Biological Activities of Novel Gyrase Inhibitors of the Aminocoumarin Class[∇]

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Thirty-one aminocoumarin antibiotics derived from mutasynthesis experiments were investigated for their biological activities. Their inhibitory activities toward *Escherichia coli* DNA gyrase were determined in two different in vitro assays: an ATPase assay and a DNA supercoiling assay. The assays gave a similar rank order of the activities of the compounds tested, although the absolute 50% inhibitory concentrations (IC₅₀s) obtained in each assay were different. To confirm that the compounds also acted as gyrase inhibitors in vivo, reporter gene assays were carried out with *E. coli* by using *gyrA* and *sulA* promoter fusions with the *luxCDABE* operon. A strong induction of both promoters was observed for those compounds that showed gyrase inhibitory activity in the biochemical assays. Compounds carrying analogs of the prenylated benzoyl moiety (ring A) of clorobiocin that were structurally very different showed high levels of activity both in the biochemical assay and in the reporter gene assay, indicating that the structure of this moiety can be varied considerably without a loss of affinity for bacterial gyrase. The experimentally determined IC₅₀s were compared to the binding energies calculated in silico, which indicated that a shift of the pyrrole carboxylic acid moiety from the O-3' to the O-2' position of the deoxysugar moiety has a significant impact on the binding mode of the compounds. The aminocoumarin compounds were also investigated for their MICs against different bacterial pathogens. Several compounds showed high levels of activity against staphylococci, including a methicillin-resistant *Staphylococcus aureus* strain. However, they showed only poor activities against gram-negative strains.

A major threat to therapy for human infections is the increase in the levels of antibiotic resistance and the continuing spread of nosocomial pathogens into the community (3, 21). Therefore, it is essential that new antibacterial drugs be developed. In this context, the reevaluation of previously discovered, but so far unexploited, classes of antibiotics has come into the focus of antibiotic research (31). The aminocoumarin antibiotics inhibit a well-validated drug target (DNA gyrase), but in contrast to the widely used fluoroquinolones, the aminocoumarins bind to the B subunit of the heterotetrameric gyrase enzyme. The binding site of the aminocoumarins overlaps with the ATP-binding site of gyrase, located on GyrB, and the aminocoumarins thereby inhibit the ATP hydrolysis required for ATP-dependent DNA supercoiling (25). In the same way, they inhibit DNA topoisomerase IV, which is a type II topoisomerase similar to gyrase and which is involved both in the control of DNA supercoiling and in the decatenation of daughter chromosomes after DNA replication. The aminocoumarins show strong activities against gram-positive pathogens like *Staphylococcus aureus* and *Staphylococcus epidermidis* (14, 15, 30, 37). Novobiocin (Albamycin; Upjohn) is the only aminocoumarin which has been licensed for the treatment of human infections, and its efficacy has been confirmed in several

clinical trials (32, 33, 38). Aminocoumarins also show the potential for use as anticancer drugs (4, 6, 20, 22, 23, 34).

Among the limitations of the aminocoumarins are their poor solubility in water and their poor oral absorption. Their low levels of activity against gram-negative bacteria were perceived as an additional drawback at the time of their discovery (15, 30); it may be argued, however, that this could also present an advantage, since gram-negative bacteria in the gut are not affected by these drugs.

Structurally, novobiocin and the closely related aminocoumarin clorobiocin (Fig. 1) are composed of a 3-dimethylallyl-4-hydroxybenzoyl moiety (ring A), a 3-amino-4,7-dihydroxycoumarin moiety (ring B) substituted with a methyl group and a chlorine atom, respectively, and a substituted deoxysugar (ring C) (Fig. 1). The 3'-OH of the deoxysugar is esterified with a carbamoyl group in the case of novobiocin and with a 5-methylpyrrole-2-carboxyl moiety in the case of clorobiocin. In contrast to the carbamoyl group of novobiocin, the 5-methylpyrrole moiety of clorobiocin is able to occupy an additional hydrophobic pocket in the GyrB subunit and to displace two water molecules (18). Thereby, clorobiocin binds more effectively to the GyrB subunit than novobiocin. The ring A moiety interacts only via hydrophobic bonds with the B subunit of gyrase and contributes only weakly to the antibacterial activity (16). However, ring A may influence the uptake of the compound into the bacterial cell (17).

