MICROBIAL HYDROXYLATION OF NOVOBIOCIN AND RELATED COMPOUNDS

O. K. SEBEK and L. A. DOLAK

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

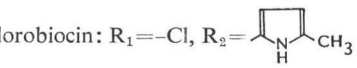
(Received for publication November 9, 1983)

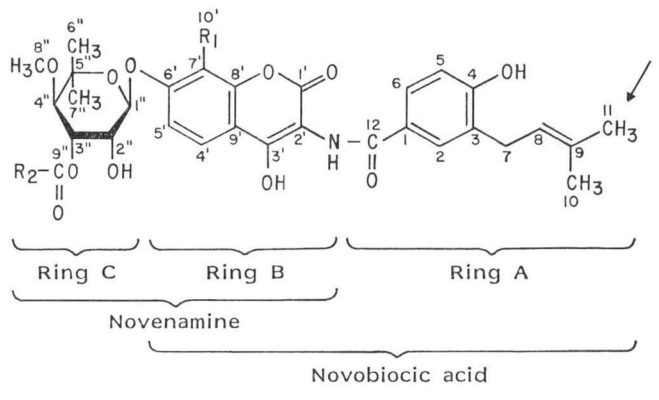
A new soil actinomycete (UC 5762, NRRL 11111) was found to transform novobiocin to 11-hydroxynovobiocin. The product was isolated by solvent extraction and column chromatography, and identified by IR, UV, ^{1}H NMR and ^{13}C NMR spectroscopy. Related structures (8,9-dihydronovobiocin, novobiocic acid and chlorobiocin) were similarly transformed to their corresponding C-11 hydroxylated analogues. The microbial process is superior to chemical (selenium dioxide) oxidation which yielded a mixture of 11-hydroxy- and 11-oxonovobiocin.

Novobiocin inhibits DNA replication by interacting with DNA gyrase[1,2], eliminates plasmids from their bacterial hosts[3,4] and is also used to differentiate staphylococci from micrococci[5] and anaerobic peptococci from peptostreptococci[6]. In clinical practice, it has been effective especially as an anti-staphylococcal agent and used as an adjunct to other antibiotics (rifampicin, fusidic acid). Since its effectiveness has been greatly reduced by a rapid development of resistance, hypersensitivity and by other untoward reactions, attempts have been made in the past to modify this antibiotic to lower such side reactions.

As seen in Fig. 1, the molecule of the antibiotic consists of three moieties: ring A (3-(3-methyl-2-butenyl)-4-hydroxybenzoic acid) is linked through an amide bond to ring B (3-amino-4,7-dihydroxy-8-methylcoumarin) which in turn is attached glycosidically to ring C (3-*O*-carbamoyl-4-*O*-methyl-5,5-dimethyl-L-lyxose).

In the past, new analogues were prepared whereby a) ring A was substituted microbiologically by

Fig. 1. Structures of novobiocin and related compounds.
Novobiocin, 8,9-dihydronovobiocin and novenamine: R_1=–CH_3, R_2=–NH_2
Chlorobiocin: R_1=–Cl, R_2= 



various benzoic acid derivatives;[7] b) novobiocin was first cleaved by bacterial hydrolysis and yielded novenamine[8] which in turn was chemically acylated to some 160 novel derivatives (L. A. DOLAK and H. HOEKSEMA, unpublished); and c) ring B was substituted by coumarin analogues by means of ring B-blocked mutants of the novobiocin-producing *Streptomyces niveus*[9,10,11].

We wish to report on yet another microbial modification of this antibiotic namely on the oxidation of the C-11 (*trans*)methyl group of the 2-butenyl side-chain of ring A[12] by a new actinomycete. Although the product is antibiotically less active than the substrate, the value of this finding is that an additional site on the novobiocin molecule has been functionalized and thus allows the synthesis of a new class of analogues of the antibiotic. Hydroxylated dihydronovobiocin, chlorobiocin and novobiocic acid were prepared from their respective substrates by the same methodology. Preliminary results of this work have been published[13].

