COUMARINS AS INHIBITORS OF BACTERIAL DNA GYRASE

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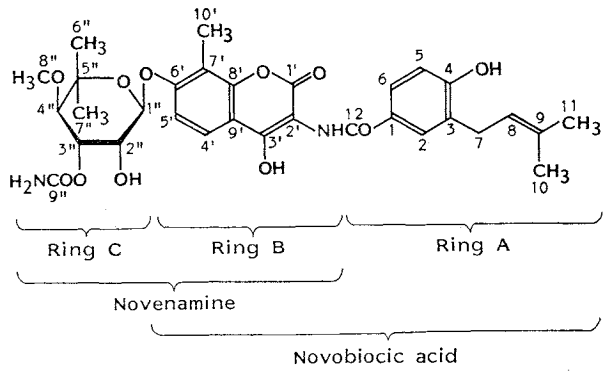
(Received for publication May 18, 1987)

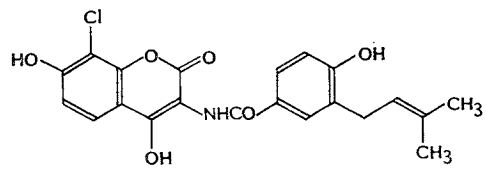
Chlorobiocic acid and 3-(carbobenzoxyamino)-4,7-dihydroxy-8-methylcoumarin were identified as two new inhibitors of *Micrococcus luteus* DNA gyrase. Both compounds possess weak antibacterial activity against whole *M. luteus* cells which indicates that they probably lack efficient transport functions to penetrate the cell envelope.

Coumarin containing antibiotics (including novobiocin, chlorobiocin and coumermycin) exert their antibacterial activity by inhibiting the function of the B-subunit of the bacterial enzyme DNA gyrase. Novobiocin consists of three distinct entities: The sugar noviose (ring C), a coumarin residue

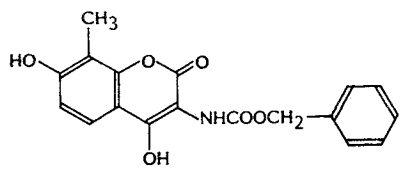
Fig. 1. Structure of novobiocin and novobiocin analogs.



Novobiocin



Chlorobiocic acid



Carbobenzoxycoumarin

(ring B) and 3'-isopentenyl-4-hydroxybenzoic acid (ring A). The subentity consisting of the noviose plus the coumarin is referred to as novenamine and the subentity consisting of the coumarin and the benzoic acid residue as novobiocic acid (Fig. 1). Based on the observation that novenamine inhibits DNA gyrase but novobiocic acid does not we concluded in a previous report that novenamine is the active moiety in novobiocin[1]. Upon examination of additional non-sugar containing novobiocin-related coumarin analogs we now found two such compounds which also inhibit DNA gyrase.

Materials and Methods

DNA Replication and Repair in Toluenized *Escherichia coli* Cells

The mutant strain *E. coli* H560 deficient in DNA polymerase I (obtained from Dr. H. HOFFMANN-BERLING) was used to prepare toluenized cells. The specific methods used to grow the cells and toluenize them as well as the assay mixtures used to assess replicative and repair DNA synthesis were as described previously[2].

DNA Gyrase

DNA gyrase isolated from *Micrococcus luteus* was obtained from Bethesda Research Labs., Gaithersburg, Maryland. The enzyme was assayed in reaction mixtures containing in a total volume of 25 μl: Tris-HCl (pH 7.5) 35 mM, $MgCl_2$ 20 mM, KCl 20 mM, EDTA·Na_2 0.1 mM, ATP 1 mM, spermidine 2 mM, mercaptoethanol 10 mM, 10% glycerol, 0.5 μg of relaxed covalently closed pBR322 DNA and 1 U of gyrase. Incubation was at 37°C for 30 minutes. Relaxed circular covalently closed DNA was prepared by digesting super-coiled pBR322 DNA with a 2-fold excess of topoisomerase I followed by ligation with T4 DNA ligase according to standard procedures. The religated product contained mostly dimers of pBR322.