Mutasynthesis, i.e., the feeding of synthetic precursor analogs to mutants of microbial producer strains of natural products, is an important and powerful tool for drug discovery and lead optimization. We recently generated a series of 31 new

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aminocoumarin compounds by addition of synthetic analogs of the ring A moiety to specific mutants of the novobiocin and clorobiocin producers (1, 2). In the current study, we utilized these compounds to investigate the structure-activity relationships of this class of gyrase inhibitors as a prerequisite for the development of aminocoumarin antibiotics with improved properties. Different biochemical and reporter gene assays, as well as a computational method and antibacterial assays, were used to examine the biological effects of these compounds, allowing a comparison of the results of these different methods. Specifically, two different biochemical assays were used, i.e., an ATPase assay and a supercoiling assay. The ATPase assay measured the ATP hydrolyzing activity of the B subunit of *Escherichia coli* DNA gyrase and its inhibition by aminocoumarin antibiotics (25). The supercoiling assay (24) measured the supercoiling activity of the intact *E. coli* gyrase heterotetramer; it exploited the fact that the formation of a DNA triplex (an alternative structure to the DNA double helix) is favored by the negative supercoiling of DNA. A plasmid containing a 20-bp insert with triplex-forming potential was partially supercoiled by the action of gyrase and subsequently captured by a biotinylated oligonucleotide which was bound to the streptavidin-coated surface of a microtiter plate. After unbound plasmids were washed out, the nucleic acids in the microtiter well were quantified by the use of a DNA-specific fluorescent dye. This assay allowed the rapid, automated determination of gyrase inhibition by the test compounds.

The experimentally obtained results were compared with the binding energies calculated from in silico docking studies by using Moloc software (www.moloc.ch). Furthermore, two reporter gene assays with living bacterial cells were used. The *gyrA* promoter responds to changes in the superhelical density of the DNA in the bacterial cell caused by gyrase inhibition. Therefore, fusions of this promoter to a reporter gene can be used to screen for inhibitors that attack at either subunit of gyrase (GyrA or GyrB). The SOS-inducible *sulA* promoter responds to agents that ultimately interfere with DNA replication (36). Inhibition of gyrase affects DNA replication and therefore leads to induction of the *sulA* promoter. The *gyrA* and *sulA* promoters were fused to the five-gene *luxCDABE* operon from *Photobacterium luminescens* for facile monitoring of kinetic responses (27).

The selected compounds were further investigated for their MICs against a panel of bacterial pathogens.

MATERIALS AND METHODS

Mutasyntetic generation of new aminocoumarins. Heterologous producer strains of novobiocin and the clorobiocin defective in the genes for the prenyltransferases NovQ or CloQ, respectively, were used for the mutasyntetic generation of new aminocoumarins. The prenyltransferase is required for the biosynthesis of the prenylated 4-hydroxybenzoate moiety (ring A) of novobiocin or clorobiocin (Fig. 1). Feeding of different synthetic ring A analogs to the *novQ*/*cloQ*-defective strains resulted in the formation of new aminocoumarin derivatives with modified substitution patterns. The range of new aminocoumarin antibiotics accessible from these mutasyntesis experiments was expanded by expression of the amide synthetase CouL or SimL, involved in the biosynthesis of other aminocoumarin antibiotics, and by two-step feeding strategies that successively used mutants of the novobiocin and the clorobiocin producer strains. Details of the generation of the new compounds and of their structural elucidation are described elsewhere (1, 2).

ATPase assay. The GyrB subunit used in the ATPase assay was purified from *E. coli* JMI09 containing the GyrB subunit expression plasmid described by Hallett et al. (12). The strain was grown at 37°C in LB broth containing 50 µg/ml ampicillin to an optical density (OD) of 0.5 (A_{595}). Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.05 mM, and cell growth was continued for 4 h. The cells were centrifuged at 10,000 × g and 4°C for 15 min. The cell pellet was resuspended in 50 mM Tris-HCl (pH 7.6), 10% (wt/vol) sucrose, 1 µg/ml RNase, 1 µg/ml DNase, and 20 µg/ml lysozyme. After incubation for 30 min at room temperature, the cells were disrupted by using a French press. The cell extract was dialyzed against TE (Tris-EDTA) buffer and applied to a heparin-Sepharose column (Pharmacia). After the column was washed with TE buffer, the column was eluted with a linear gradient of 0 to 1.0 M NaCl in TE buffer. Fractions containing the GyrB subunit were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, pooled, dialyzed against TE buffer, and loaded onto a fast-performance liquid chromatography Mono Q HR5/5 column (Pharmacia). After the column was washed with TE buffer, the column was eluted with a linear gradient of 0 to 0.2 M NaCl in TE buffer and then a linear gradient of 0.2 to 1.0 M NaCl in TE buffer. Fractions containing the GyrB subunit were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; pooled; dialyzed against 50 mM Tris-HCl (pH 8), 100 mM KCl, 5 mM dithiothreitol (DTT), 1 mM EDTA, and 10% (wt/vol) glycerol; frozen in liquid nitrogen; and stored at -80°C.

