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Inhibition of DNA gyrase and DNA topoisomerase IV of *Staphylococcus aureus* and *Escherichia coli* by aminocoumarin antibiotics

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Objectives: Aminocoumarin antibiotics are potent inhibitors of bacterial DNA gyrase. We investigated the inhibitory and antibacterial activity of naturally occurring aminocoumarin antibiotics and six structural analogues (novclobiocins) against DNA gyrase and DNA topoisomerase IV from *Escherichia coli* and *Staphylococcus aureus* as well as the effect of potassium and sodium glutamate on the activity of these enzymes.

Methods: The inhibitory concentrations of the aminocoumarins were determined in gyrase supercoiling assays and topoisomerase IV decatenation assays. Both subunits of *S. aureus* topoisomerase IV were purified as His-Tag proteins in *E. coli*. The MIC was tested *in vivo* for the control organisms *E. coli* ATCC 25922 and *S. aureus* ATCC 29213.

Results: DNA gyrase is the primary target *in vitro* of all investigated aminocoumarins. With the exception of simocyclinone D8, all other aminocoumarins inhibited *S. aureus* gyrase on average 6-fold more effectively than *E. coli* gyrase. Potassium glutamate is essential for the activity of *S. aureus* gyrase and increases the sensitivity of *E. coli* gyrase to aminocoumarins ≥10-fold. The antibacterial activity of the tested compounds mirrored their relative activities against topoisomerases.

Conclusions: The study provides insights about the substituents that are important for the inhibitory activity of aminocoumarins against the target enzymes, which will facilitate the rational design of improved antibiotics.

Keywords: potassium glutamate, MICs, supercoiling assays, decatenation assays

Introduction

DNA type II topoisomerases, which transiently cleave both strands of DNA,[1,2] are ubiquitous enzymes responsible for controlling the topological state of DNA. Bacterial type II topoisomerases, i.e. DNA gyrase and DNA topoisomerase IV (topo IV), are heterotetrameric enzymes[1] ($GyrA_2GyrB_2$ and $ParC_2ParE_2$, respectively). They are essential to the cell, which makes them attractive targets for developing more effective antimicrobials to fight the ever-increasing threat of resistance to existing antibiotics in pathogenic bacteria, particularly methicillin-resistant *Staphylococcus aureus* (MRSA).[3–6] DNA gyrase introduces negative supercoils into DNA, which is essential for DNA replication, elongation and transcription. In contrast, the primary function of topo IV is the decatenation of multiply linked daughter chromosomes during the terminal stages of DNA replication. The activity of both enzymes is energetically driven by the hydrolysis of ATP, catalysed by the GyrB subunit and ParE subunit, respectively.[1,2,7]

The aminocoumarin antibiotics (Figure 1), natural products of various *Streptomyces* strains,[8] are powerful inhibitors of gyrase, binding to this target with higher affinity than modern fluoroquinolones.[3] Aminocoumarins bind to the GyrB subunit of gyrase or the ParE subunit of topo IV, competing with the binding of ATP.[3,9,10] The interest in aminocoumarins has been stimulated by recent biochemical and X-ray crystallographic evidence showing that the aminocoumarin antibiotic simocyclinone D8 (Figure 1) inhibits gyrase by a completely new mode of action, interacting with two separate pockets of the enzyme and thereby preventing its binding to DNA.[11–13]

In vitro investigations with *S. aureus* gyrase are complicated by the fact that this enzyme requires high concentrations of potassium glutamate (K-Glu) for its activity (400–800 mM K-Glu). This is in contrast to *Escherichia coli* gyrase, which catalyses the supercoiling reaction at an optimal concentration of 100–200 mM K-Glu.[14,15]

In the present study, we aimed to perform a detailed comparison of the effect of aminocoumarin antibiotics on gyrase and topo IV from *E. coli* and *S. aureus*. We established the conditions for assaying the inhibitory effects of aminocoumarin antibiotics against these enzymes, and subsequently determined the *in vitro* target preference of naturally occurring aminocoumarin

