A NEW ANTIBIOTIC, CALVATIC ACID

Sir:

Calvatic acid is a new antibiotic isolated from fermentation of a mushroom, *Calvatia craniformis* (SHW.) FR. After we had completed this study, we read the recent publication by GASCO *et al.*[1] on the same compound which is produced by *Calvatia lilacina* (BERK.) HENN. P. In this report, the production, isolation, physico-chemical properties, structural determination, total synthesis and biological activities of calvatic acid (**I**) and related synthetic compounds are presented.

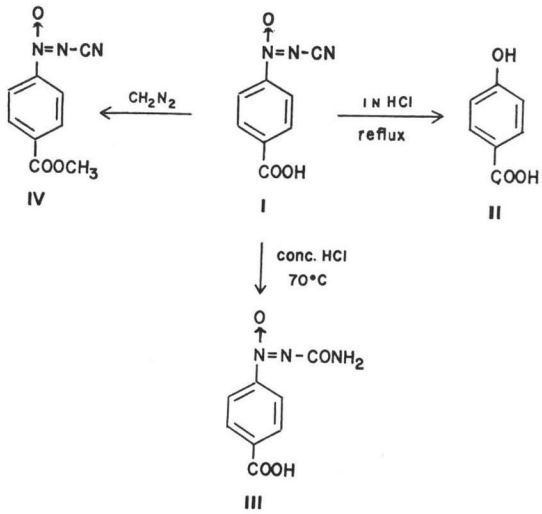
1. Production and Isolation

Inoculum was prepared by adding about 0.25 cm^2 of the mycelial growth of *Calvatia craniformis* (SHW.) FR. from a slant culture to a medium consisting of 2.0% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% KH_2PO_4 and 0.3%, $MgSO_4 \cdot 7H_2O$. It was grown in stationary culture at 28°C for 30 days and then in shake-culture on a reciprocal shaker at 28°C for 10 days. Ten ml of the inoculum was added to a 500-ml flask containing 125 ml of the above medium and was shake-cultured at 28°C for 10 days.

The culture filtrate (15.9 liters) was adjusted to pH 2.0 and extracted with *n*-butanol. The butanol extract was concentrated under reduced pressure, yielding a brownish oily substance. The material thus obtained was dissolved in a minimum amount of methanol and triturated with ethyl acetate. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure, yielding a brownish oil. It was further purified by column chromatography on silica gel with benzene-butanol (10:1 in volume) and chloroform. Purified **I** thus obtained was dissolved in acetone and *n*-hexane was added until the solution became slightly turbid. On keeping the solution in a refrigerator, colorless needle crystals of **I** formed. After vacuum desiccation at room temperature, 30 mg of faint yellow powder of **I** was obtained. m.p. 182~183°C (decomp.).

2. Physical and Chemical Properties and Structural Determination

Compound **I** is a monobasic acid with pKá 3.2. The molecular formula, $C_8H_5N_3O_3$ was established by elemental analysis (Found: C, 50.93; H, 2.79; N, 21.39; O, 24.89, Calcd. for $C_8H_5N_3O_3$ (M.W. 191): C, 50.26; H, 2.64; N, 21.99; O, 25.11.) and mass spectrometry (*m/e* 191). Compound **I** is soluble in methanol, ethanol, acetone, dimethylsulfoxide, slightly soluble in ethyl acetate, butyl acetate, chloroform, benzene and ethyl ether, and insoluble in hexane. It shows positive color reactions with picrylchloride-ammonia, nitroprusside-potassium ferricyanide and potassium permanganate, but negative with SAKAGUCHI, EHRLICH, LIEBERMANN, RYDON-SMITH and ninhydrin. The UV spectrum has an absorption maximum at 306 nm (ε 13,000) in methanol, at 304 nm (ε 15,600) in 50% aqueous methanol containing 0.01 N HCl, and at 276 nm (ε 12,000) in 50% methanol containing 0.01 N NaOH. The IR spectrum (KBr) shows absorption bands at 3450, 3100~2500, 2160 (CN), 1690 (COOH), 1600, 1475, 1425, 1325, 1315, 1295, 1110, 1015, 940, 875, 810, 780 and 700 cm^{-1}. The NMR spectrum in deuterodioxane shows a typical peak pattern for a *para*-substituted benzene centered at δ 8.30.