The ATPase assay was performed in 96-well microtiter plates, similar to a previously published method for Hsp90 (35). The reaction mixture contained 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM MgCl₂, 5 mM DTT, 1 mM EDTA, and 1 mM ATP. In each well of a 96-well microtiter plate, 25 μ l assay buffer was incubated with the *E. coli* GyrB subunit at a final concentration of 100 nM. Wells without the GyrB subunit were used as negative controls. Inhibitors, dissolved in dimethyl sulfoxide (DMSO), were added to the reaction mixtures at concentrations between 0.008 and 10 μ M, such that the final DMSO concentration was 4% (vol/vol). Wells not containing any inhibitors were used as positive controls. The plates were incubated at room temperature for 1 h. To stop the reaction, 80 μ l of malachite green reagent, prepared as described by Rowlands et al. (35), was added to each well, followed by the addition of 10 μ l of 34% aqueous sodium citrate to prevent nonenzymatic ATP hydrolysis. After 15 min at room temperature, the A_{620} was measured.

Supercoiling assay. A supercoiling assay, based on DNA triplex formation (24), was performed in black streptavidin-coated 96-well microtiter plates (Pierce). It contained 1 μ g relaxed pNO1 DNA, 35 mM Tris-HCl (pH 7.5), 24

mM KCl, 4 mM MgCl₂, 2 mM DTT, 1.8 mM spermidine, 1 mM ATP, 6.5% (wt/vol) glycerol, and 0.1 mg ml⁻¹ bovine serum albumin in a 30-μl volume. Inhibitors were added to the reaction mixtures at concentrations between 0.01 and 100 μM, such that the final DMSO concentration was 3% (vol/vol). The reactions were initiated by the addition of 10 nM GyrA and 9.3 nM GyrB, and the reaction mixtures were incubated at 37°C for 30 min. Termination, detection, and readout were carried out as described previously (24). SigmaPlot (version 10) software was used for data analysis. Selected compounds were also assayed by the conventional gel electrophoresis method to confirm the results (24).

Reporter gene assay. The luciferase operon *luxCDABE* of *Photobacterium luminescens* was placed under the control of the *gyrA* promoter of *E. coli* K-12 and cloned into low- to medium-copy-number plasmid pACYC184. The resulting plasmid was introduced into the hyperpermeable strain *E. coli* DC2 (5), in order to minimize problems of antibiotic uptake across the bacterial cell envelope. A similar construct was prepared by using the promoter of *sulA* from *E. coli* K-12. Therefore, plasmid pCGLS11 (9), which contains the genes coding for the luciferase operon (*luxC*, *luxD*, *luxA*, *luxB*, and *luxE*), was digested with EcoRI, and the fragment containing the whole luciferase operon was cloned into the vector pACYC184 (New England Biolabs). The resulting plasmid was linearized by partial digestion with EcoRI, filled in with the Klenow polymerase, and religated to destroy the EcoRI site 3' of the *luxE* gene. To delete the promoter of the chloramphenicol resistance gene, the resulting plasmid was digested with PvuII, and the band of 10.8 kb was isolated and circularized by ligation. The final plasmid, pLuxCDABE, was obtained by digesting the construct obtained as described above with EcoRI and XcmI. The band of 10.35 kb was ligated with two oligonucleotides, oligonucleotide 1 (5'-AATTCGAGCTCGGTACCCGGG CTGCAGCCATTAA-3') and oligonucleotide 2 (5'-TTAATGGCTGCAGCC CGGGTACCGAGCTCG-3'), to introduce a polylinker in front of the luciferase operon. The promoters *gyrA* and *sulA* of *E. coli* were amplified from the genomic DNA of *E. coli* strain DH10B by PCR. The PCR primers *gyrA*_1 (5'-CGACA TCGGGTACCTTTTGGCC-3') and *gyrA*_2 (5'-TCCCTCTACTGCAGCCCG GAT-3'), as well as primers *sulA*_1 (5'-GGTCAGGCGGTACCTGCCAAC-3') and *sulA*_2 (5'-CATAATCACTGCAGCCCCTGT-3'), were used for the amplification of the corresponding promoter. The PCR fragments were digested with KpnI and PstI and cloned into the same sites of pLuxCDABE.

E. coli DC2 (5) cells with the corresponding plasmid containing the promoter of the *E. coli* *sulA* or *gyrA* gene fused to the *luxCDABE* operon were cultured overnight at 37°C in LB medium with 2 μg/ml tetracycline. The overnight culture was diluted 1:100 with LB medium without tetracycline and incubated for 1.5 h at 37°C. Aliquots (100 μl) of this preculture were placed into microtiter plate wells, and inhibitors were added from stock solutions in DMSO, such that the final DMSO concentration was 1%. Novobiocin and clorobiocin were used as controls. After incubation of the microtiter plates at 37°C for 5 h, the *A*₅₉₅ and the bioluminescence were determined. For normalization of the light output with the bacterial growth, the light output was divided by the OD of the culture at 595 nm. The induction factors for a certain reporter gene construct with a given antibacterial compound were obtained by dividing the normalized light output in the presence of a compound through the normalized output in the absence of the compound.