Materials and Methods

Growth Conditions and TLC Analysis

Soil actinomycetes were isolated and inoculated into 125-ml Erlenmeyer flasks containing 10 ml of a medium (TYG) consisting of 0.5% Bacto tryptone (Difco), 0.3% Bacto yeast extract (Difco) and 1% glucose in deionized water, and adjusted to pH 7.1. The flasks were incubated at 250 rpm on an orbital New Brunswick shaker at 28°C. To those isolates which showed a heavy growth after 2~3 days of incubation, sterile solutions of novobiocin (100~750 μg/ml final concentration) were added and the incubation was continued. Aliquots were withdrawn at different time intervals and 10~20 μliter portions analyzed by TLC (silica gel sheets, Eastman Kodak Chromatogram 13171; ethyl acetate - methanol, 4: 1, v/v). Bioactive materials were located by plating dry sheets onto nutrient agar seeded with *Micrococcus luteus* UC 130 and incubated at 28°C overnight. Dihydronovobiocin and chlorobiocin were examined in the same way, and the antibacterially inactive novobiocic acid and its metabolite were located by spraying the silica gel sheets with permanganate-periodate reagent.

Preparation of [*U*-^{14}C]Novobiocin and its Conversion to the ^{14}C-Labeled Product

Novobiocin-producing *S. niveus* UC 2094 was grown in 100 ml of the above TYG medium in a shaken 250-ml flask for 24 hours. A total of 250 μCi (10.8 mg) of D-[*U*-^{14}C]glucose (NEC-042, New England Nuclear, lot No. 618–181) mixed with 100 mg of carrier glucose was then added and the incubation was continued. The bioactivity reached a peak after additional 172 hours of incubation. The beer was then acidified (pH 3.0) and extracted with 4×50 ml portions of ethyl acetate. The combined solvent layers were dried with $MgSO_4$, filtered and the solvent was removed on a rotary evaporator. The residual oil was redissolved in 5 ml of ethyl acetate and the bioactivity detected in 20 μliter aliquots by TLC as above. To locate the radioactive material, 4 mm-wide strips of the silica gel were scraped off from the sheet into individual vials which contained 15 ml of scintillation solvent, and by counting the radioactivity in a Packard Tricarb Model 314EX2A liquid scintillation spectrometer at −8°C[14]. The radioactive material which corresponded to novobiocin (Rf 0.46) was freed from organic impurities with methanol washes, the clear filtrate brought to dryness on a rotary evaporator and the solids were dissolved in 5 ml of water. An aliquot (0.5 ml) was mixed with 1 ml of carrier novobiocin (5 mg/ml) and added to 10 ml of a 3-day mycelium of UC 5762 (500 μg [^{14}C]novobiocin/ml final concentration). The mixture was incubated in a 125-ml flask, samples were withdrawn at different intervals and analyzed as above by TLC, bioactivity and scintillation spectrometry.

Isolation of the Novobiocin Product

Ten liters of UC 5762 were grown in a New Brunswick fermentor at 28°C with aeration (2-liter air/minute) and agitation (300 rpm) for 3 days at which time a solution of 1.5 g of novobiocin (sodium salt) in 15 ml of H_2O was added. After three additional days of incubation, more than 90% of the novobiocin was utilized and the product appeared (TLC) as the only other bioactive material. The acidified

(pH 5.0) beer was filtered through Dicalite 4200 and the filtrate and the mycelial cake were extracted with ethyl acetate (2×3.3 and 2×2 liters respectively). The extracts were combined, washed with brine, dried with $MgSO_4$, filtered and concentrated to a brown oil in a rotary evaporator. The oil was dissolved in 4 ml of ethyl acetate - methanol (10: 1) and the solution injected onto a size C Merck prepacked silica gel column. The column was developed with a gradient (9: 1~3: 1) of ethyl acetate - methanol at 7 ml/minute. The eluate was collected in 25 ml fractions and monitored by TLC (Analtech silica gel plates, ethyl acetate - methanol, 4: 1) with phosphor quenching and permanganate spray. Fractions Nos. 54~74 (which contained the biotransformation product) were combined, evaporated and yielded 130 mg of solids.

