MICROBIAL *O*-CARBAMYLATION OF NOVOBIOCIN

M. S. KUO, D. A. YUREK, D. G. CHIRBY, J. I. CIALDELLA and V. P. MARSHALL

Upjohn Laboratories, The Upjohn Company, Kalamazoo, MI 49001, U.S.A.

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Novobiocin was inactivated by *Streptomyces niveus* US 2094 in fermentation. The inactivation product was isolated and characterized by NMR and MS as 2″-*O*-carbamylnovobiocin. The MICs of novobiocin and 2″-*O*-carbamylnovobiocin were determined for *S. niveus* strains.

Microorganisms and their enzymes have been effective agents in the synthesis of analogues of members of various classes of antibiotics[1] (for a review, see ref 1 and references cited therein). The direct transformation of antibiotics can include oxidation, reduction, alkylation, dealkylation, acylation, deacylation, hydrolysis, phosphorylation, nucleotidylation, and glycosylation.[2] It has been proposed that these biotransformation reactions can serve as mechanisms to reduce excessive antibiotic concentrations toxic to the producer. Furthermore, recent evidence has suggested that resistance genes of pathogenic bacteria may originate in antibiotic-producing microorganisms.[3~6]

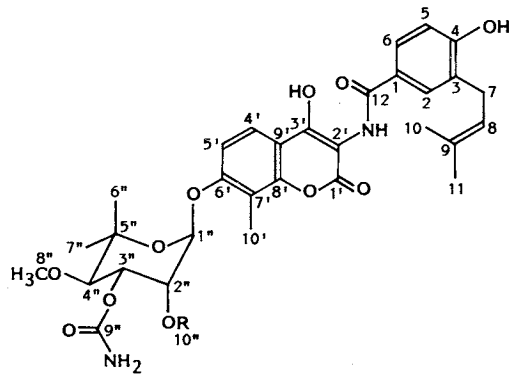
Novobiocin (Fig. 1; structure **1**) is a clinically useful, naturally occurring antibiotic. Its effectiveness, however, has been reduced by rapid development of resistance. Structurally, it consists of a noviose sugar, (C ring), a coumarin (B ring), and a substituted benzoic acid moiety (A ring) linked by glycosidic and amide bonds. Novobiocin has been previously reported[7] to be bioconverted at the 11-position to become 11-hydroxynovobiocin by *Sebekia benihana* UC 5762. 11-Hydroxynovobiocin was reported[7] to retain 30% of its bioactivity. In the current report, we present data indicating that novobiocin is carbamylated and inactivated by *Streptomyces niveus* and that the product of the carbamylation is 2″-(*O*-carbamyl)novobiocin (Fig. 1; structure **2**).

Experimental

Microbiological Methods

S. niveus strains UC 2090, UC 2094, and UC 11039 were stored over liquid N_2 in the culture collection of The Upjohn Company. *S. niveus* was inoculated into novobiocin seed and production media such as those described by SMITH *et al.*,[8] and REUSSER *et al.*,[9] respectively. The inoculated 100 ml volumes of seed medium were shaken in wide-mouth 500-ml fermentation flasks at 250 rpm for 72 hours at 28 °C. The mature seed cultures were used as inoculum for the fermentation medium. The fermentation medium was employed in the manner described for the seed medium. The fermentation was continued for 72 hours when filter sterilized, aqueous novobiocin was added to final concentra-

Fig. 1. Structure of novobiocin (**1**) and 2″-*O*-carbamylnovobiocin (**2**).



Novobiocin (**1**) R=H
2″-*O*-Carbamylnovobiocin (**2**) R=$CONH_2$

tions of 0, 125, 250, and 500 μg/ml of fermentation volume. The fermentation process was continued for another 5 days under the same conditions. Samples were taken for assay between 3 and 10 days of fermentation.

The MICs of novobiocin (compound **1**) and 2″-*O*-carbamylnovobiocin (compound **2**) vs. *S. niveus* strains were determined by growth inhibition in Trypticase soy broth (TSB). *S. niveus* was inoculated into 100 ml volumes of TSB contained in 500-ml fermentation flasks and was shaken for 72 hours at 250 rpm at 28 °C. Aliquots (50 μl) of the 72 hours cultures were inoculated into 5 ml volumes of TSB contained in 25-ml Erlenmeyer flasks. Ethanolic solutions of **1** and **2** were added aseptically to the uninoculated flasks at final concentrations ranging between 1 and 1,000 μg/ml. Controls were performed in the presence and absence of ethanol. Growth inhibition was determined after incubation at 28 °C (250 rpm) for 96 hours.

