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Design, Synthesis, and Structure–Activity Relationship Studies of ATP Analogues as DNA Gyrase Inhibitors

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Abstract—We report herein the design and synthesis of ATP-analogues, namely 4-amino-pyrazolo[3,4-*d*]pyrimidines and 4-amino-pyrazolo[1,5-*a*][1,3,5]triazines, with DNA gyrase inhibitory activity. Among these series, some compounds exhibited promising antibacterial activity. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

In recent years emergence and spread of resistance have caused serious problems in the effective treatment of infectious diseases.[1] Multiresistant pathogens were isolated especially in the clinics. We therefore searched for new and effective antibiotics, which overcome resistance.

DNA topoisomerases control the topological state of DNA. They are involved in e.g. DNA transcription and replication and therefore are essential for cell viability.[2] Like eucaryotic topoisomerases II bacterial DNA gyrase can relax positive supercoiled DNA, but it is unique in its ability to introduce negative supercoils into DNA, processes which consume ATP. The active DNA gyrase complex consists out of a tetramer A_2B_2 of the subunits A and B each of which of about 90 kDa. Quinolones, which are successfully used as broad spectrum antibiotics in the clinic, target DNA gyrase A by interfering with DNA cleavage and religation reaction. The coumarin containing antibiotics such as novobiocin (**3**), known for more than 40 years, bind to DNA gyrase B competing with ATP. They demonstrated in vivo efficacy against several bacterial pathogens. However, the coumarins suffer from several disadvantages[4] such as toxicity and fast emergence of resistance. We are designing inhibitors for the ATP-binding-pocket of subunit B.[3]

Modelling

Investigation of the X-ray structure of the complex of the nonhydrolyzable analogue of ATP, ADPNP, with the 43 kDa fragment of DNA gyrase B[5] reveals that the aminopyrimidine ring of ATP is recognised by the enzyme via a H-bond network to asp73 and a tightly bound water molecule, which is held in addition by two amino acids (Fig. 1). The other two water molecules depicted fill the empty space between ATP and the lipophilic cavity of the enzyme. An inhibitor, which mimics ATP but could eventually replace these two water molecules, should bind specifically to gyrase B with higher affinity than ATP. Selectivity of the designed inhibitors against other ATP-binding enzymes is probably guaranteed by the fact that gyrase shows an unusual binding mode for ATP, which is shared by few other enzymes.[6] In addition high and selective affinity to gyrase B should be achieved by replacing the sugar moiety with substituents which can interact with ile94, and by placing substituents at the free position of the pyrimidine ring which are capable of interacting with arg136 and a salt bridge located in this region. As shown by X-ray crystallography,[7] the 4-hydroxy-coumarin moiety of the antibiotic novobiocin (**3**) binds to these residues (Fig. 1).

By superpositioning ATP (**1**) with aminotriazine **4**, a recently described DNA gyrase B inhibitor,[8] we generated 4-amino-pyrazolo[3,4-*d*]pyrimidines **5** and 4-amino-pyrazolo[1,5-*a*][1,3,5]triazines **6** as potential DNA gyrase inhibitors (Fig. 2). These would preferentially bear an aryl-group in the 1- and 8-position of **5** and **6**, respectively, and an aryl-group linked via a heteroatom X at position 6 and 2. Residues R^1 and R^4 should be

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hydrogen or small substituents in order to maximise lipophilic interactions with the enzyme and eventually replace the water molecules mentioned above.

Chemistry

3-Aminopyrazoles **8** were prepared by reacting alkoxymethylene malononitriles **9** or formylated benzylnitriles prepared from **7** with hydrazine in alcohol (Scheme 1). They were converted with ethoxycarbonylisothiocyanate to the corresponding thiourea derivatives, which were cyclized onto the pyrazole with sodium methoxide. Methylation of the thiol with methyl iodide yielded hydroxytriazines **10**. Chlorination in refluxing phosphorus oxychloride in the presence of a base, subsequent substitution with ammonia in DMF, oxidation with MCPBA yielded the corresponding sulfones, which could be substituted easily with phenolates and thiolates in DMF to yield pyrazolo[1,5-*a*][1,3,5]triazines **6**.

