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Original article

Partial resistance of *E. coli* mutants against 2,4-diamino-5-benzylpyrimidines by interactions with bacterial membrane lipopolysaccharides. Derivation of quantitative structure–binding relationships

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Abstract – A series of previously synthesized 2,4-diamino-5-benzylpyrimidines, inhibitors of bacterial dihydrofolate reductase (DHFR) showed decreased inhibition of *E. coli* cultures, despite increased inhibitory activity against DHFR. Preliminary studies using *E. coli* mutants with different degrees of outer membrane deficiencies suggested that the decrease in activity was partly due to inactivation because of binding to outer membrane constituents. In the present study antibacterial activities of the benzylpyrimidines have been systematically determined as a function of cell membrane defects in *E. coli* using bacterial growth kinetic techniques. It has been shown that the observed differences in activity were not due to different binding affinities to the target enzyme of the mutants. Lipopolysaccharides have been extracted from the mutants and used in binding studies by ultrafiltration, photometric and NMR techniques. The observed differences in binding affinity to the lipopolysaccharides have been related to the differences in the lipophilic properties and molecular weight of the substituents. Quantitative structure–activity relationships have been derived. The results of the study show the importance of drug–membrane interactions for the rational development of antibacterials. © 2000 Éditions scientifiques et médicales Elsevier SAS

***E. coli* resistance / lipopolysaccharides / 2,4-diamino-5-benzylpyrimidines / binding studies / QSAR**

1. Introduction

Recently we reported on a new 4,4′-diaminodiphenyl substituted 2,4-diamino-5-benzylpyrimidine (K-130), synthesized in our laboratories, which binds to and inhibits simultaneously two enzymes in the folate synthesizing pathway, namely dihydropteroic acid synthase (DHPS) and dihydrofolate reductase leading to autosynergism in whole cell mycobacteria, such as *M. lufu* and *M. leprae* [1–3]. The binding affinity to DHFR derived from *M. lufu* as well as from *E. coli* was eight times stronger as compared to trimethoprim (TMP) [1, 2]. While in the case of mycobacteria a significant decrease in the minimum inhibitory concentration (MIC) was observed, MIC towards *E. coli* was disappointingly high.

It is assumed that K-130, possessing a molecular weight of about 600, cannot use the porin pathway and can reach the intracellular cytoplasm only by diffusion through the highly hydrophilic and negatively charged outer core of *E. coli*. The latter is considered to be the decisive barrier for permeation of drug molecules [4]. The outer core consists of lipopolysaccharides (LPS) which could hinder the passage of charged and lipophilic compounds such as benzylpyrimidines. A preliminary study using *E. coli* mutants revealed an increase in the inhibitory power of K-130 corresponding to an increase in outer membrane deficiency of the mutants [5]. Such a phenomenon has already been reported by Schlecht and Westphal [6], Schlecht and Schmidt [7] and Nikaido [8, 9] for a large but heterogeneous series of chemotherapeutics and various Gram-negative strains, and has been reviewed by Vaara [10].

In the present study, mutant strains of *E. coli* with defined cell wall deficiencies of LPS, but still with full vitality, were used to evaluate the inhibitory effect of the benzylpyrimidines. LPS from the different mutants were isolated for direct interaction studies with the com-

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Table I. Chemical composition of the K-oligosaccharide region of the selected *E. coli* mutants [29].

Mutant	Galac-tose	Glucose	Heptose	KDO	Phos-phate	Gene-defect
F470	2	3	3	1	3.5	rfb
F614	2	3	3	1	3.5	rfa
F612	–	1	3	1	2.5	rfa
F588	–	–	2	1	–	rfa
F515	–	–	–	1	–	rfa

pounds. In addition the inhibitory power of the benzylpyrimidines against the target enzyme was determined to exclude differences in binding affinity to the target DHFR in the studied mutants.

2. Results and discussion

2.1. Selection of E. coli *mutants*

LPS of wild type *E. coli* consists of three major parts: O-specific side chain; core oligosaccharide and lipid A. The strong hydrophilic O-specific side chain is missing totally in all selected mutants except *E. coli* 055:B5 L2880 used in NMR-binding experiments. *Table I* and *figure 1* represent the chemical composition and the scheme of the core oligosaccharide region of the selected *E. coli* mutants. In addition, a mutant with an rfb-gene defect (*E. coli* F470) was also included in this study as it misses the enzyme responsible for the synthesis of the repeating units of the polysaccharides. Chemotypically, all selected mutants belong to the R1-core type. The complete *E. coli* R1-core, however, is only present in the R1-mutants F470 and F614. The absence of special sugars leads to a steady decrease in the hydrophilic properties of the cell wall in the sequence F470, F614, F612, F588, F515 (*figure 1*). The content of phosphate groups of the oligosaccharide component decreases in the same sequence.

