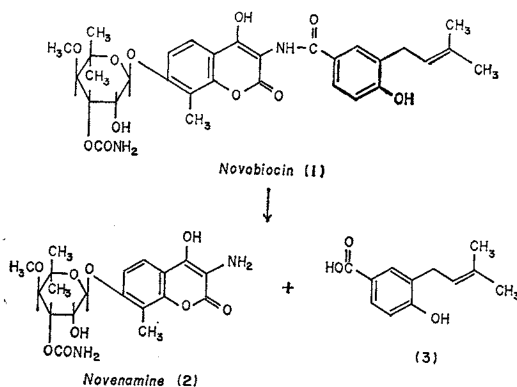
ENZYMIC FORMATION OF NOVENAMINE, A NOVIOSYL-AMINOCOUMARIN MOIETY OF NOVOBIOCIN

Sir:

The antibiotic novobiocin (**1**) contains a noviosyl-aminocoumarin moiety (**2**) which is a useful intermediate for preparing diverse semisynthetic analogues of the antibiotic. To date, however, no adequate method has been described which would yield **2**. Under conventional hydrolytic conditions the amide bond linking this moiety to the 3-[3-methylbuten-2-yl]-4-hydroxybenzoic acid fragment (**3**) is the most stable of the four solvolyzable bonds of novobiocin. The selective cleavage of the amide bond was achieved only when novobiocin was reacted with acid anhydrides but the resulting oxazole proved to be a very stable compound. It could not be converted into the free amine without concurrent hydrolysis of the glycosidic bond between the sugar moiety [3-(O)-carbamoylnoviose] and the aminocoumarin[1].

Since microbial emzymes have been known to transform a number of antibiotics in different ways[2], we have attempted to hydrolyze the amide bond of novobiocin by microbial means. Many well-defined and identified microorganisms failed to carry out this reaction but eventually we have isolated a Gram-negative non-motile bacterium* which hydrolyzed novobiocin in the desired fashion. The two reaction products were isolated and identified as 3-[3-methylbuten-2-yl]-4-hydroxybenzoic acid (**3**), and as 3-amino-4-hydroxy-7-noviosyl-8-methylcoumarin which we have named novenamine (**2**).



The process consists of three phases: 1) The bacterium is grown under aerobic conditions, harvested, and a heavy cell suspension is prepared, 2) novobiocin is incubated with this cell suspension under anaerobic conditions, and 3) novenamine, a product of the incubation, is isolated.

The bacterium was grown in a 250-ml Erlenmeyer flask containing 100 ml of a medium which consisted of 800 mg of enzymatic digest of casein (NZAmine Type B, Sheffield Chemical Co., Norwich, N. Y.), 50 mg KH_2PO_4, 20 mg $MgSO_4 \cdot 7H_2O$, 50 mg NaCl, 1 mg $FeSO_4 \cdot H_2O$, in 100 ml of deionized water, adjusted to pH 7.2~7.6 with 1 N NaOH, and incubated at 28°C on a rotary shaker (200~280 rpm). When the cells reached the late logarithmic or early stationary phase (15~18 hours), they were centrifuged, washed and resuspended in a small (about 5 ml) volume of deionized water. One-ml portions of the suspension were mixed with novobiocin solution in a test tube and incubated under anaerobic conditions (nitrogen) at 28°C overnight. The cells were then centrifuged off and the supernatant was analyzed for the novobiocin and novenamine contents. When novobiocin was incubated in varying concentrations with a constant number of cells, it was converted completely to novenamine in concentrations of up to 10 mg/ml, and was hydrolyzed less efficiently at higher concentrations (Table 1).

Table 1. Effect of novobiocin concentration on the cleavage efficiency of novobiocin

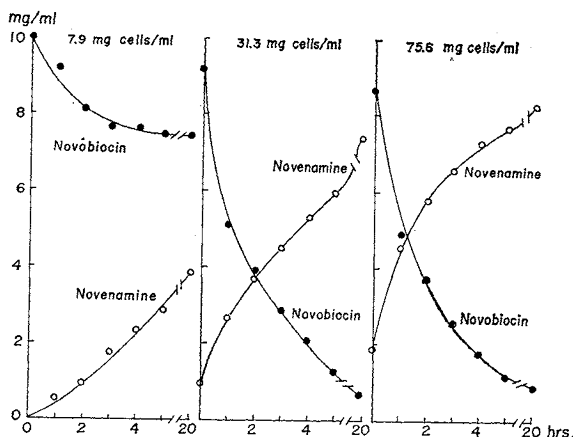
Novobiocin (mg/ml) initial concentration	Novenamine formed %
10	100.0
25	91.7
50	44.4
75	4.9
100	0.0

One ml of cell suspension (68.3 mg cells, dry weight) contained the above initial concentrations, pH 7.3. Incubated with mild shaking under anaerobic (N_2) conditions at 28°C for 22 hours.

* This bacterium (NRRL B-3652) has been deposited at the ARS Culture Collection, U. S. Department of Agriculture, Peoria, Illinois, U.S.A.

Fig. 1. Effect of cell concentration on the rate of novobiocin cleavage.

Ten ml of cell suspension in each tube contained 10 mg novobiocin/ml, pH 7.3. Incubated with mild shaking under anaerobic (N_2) conditions at 28°C, samples taken as indicated and analyzed.