Results and Discussion

Novobiocin Biotransformation

Two-hundred and five randomly selected soil actinomycetes which had been found to produce no antibiotics, were tested for their ability to modify novobiocin. A number of them inactivated the antibiotic but six of them transformed it in each case to one bioactive product which was more polar (Rf 0.24) than the substrate itself (Rf 0.46). Isolate UC 5762 (*Sebekia benihana*, NRRL 11111, Dietz and Li, n. sp.,[15]) carried out this transformation more rapidly and more efficiently than the others and was used for subsequent work. The transformation was dose-dependent since 100 μg of the antibiotic/ml was completely converted to the new product in 40 hours at a rate indicated in Fig. 2, while up to 7 days were needed for a complete conversion of 500~750 μg novobiocin/ml. The transformation remained incomplete at higher concentrations even after 14 days of incubation.

Instead of using pure novobiocin as a substrate, an alternative two-step microbial sequence was also found workable. Novobiocin was first produced by *S. niveus* UC 2094[16,17]. Clear beer was mixed with washed 3-day mycelium of the novobiocin convertor UC 5762 and incubated as above for four additional days. The TLC analysis of this process showed again that novobiocin formed in the first step was

Fig. 2. Biotransformation of novobiocin.

Novobiocin: 100 μg/ml (80 μliters of clear beer applied). Activity: detected by *M. luteus* UC 130. Activity at origin not related to novobiocin, see the 0 hour sample; length of incubation: 0= 0 hour, 1=10 hours, 2=24 hours, 3=40 hours.

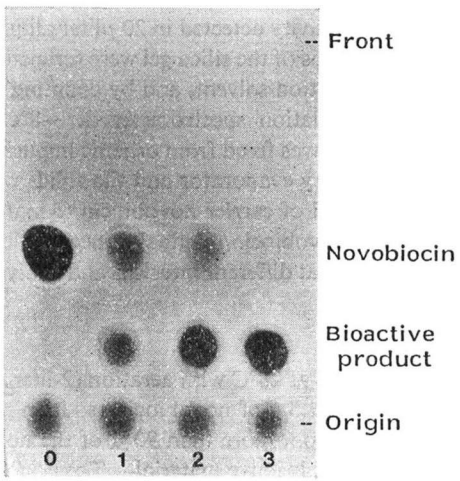
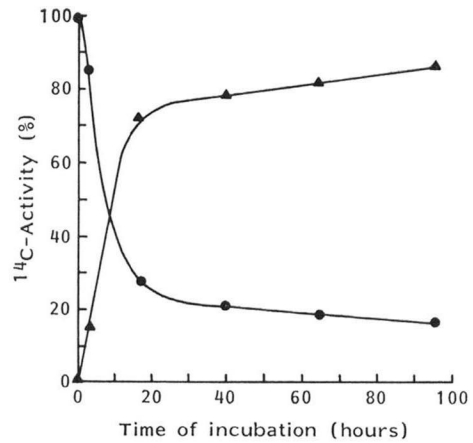


Fig. 3. Rate of [*U*-^{14}C]novobiocin biotransformation.

Substrate (500 μg/ml final concentration) was added to 3-day mycelium of UC 5762, samples taken at indicated intervals.

●=[^{14}C]Novobiocin, ▲=^{14}C-bioactive product



completely converted to the new product.

A comparison of the activities (zone sizes) in samples taken at 3 different time intervals indicated (Fig. 2) that the product was somewhat less active than the substrate. In order to determine if any antibacterially inactive products were also formed, the transformation was monitored by means of [*U*-^{14}C]novobiocin prepared from [*U*-^{14}C]glucose as described in Materials and Methods. The TLC data showed that the radioactive novobiocin was transformed only to one product which was inhibitory to *M. luteus* and that no other metabolites were formed. The transformation proceeded at a rapid rate and was essentially complete in 36~48 hours (Fig. 3).

Identification of the Novobiocin Product

An ethanolic solution of the combined solids (130 mg) from fractions 54~74 (obtained as described in Materials and Methods), had a UV spectrum identical to that of novobiocin over a wide pH range. No changes were therefore likely to have occurred in the aromatic rings of the new compound. The ^{1}H NMR, spectrum (DMSO-d_6, 60 MHz) showed a singlet at δ 1.6 ppm which was assigned to one of the allylic methyls (C-10, C-11) of the 3-methyl-2-butenyl(isopentenyl) side chain of ring A.