Degradation of **I** in 1 N HCl containing 10% methanol under reflux for 10 hours gave a faint yellow powder melting at 210~211°C, which was shown to be *p*-hydroxy-

benzoic acid (**II**) by IR spectrum and melting point. Treatment of **I** with conc. HCl at 70°C for 1 hour afforded the hydrated product (**III**), m.p. 230~235°C (decomp.), which was identical with the synthetic product (IR, UV) described in the following section. Treatment of **I** with diazomethane gave a crystalline methyl ester (**IV**), m.p. 108~109°C, (ν $c=o$ 1725 cm^{-1}; 3 H at δ 3.99 in $CDCl_3$). High resolution mass spectrum of **IV** showed *m/e* 205.0484 ($C_9H_7N_3O_3$ M^+), 189.0528 ($C_9H_7N_3O_2$, M^+-O), 165.0420 ($C_8H_7NO_3$, M^+-CN_2) and 135.0453 ($C_8H_7O_2$, M^+-CN_3O). These fragmentation processes together with all of the above-mentioned results suggested the structure **I** (*p*-carboxyphenylazoxycyanide) for calvatic acid.

3. Total Synthesis

Total synthesis of **I** was completed in 4 steps starting from *p*-hydrazinobenzoic acid hydrochloride (**V**).

Treatment of **V** with urea[2] in water at 120°C for 12 hours under reflux afforded **VI** (m.p. 246~247°C, decomp.) in 63 % yield. Oxidation of **VI** with potassium permanganate[3] in 50 % acetic acid gave **VII** (m.p. 214~216°C, decomp.) in 70 % yield. Compound **VII** was treated with formic acid and hydrogen peroxide[4] to give **III** (m.p. 230~235°C, decomp.) in 79 % yield. Compound **III** was treated with thionyl chloride[5] in dimethylformamide, affording calvatic acid as faint brownish powder (m.p. 184~185°C, decomp.) in 88 % yield. The identity of the synthetic calvatic acid with the natural material was confirmed in all respects so far tested including biological activity.

Phenylazoxycyanide and its other substituted derivatives were synthesized by similar method. They also showed antibacterial and antifungal activities, which will be reported in another paper in detail.

4. Biological Activities

Antimicrobial spectra of calvatic acid (**I**) and its methyl ester (**IV**) together with those of phenylazoxycyanide and its *p*-methyl and *p*-chloro derivatives determined by agar dilution method are shown in Table 1. As shown in the table, calvatic acid inhibited growth of Gram-positive bacteria (3.12~6.25 mcg/ml) and some Gram-negative bacteria. It showed no activity against most yeasts and fungi at 100 mcg/ml. Phenylazoxycyanide and its synthetic derivatives showed a broader antimicrobial spectra.

Calvatic acid showed antitumor activity. It inhibited the growth of YOSHIDA sarcoma in cell culture by 50 % at 1.56 mcg/ml. It also showed growth inhibition of mouse Leukemia 1210. The intraperitoneal administration of 400, 200 and 100 mcg/mouse/day $\times 10$ of calvatic acid to mice which have been intraperitoneally inoculated with 1×10^5 cells of mouse Leukemia 1210 prolonged the survival time to 153 %, 147 % and 138 % of the control, respectively.

The LD_{50} (i.p.) of calvatic acid and phenylazoxycyanide in mouse was in the range of 125~250 mg/kg, while that of *p*-tolylazoxycyanide was more than 250 mg/kg and that of *p*-Cl-phenylazoxycyanide was in the range of 62.5~125 mg/kg.