We investigated the reaction of aminopyrazoles **8** with the commercially available dimethyl *N*-cyanodithioimino carbonate **11** to yield in one-step the triazine

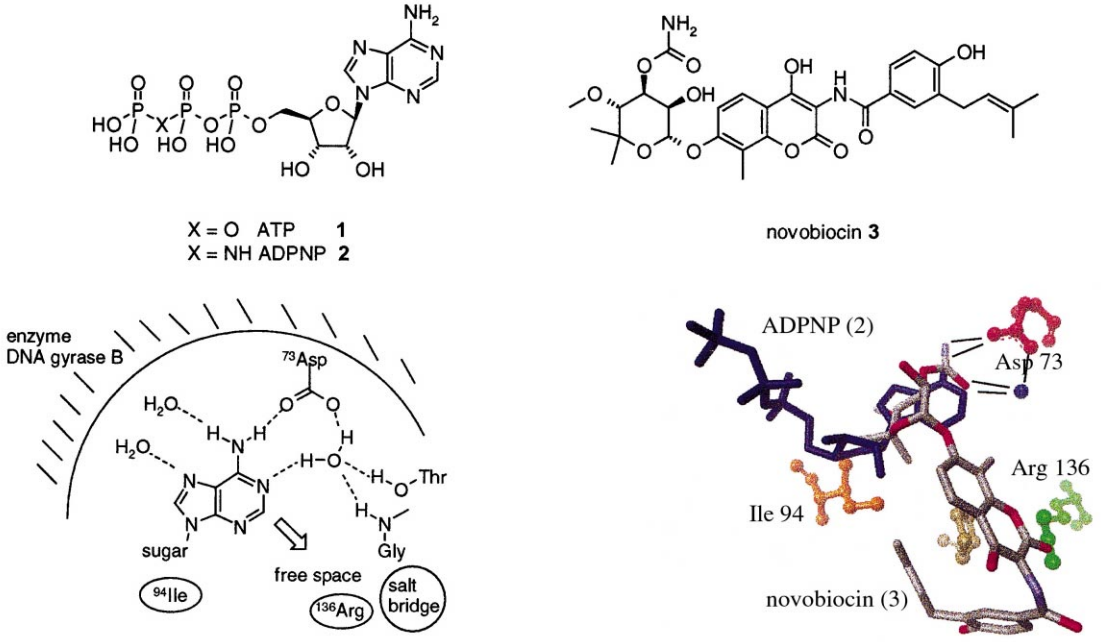


Figure 1. Schematic representation of ATP bound to DNA gyrase B; superposition of ADPNP (**2**, blue) with novobiocin (**3**, grey).

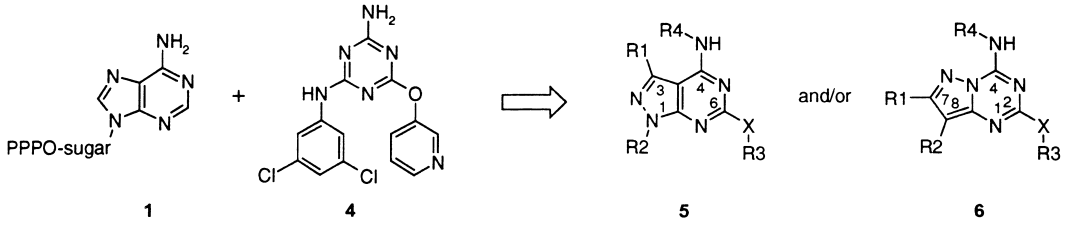
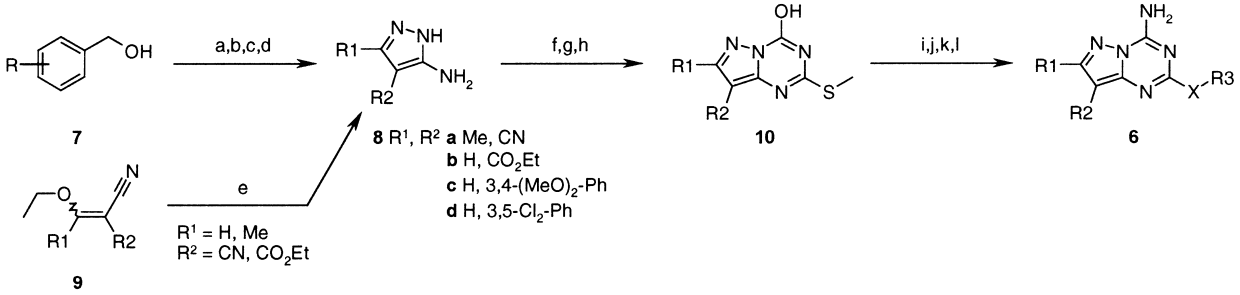


Figure 2.