HPLC Methods

A Varian 5500 HPLC employing a 250 × 4.6 mm Econosphere 5 μm silica cartridge column and guard cartridge were used to separate novobiocin products. Elution was with isocratic *n*-BuCl (50% H_2O saturated)-THF-MeOH-acetic acid (88:5:4:3) as reported by TSUJI *et al.*[10] Detection was at 340 nm using a Varian 9060 diode array detector. The detector signal was recorded and integrated by a HP 3392A integrator. In this system, **1** elutes at 9.4 minutes while **2** elutes at 12.7 minutes. Other peaks observed include descarbamylnovobiocin at 5.7 minutes and isonovobiocin at 8.3 minutes.

Column Chromatography Methods

A size "C" Lobar silica column was used for preparative runs. 2.5~3.5 g of the dried mother liquor remaining after crystallization of novobiocin from the fermentation extract (10-liter broth)[11] were dissolved in 50 ml of 10% THF-eluting solvent. Undissolved residues were washed twice in the same mixture and filtered. The filtrate was added to the initial sample prior to injection onto the column. The desired component migrated very slowly as a yellow band. After application of 8 liters of eluting solvent, the percentage of methanol was raised to 8% for another 2 liters and then to 12%. The yellow band was resolved into two bands, the first eluting after elution using 7 liters of the 12% MeOH solvent mixture. HPLC indicated that this band contained the compound of interest. 237 mg of dried powder were collected after pooling fractions and removing organic solvents under reduced pressure followed by the addition of water and lyophilization to remove acetic acid. Two additional runs were made in a similar fashion and the appropriate fractions were collected, dried, and combined with a total yield of 750 mg.

TLC Methods

The combined Lobar preparations were then further purified by preparative TLC (Analtech silica, 2,000 μm) using 10% MeOH-$CHCl_3$ as the developing solvent. The band between Rf 0.2 and 0.3 was collected and shown to be the component of interest by HPLC. Other bands observed at Rf 0.5 and 0.6 were faint and were presumed to be **1** and isonovobiocin, respectively.

Spectroscopy Methods

NMR spectra were recorded on a Bruker AM-300 spectrometer operated at 300 and 75 MHz for the ^1H and ^{13}C nuclei, respectively. Spectra were run in DMSO with and without trace amounts of D_2O and were reported as parts per million relative to TMS. FAB-MS spectra were obtained on a MAT CH-5 spectrometer operated in positive-ion mode. The 2D ^1H-^1H and ^1H-^{13}C COSY were obtained by the standard sequences. The multiplicities of carbon signals were determined by DEPT experiments using standard sequences provided by the Bruker instrument. The 2D long-range ^1H-^{13}C COSY were obtained by using the correlation *via* long range coupling pulse sequence.[12]

Results and Discussion

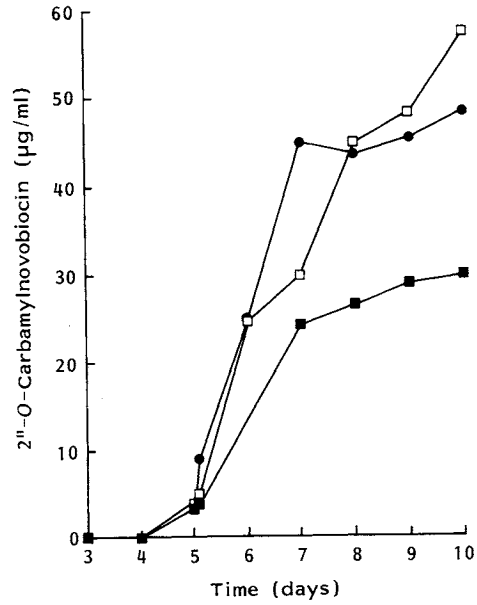
Production of 2″-*O*-Carbamylnovobiocin

Compound **2** was originally detected as a minor impurity in production scale fermentation. To provide enough material for this study, the production of **2** by *S. niveus* UC 2094, was studied under different