In the spectrum of novobiocin, the peak at δ 1.6 ppm (C-10, C-11) integrates 1:1 relative to the signal at δ 1.2 (C-6″, C-7″, ring C). In the product, the peaks at δ 1.6 ppm and δ 1.2 ppm integrated 1:2 which indicates that one of the allylic methyl groups was involved in the biotransformation. This conclusion was further supported by the appearance of a peak at δ 3.9 ppm which is not present in novobiocin and is consistent with the chemical shift predicted for an allylic methylene bearing a hydroxyl group. Thus it was concluded that one of the allylic methyl groups (C-10 or C-11) in novobiocin was hydroxylated.

As in novobiocin, the ^{13}C NMR spectrum of the new compound had 31 lines thus showing that the carbon skeleton of the new compound remained intact. Since the ^{13}C NMR spectrum of novobiocin has been completely assigned[12], a comparison of the two spectra (Table 1) showed that it was the *trans* methyl group (C-11) of novobiocin which was hydroxylated. The triplet for C-11 in the new product

Table 1. The ^{13}C NMR assignment for hydroxynovobiocin and novobiocin.

Carbon No.	Novobiocin (ppm)	Hydroxy-novobiocin (ppm)	Carbon No.	Novobiocin (ppm)	Hydroxy-novobiocin (ppm)
1	123.6	125.1	5′	110.3 d	108.8 d
2	129.9 d	129.9 d	6′	157.1*	156.5*
3	127.6	127.0	7′	110.3	112.0
4	158.8*	162.1*	8′	150.4	151.4
5	114.6 d	114.4 d	9′	113.0	115.8
6	127.6 d	127.3 d	10′	8.3 q	8.6 q
7	28.5 t	27.9 t	1″	98.7 d	98.8 d
8	122.5 d	123.0 d	2″	69.0 d	69.1 d
9	131.7	135.8	3″	70.7 d	70.6 d
10	17.7 q	13.8 q	4″	81.1 d	81.2 d
11	25.5 q	66.5 t	5″	78.3	78.1
12	167.0	167.2	6″	28.2 q	28.8 q
1′	158.2*	158.2*	7″	22.8 q	22.9 q
2′	101.9	99.5	8″	61.0 q	61.1 q
3′	160.7	166.6	9″	156.5*	156.1*
4′	121.9 d	122.0 d			

* Assignments of carbons may be interchangeable.

appeared at δ 66.5 ppm while the rest of the spectrum both in acidic and basic solutions was identical to that of novobiocin.

Bioactivity of 11-Hydroxynovobiocin

11-Hydroxynovobiocin was tested against *Staphylococcus aureus* UC 80, *Bacillus subtilis* UC 564, *B. cereus* UC 3145, *M. luteus* UC 130, *Klebsiella pneumoniae* UC 57 and *Mycobacterium avium* UC 159, and was found to retain about 30% of the activity of novobiocin.

11-Hydroxydihydronovobiocin

Three grams of 8,9-dihydronovobiocin (calcium salt) in a final concentration of 300 μg/ml of beer were converted by UC 5762 in 3 days to a more polar product (Rf 0.20, ethyl acetate - methanol, 10:1) which was isolated by column chromatography. The ^{13}C NMR spectrum of this product showed a triplet at δ 69 ppm which indicated that by analogy to novobiocin one of the methyl groups (at C-10 or C-11) of the substrate was hydroxylated.

11-Hydroxychlorobiocin

Chlorobiocin was treated in the same way. This antibiotic is a chlorine-containing novobiocin analogue produced by three different streptomycetes[18] and contains the ring A moiety. When incubated with UC 5762, it was completely transformed to an antibacterially active product which was more polar (Rf 0.15) than the substrate (Rf 0.36). Although the amount of the isolated product was not sufficient for a conclusive identification, it is suggested by analogy with the above data that chlorobiocin was transformed in the same way as the above compounds and yielded 11-hydroxychlorobiocin.

