OXAMICETIN, A NEW ANTIBIOTIC OF BACTERIAL ORIGIN I. PRODUCTION, ISOLATION AND PROPERTIES

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A new antibiotic, oxamicetin, was isolated from the fermentation broth of *Arthrobacter oxamicetus* sp. nov. It is a basic antibiotic and its hydrochloride salt crystallizes as colorless needles. The physico-chemical and biological properties of oxamicetin suggest that it is closely related to amicetin, a *Streptomyces* antibiotic.

In the course of our search for new antibiotics, a bacterial strain No. B302-B75 which was isolated from a soil sample collected at Kominato, Chiba, Japan, was found to produce a new antibiotic (designated Bu-1848 in our antibiotic file). This antibiotic was extractable into organic solvents from the fermentation broth and showed moderate activity against a variety of microorganisms including Gram-positive, Gram-negative and acid-fast bacteria. As reported in a companion paper,[1] the structure of the antibiotic was found to be closely related to amicetin[3,4] but with an additional hydroxyl group in the molecule, and therefore the name oxamicetin was given to this antibiotic. It is interesting that a bacterial culture can also elaborate an antibiotic of this group which has exclusively been produced by *Streptomyces*. This paper reports the production, isolation, physico-chemical and biological properties of oxamicetin. Taxonomy of the producing organism[2] and the chemistry[1] of oxamicetin are reported in separate papers.

Production of Oxamicetin

Bacterial strain No. B302-B75, which was found to be a new species of Genus *Arthrobacter* and named *Arthrobacter oxamicetus* sp. nov.[2], grows well at 20~30°C on agar slant. An agar slant was used to inoculate liquid vegetative medium containing the following ingredients: 2% glycerol, 1% Pharmamedia, 1% corn steep liquor, 0.3% $(NH_4)_2SO_4$, 0.4% $CaCO_3$ and 0.003% $ZnSO_4 \cdot 7H_2O$. The seed culture was incubated at 28°C for 2 days on a rotary shaker (250 rpm), and for production 2 ml of the growth was added to 100 ml of the same medium in a 500-ml Erlenmeyer flask.

The progress of the fermentation was followed by a paper disc-agar diffusion assay using *Bacillus subtilis* PCI 219 as the test organism. In one of the shake culture experiments, antibiotic production began on the 2nd day (potency: 180 mcg/ml, pH 6.9) and reached a maximum on the 3rd day (250 mcg/ml, pH 7.7). Antibiotic production was also carried out in 10-liter jar fermentors and 100-liter pilot tanks with the same medium as in the shaking fermentation, giving a peak potency of about 200 mcg/ml after 50~60 hours.

Isolation and Purification

The fermentation broth (50 liters) was filtered at pH 3 and the filtrate was extracted at pH 8.2~

8.4 with about one-third volume of *n*-butanol (15 liters). The extract was stirred with 5 liters of acidic water, the pH being adjusted to 2.0 with dilute hydrochloric acid. The active aqueous extract was then made alkaline (pH 8.2~8.4) and extracted again with 3 liters of *n*-butanol. The butanol extract was washed with water, concentrated *in vacuo* and then lyophilized to give about 6 g of yellowish powder (potency: *ca.* 500 mcg/mg). Alumina thin-layer chromatography (TLC) with 80% methanol indicated the presence of slower moving impurity in the crude preparation.

Further purification was effectively accomplished by alumina column chromatography. Crude oxamicetin (3 g) was dissolved in 50 ml of 0.5 N methanolic HCl and the solution was applied to a column of acid-treated alumina, which was then eluted fractionally by methanol. The active fractions were combined and concentrated *in vacuo* to dryness to afford 1.2 g of oxamicetin hydrochloride of 90~95% purity. The solid was dissolved in a small amount of water containing a drop of hydrochloric acid and acetone was added to the solution to the cloud point. Overnight storage of the solution in the cold gave colorless needle-like crystals of oxamicetin hydrochloride. An aqueous solution of the crystalline hydrochloride was adjusted to pH 8.4 and extracted with *n*-butanol. The extract was evaporated to afford white solid of oxamicetin base which was crystallized from aqueous methanol at room temperature.

Physico-chemical Properties of Oxamicetin

Oxamicetin free base is readily soluble in acidic water, methanol, ethanol and *n*-butanol, slightly soluble in neutral and alkaline water, but practically insoluble in acetone, ethyl acetate, ether and other common organic solvents.

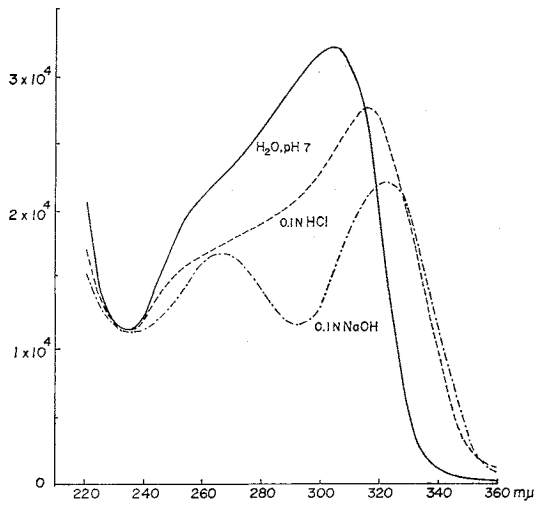
Crystalline oxamicetin base melts at 176~179°C. Molecular weight determined by vapor pressure osmometry was 639. Elemental analysis of the base agreed with a formula of $C_{29}H_{42}N_6O_{10}\cdot H_2O$ (MW 652.69).