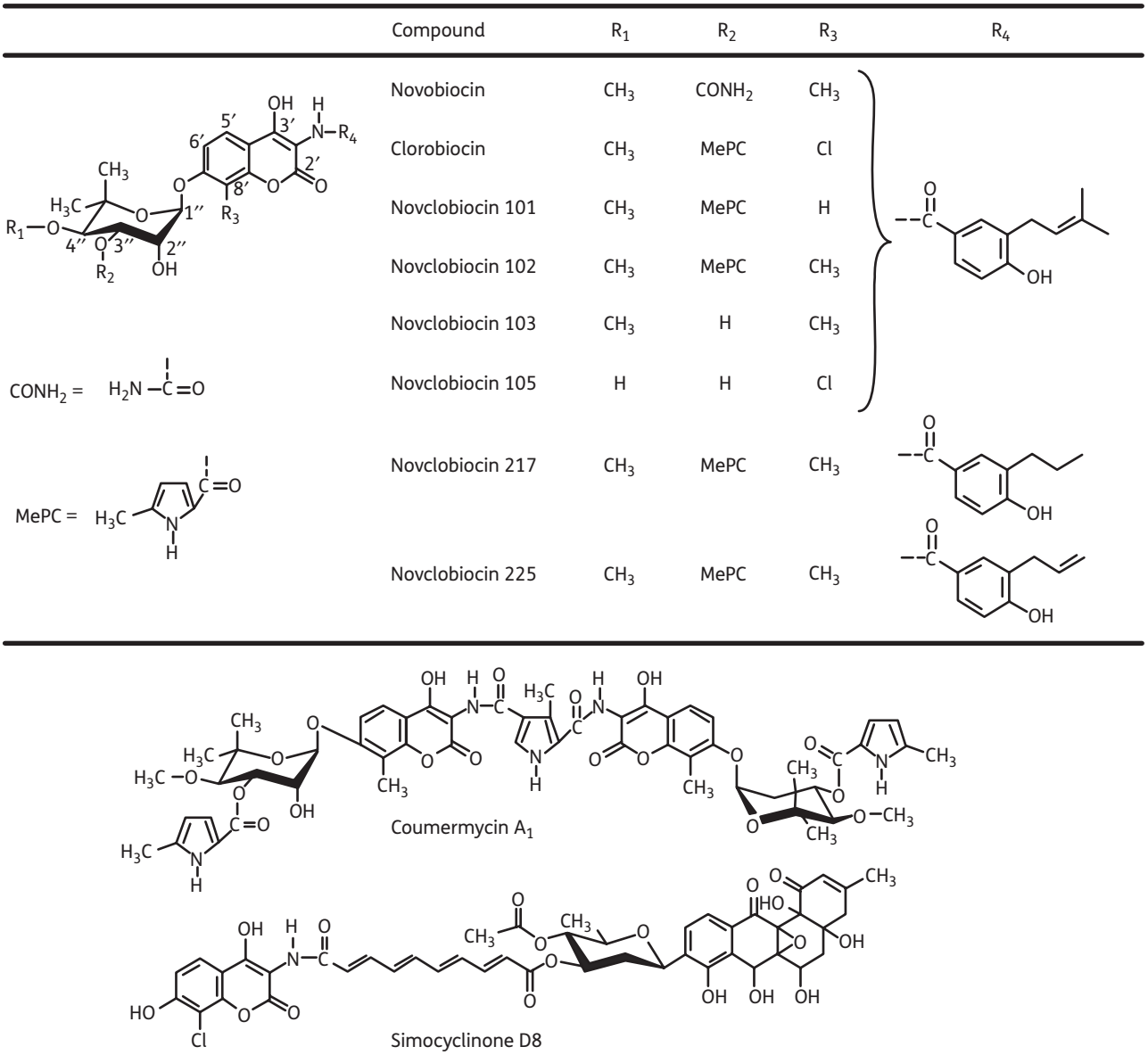

Figure 1. Chemical structures of aminocoumarin antibiotics.

antibiotics and of several derivatives thereof (novclobiocins), obtained from mutasynthesis and metabolic engineering experiments.[16–18] The results will be useful for the further investigation of type II topoisomerase inhibitors, both from the aminocoumarin class and from other structural classes.[4]

Materials and methods

DNAs and proteins

Nucleic acids were manipulated using standard methods[19] or instructions provided by the manufacturers of restriction enzymes and kits. Relaxed pBR322 DNA and kDNA (from *Crithidia fasciculata*), *E. coli* gyrase and topo IV, and *S. aureus* gyrase were obtained from Inspiralis (Norwich, UK).

Chemicals

New aminocoumarin compounds were obtained by metabolic engineering, mutasynthesis and chemoenzymatic synthesis, as described previously.[16,20–22] These compounds were dissolved in a small volume of DMSO and the solutions were diluted with water to a final concentration of 5% DMSO. K-Glu was purchased from Fluka. Sodium glutamate (Na-Glu), novobiocin and coumermycin A_1 were purchased from Sigma–Aldrich. Simocyclinone D8 was a gift from H.-P. Fiedler (University of Tübingen, Germany).

Cloning, protein expression and purification

S. aureus gyrase, as well as *E. coli* topo IV and gyrase, were available commercially, but it was necessary to generate *S. aureus* topo IV for our study (recently, *S. aureus* topo IV has also become available commercially from Inspiralis).

For construction of ParC and ParE overexpression plasmids, *S. aureus* RN4220 *parC* and *parE* (GenBank D67075) were PCR-amplified using plasmids pET11c-S*aparC* and pET11c-S*aparE*, kindly provided by H. Hiasa (University of Minnesota, MN, USA), as a template.[15] The following oligonucleotides were used as primers: N-terminus of ParC, 5′-AAA GGC ATG CAT AGT GAA ATA ATT CAA GAT TTA TCA CTT-3′; C-terminus of ParC, 5′-GGA AGA TCT GCT AAT ATA CAT GTC TAT TAC TTC AC-3′; N-terminus of ParE, 5′-AAA GGC ATG CAT AAT AAA CAA AAT AAT TAT TCA GAT GAT TCA ATA-3′; and C-terminus of ParE, 5′-GGA AGA TCT GAT TTC CTC CTC ATC AAA TTG ATC-3′. *parC* and *parE* were cloned into the SphI and BglII restriction sites of the pQE70 (QIAGEN) expression vector, and transformed into *E. coli* BL21/pREP4 cells (Novagen; QIAGEN). For protein expression, cultures were grown at 37°C in 1 L of Luria–Bertani medium[23] to OD_{600}=0.7; 1 mM IPTG was added and growth continued for 4 h. After centrifugation, cell pellets were resuspended in 25 mL of lysis buffer [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 1% Tween 20, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM dithiothreitol (DTT), 20 mM imidazole and 0.5 mg/mL lysozyme] and sonicated to release soluble proteins. Insoluble material was removed by centrifugation and the supernatant loaded onto a 5 mL His-Trap™ HP column (GE Healthcare) that had been equilibrated previously with buffer A [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol and 20 mM imidazole]. The column was eluted with a linear gradient from 20 to 250 mM imidazole (30 min; 1 mL/min) and the eluates were dialysed against buffer B [25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM DTT and 15% glycerol]. Ni^{2+}-affinity chromatography resulted in ∼7 mg of purified ParC and 5 mg of purified ParE per litre of culture. Absorbance at 280 nm was measured to calculate the protein concentration. SDS–PAGE of the purified topo IV subunits revealed bands with apparent molecular weights of 96.2 and 77.8 kDa, corresponding to ParC and ParE, respectively. Mixing of equimolar amounts of the two subunits resulted in active topo IV.

