A NEW MICROBIAL PRODUCT, OUDENONE, INHIBITING TYROSINE HYDROXYLASE

Sir:

Considering the ability of microorganisms to produce various types of compounds, we undertook a systematic study of specific enzyme inhibitors of microbial origin. We thought that microorganisms which produced proteinases in their culture liquids might produce the inhibitors. We thought also that inhibitors of enzymes involved in norepinephrine biosynthesis would be useful for analysis of the role of this amine in hypertensive disease. Thus, first we studied inhibitors of microbial origin against these enzymes. As reported in previous papers, leupeptins[1,2,3,4] inhibiting trypsin and papain, pepstatin[5,6] inhibiting pepsin, and chymostatin[7] inhibiting chymotrypsins and weakly papain were isolated, and production of these inhibitors was found distributed widely among *Actinomycetes*. We isolated aquayamycin[8,9,10] from *Streptomyces misawaensis* and chrothiomycin[11] from *Streptomyces pluricoloresces*, both of which inhibited tyrosine hydroxylase and dopamine β-hydroxylase. A compound which inhibited dopamine β-hydroxylase was isolated from *Fusarium* sp. and found to be fusaric acid[12,13,14]. In this paper, we report isolation of a new compound named oudenone inhibiting tyrosine hydroxylase and exhibiting a hypotensive effect.

The oudenone-producing strain was isolated under a needle-leaved tree at Kirizumiyama, Gumma Prefecture, and designated strain 10 F in the authors' laboratory. The morphological properties of the fruit body which was formed on a saw-dust medium (10 % of saw-dust was mixed with 60 ml of a medium containing 2.0 % glucose and 0.5 % dry yeast, and sterilized at 120°C for 20 minutes) in a flask of 500 ml volume were similar to those of *Oudemansiella* (*Mucidula*) and among the known species of this genus this strain was most closely related to *Oudemansiella radicata*.

This strain can be transferred on an ager medium consisting of 2.0 % glucose, 0.5 % dry yeast and 1.5 % agar sterilized at 120°C for 20 minutes (pH after the sterilization was 5.6). The mycelial growth on this agar medium was inoculated to the saw-dust medium described above and cultured for 10~20 days at 27°C. Then a sterilized solution consisting of 2.0 % glucose and 0.5 % dry yeast (250 ml/flask containing the mycelial growth on the saw-dust (10 g) medium described above) was added to obtain suspension of the mycelium. This mycelium suspension was used as the inoculum. Oudenone was produced in the shaking culture and fermentation tanks in media containing various kinds of carbon sources and nitrogen sources. Media containing 2.0 % glucose and 1.0 % corn steep liquor (pH 5.6), or containing 2.0 % glucose, 1.0 % soybean meal and 0.5 % dry yeast (pH 5.6) are examples of media suitable for the production. During 5~8 days of the shaking culture at 27°C, pH turned to 4.4~4.8, thereafter in creased to 5.0~5.6, and the production reached a maximum on 10~15 days of the shaking culture. In fermentation tanks containing 150 liters of a medium consisting of 2.0 % glucose, 0.5 % peptone, 0.3 % dry yeast, 0.3 % KH_2PO_4 and 0.1 % $MgSO_4 \cdot 7H_2O$ under aeration and stirring at 27°C, the growth reached a maximum at about 80 hours and the production reached a maximum (607 mcg/ml) at about 90 hours, when the pH was 4.8.

Oudenone can be determined by measuring the tyrosine hydroxylase activity by the method described in a previous paper[9]. It can also be determined by spectrophotometric method based on the UV maximum at 246 mμ at pH 7.0 which disappears at acid pH. The method is as follows: a test solution is diluted with phosphate buffer of pH 7.0 or with 0.1 N HCl and the optical density (a) at 246 mμ of the phosphate buffer solution and that (b) of the solution diluted with 0.1 N HCl are read and the concentration of oudenone (mcg/ml) in the test solution is calculated by $(a-b)n \times 10^4/(1{,}000-60)$, where n is the dilution.

