table II). Antitumor activity *in vivo* was assayed using the solid form of Ehrlich ascites.[3] Ehrlich ascites tumor cells (2×10^6 cells) were inoculated subcutaneously to ddy mice and treated with **I** by the intraperitoneal route once daily at the first, third and fifth day after inoculation. *o*-Acetylbenzeneamidinocarboxylic acid inhibited the solid tumor growth when the mice were sacrificed at the eleventh day and the tumor was compared with the control group. A 30% inhibition of tumor growth was observed at dose levels of 30 mg/kg three times (Table III).

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