DNA gyrase supercoiling assays and topo IV decatenation assays

1 U of enzyme (gyrase or topo IV, Inspiralis) converts 0.5 μg of relaxed pBR322 DNA to the supercoiled form (gyrase)[24] or decatenates 200 ng of kinetoplast DNA (topo IV). Enzyme activity was detected by incubation for 45 min at 37°C in a total reaction volume of 30 μL. Standard reaction mixtures for the gyrase supercoiling assays contained 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 700 mM K-Glu, 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM spermidine, 1 mM ATP, 6.5% (w/v) glycerol and 0.1 mg/mL albumin. Topo IV activity was measured by using a decatenation assay that monitored the ATP-dependent unlinking of DNA minicircles from kDNA containing 40 mM HEPES-KOH (pH 7.5), 100 mM K-Glu, 10 mM magnesium acetate, 10 mM DTT, 1 mM ATP and 50 μg/mL albumin. Typically, the supercoiling assays contained 1 U of gyrase (corresponding to 20 ng of *E. coli* or *S. aureus* enzyme, respectively) and decatenation assays contained 1 U of topo IV (corresponding to 18.7 or 200 ng of *E. coli* or *S. aureus* enzyme, respectively).

The reactions were stopped by adding an equal volume of stop buffer [40% sucrose, 100 mM Tris-HCl (pH 7.5), 100 mM EDTA and Bromophenol Blue], followed by extraction with 1 volume of chloroform/iso-amyl alcohol (24:1). Then, 20 μL of the aqueous phase of each sample was analysed on 1% agarose gels for 4 h at 80 V in Tris/acetate/EDTA buffer and visualized after staining with ethidium bromide.

Prior to gel electrophoresis, samples of the supercoiling assays were subjected to a buffer exchange with 10 mM Tris-HCl (pH 8.0), performed by dialysis with MF™-membrane filters (Millipore, 0.025 μm VSWP). The aqueous phase of the assay mixtures was pipetted onto the floating membranes in a Petri dish. After 3 h, the assay mixtures were removed from the membranes and mixed with 15 μL of loading buffer (50% water, 49.75% glycerol and 0.25% Bromophenol Blue). The IC_{50} was defined as the concentration causing 50% inhibition of the supercoiling or of the decatenation reaction.

The IC_{50} for inhibition of supercoiling and decatenation, respectively, can be visually assessed as the concentration of compound that leads to a 50% reduction in the supercoiling or minicircle band, respectively. IC_{50} values are averages from at least three separate experiments. For testing the effect of K-Glu on the activity of gyrase and topo IV, buffers were mixed without this component.

MIC determination

The broth microdilution procedure recommended by Wiegand *et al.*[25] was used to determine the MICs. *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were used as test strains, and were obtained from the Pasteur Institute (Paris, France).

Results

Removal of K-Glu from the assays for DNA gyrase activity

We established assay conditions for the measurement of the inhibition of *E. coli* and *S. aureus* DNA gyrase and topo IV by aminocoumarin antibiotics. As described by Morgan-Linnell *et al.*[26] and Pan and Fisher,[27] for investigation of the inhibitory action of agents against topo IV the decatenation assay is most appropriate, while the most relevant assay for agents like aminocoumarins, acting on gyrase, is the inhibition of supercoiling. The

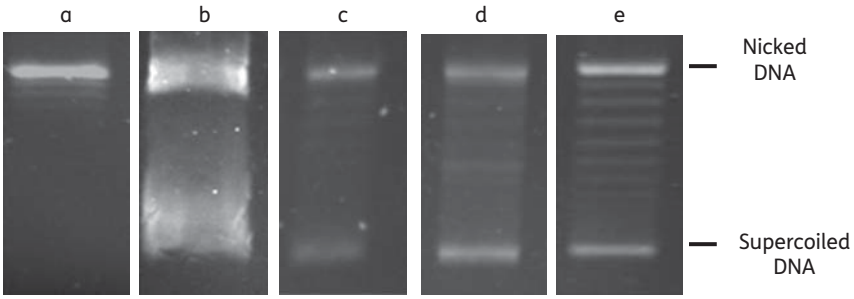

Figure 2. Electrophoretic analysis of *S. aureus* DNA gyrase supercoiling assays using different methods for K-Glu removal. Lane a, assay without K-Glu (the enzyme is inactive under these conditions); lanes b–e, assays with 700 mM K-Glu; lane b, without removal of K-Glu; lane c, addition of 700 mM 18-Crown-6; lane d, desalting with QIAprep Spin Miniprep Kit for plasmid DNA purification (QIAGEN); and lane e, dialysis against 10 mM Tris-HCl buffer (pH 8.0) with 0.025 μM membrane filters MF™ VSWP (Millipore).