Antibacterial Activity

To assess antibacterial activity against whole *M. luteus* cells 0.015 μM of drug was dispensed on paper discs and the discs were placed on agar plates seeded with the indicator organism. Following incubation overnight the diameters of the resulting zones of inhibition were read in mm.

Results

Each of the compounds was initially tested in a system assessing replicative DNA synthesis. The system is based on permeabilized *E. coli* cells derived from a mutant deficient in DNA polymerase I, the repair enzyme. In this milieu DNA synthesis is dependent on the four deoxyribonucleotides, the chromosome-replicative enzyme complex in the cell and ATP as an energy source. This enzyme complex includes DNA gyrase which is essential for replicative DNA synthesis. The system should thus allow the tentative identification of DNA gyrase inhibitors although it will also respond to other inhibitors such as DNA binding agents and inhibitors of polymerase III.

The same system can be run in the repair mode. In this case no ATP is required and DNase is supplied to induce damage to the chromosomal template thus initiating repair DNA synthesis. This system does not require DNA gyrase functions and specific gyrase inhibitors have little or no effect on this system. An inhibitor of DNA gyrase should inhibit the replicative but not the repair system or the latter to a much lesser extent. Hence inhibitors found active in the DNA replication system were further tested in the repair system.

The third systemapplied was a direct test on the isolated gyrase in which case relaxed covalently closed pBR322 plasmid DNA served as the substrate of the enzyme. This system identifies gyrase inhibitors as well as DNA binding agents, the latter interacting with the DNA substrate.

Based on these three systems specific DNA gyrase inhibitors can be identified in that they should

Table 1. Effect of novobiocin-related coumarins on replicative and repair DNA synthesis.

Compound	Replicative DNA synthesis (% inhibition)	Repair DNA synthesis (% inhibition)	Activity vs. whole cell (mm)
Novobiocin	81	33	34
Chlorobiocic acid	77	14	19
Carbobenzoxycoumarin	60	7	16
3-Amino-4,7-dihydroxy-8-methylcoumarin (ring B)	0	—	0

Reaction mixtures (replication) contained in a total volume of 0.3 ml: Tris-HCl buffer (pH 8.0) 50 mM; KCl 0.1 M; $Mg(OAc)_2$ 10 mM; ATP 2 mM; dCTP, dGTP, dATP, 0.5 mM each; [^{3}H]TTP 0.02 mM containing 2.5 μCi/sample and approximately 2×10^8 *Escherichia coli* cells/sample. To assess DNA repair the samples contained no ATP but 0.15 μg of pancreatic DNase per sample. Incubation of the reaction mixtures was for 30 minutes at 37°C. In the replication assay the control samples incorporated approximately 15,500 cpm, in the repair assay 29,900 cpm, respectively. Drug concentrations were 0.05 mM. Antibacterial activity was assessed vs. *Micrococcus luteus* as the indicator organism. The data represents zones of growth inhibition in mm.

inhibit replicative DNA synthesis, have little or no effect on DNA repair synthesis but inhibit the gyrase assay.