MIC determination. The broth microdilution procedure recommended by the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (28) was used for determination of the MICs. The following strains were used: (i) strains with unaltered permeability, which included *Staphylococcus aureus* ATCC 29213, *Streptococcus pneumoniae* ATCC 49619, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212 (28); *Staphylococcus aureus* 80CR5 (7); *E. coli* UB1005 (5); *Pseudomonas aeruginosa* K799 (wild type) (5, 19); and *Streptococcus pneumoniae* TUPELO (26) and (ii) hyperpermeable strains *E. coli* DC2 and *Pseudomonas aeruginosa* K799/61 (5). All test strains were obtained from the bacterial collection of Basilea Pharmaceutica AG (Basel, Switzerland). The MICs were determined as described previously (10).

For testing of the aminocoumarins, were dissolved in DMSO; the maximum final concentration of DMSO in the assays was 2% (vol/vol). All the bacteria grew well in 2% DMSO in the absence of antibiotics.

In silico docking studies. The binding energies of the aminocoumarin structures to the GyrB subunit were calculated by using Moloc software (www.moloc.ch). First, the compounds were docked manually by using the structure of the *E. coli* GyrB 24-kDa domain in complex with clorobiocin (PDB code 1kzn) (16). If applicable, a binding mode similar to that of clorobiocin was used as the starting point. Subsequently, the complexes were optimized by using Moloc software with standard force field and optimization parameters. The positions of the protein residues and water molecules were kept invariable. The calculated binding energies were corrected by the energy term related to the conformational strain.

This energy term was estimated from the differences between the conformational energies of the compounds in the predicted binding conformation and after optimization in vacuum.

RESULTS

Panel of test compounds. The 31 aminocoumarin compounds tested in this study are listed in Table 1. In contrast to the 3-dimethylallyl-4-hydroxybenzoyl moiety (ring A) of clorobiocin, they contained 11 different structural analogs of that moiety. Other structural differences from the structure of clorobiocin included the nature of the substituent at position 8' of the aminocoumarin moiety, representing either chlorine, methyl, or hydrogen, and the position of the acyl substituent at the deoxysugar, being attached either to the 3'-OH group or to the 2'-OH group. All these compounds were derived from mutasynthetic feeding experiments, and the structures of all compounds have been confirmed by nuclear magnetic resonance and mass spectrometry (1, 2). Compounds 241, 311, 312, and 313 have been identified and partially investigated for their biological activities in previous studies (10), whereas the other 27 compounds were investigated here for the first time.

Activities against *Escherichia coli* DNA gyrase. The test compounds were investigated in vitro for their inhibitory effects on *E. coli* DNA gyrase in comparison with the inhibitory effects of the natural antibiotics novobiocin and clorobiocin. Two different assays were used: an ATPase assay and a supercoiling assay.

The ATPase assay measured the ATP hydrolyzing activity of the B subunit of *E. coli* DNA gyrase and its inhibition by aminocoumarin antibiotics (25). The assay buffer contained ATP, the gyrase subunit B, and different concentrations of the respective aminocoumarin. The supercoiling assay, based on DNA triplex formation (24), measured the activity of the intact *E. coli* gyrase heterotetramer.

All 31 test compounds were investigated by use of the ATPase assay. In accordance with previously reported results (8, 10), the five compounds lacking a pyrrole unit attached to the deoxysugar (i.e., novclobiocin compounds 204, 219, 315, 606, and 704) were found to be completely inactive. Therefore, these compounds were excluded from further investigation. Three compounds (novclobiocin compounds 372, 604, and 703) were not available in sufficient amounts, so 23 compounds were also investigated by use of the supercoiling assay.

Table 2 shows the 50% inhibitory concentrations (IC₅₀s) obtained by both assays as well as the ratios of the values from the two assays. In Table 2, the compounds are ranked according to their activities in the ATPase assay. As is immediately obvious, the rank order of activities obtained by both assays was similar; however, the absolute IC₅₀s obtained by the two assays showed clear differences (see Discussion).

In both assays, novclobiocin compounds 201 and 601 were the most active of the new compounds, with novclobiocin compounds 217 and 371 being only slightly less active. These four compounds carried completely different analogs of the ring A moiety: novclobiocin compound 201 carried a 3,5-dimethyl-4-hydroxybenzoyl group, novclobiocin compound 601 carried a benzoyl group, novclobiocin compound 217 carried a 3-propyl-4-hydroxybenzoyl group, and novclobiocin compound 371 carried a 4-methylthiobenzoyl group. These results support earlier