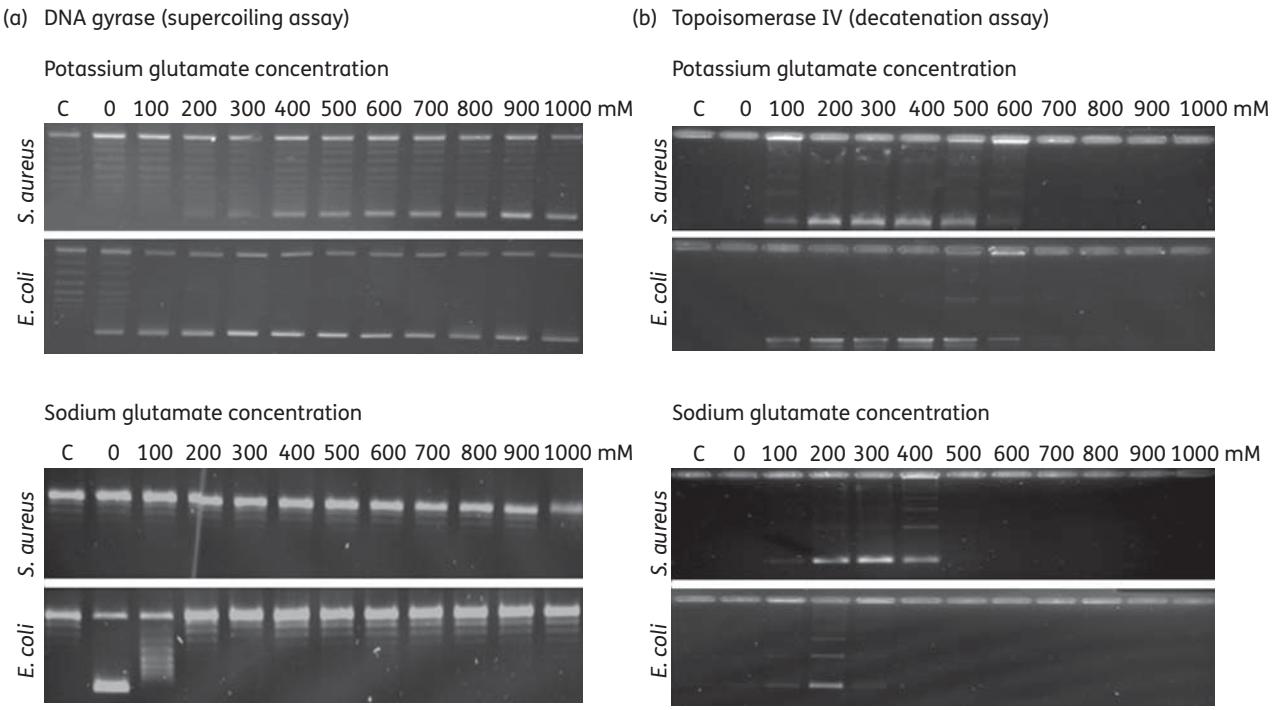

Figure 3. Effect of K-Glu and Na-Glu on *S. aureus* and *E. coli* gyrase in supercoiling assays (a) and on topo IV in decatenation assays (b). The supercoiling reaction mixtures contained relaxed pBR322 DNA, DNA gyrase, and the indicated concentrations of K-Glu and Na-Glu. The decatenation reaction mixtures contained kDNA, topo IV, and the indicated concentrations of K-Glu and Na-Glu. The first lane, labelled 'C', contains a control assay without enzyme. In the DNA gyrase assays, the lower band shows supercoiled DNA. In the topo IV assays, the lower band shows decatenated DNA.

activity of *S. aureus* gyrase is dependent on high concentrations of K-Glu.[14,15] However, we found that K-Glu concentrations >400 mM impaired the resolution in the gel electrophoresis analysis of the supercoiling assays (Figure 2, lane b). We tested different methods for desalting the samples before loading them onto agarose gels. The addition of 700 mM 18-Crown-6 (Fluka) after the reaction, intended to complex the potassium cations, led to an undesirable reduction of the intensity of the bands on the agarose gel (Figure 2, lane c). Purification of the assay products with the QIAprep Spin Miniprep Kit for plasmid DNA purification (QIAGEN) provided better results (Figure 2, lane d). By far the best resolution and visualization of the different topoisomers of relaxed DNA was achieved by dialysis against 10 mM Tris-HCl buffer (pH 8.0) using membrane filters (0.025 μm pore diameter) (Figure 2, lane e), and therefore this method was used for all further gyrase assays. No dialysis was required for topo IV activity tests. Kinetoplast DNA was used as the substrate in these assays and the resolution in agarose gel electrophoresis was satisfactory without the removal of K-Glu (Figure 3b). Furthermore, *S. aureus* topo IV required a lower concentration of K-Glu for activity than gyrase (see below).