Scheme 1. (a) PBr_3, Et_2O, 0 °C to rt, 6h, 96%; (b) KCN, EtOH, H_2O, refl., 4 h, 78%; (c) NaOMe, HCO_2Et, benzene, rt, 2 h, 87%; (d) N_2H_4, EtOH, HOAc, refl., 6 h, 50%; (e) N_2H_4, EtOH, refl., 2–5 h, 92–94%; (f) EtOCONCS, acetone, rt, 4 h, 68–89%; (g) NaOMe, MeOH, rt, 4–12 h, 66–90%; (h) MeI, NaOH, MeOH, H_2O, rt, 4–12 h, 66–90%; (i) $POCl_3$, NEt_2iPr, refl., 2–4 h, 78–94%; (j) concd aq NH_3, DMF, rt, 3 h, 50–93%; (k) MCPBA, THF, rt, 4 h, 48–100%; (l) NaH, HXR^3, DMF, rt–90 °C, 3–12 h, 35–87%.

derivatives **12** (Scheme 2). This would shorten the synthesis of **6** by four steps (Scheme 1, steps f–j). Results from similar reactions described in the literature[9] were not conclusive as to the outcome of regioselectivity. With aryl substituents at the pyrazole ring, we obtained the desired product **12**. In contrast, a nitrile or ester group at the same position afforded the opposite regiochemistry (**13**). Whereas **12** was easily oxidizable with MCPBA, compound **13** resisted oxidation with MCPBA even under forcing conditions.

The synthesis of the pyrazolo[3,4-*d*]pyrimidines **5** (Scheme 3) started with the preparation of 1-aryl-5-aminopyrazoles **15** from ethoxymethylene malononitrile derivatives **8** and arylhydrazines **14**. Condensation with benzoylisothiocyanate prepared in situ, subsequent cyclization with sodium hydroxide and methylation with methyl iodide yielded the pyrimidones **16**. Refluxing with phosphorus oxychloride in the presence of a base followed by substitution with an amine or ammonia and finally oxidation with MCPBA afforded the sulfones, which could be easily substituted with phenolates and thiolates to yield the products **5**.

As discussed above we were hoping to reach the coumarin-binding site of novobiocin (**3**) with our new inhibitors to gain affinity through π-stacking to a salt bridge and interactions with the basic arg136. The benzamide residue on the coumarin ring is necessary for the good antibacterial activity of novobiocin (**3**) but is fairly large and lipophilic. Recently novobiocin-derivatives with smaller and more hydrophobic coumarin-residues **17**–**19** (Fig. 3) were described that were equally active to novobiocin (**3**).[10] We synthesised these coumarins **17**–**19** in order to incorporate them in our new inhibitors as substituents at the aminopyrimidine ring.

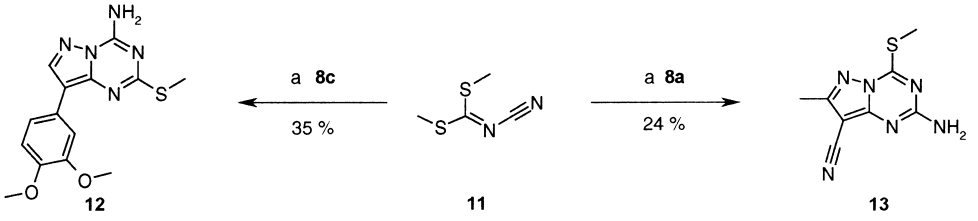
Biology

The compounds synthesised were tested in a coupled, spectrophotometic ATPase assay (using DNA gyrase, pyruvate kinase and lactate dehydrogenase), which monitored the disappearance of NADH as a function of ATP-consumption, and for validation in the supercoiling assay.[11] The effect was measured as maximum non-effective concentration (MNEC) defined as the highest inhibitor concentration, which showed no DNA gyrase inhibition. The in vitro antibiotic activity was measured as minimal inhibitory concentration (MIC) in 2-fold agar dilutions (Müller-Hinton agar, inoculum 10^4 CFU/spot).

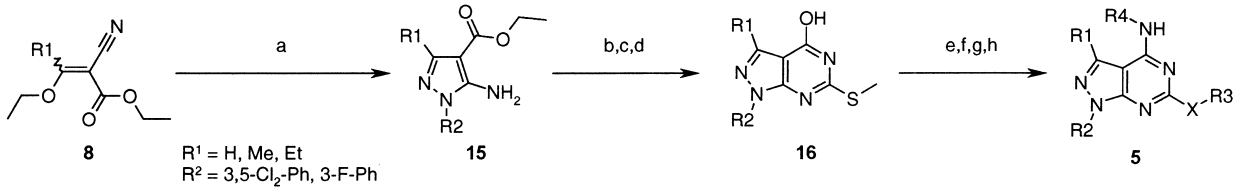
Results and Discussion

The pyrazolotriazines with a 4-hydroxy-coumarin (**6b,c**) or a 3-mercapto benzoic acid substituent (**6d,e**) were good enzyme inhibitors (Table 1) proving our modelling hypothesis.