TABLE 1. Chemical structures of the compounds tested

Novclobiocin compound	R ₁	R ₂	2''	3''
201		Cl	Pyr ^a	OH
202		Cl	OH	Pyr
203		H	Pyr	OH
204 ^b		CH ₃	OH	OH
217 ^b		CH ₃	Pyr	OH
218 ^b		CH ₃	OH	Pyr
219 ^b		CH ₃	OH	OH
225 ^b		CH ₃	Pyr	OH
226 ^b		CH ₃	OH	Pyr
241		Cl	Pyr	OH
311		Cl	Pyr	OH
312		Cl	OH	Pyr
313		H	Pyr	OH
314 ^b		CH ₃	Pyr	OH
315 ^b		CH ₃	OH	OH
371		Cl	Pyr	OH
372		Cl	OH	Pyr
386 ^b		CH ₃	Pyr	OH
387 ^b		CH ₃	OH	Pyr
601		Cl	Pyr	OH
602		Cl	OH	Pyr
603		H	Pyr	OH
604 ^b		CH ₃	Pyr	OH
606 ^b		CH ₃	OH	OH
701		Cl	Pyr	OH
702		Cl	OH	Pyr
703 ^b		CH ₃	Pyr	OH
704 ^b		CH ₃	OH	OH
731		Cl	Pyr	OH
741		Cl	Pyr	OH
742		H	Pyr	OH

^a Pyr, pyrrole =

^b Structures resulted from two-stage feeding experiments.

TABLE 2. IC₅₀s determined by ATPase and triplex formation assays

Compound	IC ₅₀ (nM) by:		Ratio ^c	Concn (μM) required for fourfold or greater induction in reporter gene assays	
	ATPase assay ^a	Triplex formation assay ^b		<i>gyrA</i> promoter fusion	<i>sulA</i> promoter fusion
Novclobiocin 201	65 ± 7	17 ± 1	3.8	13	6.3
Novclobiocin 601	72 ± 6	<5 ± 0.7	ND ^d	3.1	3.1
Clorobiocin	73 ± 5	<5 ± 0.2	ND	1.6	<1
Novclobiocin 241	73 ± 6	41 ± 11	1.8	3.1	3.1
Novclobiocin 217	84 ± 5	19 ± 6	4.4	3.1	1.6
Novclobiocin 371	84 ± 5	20 ± 10	4.2	3.1	12.5
Novclobiocin 314	86 ± 8	25 ± 2	3.4	12.5	12.5
Novclobiocin 701	90 ± 20	34 ± 6	2.7	6.3	3.1
Novclobiocin 703	106 ± 24	ND	ND	NI ^e	50
Novclobiocin 225	116 ± 6	34 ± 6	3.4	1.6	3.1
Novclobiocin 311	122 ± 8	22 ± 6	5.6	25	6.3
Novclobiocin 604	131 ± 4	ND	ND	6.3	6.3
Novclobiocin 741	137 ± 15	32 ± 4	4.3	25	25
Novclobiocin 386	152 ± 27	44 ± 8	3.5	25	50
Novobiocin	154 ± 10	200 ± 20	0.8	3.1	6.3
Novclobiocin 313	185 ± 10	75 ± 10	2.5	25	25
Novclobiocin 603	219 ± 10	50 ± 10	4.4	50	50
Novclobiocin 203	229 ± 39	120 ± 20	1.9	>100	NI
Novclobiocin 731	263 ± 53	43 ± 4	6.1	6.3	3.1
Novclobiocin 742	276 ± 36	280 ± 70	1.0	50	50
Novclobiocin 372	280 ± 19	ND	ND	NI	12.5
Novclobiocin 602	297 ± 42	110 ± 40	2.7	>100	50
Novclobiocin 202	376 ± 172	320 ± 70	1.2	100	100
Novclobiocin 702	598 ± 144	1,200 ± 200	0.5	>100	NI
Novclobiocin 218	758 ± 67	140 ± 30	5.4	25	100
Novclobiocin 387	784 ± 43	190 ± 40	4.1	>100	100
Novclobiocin 312	891 ± 82	460 ± 70	1.9	NI	100
Novclobiocin 226	2,787 ± 512	460 ± 100	6.1	NI	100
Novclobiocin 204	>10,000	ND	ND	NI	NI
Novclobiocin 219	>10,000	ND	ND	3.1	NI
Novclobiocin 315	>10,000	ND	ND	NI	NI
Novclobiocin 606	>10,000	ND	ND	NI	NI
Novclobiocin 704	>10,000	ND	ND	NI	NI

^a The values represent the means ± standard deviations. Grafit 5 software was used to calculate IC₅₀s from single data points obtained at eight different concentrations.

^b The values represent the means ± standard errors of the means. Sigma Plot (version 10) software was used to calculate IC₅₀s from duplicate or triplicate data points obtained at eight different concentrations.

^c Ratio of the IC₅₀ by the ATPase assay to the IC₅₀ by the triplex formation assay.

^d ND, not determined.

^e NI, no induction was observed.

observations (16) that the ring A moiety lies outside of the principal binding pocket and makes only weak interactions with the bacterial gyrase, allowing considerable structural variability without a loss of activity.