Effect of K-Glu on the activity of DNA gyrase and topo IV of E. coli and S. aureus

Previous investigations had shown that K-Glu has an important influence on the activities of bacterial type II topoisomerases. The optimal K-Glu concentration is different for the *S. aureus* and *E. coli* enzymes, and different for DNA gyrase and topo IV. Even for the same enzyme of a given origin, the optimal K-Glu concentration varies for different assay types, e.g. for the supercoiling and the DNA cleavage assay in the case of *S. aureus* gyrase, or for the decatenation and the relaxation assay in the case of topo IV.[14,15,28] We investigated the influence of K-Glu and Na-Glu at concentrations of 0–1000 mM on the four enzymes relevant to our study, i.e. gyrase and topo IV from both *S. aureus* and *E. coli*, using the supercoiling assay for gyrase and the decatenation assay for topo IV (Figure 3).

S. aureus gyrase and topo IV were both inactive in the absence of K-Glu. Gyrase activity was detectable at 300–1000 mM K-Glu, with an optimal concentration of ~900 mM. Topo IV activity was detectable at 100–500 mM K-Glu, with optimal activity at 200–400 mM. These data are in good agreement with the results of previous studies on the influence of K-Glu on *S. aureus* topoisomerases.[14,15,28,29]

E. coli gyrase is active in the absence of K-Glu and this may be the reason that only sparse systematic data are available on the influence of K-Glu on type II topoisomerases of *E. coli*.[15] Our investigations confirmed that *E. coli* gyrase does not require K-Glu for activity, but also showed that inclusion of 200–500 mM K-Glu moderately stimulates the activity of this enzyme. Using the enzyme amount in our standard decatenation assay, the activity of *E. coli* topo IV was not detected in the absence of K-Glu, but was clearly observed in the presence of 100–500 mM of this salt; the optimal concentration range was 200–400 mM

(Figure 3). Therefore, also *E. coli* topo IV is clearly stimulated by K-Glu.

Blanche *et al.*[14] reported that for stimulation of *S. aureus* gyrase in the supercoiling assay, K-Glu could be replaced by its enantiomer, e.g. the potassium salt of D-Glu, but not by Na-Glu or KCl. Our investigations confirmed that *S. aureus* gyrase is inactive in the presence of Na-Glu, irrespective of the concentration (Figure 3). In clear contrast, *S. aureus* topo IV is stimulated by Na-Glu (200–400 mM), although less than by K-Glu. Also, *E. coli* topo IV is weakly stimulated by 200 mM Na-Glu. Notably, *E. coli* gyrase is completely inhibited by Na-Glu at concentrations of ≥200 mM (Figure 3). To test whether potassium is responsible for the catalytic activity of *S. aureus* gyrase, we performed a similar series of supercoiling assays with 0–1000 mM KCl or NaCl, because it has been shown that a monovalent cation is required for the ATPase activity of this family of enzymes with some specificity for the cation.[30] *S. aureus* gyrase was inactive regardless of the concentration of KCl or NaCl, while the *E. coli* enzyme lost activity at ≥100 mM of either KCl or NaCl (data not shown). Since *E. coli* gyrase does not require and is not affected by K-Glu, the lack of activity observed when adding Na-Glu could indicate that the enzyme prefers potassium and that sodium is competing for binding to the enzyme. To test this hypothesis, we performed supercoiling assays in the presence of 100 mM Na-Glu and increasing concentrations of K-Glu up to 400 mM, but K-Glu did not restore enzyme activity.

K-Glu modulates the sensitivity of E. coli DNA gyrase to aminocoumarin antibiotics

Previous studies have shown that the sensitivity of DNA gyrase to quinolones is modulated by K-Glu.[15,29] We now tested the influence of K-Glu on the sensitivity of *E. coli* gyrase to aminocoumarin antibiotics. *E. coli* gyrase was ~10-fold more sensitive to novobiocin, clorobiocin and novclobiocin 101 (Figure 1) in the presence of 700 mM K-Glu than in the absence of this salt (Figure 4). Unexpectedly, the effect of K-Glu was much more pronounced in the case of novclobiocin 103, which is an analogue of novobiocin lacking the acyl substituent, which is attached in the naturally occurring antibiotic (Figure 1) to the 3-OH group of the deoxysugar moiety. *E. coli* gyrase was only weakly inhibited by this compound in the absence of K-Glu, but sensitivity increased by a factor of 150 in the presence of K-Glu. X-ray crystallographic studies have shown that the carbamoyl group of novobiocin occupies a distinct binding pocket in the GyrB protein, providing an important interaction with the target.[3] Apparently, the relative importance of this particular interaction in the overall binding of the antibiotic is reduced in the presence of K-Glu. The concentration of K-Glu is therefore an important consideration when the inhibitory effects of aminocoumarins on gyrase from *E. coli* and *S. aureus* are compared. We decided to subsequently use 700 mM K-Glu in assays of gyrases from both organisms, i.e. the concentration recommended by Blanche *et al.*[14] for assays of *S. aureus* gyrase.

The other three enzymes in our study, i.e. gyrase from *S. aureus* and topo IV from both *S. aureus* and *E. coli*, did not show detectable activity in the absence of K-Glu under our assay conditions; therefore, the influence of K-Glu on their sensitivities to aminocoumarins could not be tested in an experiment similar to that described above for *E. coli* gyrase. We decided to use 100 mM K-Glu in subsequent assays for topo IV from both organisms, which is the concentration used in several previous studies[16,31] and which is recommended by the commercial suppliers of topo IV, i.e. Inspiralis and Topogen (Columbus, OH, USA); our data show that the enzyme activity is higher at 200–400 mM K-Glu (Figure 3).