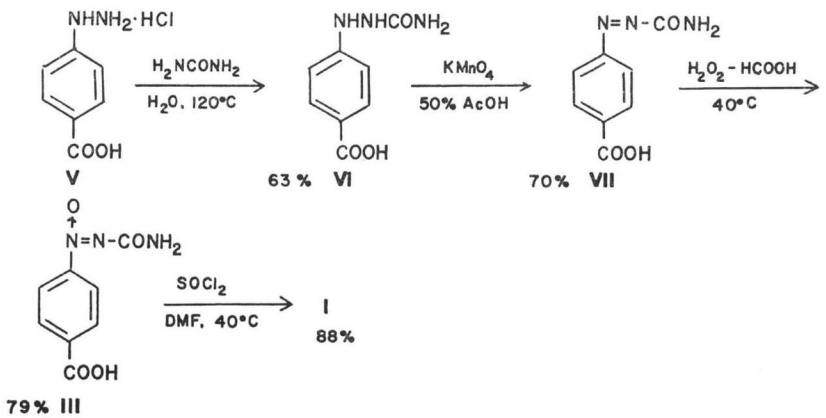


Table 1. Antibacterial and antifungal spectra of phenylazoxycyanide and its *para*-substituted derivatives.

Test microorganisms	MIC (mcg/ml)				
	p-Substituted phenylazoxycyanide				
	−H	−COOH(I)	−$COOCH_3$(IV)	−CH_3	−Cl
Staphylococcus aureus FDA 209P	6.25	6.25	−	12.5	3.12
〃 〃 Smith	3.12	6.25	6.25	12.5	3.12
〃 〃 Terajima	−	1.56	−	−	−
Sarcina lutea PCI 1001	12.5	3.12	6.25	25	6.25
Micrococcus flavus FDA 16	12.5	3.12	3.12	25	6.25
Corynebacterium bovis 1810	12.5	3.12	3.12	25	6.25
Bacillus anthracis	3.12	6.25	<0.78	3.12	1.56
〃 *subtilis* NRRL B-558	12.5	12.5	−	12.5	3.12
〃 *cereus* ATCC 10702	3.12	3.12	−	12.5	3.12
Escherichia coli NIHJ	6.25	6.25	6.25	12.5	6.25
〃 〃 K-12	6.25	25	6.25	12.5	6.25
Salmonella typhosa T-63	6.25	12.5	6.25	12.5	3.12
Shigella sonnei 191-66	−	6.25	−	−	−
Proteus vulgaris OX 11	3.12	6.25	3.12	12.5	6.25
〃 *rettgeri* GN 311	6.25	100	−	50	6.25
Pseudomonas aeruginosa A3	100	>100	50	>100	100
〃 *fluorescens*	>100	>100	−	100	50
Klebsiella pneumoniae PCI 602	6.25	12.5	6.25	12.5	6.25
Mycobacterium smegmatis ATCC 607	25	>100	3.12	25	12.5
〃 *phlei*	−	>100	−	−	−
Aeromonas salmonicida ATCC 14174	12.5	<0.78	−	3.12	3.12
Vibrio anguillarum NCBM 6	6.25	<0.78	−	1.56	1.56
Candida albicans 3147	50	>100	−	6.25	3.12
〃 Yu-1200	50	>100	−	6.25	3.12
〃 *pseudotropicalis* NI 7494	3.12	>100	−	<0.78	3.12
〃 *krusei* NI 7492	3.12	100	−	1.56	1.56
Cryptococcus neoformans NI 7496	3.12	>100	−	1.56	1.56
Saccharomyces cerevisiae	3.12	>100	−	1.56	<0.78
Pyricularia oryzae	25	>100	−	6.25	3.12
Helminthosporium oryzae	25	>100	−	25	12.5
Xanthomonas oryzae	12.5	3.12	−	3.12	1.56
Pellicularia filamentosa	6.25	100	−	6.25	25
Trichophyton sateroides	25	>100	−	25	12.5

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(Received October 29, 1974)

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