2.2. Selection criteria for the tested 2,4-diamino-5-benzylpyrimidines

Most of the benzylpyrimidines had been synthesized in our laboratory aiming to increase their affinity towards isolated DHFR and partly to inhibit simultaneously DHPS of bacteria [1, 11, 12]. They showed high inhibitory activities against the isolated enzymes and also against whole cell mycobacteria [13]. Despite the high affinity to *E. coli* derived DHFR, however, only a weak inhibitory activity was observed against *E. coli* cultures (*table II*). It was suggested that this discrepancy could be due to different degrees of binding to outer membrane constituents. To test this hypothesis, from the available derivatives, were selected those which showed a large variation in the substitution of the benzyl moiety, especially large variation in lipophilicity. The compounds and their inhibitory activity, I_{50}, against the *E. coli* mutants together with their molecular weight and a descriptor of lipophilicity (log k′), are listed in *table II*.

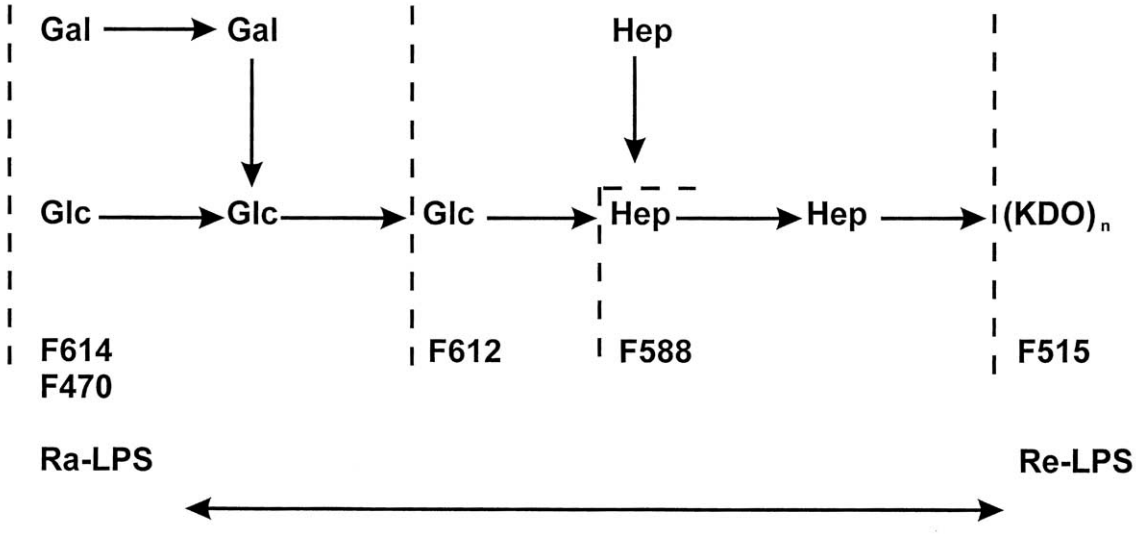


Figure 1. Scheme of the outer rough core region of the selected *E. coli* mutants.

Table II. Code, structure, I_{50}-values against various *E. coli* mutants, and physico-chemical properties of the tested 2,4-diamino-5-benzylpyrimidines.