Calc'd: C 53.36, H 6.80, N 12.89.
Found: C 53.53, H 6.45, N 13.00.

Crystalline oxamicetin hydrochloride melts at 205~210°C with decomposition. Potentiometric titration of the hydrochloride in 50% aqueous ethanol showed the presence of three titrable group in the molecule; one acidic group with a pKa' of 11.2 and two basic groups with pKa' values of 6.70, the titration equivalent being 748. It analyzed well for $C_{29}H_{42}N_6O_{10}\cdot 2HCl\cdot 2H_2O$ (MW 743.6).

Calc'd: C 46.84, H 6.51, N 11.30.
Found: C 47.22, H 6.58, N 11.29.

Fig. 1. Ultraviolet spectra of oxamicetin



Optical rotation of oxamicetin hydrochloride is: $[\alpha]_D^{25}+66°$ (*c* 0.4, water). The ultraviolet absorption spectrum is shown in Fig. 1. Absorption maxima are observed at 305 mμ (ε: 31,7000) in water, 316 mμ (ε: 26,600) in 0.1 N HCl and 322 mμ (ε: 21,900) in 0.1 N NaOH. The infrared spectrum of oxamicetin

hydrochloride in KBr pellet is shown in Fig. 2, and closely resembles that of amicetin. The NMR spectrum(Fig. 3) indicated the presence of three C-CH_3 groups, one dimethylamino group and six aromatic or vinylic protons. It also showed two signals in the anomeric proton region, centered at δ 5.32 ppm (doublet, J=3.5 Hz) and δ 5.70 ppm (broad doublet, J=10.5 Hz). The chemical shift of the former signal was clearly distinguishable from that of amicetin (5.09 ppm).

Oxamicetin gives positive reaction with ninhydrin, DRAGENDORFF, anthrone and EHRLICH reagents. FEHLING, TOLLENS and ferric chloride reactions were negative. It gives the following

Fig. 2. Infrared spectrum of oxamicetin hydrochloride (KBr)

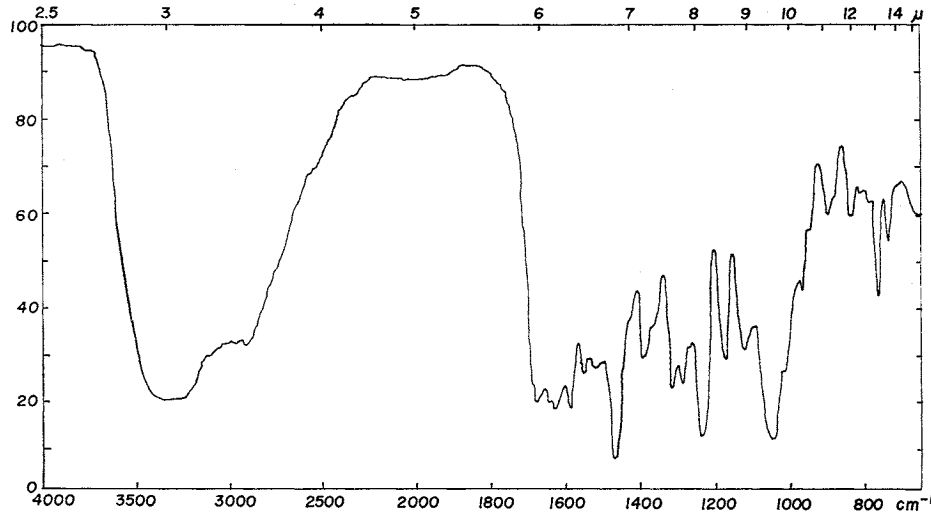


Fig. 3. NNR spectrum of oxamicetin hydrochloride (60 MHz, in D_2O)