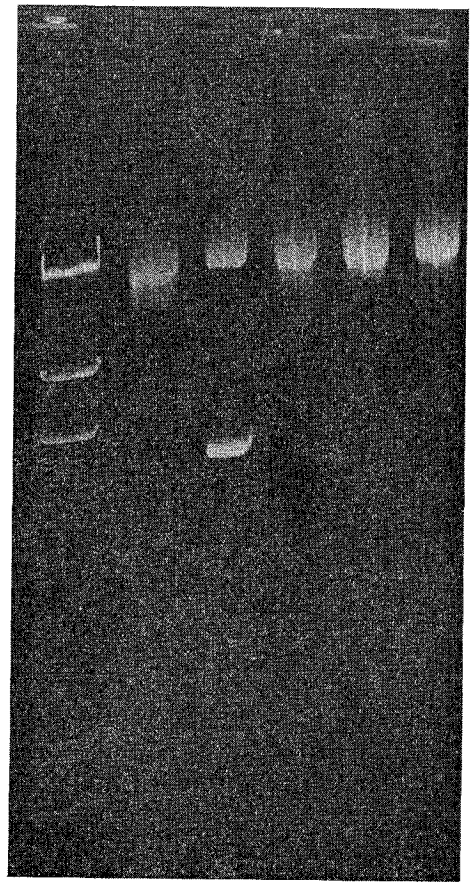
Several novobiocin-related coumarins were first tested for inhibition of the replication system. Two of these compounds, chlorobiocic acid and 3-(carbobenzoxyamino)-4,7-dihydroxy-8-methylcoumarin, significantly inhibited this system (Table 1). For the sake of brevity the latter compound will be referred to as carbobenzoxycoumarin in this communication. Chlorobiocic acid inhibited to the extent of 77%, carbobenzoxycoumarin to the extent of 60%, respectively. The coumarin residue as present in novobiocin (ring B) (3-amino-4,7-dihydroxy-8-methylcoumarin) did not inhibit. The structures of these compounds are shown in Fig. 1.

The two inhibitors of DNA replication mentioned above were further tested in the DNA repair system where only negligible inhibitions were found (Table 1). The specific DNA gyrase test revealed that both chlorobiocic acid as well as carbobenzoxycoumarin definitely inhibit DNA gyrase and thereby represent novel inhibitors of DNA gyrase (Fig. 2).

Chlorobiocic acid and carbobenzoxycoumarin both possess weak inhibitory activity against whole *M. luteus* cells in an agar diffusion test system. The unsubstituted coumarin struc-

Fig. 2. Sensitivity of DNA gyrase to novobiocin analogs.

Lanes from left to right: DNA-*Hin*d III digest, marker; relaxed pBR322 DNA, no gyrase; relaxed pBR322 DNA plus gyrase; novobiocin; chlorobiocic acid; carbobenzoxycoumarin. Drug concentration, 0.5 mM.



ture (ring B) showed no antibacterial activity. Since chlorobiocic acid as well as the carbobenzoxycoumarin appear nearly as potent as novobiocin as *M. luteus* gyrase inhibitors but possess very weak antibacterial activity against whole *M. luteus* cells, it follows that the former two compounds probably can not penetrate the bacterial cell envelope efficiently which means that they lack good transport functions across the cell membrane.

Discussion

In earlier studies with the novobiocin fragments novenamine and novobiocic acid, novenamine proved active and novobiocic acid inactive against DNA gyrase. Based on this observation we then concluded that novenamine was the active moiety in novobiocin[1]. The studies presented here require a modification of this claim. Chlorobiocic acid and carbobenzoxycoumarin both lack the sugar moiety present in novenamine and are still very potent inhibitors of DNA gyrase. The unsubstituted coumarin residue (ring B) *per se* possesses no inhibitory activity. On the other hand this coumarin moiety is the only entity present in all the compounds found active such as novenamine, chlorobiocic acid and carbobenzoxycoumarin. This suggests but does not definitely prove that the coumarin moiety might be the essential entity required to interact with gyrase. Assuming the correctness of this hypothesis and considering the fact that the unsubstituted coumarin (ring B) does not inhibit gryase would point to the importance of proper substituent groups to impart inhibitory activity to the coumarin moiety. Novobiocic acid differs structurally from chlorobiocic acid in that a chlorine atom replaces a methyl group in the former to give the latter. As novobiocic acid lacks gyrase inhibitory activity while chlorobiocic acid is quite active, this drastic difference in biological activity can be ascribed to the difference imparted by replacement of a methyl group with a chlorine atom.

The results presented do not exclude the possibility that despite the presence of the coumarin moiety (ring B) in all the active compounds the two new gyrase inhibitors described represent new inhibitors in their own right.

References

1) REUSSER, F. & L. DOLAK: Novenamine is the active moiety in novobiocin. J. Antibiotics 39: 272~274, 1986
2) REUSSER, F.: Ficellomycin and feldamycin; inhibitors of bacterial semiconservative DNA replication. Biochemistry 16: 3406~3412, 1977