The position of the 5-methylpyrrole moiety at the deoxy-sugar is very important for activity. Compounds carrying this group at position 3'' of the deoxysugar were, on average, sevenfold more active in the ATPase assay than the corresponding compounds which carried the 5-methylpyrrole moiety at position 2''.

In contrast, it was of minor importance whether the position 8' of the aminocoumarin moiety was substituted by a methyl group or a chlorine atom. Both kinds of compounds showed similar IC₅₀s in the ATPase assay (novclobiocin compound 311 versus novclobiocin compound 314, novclobiocin compound 601 versus novclobiocin compound 604, and novclobiocin compound 701 versus novclobiocin compound 703). However, if there was no substituent at position 8' of the aminocoumarin moiety, the activity was consistently lower than for the methyl-

or chlorine-substituted compounds (novclobiocin compound 201 versus novclobiocin compound 203, novclobiocin compounds 311 and 314 versus novclobiocin compound 313, and novclobiocin compounds 601 and 604 versus novclobiocin compound 603).

Reporter gene assays with *gyrA* and *sulA* promoter fusions. To further investigate the mode of action of the new aminocoumarin antibiotics, two cell-based reporter gene assays that used fusions of the *luxCDABE* operon to the *gyrA* or the *sulA* promoter, respectively, were used (see Introduction).

As shown in Fig. 2, increasing concentrations of novobiocin and clorobiocin resulted in increasing growth inhibition (decreasing OD). At concentrations of the antibiotic that caused marked but not complete growth inhibition, a strong induction of light emission was observed both from the *gyrA* promoter fusion and from the *sulA* promoter fusion. As expected, the concentrations required differed according to the potency of the inhibitor. For example, in order to reach at least a fourfold induction of light emission, 3 to 6 μM of novobiocin but only

FIG. 2. Growth inhibition and reporter gene induction in the *gyrA* and *sulA* reporter gene assays. See the text for explanations.

TABLE 3. Calculated binding energies of selected structures

Compound	Energy ^a	
	<i>E</i> (uncorr)	<i>E</i> (corr)
Novclobiocin 731	-67.1	-56.3
Novclobiocin 701	-61.2	-55.2
Novclobiocin 311	-66.2	-54.2
Novclobiocin 313	-64.1	-53.1
Novclobiocin 601	-61.9	-52.9
Novclobiocin 603	-59.9	-52.9
Clorobiocin	-68.5	-52.5
Novclobiocin 741	-63.5	-52.5
Novclobiocin 314	-66.0	-52.0
Novclobiocin 386	-66.0	-52.0
Novclobiocin 217	-66.4	-51.4
Novclobiocin 225	-66.7	-50.7
Novclobiocin 604	-61.6	-46.9
Novclobiocin 203	-62.8	-45.8
Novclobiocin 201	-64.9	-44.9
Novclobiocin 371	-64.7	-44.7
Novclobiocin 219	-50.7	-36.7

^a Energies (*E*) are given in Moloc energy units. *E*(uncorr), ligand/protein interaction energy; *E*(corr), energy corrected for conformational strain.

0.8 to 1.6 μ M of clorobiocin were required. Higher concentrations of novobiocin further increased the induction, until at concentrations of 50 to 100 μ M, light emission was reduced due to the effect of the antibiotic on cell viability. Higher concentrations of clorobiocin, in contrast, quickly reduced growth and light emission, reflecting the fact that clorobiocin is an extremely potent inhibitor of two vital targets in the cell, gyrase and topoisomerase IV (8).

The new aminocoumarin compounds, which were active as gyrase inhibitors in vitro, also showed induction of the *gyrA* and *sulA* promoters in the reporter gene assays. Figure 2 shows, as examples, the light emission and growth inhibition curves of a highly active inhibitor (novclobiocin compound 601), a less potent inhibitor (novclobiocin compound 603) and an inactive compound (novclobiocin compound 606).

Table 2 lists for each of the new aminocoumarins the lowest test concentration which resulted in at least fourfold induction of light emission in the respective assay. With very few exceptions, there was a good correlation between the gyrase inhibitory activities in vitro and the *gyrA* and *sulA* promoter-inducing activities in the cell-based reporter gene assay. This confirms that nearly all test compounds were active as gyrase inhibitors in the bacterial cell. Only a very few compounds with inhibitory activities in the ATPase assay showed a low level of or no activity in the cell-based reporter gene assays, e.g., novclobiocin compound 203, which also showed a low level of antistaphylococcal activity (see Table 4). The reason for the low level of in vivo activity of novclobiocin compound 203 is unknown. Therefore, the reporter gene assays, which require minimal amounts of the respective antibiotic and which can be carried out in an automated fashion, are suitable for the screening for novel gyrase inhibitors, although they may not detect every compound which shows some gyrase inhibition in vitro.