Inhibition of DNA gyrase and topo IV from E. coli and S. aureus by different aminocoumarin antibiotics

Using the assay conditions established in the preceding experiments, we investigated the inhibitory effect of different aminocoumarin antibiotics on DNA gyrase and topo IV from *E. coli* and *S. aureus*. We tested the three 'classical' aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1, as well as the structurally different simocyclinone D8 (Figure 1). We also included several novobiocin and clorobiocin derivatives (termed 'novclobiocins'), which we had obtained in previous mutasynthesis and metabolic engineering experiments.[16,20–22] Novobiocin, clorobiocin and coumermycin A_1 were on average 6-fold more active against *S. aureus* gyrase than against *E. coli* gyrase (Table 1). The inhibitory concentrations were in the range of 6–10 nM, i.e. three orders of magnitude lower than reported for fluoroquinolones, such as ciprofloxacin, ofloxacin or sparfloxacin.[27,32,33] This confirms the potency of aminocoumarins as gyrase inhibitors in Gram-positive pathogens. However, the activity of the aminocoumarins against *S. aureus* topo IV was much weaker (Table 1). These biochemical data suggest that gyrase is the primary target of the tested aminocoumarins in *S. aureus*. This is supported by the observation by Fujimoto-Nakamura *et al.*,[34] who showed that cultivation of *S. aureus* in the presence of novobiocin results first in the selection of mutants with altered gyrase. The use of higher concentrations of novobiocin additionally results in mutations of topo IV as a second step in the emergence of resistance.

Simocyclinone D8, an aminocoumarin antibiotic with a completely new mode of action,[11,12] showed similar activity to novobiocin against *E. coli* gyrase, but was much less active against *S. aureus* gyrase. Its effect on *S. aureus* topo IV was weak and it was essentially inactive against the corresponding *E. coli* enzyme (Table 1).[13]

Novclobiocin 101, which is very similar in structure to clorobiocin but lacks the chlorine in position 8 of the aminocoumarin moiety (Figure 1), showed 8-fold lower inhibitory activity against *S. aureus* gyrase than clorobiocin (Table 1). If the chlorine atom of clorobiocin is replaced by a methyl group (novclobiocin 102), there is 2-fold reduction in activity compared with clorobiocin. These biochemically determined ratios of activities between clorobiocin, novclobiocin 101 and novclobiocin 102 are identical to those determined earlier in a disc diffusion assay against *Bacillus subtilis*.[21]

When the acyl substituent at position 3 of the deoxysugar of novobiocin is removed, resulting in novclobiocin 103, the activity against *S. aureus* gyrase is reduced by a factor of 100. When additionally the methyl group of the 4-methoxy group at the deoxysugar moiety of clorobiocin is removed, activity is lost completely (novclobiocin 105).

Novclobiocins 217 and 225 are clorobiocin derivatives in which the alkyl side chains of the 4-hydroxy-benzoyl moieties