The pyrazolopyrimidines **5** followed a similar trend to the pyrazolotriazines **6** (Table 2). Again, the 4-hydroxy-coumarin (**5m**–**q**) and 3-mercaptobenzoic acid derivatives (**5g**–**5l**) were the most active enzyme inhibitors. The residue R^1 (H, Me, Et) had a strong influence on



Scheme 2. (a) CH_2Cl_2, NEt_2iPr, 50 °C, 3d.



Scheme 3. (a) R_2NHNH_2*HCl **14**, NEt_3, MeOH, refl., 3 h, 58–97%; (b) PhCOCl, KSCN, acetone, refl., 3–12h, 78–100%; (c) NaOH, MeOH, refl., 2 h, 54–100%; (d) MeI, NaOH, MeOH, H_2O, rt, 2–12h, 63–100%; (e) $POCl_3$, NEt_2iPr, refl., 1 h, 78–99%; (f) NH_3gas or NH_2R^4, DMF or THF, rt, 12h, 59–99%; (g) MCPBA, THF, 0 °C to rt, 12 h, 64–92%; (h) NaH, HXR^3, DMF, 70 °C, 11–97%.

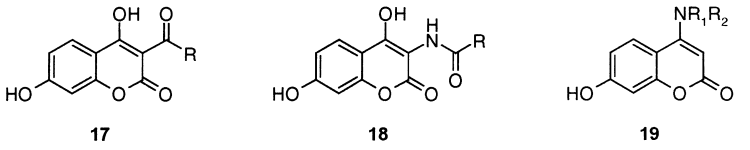
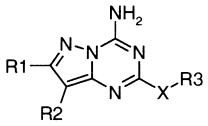
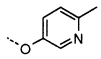
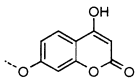
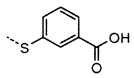


Figure 3.

Table 1. In vitro *E. coli* DNA gyrase supercoiling activity (MNEC (μg/mL))

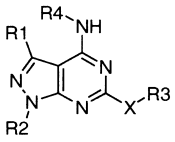


6

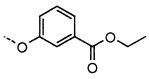
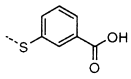
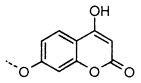
R^1	R^{2a}	-X-R^3 (compound no.)			
					
Me	-CN		8	**6b**	0.5	**6d**
H	3,4-$(MeO)_2$-Ph-		0.1	**6c**		
	3,4-$(Cl)_2$-Ph-	>63 (<16[b]) **6a**			1	**6e**

[a]The numbering refers to the position of the substituent on the benzene ring.
[b]The activity was determined with the ATPase assay.

Table 2. In vitro *E. coli* DNA gyrase supercoiling activity (MNEC (μg/mL))



5a-q

$R^{2\ a}$	R^1	R^4	-X-R^3 (compound no.)							
										
3,5-$(Cl)_2$-Ph-	H	H	63–125[b]	**5a**	>125	**5d**	1	**5g**	0.5	**5m**
	Me	H	31–63[b]	**5b**	63–125[b]	**5e**	1	**5h**	0.25	**5n**
	Et	H	63–125[b]	**5c**	>16 (</[b])	**5f**	0.25	**5i**	0.06	**5o**
		c-Pr					8	**5j**	4	**5p**
		Et					8	**5k**		
3-F-Ph-	Et	H					8	**5l**	1	**5q**

[a]The numbering refers to the position of the substituent on the benzene ring.
[b]The activity was determined with the ATPase assay.

the activity. The ethyl group was obviously best capable of optimising the lipophilic interactions with the enzyme (e.g. **5m,n,o**).

Additional substituents R^4 on the amino group decreased the activity (**5i–k,o,p**). The 3,5-dichlorophenyl group proved to be the best substituent in the 1-position (**5i,l** and **5o,q**). The best series was found to be 4-amino-1-(3,5-dichlorophenyl)-3-ethyl pyrazolopyrimidines. We were able to obtain an X-ray structure of **5i** complexed with the 23 kDa fragment of DNA gyrase B (loop-deletion mutant of *S. aureus* enzyme;[12] Fig. 4). Our modelling hypothesis was thus confirmed: specific H-bonds to asp73 and to a water molecule, π-stacking to the salt bridge, ionic interactions with arg136, lipophilic interactions with ile94 and replacement of one H_2O by the ethyl group.