Hydroxynovobiocic Acid

This acid (1,050 mg, 750 μg/ml of beer) was incubated with UC 5762 for 7 days and the pool of the acidified (pH 3.0) beer was extracted with ethyl acetate. Upon evaporation of the solvent, the resulting yellow solid (1.0 g) was injected into a HPLC column (2.5×121.9 cm) and eluted isocratically (4 ml/minute, 24 ml/fraction). Fractions Nos. 23~26 were pooled, concentrated and yielded 500 mg of a homogeneous (TLC) product. The ^1H NMR spectrum of this material differed from that of the substrate novobiocic acid in two respects: (1) there was a new singlet (2 protons) at δ 3.9 ppm, and (2) integration of the singlet at δ 1.7 ppm showed the presence of a single methyl group. This indicated that the hydroxylation had occurred at the C-11 allylic methyl group.

Anal Calcd for $C_{22}H_{21}NO_8$: C 64.20, H 5.12, N 3.41.
Found: C 63.59, H 5.06, N 3.42.

The hydroxylating enzyme is moderately substrate-specific since novobiocin, dihydronovobiocin, and chlorobiocin were completely hydroxylated within 48 hours while it took 7 days to hydroxylate novobiocic acid. Ring A novobiocin (see Fig. 1) was modified only to a limited extent even after 10 days of incubation. On the other hand, it was reported that lapachol, a naturally occurring naphthoquinone derivative and structurally similar to novobiocic acid, was hydroxylated by UC 5762 in the same manner as novobiocins in moderate yields within 24~48 hours[19].

Chemical Synthesis of 11-Hydroxy- and 11-Oxonovobiocin

When novobiocin was oxidized with selenium dioxide, it yielded a mixture of 11-hydroxy- and 11-oxonovobiocin. The success of the chemical preparation of the former depends on the optimization of the reaction conditions and on the chromatographic separation of the two products from the unreacted novobiocin. This route is not applicable to the chemical preparation of 11-hydroxydihydronovobiocin

due to the lack of allylic methyl groups in the molecule (see Fig. 1).

11-Hydroxynovobiocin was prepared by adding a slurry of selenium dioxide (500 mg, 5 mmol) in 100 ml of 95% ethanol to a refluxing solution of novobiocin (6.1 g, 10 mmol) in 100 ml of 95% ethanol over a 45-minute period. After additional 60 minutes of reflux, the hot reaction mixture was filtered and the solvent was removed on a rotary evaporator. The solid residue was dissolved in 20 ml of chloroform - methanol (19: 1), the solution injected onto a 230~400 mesh silica gel column (2.5×100 cm) and eluted with the same solvent mixture. Fractions containing the desired product and free from the unreacted novobiocin and 11-oxonovobiocin, (the aldehydic co-product) were pooled and concentrated. The residue was dissolved in 50 ml of methanol and the solution poured into 200 ml of water. The resulting precipitate was collected and dried at 60°C and gave 11-hydroxynovobiocin (2.0 g, yield 31%) identical in all respects to that obtained microbiologically.

11-Oxonovobiocin was obtained by refluxing novobiocin (20 g, 32.7 mmol) in 200 ml of 95% ethanol containing selenium dioxide (10 g, 90.9 mmol) for 3 hours. The reaction mixture was cooled to room temperature, filtered and the solvent removed on a rotary evaporator. The residue was dissolved in 100 ml of ethyl acetate - methanol (20: 1), filtered and injected as above onto a 230~400 mesh silica gel column (15×150 cm). The product was eluted with the same solvent mixture. The fractions containing the aldehyde were pooled, concentrated, and the residue was re-chromatographed on another silica gel column (2.5×100 cm) with a chloroform - methanol (19: 1) mixture. The pure aldehyde (yield 50%) had Rf 0.27 (silica gel TLC, Merck, the same chloroform - methanol mixture) and the ^{1}H NMR spectrum showed an aldehyde singlet at δ 9.5 ppm and one allylic methyl peak at δ 2.2 ppm. It exhibited 50% of the hydroxynovobiocin activity when tested against the above bacteria.

Acknowledgment

We gratefully acknowledge the assistance of Mrs. J. M. SWANSON in these experiments.