In silico docking studies and correlation with experimentally obtained activities. The energies of binding of a subset of the new aminocoumarin structures to the GyrB subunit were

calculated by using Moloc software (www.moloc.ch). As the first step, the compounds were docked manually by using the structure of the *E. coli* GyrB 24-kDa domain in complex with clorobiocin (PDB code 1kzn) (16). Interestingly, no suitable initial binding mode was found for derivatives acetylated in position 2'' of the deoxysugar sugar; therefore, these compounds were omitted from the calculation. Subsequently, the complexes were optimized by using Moloc software (see Materials and Methods). The results are summarized in Table 3.

Low binding energies (i.e., high negative numbers) reflect the tight binding of the compound to the bacterial gyrase. As a consequence, a compound with a low binding energy should also have a low IC₅₀. Comparison of the experimental values (Table 2) and the calculated values (Table 3) indicates that the majority of the compounds were correctly classified as stronger or weaker inhibitors. For example, the calculated binding energy of novclobiocin compound 219, which does not carry an acyl moiety at the deoxysugar, was clearly higher than that of the other compounds, reflecting the inactivity of this compound in the ATPase assay. An exception is compound 201, which showed high activity in the ATPase and supercoiling assays, while only weak binding to GyrB was predicted for this compound in silico. Analysis of the uncorrected energy values indicates that this is most likely based on the overestimation of the energy term for conformational strain.

Antibacterial activities of the new compounds. The MICs of 13 compounds, available in sufficient amounts, against a panel of clinically relevant gram-positive and gram-negative bacteria were determined (Table 4).

Six of the new compounds showed MICs of ≤ 0.25 μ g/ml for *Staphylococcus aureus* ATCC 29213. Even against methicillin-resistant strain *Staphylococcus aureus* ATCC 43300, similar activities were found. Novobiocin resistance is very uncommon in staphylococci (14), but nevertheless, we included novobiocin-resistant strain *Staphylococcus aureus* 80CR5 in our test panel. However, the new compounds were not active against this strain.

Most of the compounds tested also showed a certain amount of activity against *Streptococcus pneumoniae* strains, including *S. pneumoniae* SL336-T (which is resistant to erythromycin) and *S. pneumoniae* TUPELO (which is resistant to vancomycin). Of the two enterococcal strains tested, only *Enterococcus faecalis* ATCC 29212 was weakly affected by the new aminocoumarins. In contrast, *Enterococcus faecium* ATCC 19434 was resistant to all the compounds tested.

As expected (10), the aminocoumarins showed only poor activities against the gram-negative strains *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Only the hyperpermeable and efflux-defective strain *P. aeruginosa* K799/61 (19) was sensitive, in clear contrast to the parental strain *P. aeruginosa* K799/WT. The hyperpermeable strain *E. coli* DC2 (5) showed only low sensitivity.

DISCUSSION

In the present study, we compared a panel of new gyrase inhibitors, prepared by mutasynthesis, using in vitro assays, reporter gene assays, and antibacterial activity testing.

The ATPase assay, which measured the inhibition of the GyrB-catalyzed hydrolysis of ATP, was adopted from a pub-

TABLE 4. Effects of the new derivatives on growth of selected microorganisms

Compound	Growth inhibition (MIC [μ g/ml]) of:														
	<i>Staphylococcus aureus</i>			<i>Streptococcus pneumoniae</i>			<i>Enterococcus faecalis</i>	<i>Enterococcus faecium</i>	<i>Escherichia coli</i>			<i>Klebsiella pneumoniae</i>	<i>Pseudomonas aeruginosa</i>		
	ATCC 29213	ATCC 43300	80CR5	ATCC 49619	SL336-T	TUPELO	ATCC 29212	ATCC 19434	ATCC 25922	UB1005	DC2	ATCC 27736	ATCC 27853	K799/WT	K799/61
Clorobiocin	≤0.06	≤0.06	4	0.25	1	1	4	>32	32	>32	2	16	8	16	≤0.06
Novobiocin	0.25	0.25	>32	1	2	2	>32	>32	>32	>32	4	>32	>32	>32	1
Novclobiocin 201	0.125	0.25	32	4	8	8	16	>32	>32	>32	16	>32	>32	>32	1
Novclobiocin 203	4	4	>32	8	16	16	16	>32	>32	>32	>32	>32	>32	>32	8
Novclobiocin 217	≤0.06	≤0.06	32	2	2	4	16	>32	>32	>32	8	>32	>32	>32	0.5
Novclobiocin 225	≤0.06	≤0.06	16	1	2	2	8	>32	>32	>32	8	>32	>32	>32	0.5
Novclobiocin 241 ^a	2	4	>32	4	8	16	32	>32	>32	>32	16	>32	>32	>32	1
Novclobiocin 311 ^a	1	1	32	2	4	8	16	>32	>32	>32	32	>32	>32	>32	1
Novclobiocin 314	≤0.06	0.25	>32	1	2	4	8	>32	>32	>32	16	>32	>32	>32	2
Novclobiocin 371	0.125	0.25	32	2	4	16	32	>32	>32	>32	8	>32	>32	>32	2
Novclobiocin 386	1	1	>32	4	4	4	8	>32	>32	>32	>32	>32	>32	>32	4
Novclobiocin 701	0.25	1	>32	8	16	16	16	>32	>32	>32	32	>32	>32	>32	4
Novclobiocin 731	0.5	0.5	>32	8	8	16	32	>32	>32	>32	16	>32	>32	>32	2