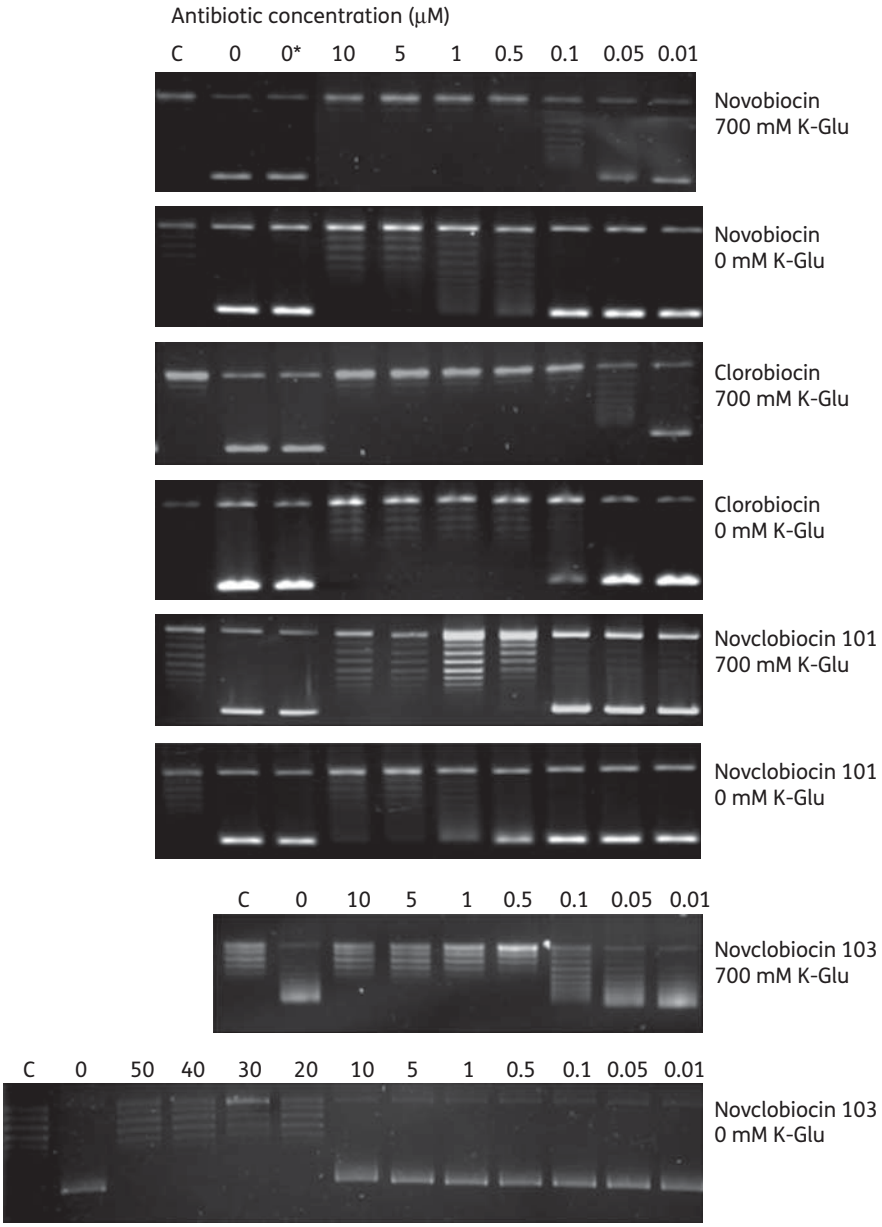

Figure 4. Influence of K-Glu on the sensitivity of *E. coli* DNA gyrase towards aminocoumarin antibiotics. The supercoiling reaction mixtures contained relaxed pBR322 DNA, DNA gyrase and the indicated concentrations of the antibiotic. The first lane, labelled 'C', contains control assays without enzyme. The lanes labelled 0 or 0* contain assays with no addition or with addition of 3 μL of solvent (5% aqueous DMSO). The other lanes contain assays to which the indicated amount of antibiotic, dissolved in 5% DMSO, has been added.

have been modified (Figure 1).[20] Our study shows that these compounds are highly potent inhibitors of *S. aureus* gyrase *in vitro*, with inhibitory concentrations of 1 nM. In contrast, their changes in the acyl side chains, which provided increased gyrase inhibitory activity, led to a lower inhibition of topo IV. It is also remarkable that *E. coli* topo IV is more sensitive to the aminocoumarin antibiotics tested than the *S. aureus* enzyme. The results indicate that the presence of the 3″-5-methyl-pyrrole-2-carbonyl (MePC) moiety and the natural occurring 3-dimethylallyl-4-hydroxy-benzoic acid side chain are of importance for topo IV inhibition.

Inhibition of E. coli and S. aureus by different aminocoumarin antibiotics

To further assess the efficacy of the novel novclobiocins, we tested them alongside the natural aminocoumarins for their ability to inhibit the growth of *E. coli* and *S. aureus* cells in liquid culture (Table 1). We broadly found that the activity of the compounds mirrored their relative activities against gyrase and topo IV. However, aminocoumarin antibiotics tend to be more active against Gram-positive strains, as shown by the very high activities of coumermycin A_1 and

Table 1. *In vitro* activity of aminocoumarin antibiotics against DNA gyrase supercoiling and topo IV decatenation (IC_{50}), and *in vivo* antibacterial activity (MIC) against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213

	E. coli				*S. aureus*			
	IC_{50} (μM)		MIC		IC_{50} (μM)		MIC	
Compound	gyrase[a]	topo IV	μM	mg/L	gyrase[a]	topo IV	μM	mg/L
Novobiocin	0.08	10	394	250	0.01	20	0.25	0.16
Clorobiocin	0.03	3	23	16	0.006	10	<0.06	<0.04
Coumermycin A_1	0.03	5	7	8	0.006	100	<0.04	<0.04
Simocyclinone D8	0.1	>10	68	63	2	17	4.3	4
Novclobiocin 101	0.3	3	>368	>250	0.05	35	0.24	0.16
Novclobiocin 102	0.03	0.3	91	63	0.01	5	<0.06	<0.04
Novclobiocin 103	0.1	>10	>439	>250	1	>50	17.6	10
Novclobiocin 105	>100	>100	>434	>250	>100	>100	>434	>250
Novclobiocin 217	0.006	8	>49	>32[b]	0.001	>50	<0.09	<0.06[b]
Novclobiocin 225	0.006	8	>49	>32[b]	0.001	>50	<0.09	<0.06[b]

The concentration (μM) of antibiotic that caused 50% inhibition (IC_{50}) of DNA gyrase supercoiling and topo IV decatenation as well as the concentration (μM and mg/L) of antibiotic that caused 50% inhibition of cell growth (MIC) are given.
[a]DNA gyrase activities were determined in the presence of 700 mM K-Glu.
[b]Data from Anderle *et al.*[20]

clorobiocin observed against *S. aureus* (Table 1). This indicates that either the uptake through the membrane or the presence of an active efflux pump limit the effectiveness of aminocoumarin antibiotics against Gram-negative organisms. Besides clorobiocin, novclobiocin compounds 217 and 225 have been identified recently as very potent inhibitors of the growth of *S. aureus* ATCC 29213 and the MRSA strain ATCC 43300, with MIC values of <0.06, equal to the parent compound clorobiocin.[35] In this study, novclobiocin 102, which carries the 3″-MePC group typical of clorobiocin and the 8′-methyl typical of novobiocin, turned out to be a potent antistaphylococcal compound with MIC values comparable to those of coumermycin A_1 and clorobiocin. Removal of the chlorine atom from the structure of clorobiocin (novclobiocin 101) reduced the antibacterial activity within the tested concentrations >15-fold for *E. coli* and >4-fold for *S. aureus*. Novclobiocin 103, carrying the methyl group at 8′ but no substituent at 3″-OH, lost >15-fold activity against *E. coli* and >250-fold activity against *S. aureus*. Novclobiocin 105, lacking a methyl group at O-4″ of L-noviose, was inactive against *E. coli* and *S. aureus*, as expected from its lack of activity against topoisomerases *in vitro*.