conditions. It was found that the production of **2**, as indicated by analytical HPLC results, can be greatly increased if **1** was added to the fermentations at day 3 and harvested at day 10. As indicated in Fig. 2, the addition of 125 μg/ml **1** to the producing fermentation generated 58 μg/ml **2** at day 10. While addition of 500 μg/ml **1** gave a higher titer of **2** initially, the peak titer value (47 μg/ml at day 10) was smaller than that observed with the lower dose addition. Both **1** and **2** were found to decompose quickly if left in the fermentation beers after day 10 (data not shown). The higher level of **1** probably had some toxic effect on the microorganism, therefore a lower titer of **2** was ultimately produced.

Fig. 2. Conversion of novobiocin (**1**) to 2″-*O*-carbamylnovobiocin (**2**) by *Streptomyces niveus* UC 2094.

Novobiocin added: ■ Control (no addition), □ 125 μg/ml (day 3), ● 500 μg/ml (day 3).



Isolation and Identification of 2″-*O*-Carbamylnovobiocin

As described in Materials and Methods, **2** was isolated by silica column chromatography, followed by preparative TLC. The purification of **2** was followed by analytical HPLC. Compound **2** was isolated as an amorphous, pale yellow material soluble in lower alcohols, ethyl acetate, basic water, chloroform, acetone, pyridine and DMSO, and slightly soluble in ether or petroleum ether. The UV absorption spectrum of **2** is very similar to that of **1**. Like **1**, the absorption maximum is red-shifted from 307 nm to 324 nm on adjusting the solution pH from neutral to acidic, indicating the existence of a phenolic group in **2**. The FAB-MS results (ions due to M+K, M+Na, and M+H were all detected, with the M+K ion being the most intense peak) indicated that the MW of **2** is 655 which is 43 a.m.u. higher than that of **1**. HR measurement of the $(M+K)^+$ ion suggested $C_{32}H_{37}N_3O_{12}K$ to be the most likely molecular formula (694.2018 measured vs. 694.2014 theory). Compared to the molecular formula of **1** ($C_{31}H_{36}N_2O_{11}$), **2** has an additional carbon, hydrogen, nitrogen, and oxygen.

In order to elucidate the nature of these extra elements, ^1H NMR studies were carried out for both **1** and **2**. The assignments of each of the signals were facilitated by either decoupling or 2D COSY and ^{13}C-^1H correlation experiments. The results are listed in Table 1. Comparing the two spectra, the following observations were made: 1) the A and B ring protons of **1** and **2** were virtually identical in terms of their chemical shift and coupling constants, indicating that those two moieties were not subjected to biotransformation; 2) the exchangeable proton assigned as 2″-OH of the noviose moiety was absent from the spectrum of **2**, and the coupling pattern of the 2″-H peak in **2** became a simple triplet vs. the multiplet pattern observed for its counterpart in **1**, indicating the hydroxyl group to be the site of the biotransformation; 3) the 2″-H peak in **2** was deshielded by 1.1 ppm from its counterpart in **1**, indicating that 2″-OH was functionalized by a carbonyl group in **2**; 4) small size deshieldings were also observed for 1″-H (0.26 ppm) and 3″-H (0.22 ppm), supporting the previous conclusion; 5) the peak area underneath the carbamide signal of **2** integrated to four protons, indicating that **2** has two additional carbamide protons.

Table 1. ^{1}H NMR data of **1** and **2**.