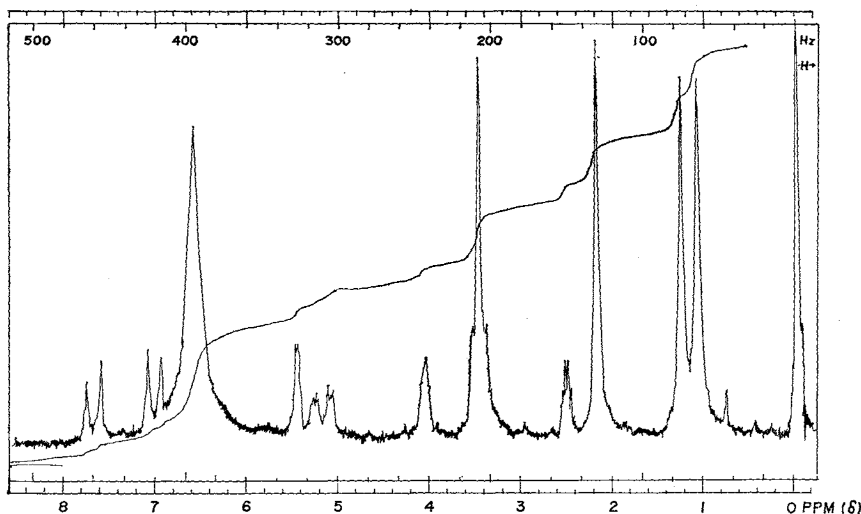
When 10 mg of the antibiotic/ml was incubated with increasing concentrations of cells, the rate of hydrolysis was proportional to the amount of the cell mass (Fig. 1).

Crude cell-free enzyme solutions also carried out the desired hydrolysis. The cells were either ruptured in the French pressure cell or sonicated (15 minutes at 6°C, 10 kc Raytheon magnetostrictive oscillator Waltham, Mass.), or incubated with lysozyme (1 mg/ml, Sigma Chemical Co., St. Louis, Mo.). In each instance the suspension was subsequently treated with the deoxyribonuclease I (50 μg/ml, Worthington Biochemical Corporation, Freehold, N.J.) and centrifuged (30,000 g for 40 minutes). The yellow opaque supernatant was dialyzed against 0.05 M phosphate buffer (pH 7.5) overnight at 6°C, and contained the desired hydrolytic enzyme.

Crystalline novenamine was isolated by the following procedure. The reaction mixture was adjusted to pH 2.0 with 6 N hydrochloric acid and filtered to remove the cells and some of the unreacted novobiocin. The second reaction product, 3-[3-methylbuten-2-yl]-4-hydroxybenzoic acid, as well as any residual novobiocin was then removed from the filtrate by extraction with butyl acetate. The aqueous layer adjusted to pH 4.0 with 2 N sodium hydroxide, was lyophilized to provide a tan powder. This powder was then triturated with absolute methanol affording a mixture of sodium chloride and white crystalline novenamine. This mixture was washed with water to yield novenamine as crystalline free base, melting from 220°C, with decomposition. Elemental analyses were consistent with the formula $C_{19}H_{24}N_2O_9$. The ultraviolet spectrum was characteristic of an aminocoumarin with maxima at 233, 282

Fig. 2. Nmr spectrum of novenamine in dimethyl-d_6-sulfoxide.

The spectrum was determined on a Varian T-60 spectrophotometer with tetramethyl silane as a reference standard.



and 297 nm ($\varepsilon_{297\,nm}$ 1.5×10^4). Titration in 60 % ethanol showed pKa's of 2.9 and 7.2. A characteristic nuclear magnetic resonance spectrum is in Fig. 2.

The second hydrolytic product, 3-[3-methyl-buten-2-yl]-4-hydroxybenzoic acid, was isolated from the butyl acetate extract in the following manner. The butyl acetate extract was washed with water, dried, and brought to dryness on a rotary evaporator. The residue was extracted with ether and the ether was evaporated. The resulting dry residue was then crystallized from acetone and water to afford the desired compound which was identical to the authentic compound[1].

The foregoing extraction procedure provided the basis for an assay by which the course of the enzymic hydrolysis could be followed quantitatively. The cells were removed from the reaction mixture by centrifugation, the clear supernatant was acidified with 3 N hydrochloric acid and extracted with butyl acetate to remove any residual novobiocin as well as 3-[3-methylbuten-2-yl]-4-hydroxy benzoic acid. These two substances were determined directly in the butyl acetate by ultraviolet absorption measurements. The aqueous layer contained novenamine as the major absorbing component. Its concentration was determined quantitatively by the absorptivity at 297 nm (ε 1.5 $\times10^4$). An alternative qualitative estimation of novenamine was made by thin-layer chromatography on silica gel G (chloroform - methanol, 95 : 5, v/v). Novenamine (Rf 0.82) forms an instantaneous purple color when sprayed with ninhydrin reagent (0.2 % ninhydrin, w/v, in pyridine or acetone).

The chemical resynthesis of novobiocin from novenamine and the hydroxybenzoic acid moiety demonstrated that a cleavage of the amide bond was indeed the only reaction that took place under the conditions described. The novobiocin was synthesized according to previously described methods[3] and isolated as a crystalline free acid. It was identical to an authentic sample of novobiocin by mixture melting point, uv, ir and nmr spectra. Its antibacterial properties were also identical to authentic novobiocin.

Novenamine has proved to be a convenient substrate for preparation of novel semisynthetic novobiocins. Although analogues of this type have also been obtained biosynthetically by adding analogues of 3-[3-methyl-buten-2-yl]-4-hydroxybenzoic acid as precursors to *Streptomyces spheroides*, only 19 of such compounds have been prepared by this method[4]. In contrast, the availability of novenamine as reported in this communication has allowed the preparation of a variety of such analogues which will be described in future reports.

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Oldrich K. Sebek
Herman Hoeksema

Research Laboratories,
The Upjohn Company,
Kalamazoo, Michigan 49001, U.S.A.

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