In the cultured broth oudenone is found both in the liquid and the mycelium. After strain 10 F was shake-cultured in 125 ml of a medium consisting of 2.0 % glucose, 0.5 %

peptone, 0.3 % dry yeast, 0.3 % KH_2PO_4 and 0.1 % $MgSO_4 \cdot 7H_2O$ the following amounts of oudenone were found in the liquid and mycelium of 10 ml of cultured broth: 5 days, 372 mcg (liq.), 185 mcg (myc.); 7 days, 845 mcg (liq.), 365 mcg (myc.); 8 days 1,300 mcg (liq.), 410 mcg (myc.); 11 days, 2,180 mcg (liq.), 342 mcg (myc.).

Oudenone is soluble in water, methanol, ethanol, *n*-butanol, ethyl acetate, chloroform and benzene and extracted into water-immissible solvents at acid pH. The partition coefficients, solvent/H_2O, were determined as follows: butanol-water, pH 2 8, pH 4 4, pH 5 2.5, pH 6 2.5, pH 7 3, pH 8 3, pH 9 0.6, pH 10 0.8; ethyl acetate-water, pH 2 5, pH 3 4, pH 4 5, pH 5 0.2, pH 6 <0.1; butyl acetate-water, pH 2 3, pH 3 2.5, pH 4 1, pH 5 0.1, pH 6 <0.1; chloroform-water, pH 4 19, pH 5 1, pH 6 0.1; methyl isobutyl ketone, pH 2 1, pH 3 1, pH 4 0.3, pH 5 0.1, pH 6 0.1 (the coefficients of methyl isobutyl ketone were determined by inhibition of tyrosine hydroxylase and the others by the spectrophotometric method).

Oudenone is stable and no decomposition was observed after heating for 30 minutes at 60°C or for 5 minutes at 100°C at pH 2.0, 7.0 or 9.0.

Oudenone can be extracted from the culture filtrate by an anion-exchange resin process, for instance, using IRA 400 resin in Cl^- form followed by elution with dilute hydrochloric acid, or by a precipitation method as the water-insoluble salt, for instance, as the cupric salt. It is also extracted by carbon adsorption process. However, most efficiently and easily it is prepared by extraction with an organic solvent at acid pH and

Fig. 1. Ultraviolet spectra of oudenone (5 mcg/ml) in neutral (phosphate buffer, pH 7.0) and acidic solution (0.1 N HCl)

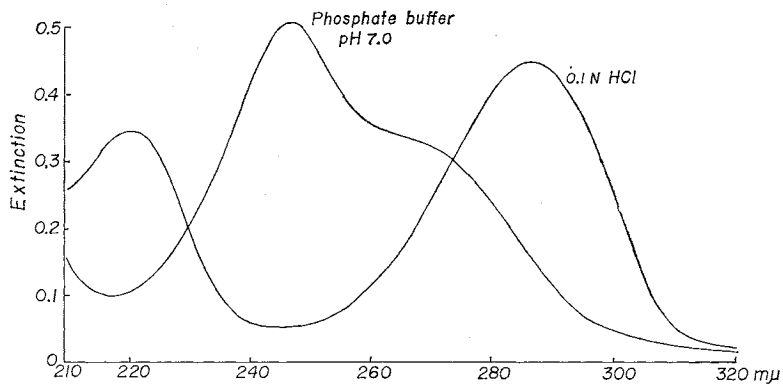
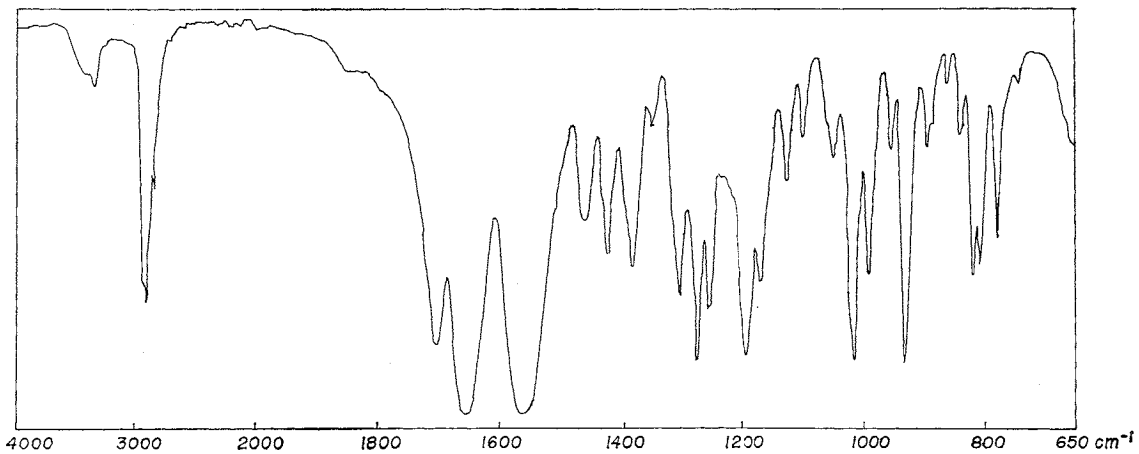