^a The compounds were already investigated by Galm et al. (10).

lished method for measuring the inhibition of the ATPase activity of heat shock protein 90 (35) and provided a rapid, easily automated first screen. The supercoiling assay, based on the differential formation of DNA triplexes in supercoiled versus relaxed DNAs (24), measures the supercoiling activity of the intact gyrase heterotetramer. In contrast to conventional supercoiling assays, which measure gyrase activity by the gel electrophoretic mobilities of relaxed and supercoiled plasmids, the triplex formation assay offers the advantage that it is sensitive over the full range of superhelical densities and is much easier to quantitate (24). As shown in Table 2, both the ATPase and the supercoiling assays provided a very similar rank order for the new compounds regarding their potencies as gyrase inhibitors. The absolute IC₅₀s, however, were clearly different for both assays, reflecting differences in the reaction conditions and protein concentrations used in the assays. It should be noted that the IC₅₀s determined for the gyrase inhibitors are strongly dependent on assay conditions, and this problem is especially prominent for the aminocoumarin antibiotics. Therefore, the published values for the IC₅₀s of the aminocoumarins vary considerably between different studies (8, 13, 17, 29). Our study shows that both biochemical assays used in this study are suitable for the screening of gyrase inhibitors but that the inhibitory concentrations obtained in both assays cannot be directly compared.

The *gyrA* reporter gene assay responds to changes in the superhelical density of DNA in the bacterial cell, caused by gyrase inhibition. Therefore, it can be used to screen for inhibitors that attack at either subunit of gyrase. The inhibition of gyrase affects DNA replication and therefore leads to the induction of the *sulA* promoter (36). The results from the *gyrA* and *sulA* reporter gene assays (Table 2) therefore showed a close correlation to each other and also correlated quite well with the data from the in vitro assays. Luciferase genes are now widely used reporter genes in prokaryotic and eukaryotic systems because they provide a simple approach for the real-time detection of gene expression and regulation (11). The use of the five-gene *luxCDABE* operon from *Photobacterium luminescens* allows facile monitoring of kinetic responses because all components necessary for light production are present in the

cell, thus obviating the need for cell lysis and substrate addition (27).

To investigate whether the new compounds have a binding mode similar to that of clorobiocin, the experimentally determined data were compared with the energies of binding to *E. coli* GyrB calculated in silico. The comparison shows that the calculated binding energies correctly rank the affinities of the majority of the compounds, which indicates that the compounds with the pyrrole moiety in position 3' of the deoxysugar most probably follow the binding mode observed for clorobiocin. In contrast, the prediction of binding energies was not possible for the compounds with the pyrrole moiety in position 2' of the deoxysugar. For these compounds, no binding mode which follows the typical pattern of interaction with the aspartate residue and water molecules could be identified (16). This observation indicates that these compounds could have untypical binding modes.

MICs were determined against *Staphylococcus* and *Streptococcus* strains as well as other relevant pathogens. The most active antistaphylococcal compounds, besides clorobiocin, were novclobiocin compounds 217 and 225, which contain nonpolar alkyl side chains attached to their benzoyl moieties. However, novclobiocin compounds 201, 314, 371, and 731, which contain ring A analogs which are structurally quite different from the genuine ring A, were also highly active, with their activities equaling or even exceeding the activity of the clinically introduced novobiocin. In contrast, novclobiocin compound 241, which also showed good in vitro activity in the ATPase and supercoiling assays as well as in the reporter gene assay, showed clearly lower antistaphylococcal activity. Since novclobiocin compound 241 contains a polar side chain attached to the benzoyl moiety, this observation may confirm earlier speculations that the nonpolar side chain attached to the benzoyl moiety of novobiocin and clorobiocin may be important for the uptake of these antibiotics into bacteria (17). Interestingly, compounds which carried cinnamic acid or 3-methoxy-4-hydroxycinnamic acid as ring A analogs (novclobiocin compounds 701 and 731) showed quite high levels of activity against staphylococci. At least for novclobiocin compound 731, this was not predicted by the in vitro activity against *E. coli*

gyrase in the ATPase assay (Table 2). Replacement of the chlorine atom at position 8' of the aminocoumarin moiety of novobiocin compound 201 with a proton (which gave novobiocin compound 203) resulted in clearly reduced antibacterial activity (novobiocin compounds 201 and 203).

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