Variations on the coumarin substituent (**5q–w**, Table 3) decreased the activity compared to the highly potent coumarin **5o**. Still the enzyme activities of the 3-acyl-4-hydroxy coumarin derivatives **5t–w** were good enough to be antibacterial active if they could penetrate well through the bacterial membrane.

All investigated pyrazolotriazines **6** were almost inactive against bacteria; they showed at best slight activity against *S. epidermidis*; except for compound **6e**, which was unfortunately inactive against *S. aureus* (Table 4). In general, the pyrazolopyrimidines **5** were better antibacterials than **6**. From the compounds listed in Table 2 only **5i** was moderately antibacterial active. All unsubstituted hydroxycoumarins; although being highly potent enzyme inhibitors (**5m–o**); were inactive against bacteria. As we anticipated 3-substituted 4-hydroxy coumarin substituents—especially with amide and urea residues (**5v,w**)—led to inhibitors with improved cell permeability and promising activity against Gram-positive bacteria including novobicin resistant strains (Table 4).

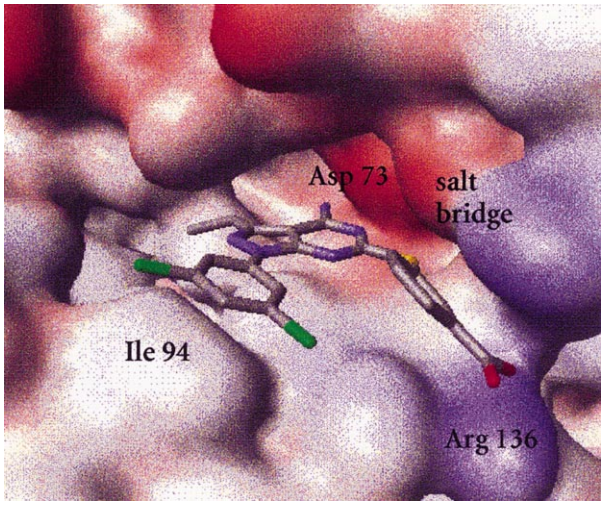
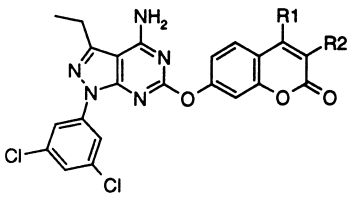


Figure 4. X-ray structure[12] of the 23kDa loop-deletion fragment of DNA gyrase B with **5i** (Weblab Viewer Pro 3.0 ®).

Table 3. In vitro *E. coli* DNA gyrase supercoiling activity (MNEC (μg/mL))



5q-w

R^1	R^2 (compound no.)									
	H		COMe		CO_2Et		NHAc		NHCONHBu	
Me	>125[b]	**5q**								
1-Me-piperazine	>125[b]	**5r**[a]								
NMe_2	63–125[b]	**5s**[a]								
OH	0.06	**5o**[a]	2	**5t**[a]	2	**5u**[a]	0.5	**5v**	0.5	**5w**

[a]The compounds showed at best slight activity against *S. epidermidis*.
[b]The activity was determined with the ATPase assay.

Table 4. In vitro *E. coli* DNA gyrase supercoiling activity (MNEC) and antibacterial activity (MIC)

Compound no.	MNEC (μg/mL)	MIC (μg/mL)[a]								
		S. aureus ATCC 25923	*S. aureus* NovoR	*S. aureus* Coum-R	*S. aureus* Smith	*S. aureus* Smith QR-54	*S. aureus* H19982	S. epidermidis ATCC 14990	*S. pyogenes* β15	*E. faecium* vanA E23-8
Novobiocin (**3**)	0.25	0.25	8	16	0.25	8	0.25	0.12	2	8
6e	1	>32	>32	>32	>32	>32	>32	4	0.5	32
5i	0.25	16	32	32	16	2	4	0.5	n.d.[b]	>32
5v	0.5	16	16	16	8	8	4	4	1	>32
5w	0.5	4	8	8	4	2	4	4	2	16

[a]Resistance is mentioned as follows: NovoR (against novobiocin, R144-I in ATCC 25923), Coum-R (against coumermycin), QR54 (against methicillin and quinolones), H19982 (against methicillin), vanA E23-8 (against ampicillin, trimethoprim, vancomycin).
[b]n.d. = not determined.

In summary, we have designed new ATP-analogues, namely 4-amino-pyrazolo[3,4-*d*]pyrimidines and 4-amino-pyrazolo[1,5-*a*][1,3,5]triazines, which exhibited potent DNA gyrase inhibition and good antibacterial activity against several resistant gram-positive bacteria.

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