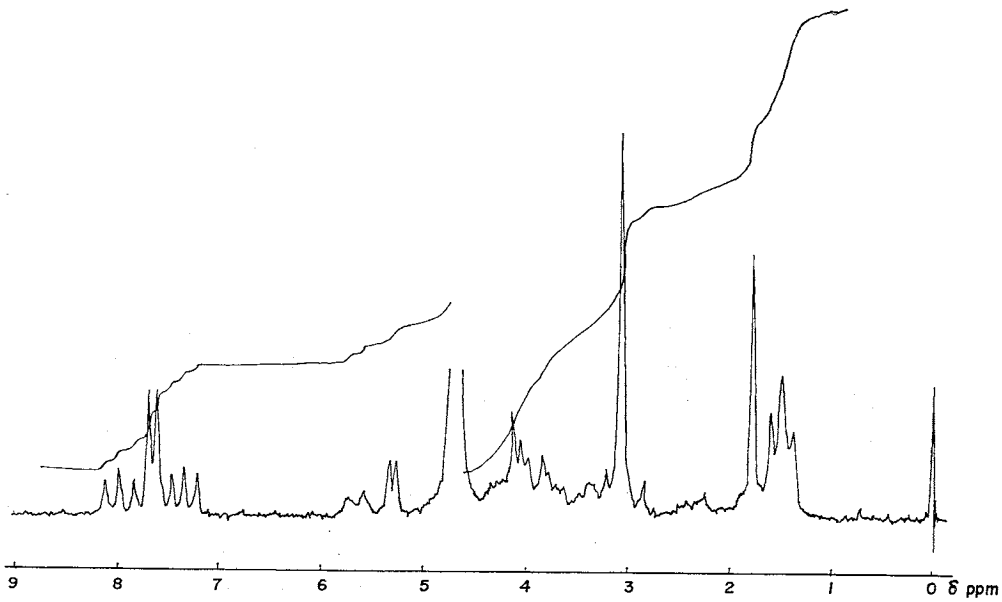


Table 1. Antibacterial spectrum of oxamicetin

Test organisms	Test medium*	MIC (mcg/ml)	
		Oxamicetin	Amicetin
Escherichia coli NIHJ	A	25	50
〃 〃 Juhl	A	100	>100
〃 〃 A 15169	A	6.3	6.3
〃 〃 A 20365 (KM, NM, SM-R)**	A	6.3	12.5
Klebsiella pneumoniae Dll	A	50	100
Proteus vulgaris A 9436	A	50	50
〃 〃 A 9526	A	25	25
〃 *morganii* A 20031	A	50	>100
〃 *mirabilis* A 9554	A	>100	>100
Shigella flexneri A 9684	A	100	100
〃 *sonnei* Yale	A	50	100
Salmonella enteritidis A 9531	A	3.1	3.1
〃 *typhosa* Yale	A	50	100
Pseudomonas aeruginosa D 15	A	>100	>100
Staphylococcus aureus Smith	A	12.5	6.3
〃 〃 #193 (PC, TC-R)	A	12.5	6.3
〃 〃 Russell (PC-R)	A	12.5	6.3
〃 〃 Terajima	A	1.6	0.8
Streptococcus pyogenes S-23	B	6.3	3.1
〃 〃 Dick	B	100	50
〃 〃 Digonnet	B	25	6.3
Diplococcus pneumoniae Type 2	B	25	3.1
Sarcina lutea PCI 1001	A	1.6	1.6
Micrococcus flavus	A	1.6	3.1
Bacillus subtilis PCI-219	A	6.3	12.5
Bacillus anthracis 115	A	25	12.5
Mycobacterium 607	C	12.5	3.1
〃 〃 (KM-R)	C	12.5	3.1
〃 〃 (KM, SM-R)	C	3.1	1.6
〃 *phlei*	C	3.1	0.8
〃 *ranae*	C	12.5	3.1

* A: nutrient agar. B: blood agar.
C: 3% glycerol, 0.3% sodium L-glutamate, 0.2% peptone, 0.31% Na_2HPO_4, 0.1% KH_2PO_4, 0.005% ammonium citrate, 0.001% $MgSO_4$, 1.5% agar.

** KM=kanamycin, NM=neomycin, SM=streptomycin, PC=penicillin G, TC=tetracycline, −R=resistant

Rf values on silica-gel TLC detected by bioautography with *Bacillus subtilis* or anthrone reagent spray: 0.25 (*n*-BuOH-AcOH-H_2O, 3:1:1), 0.72 (EtOH-28% NH_4OH-H_2O, 8 : 1 : 1). Alumina TLC developed with 80% methanol was found suitable to differentiate oxamicetin (Rf: 0.54) from amicetin (Rf: 0.61).

Biological Activity

The minimum inhibitory concentration (MIC) of oxamicetin was determined by the serial agar dilution method. The results are shown in Table 1 along with those obtained with amicetin which

was used as a reference.

Oxamicetin exhibits an antibacterial spectrum similar to that of amicetin. It is somewhat more active than amicetin against Gram-negative bacteria, but less active than the latter against Gram-positive and acid-fast bacteria.

The *in vivo* activity of oxamicetin was assessed in experimental infections in mice with two pathogenic bacteria, *Staphylococcus aureus* Smith and *Escherichia coli* NIHJ. Mice were inoculated intraperitoneally with a $100 \times LD_{50}$ dose of the pathogen, and oxamicetin was administered subcutaneously just after the bacterial challenge. Oxamicetin protected mice from the infection, giving subcutaneous CD_{50} of 100 mg/kg against *S. aureus* Smith and 120 mg/kg against *E. coli* NIHJ. No *in vivo* activity was seen when the antibiotic was given orally.

The acute toxicity of oxamicetin was determined in mice, the intravenous LD_{50} being 200 mg/kg. It was nontoxic at 400 mg/kg when given subcutaneously.

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