Fig. 2. Infrared absorption spectrum of oudenone (KBr).



reextraction from the solvent with water at neutral pH. The following is an example: 100 liters of the culture filtrate containing oudenone at 500 mcg/ml were extracted with 100 and 60 liters of *n*-butanol successively; the butanol extracts were combined and concentrated to a syrup under reduced pressure; the syrup was dissolved in 4 and 1 liter of ethyl acetate successively and oudenone in the ethyl acetate extracts was transferred into 1.5 liters of water adjusted to pH 7.0 with 10 N NaOH; the aqueous solution was extracted with 3 and 1 liter of ethyl acetate at pH 3 with 10 N HCl; evaporation of the ethyl acetate extracts under reduced pressure yielded oudenone crystals and syrup; recrystallization from warm hexane (3 liters) yielded 29 g of crystalline oudenone.

Oudenone is recrystallized from hexane or mixture of hexane and benzene and obtained as white plate crystals, m.p. 77~79°C. The formula of $C_{12}H_{16}O_3$ was determined by high resolution mass spectroscopy and elemental analysis. It is optically active: $[\alpha]_D^{20}$ −10.6° (*c* 0.5, ethanol). As shown in Fig. 1, oudenone in phosphate buffer of pH 7.0 shows a maximum at 246 mμ ($E_{1cm}^{1\%}$ 1,000) and its hydrochloric acid solution shows maxima at 221 mμ ($E_{1cm}^{1\%}$ 625) and 285 mμ ($E_{1cm}^{1\%}$963). Carbonyl bands are seen in the infrared spectrum which is shown in Fig. 2.

The structure and the synthesis of oudenone will be reported in another paper. Oudenone has no carboxyl group, but it is acidic with a pKa' 4.1 as shown by titration. The sodium salt (m.p. 145~148°C), the potassium salt (m.p. 116~117°C), the calcium salt (m.p. 167~169°C), the magnesium salt (m.p. 252~255°C) and the barium salt (m.p. 135~138°C) were pre-

Fig. 3. LINEWEAVER-BURK plot of L-tyrosine concentration against rate of 3,4-dihydroxyphenylalanine formation with or without oudenone (4.8×10^{-5}~2.4×10^{-4} M).

Reaction mixture contained 200 μmoles of acetate buffer, pH 6.0, various concentrations of L-tyrosin labelled with L-tyrosin-^{14}C, 100 μmoles of mercaptoethanol, 1 μmole of $DMPH_4$, 2.5 μmoles of $FeSO_4$, enzyme preparation, and distilled water of final volume of 1.0 ml.

The Km value obtained from the figure is 1.0×10^{-4} M.