References

1) GELLERT, M.; M. H. O'DEA, T. ITOH & J.-I. TOMIZAWA: Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. USA 73: 4474~4478, 1976
2) LIU, L. F. & J. C. WANG: *Micrococcus luteus* DNA gyrase: Active components and a model for its supercoiling of DNA. Proc. Natl. Acad. Sci. USA 75: 2098~2102, 1978
3) MCHUGH, G. L. & M. N. SWARTZ: Elimination of plasmids from several bacterial species by novobiocin. Antimicrob. Agents Chemother. 12: 423~426, 1977
4) TAYLOR, D. E. & J. G. LEVINE: Characterization of a plasmid mutation affecting maintenance, transfer and elimination by novobiocin. Mol. Gen. Genet. 174: 127~133, 1979
5) MITCHELL, R. G. & A. C. BAIRD-PARKER: Novobiocin resistance and the classification of staphylococci and micrococci. J. Appl. Bacteriol. 30: 251~254, 1967
6) WREN, M. W. D.; C. P. ELDON & G. H. DAKIN: Novobiocin and the differentiation of peptococci and peptostreptococci. J. Clin. Pathol. 30: 620~622, 1977
7) WALTON, R. B.; L. E. MCDANIEL & H. B. WOODRUFF: Biosynthesis of novobiocin analogues. Devs. Indust. Microbiol. 3: 370~375, 1962
8) SEBEK, O. K. & H. HOEKSEMA: Enzymic formation of novenamine, a noviosylaminocoumarin moiety of novobiocin. J. Antibiotics 25: 434~436, 1972
9) BIRCH, A. J.: Partial synthesis of some novobiocin analogues. *In* Advans Antimicrobial Antineoplastic Chemotherapy. M. HEJZLAR *et al., Ed.*, 1/2, pp. 1023~1024, Urban & Schwarzenberg, Munich, 1972
10) LEMAUX, P. G. & O. K. SEBEK: Use of a mutant for the biosynthesis of a novobiocin analogue. Abstrs. 13th Intersci. Conf. Antimicrob. Agents Chemother., Abstr. No. 49, Washington, D.C., 1973
11) SEBEK, O. K.: On the biosynthesis and biomodifications of novobiocin. The 2nd Internatl. Symp. Genet.

Indust. Microorg., p. 14, 1976
12) DOLAK, L. A.: The ^{13}C NMR spectrum of novobiocin. J. Antibiotics 29: 710~713, 1976
13) SEBEK, O. K. & L. A. DOLAK: Further studies on the biomodification of novobiocin. Abstrs. 77th Ann. Meet. Amer. Soc. Microbiol., O 26, p. 249, New Orleans, 1977
14) THOMAS, R. C.; D. J. DUCHAMP, R. W. JUDY & G. J. IKEDA: Metabolic fate of talazamide in man and in the rat. J. Med. Chem. 21: 725~732, 1978
15) DIETZ, A. & G. P. LI: *Sebekia*, a new genus of the family *Actinoplanaceae*. Abstrs. 82nd Ann. Meet. Amer. Soc. Microbiol., 163, Atlanta, 1982
16) KOMINEK, L. A.: Biosynthesis of novobiocin by *Streptomyces niveus*. Antimicrob. Agents Chemother. 1: 123~134, 1972
17) KOMINEK, L. A. & O. K. SEBEK: Biosynthesis of novobiocin and related coumarin antibiotics. Devs. Indust. Microbiol. 15: 60~69, 1974
18) NINET, L.; F. BÉNAZET, Y. CHARPENTIÉ, M. DUBOST, J. FLORENT, D. MANCY, J. PREUD'HOME, T. L. THRELFALL, B. VUILLEMIN, D. E. WRIGHT, A. ABRAHAM, M. CARTIER, N. DE CHEZELLES, C. GODARD & J. THEILLEUX: La clorobiocine (18.631 R.P.), Nouvel antibiotique chloré produit par plusieurs espèces de streptomyces. Compt. Rend. Acad. Sci. Ser. C 275: 455~458, 1972
19) OTTEN, S. L. & J. P. ROSAZZA: Microbial transformations of natural antitumor agents. 17. Conversions of lapachol by *Cunninghamella echinulata*. J. Natur. Prod. 44: 562~568, 1981