Discussion

The present work aimed at elucidating the contribution of different aminocoumarin structural elements to inhibitory activity against gyrase and topo IV of Gram-positive and Gram-negative bacteria. We established an optimized protocol for supercoiling and decatenation assays, and we determined the inhibitory and antibacterial activity of four naturally occurring aminocoumarin antibiotics and of several novclobiocins, compounds derived from clorobiocin and novobiocin, against gyrase and topo IV from *S. aureus* and *E. coli*.

In the supercoiling assay, *S. aureus* gyrase requires the presence of high concentrations of K-Glu, whereas *E. coli* gyrase has so far been assayed in the absence of this salt. For the first time, our study shows that the sensitivity of *E. coli* gyrase towards the inhibition by aminocoumarins is considerably increased by K-Glu. Therefore, the concentration of K-Glu used in the assays needs to be considered when comparing the effect of inhibitors on *E. coli* and *S. aureus* gyrase. The precise role of K-Glu in the catalytic mechanism of topoisomerases is unclear. Hiasa *et al.*[15] provided evidence that K-Glu is not required for the binding of DNA to the catalytic domain of gyrase, but rather for its binding to the C-terminal domain of GyrA and the resulting wrapping of DNA, which enables gyrase to catalyse the supercoiling reaction.

Another theory is that gyrase requires potassium for activity and it has been shown that it stabilizes the GyrB subunit.[36] Gyrase belongs to the GHKL family of enzymes (represented by gyrase, Hsp90, certain protein kinases and the DNA mismatch protein MutL), and crystallographic and biochemical studies have revealed a distinct binding site for monovalent cations such as K^+ on these enzymes, which is important for catalytic activity.[30] This may explain the difference of the effects of K^+ and Na^+ on the type II topoisomerases. Assays with Na-Glu instead of K-Glu showed no stimulatory effect for *S. aureus* gyrase and we even found an inhibitory effect for *E. coli* gyrase. Since this enzyme does not require K-Glu, the most feasible explanation is that the sodium added with Na-Glu outcompetes the potassium present in the reaction buffer (24 mM KCl); this would explain the weak activity observed when adding only 100 mM Na-Glu, a concentration at which a proportion of gyrase could still be stimulated by potassium (Figure 3), and it would also explain the lack of activity of *E. coli* enzyme with increasing concentrations of NaCl. The results obtained with the competition assays between Na-Glu

and KCl or K-Glu did not support this hypothesis, and more experiments are required to further elucidate gyrase's requirement for potassium and glutamate.

Assays of the inhibitory activity of aminocoumarin antibiotics reinforced the previous knowledge and provided further insights about the relevance of the different substituents for antibiotic activity. Out of the four naturally occurring aminocoumarin antibiotics, clorobiocin and coumermycin A_1 had the highest inhibitory activity against all the enzymes tested, and clorobiocin had the lowest IC_{50} towards topo IV of both *E. coli* and *S. aureus*. Manipulation of the substituents[16] at position 3″-OH of noviose confirmed that the presence of MePC is essential for high inhibition of both gyrase and topo IV (Figure 1). Furthermore, elimination of the methyl group at position 8″ of noviose rendered the compound (novclobiocin 105) completely inactive against all the enzymes tested, which indicates that hydrophobic contacts between this methyl group and a hydrophobic patch of the enzymes play a prominent role.[9,37] One of the most exciting results is the identification of clorobiocin and novobiocin derivatives with stronger antibiotic activity than the natural compounds. In this way, modification of the ring A, attached to the amino group of the coumarin ring, provided higher activity against gyrase, particularly against *E. coli* enzyme, although with loss of activity against topo IV from both microorganisms. Therefore, our experiments suggest that the most active compounds against *S. aureus* and *E. coli* type II topoisomerases contain an 8″-CH_3 group, an MePC moiety at position 3″, and an 8′-CH_3 group.

These biochemical data suggest that gyrase is the primary target of all investigated aminocoumarin antibiotics *in vitro*. Fujimoto-Nakamura *et al.*[34] have shown that cultivation of *S. aureus* in the presence of novobiocin results not only in the selection of mutants with altered gyrase, but also in mutations of topo IV as a second step in resistance development. Therefore, topo IV may not be completely irrelevant as a target of aminocoumarins in *S. aureus*, at least at higher antibiotic concentrations.

Another group of topoisomerase inhibitors are the fluoroquinolones. Resistance against the fluoroquinolones is rapidly emerging, usually by mutations near the active site tyrosines of GyrA or ParC.[38] Agents that target type II topoisomerases in a different way than fluoroquinolones would offer a possibility to overcome this resistance while still exploiting the same validated target. The combination of fluoroquinolones with aminocoumarins, which act at very distinct sites on the enzymes, may offer a strategy to provide effective antibacterial therapy[39] with a reduced risk of resistance development. *In vitro* studies have shown that *S. aureus* mutants that are simultaneously resistant to both fluoroquinolones and aminocoumarins arise only at very low frequency, and might therefore not be selected if those agents were used as combination therapy.[40] These considerations may warrant the development and evaluation of new aminocoumarin antibiotics. The assay methods developed here can be useful in such an approach.

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Transparency declarations

None to declare.

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