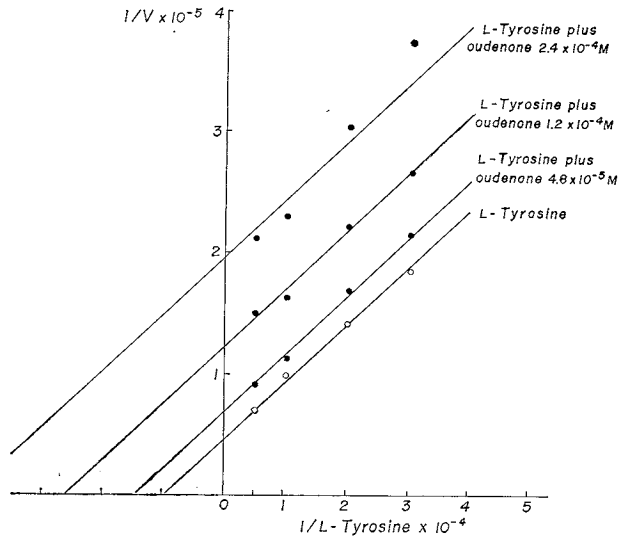
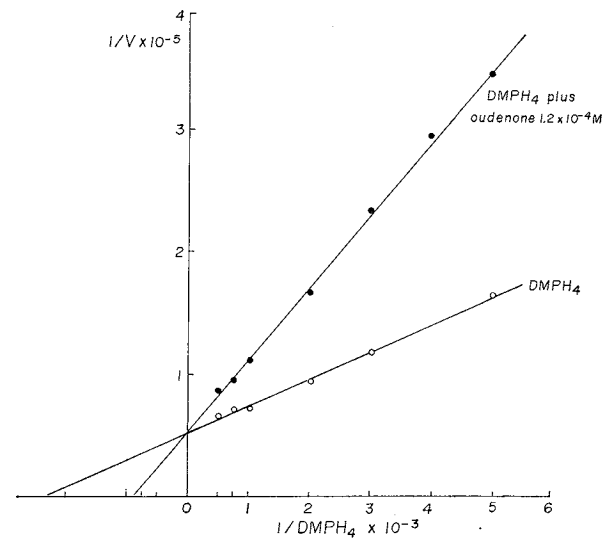


Fig. 4. LINEWEAVER-BURK plot of $DMPH_4$ concentration against rate of 3,4-dihydroxyphenylalanine formation with or without oudenone (1.2×10^{-4} M).

The reaction mixture contained 200 μmoles of acetate buffer, pH 6.0, various concentrations of $DMPH_4$, 0.1 μmole of L-tyrosine containing 1.1×10^5 c.p.m. of L-tyrosine-^{14}C, 100 μmoles of mercaptoethanol, 2.5 μmoles of $FeSO_4$, enzyme preparation, and distilled water to final volume of 1.0 ml.

The Km value obtained from the figure is 4.26×10^{-4} M and the Ki value is 7.58×10^{-5} M.



pared. Oudenone gives positive reactions to 2,4-dinitrophenyl hydrazine, hydroxamic acid-ferric chloride and tetrazolium, but negative to FEHLING and TOLLENS.

No inhibition was observed at 100 mcg/ml of oudenone against *Staphylococcus aureus* (209P), *Escherichia coli* (K-12), *Salmonella typhosa*, *Shigella dysenteriae*, *Klebsiella pneumoniae*, *Bacillus anthracis*, *B. subtilis*, *Mycobacterium* 607 on nutrient agar and no inhibition against *Pyricularia oryzae*, *Candida albicans*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Penicillium chrysogenum* and *Aspergillus niger* on 1.0 % glucose nutrient medium. However, oudenone at not less than 12.5 mcg/ml showed inhibition against *Pyricularia oryzae* growing in rice plant juice medium (pH 5.0).

Fig. 5. Effect of orally administered oudenone on blood pressure of spontaneously hypertensive rats.
Age of rats: 16~30 weeks.
Percent change of blood pressure is the average of two rats.