Atom No.	^{1}H NMR				^{13}C NMR	
	1		**2**		**1**	**2**
	Chemical shift[a]	Multiplicity (J=Hz)	Chemical Shift	Multiplicity (J=Hz)	Chemical shift, multiplicity	
1	—	—	—	—	123.64, s	124.16, s
2	7.73	s	7.73	s	129.61, d	129.82, d
3	—	—	—	—	127.61, s	127.34, s
4	—	—	—	—	158.15, s	158.38, s
4-OH	9.98	s	9.98	s	—	—
5	6.92	d (8.8)	6.92	d (9)	114.56, d	114.31, d
6	7.73	d (8.8)	7.73	d (9)	127.63, d	127.34, d
7	3.25	d (7.8)	3.25	d (8)	28.16, t	28.07, t
8	5.30	t (7.8)	5.30	t (8)	122.53, d	122.57, d
9	—	—	—	—	131.69, s	131.53, s
10	1.70	s	—	s	17.69, q	17.70, q
11	1.70	s	—	s	25.54, q	25.56, q
12	—	—	—	—	167.03, s	166.64, s
1′	—	—	—	—	160.69, s	160.58, s
2′	—	—	—	—	101.90, s	101.72, s
2′-NH	11.2	s	11.2	s	—	—
3′	—	—	—	—	158.78, s	159.11, s
3′-OH	9.15	s	9.15	s	—	—
4′	7.75	d (9.1)	7.75	d (9)	121.94, d	121.88, d
5′	7.16	d (9.1)	7.16	d (9)	110.30, d	110.66, d
6′	—	—	—	—	157.11, s	156.82, s
7′	—	—	—	—	112.99, s	113.04, s
8′	—	—	—	—	150.43, s	150.57, s
9′	—	—	—	—	110.29, s	110.16, s
10′	2.21	s	2.21	s	8.30, q	8.23, q
1″	5.54	d (2.4)	5.70	d (2)	98.68, d	95.86, d
2″	4.08	m	5.30	t (2)	68.99, d	68.33, d
2″-OH	5.62	d (5.4)	—	—	—	—
3″	5.20	dd (10.2, 2.4)	5.42	dd (10, 2)	70.69 d,	70.04, d
4″	3.50	d (10.2)	3.51	d (10)	81.65, d	81.06, d
5″	—	—	—	—	78.26, s	78.28, s
6″	1.30	s	1.25	s	22.78, q	22.96, q
7″	1.05	s	0.98	s	28.52, q	27.67, q
8″	3.48	s	3.68	s	61.04, q	60.92, q
9″	—	—	—	—	156.48, s	155.66,[b] s
9″-NH_2	6.73, 6.53	s	6.7	br	—	—
10″	N.A.		—	—	N.A.	156.68,[b] s
10″-NH_2	N.A.		6.7	br	N.A.	—

[a] In ppm relative to TMS.
[b] Assignments could be interchanged.
N.A.: Not applicable.

All the evidence presented thus far suggests that **2** is 2″-*O*-carbamylnovobiocin. Furthermore, from the observed coupling constants, it can be deduced that the noviose sugar moiety assumes a near chair conformation with equatorial 1″ and 2″ and axial 3″ and 4″ protons.

To further confirm the structure of **2**, ^{13}C NMR spectra of **1** and **2** were taken and compared (results shown in Table 1). The major difference was the appearance of a new signal in the carbamyl region

(155~157 ppm). Interestingly, no other significant differences were observed for the rest of the carbon signals except for the anomeric carbon C-1″. The 2.8 ppm difference can be rationalized by the so-called "γ-effect".[13] In order for the γ-effect to be operative, the carbon in question would require a ^{13}C-^{1}H bond in close vicinity to the perturbing group. The replacement of a hydroxyl proton by a carbamyl group, therefore, caused an upfield shift of the C-1″ by polarizing its ^{13}C-^{1}H bond through space. Notice that C-3″, although also situated three bonds away from the carbamyl carbon, did not show any upfield shift since its ^{13}C-^{1}H bond is located *trans* to the carbamyl group. By the same analysis, the chemical shift value of C-4″ of **1** should be roughly 2.8 ppm more upfield than its counterpart in the 3″-*O*-descarbamylnovobiocin. Indeed, the C-4″ was found to resonate at 81.0 and 83.4 ppm for **1** and 3″-*O*-descarbamylnovobiocin, respectively.

The 2″-*O*-carbamylnovobiocin was found to be devoid of any antibacterial activity vs. *S. niveus* at 3 mg/ml. Carbamylation is a previously unreported means of microbial inactivation of novobiocin. As antibiotic producing microorganisms such as *S. niveus* are considered to be potential sources of genes conferring antibiotics resistance, this transformation may have significance beyond *S. niveus*. As might be expected, a producing strain (UC 11039) is highly resistant to novobiocin (MIC > 1 mg/ml), while another strain of *S. niveus*, UC 2090, more closely resembling the *S. niveus* wild type, is more sensitive (MIC 250 μg/ml). Both organisms are highly resistant to 2″-*O*-carbamylnovobiocin (MIC's > 1 mg/ml).

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