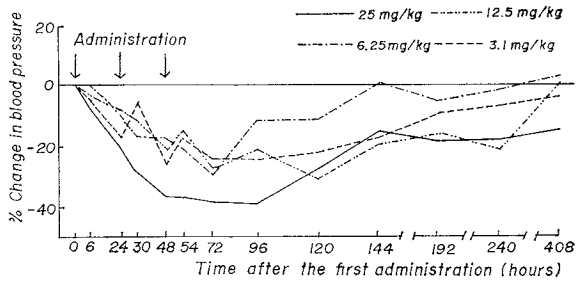


Table 1. Effect of oudenone on blood pressure of spontaneously hypertensive rats

Does (mg/kg)	Rat No.	Body Wt. (g)	Age (Weeks)		Blood pressure and percent depression					
					0 hr.	1 hr.	3 hrs	6 hrs	22 hrs	48 hrs
100	BM-15	360	20	B.P.	170±0	123±4	118±4	140±0	95±5	120±0
				Dep. %		28	31	18	44	29
25	BM-18	360	20	B.P.	190±0	163±8	155±5	140±0	125±5	160±0
				Dep. %		14	18	26	34	16
6.25	BM-6	400	34	B.P.	183±4	125±10	145±5	120±0	115±5	160±0
				Dep. %		32	21	34	37	13
3.13	BM-32	220	12	B.P.	173±4	148±4	140±0	128±4	135	135
				Dep. %		14	19	26	22	22
1.56	BM-24	240	12	B.P.	170±0	170±0	167±4	163±4	173±4	160
				Dep. %		0	1.8	4,1	−1.8	5.9

Route : I. P.
B.P. : Blood pressure, mmHg; each value is the average of three determinations.
Dep. %: Percent depression.

The effect of oudenone on tyrosine hydroxylase was tested as follows: the reaction mixture contained 0.1 μmole of L-tyrosine containing L-tyrosine-^{14}C (1.1×10^5 cpm), 1.0 μmole of 2-amino-4-hydroxy-6, 7-dimethyltetrahydropteridine, 0.1 ml of tyrosine hydroxylase solution (1 mg as protein/ml)[8], 200 μmoles of acetate buffer (pH 6.0), 100 μmoles of 2-mercaptoethanol, 1, 12.5, 25, 50 or 100 mcg of oudenone and distilled water (the final volume was 1.0 ml); after 15 minutes at 30°C, the reaction product (3, 4-dihydroxyphenylalanine) was determined. The following percentage inhibition was observed: 30.6 % by 12.5 mcg/ml, 39.0 % by 25 mcg/ml, 57.6 % by 50 mcg/ml and 74.1 % by 100 mcg/ml. The effect of oudenone was not influenced by addition of ferrous salt to the reaction mixture. In the reaction mixture which was same as that described above but with added Fe^{++} at 2.5×10^{-3} M, the kinetics was studied, and by the LINEWEAVER-BURK plot of the results (Figs. 3 and 4) the uncompetitive relation between oudenone and tyrosine and the competitive relation between 2-amino-4-hydroxy-6, 7-dimethyltetrahydropteridide were observed.

An inhibitor of tyrosine hydroxylase can be expected to have hypotensive activity. It was shown by the test against spontaneously hypertensive rats. The results of the experiments are shown in Table 1 and Fig. 5. Intraperitoneal injection of not less than 3.13 mg/kg showed a strong hypotensive ef-

fect, and oral administration also showed a hypotensive effect. Using ^{3}H-oudenone (3.4×10^{4} dpm/mcg), the absorption of oudenone after the oral administration and excretion in urine were confirmed. Oudenone has low toxicity. When oudenone is injected intravenously or intraperitoneally to mice, LD_{50} was 138 mg/kg or 163 mg/kg respectively, and when its sodium salt was injected intravenously or intraperitoneally LD_{50} was 1,000 mg/kg or 1,850 mg/kg respectively. LD_{50} by oral administration of oudenone or its sodium salt was 1,100 mg/kg or 2,000 mg/kg respectively.

As shown by the properties described above and the structure which will be published in another paper, oudenone is a new compound showing hypotensive effect.

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