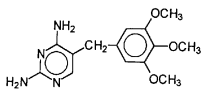
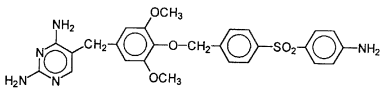
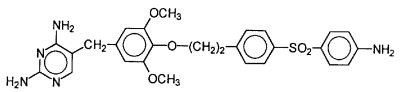
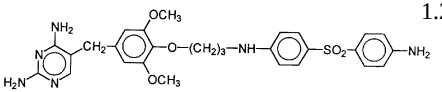
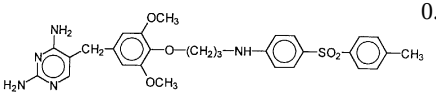
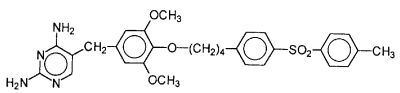
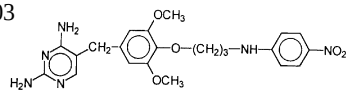
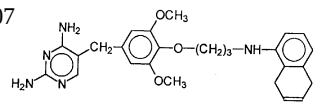
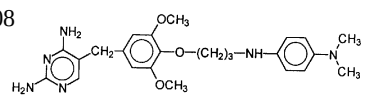
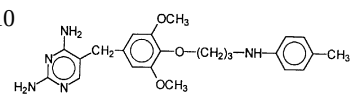
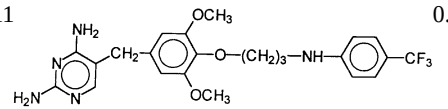
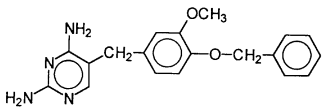
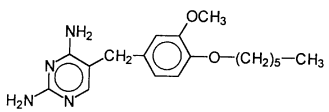
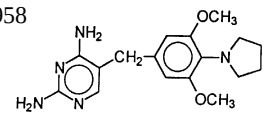
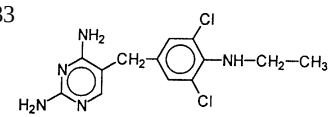
Compound	Formula	I_{50} F515 [(mol/l]	I_{50} F588 [(mol/l]	I_{50} F612 [(mol/l]	I_{50} F614 [(mol/l]	M.W.	log k′	log P_{ClogP}	log P_{VG}	log $k'_{calc.}$
TMP		0.80 ± 0.07	1.79 ± 0.09	1.34 ± 0.23	1.31 ± 0.10	290.32	0.55	0.91	1.05	0.46
K-96		at 10 ± mol/L about 20 % inhibition	no activity up to 10 ± mol/l	n.d.[a]	n.d.[a]	521.61	n.d.	n.c.[b]	n.c.[b]	n.c.[b]
K-107		3.60 ± 0.15	10.92 ± 1.38	n.d.[a]	n.d.[a]	550.65	1.64	1.89	2.53	1.98
K-130		1.22 ± 0.08	8.10 ± 0.59	14.67 ± 1.62	13.73 ± 1.12	564.68	2.09	1.99	2.58	2.06
K-150		0.84 ± 0.06	14.15 ± 1.15	20.35 ± 0.69	16.22 ± 1.12	563.69	3.80	3.50	3.83	3.50
KC-142		2.45 ± 0.59	–[c]	–[c]	–[c]	562.68	n.d.[a]	4.28	5.16	4.63
KC-1303		0.39 ± 0.04	1.17 ± 0.18	2.05 ± 0.48	2.83 ± 0.24	454.49	2.39	2.35	2.49	2.18
KC-1307		1.94 ± 0.26	9.59 ± 0.99	18.55 ± 2.40	13.44 ± 1.28	463.58	n.d.[a]	3.05	3.76	3.24
KC-1308		2.57 ± 0.03	3.16 ± 0.32	2.22 ± 0.47	3.29 ± 0.41	452.56	2.02	2.16	2.80	2.26
KC-1310		1.07 ± 0.15	6.34 ± 0.47	n.d.[a]	n.d.[a]	423.52	2.69	2.46	3.01	2.52

Table II. (Continuation)

Compound	Formula	I_{50} F515 [(mol/l]	I_{50} F588 [(mol/l]	I_{50} F612 [(mol/l]	I_{50} F614 [(mol/l]	M.W.	log k′	log P_{ClogP}	log P_{VG}	log $k'_{calc.}$
KC-1311		0.75 ± 0.03	7.23 ± 0.60	14.36 ± 3.24	22.10 ± 1.92	477.48	n.d.	3.37	3.42	3.20
GH-101		3.02 ± 0.14	16.01 ± 1.02	30.38 ± 3.88	35.36 ± 2.62	336.40	n.d.	2.82	3.07	2.74
GH-306		3.03 ± 0.28	9.59 ± 0.76	32.00 ± 5.42	42.74 ± 2.27	330.44	3.00	3.69	3.30	3.29
RO-18958		0.32 ± 0.02	1.30 ± 0.16	4.57 ± 0.92	5.28 ± 0.39	353.43	2.24	2.60	2.12	2.09
A-44733		11.69 ± 1.86	22.03 ± 2.97	55.09 ± 11.40	73.74 ± 6.49	312.22	2.22	2.32	2.76	2.31

[a] n.d.: not determined; [b] n.c.: not calculated; [c] inactive up to 15 µmol/L. I_{50} values are in µmol/L.

2.3. Bacterial growth kinetics of E. coli *in the absence and presence of 2,4-diamino-5-benzylpyrimidines, determination of I_{50} values*

To study the concentration- and time-dependent onset of inhibition and to determine the concentration causing 50% inhibition we followed up bacterial generation rates using a Coulter Counter technique [14, 15] (see also Experimental protocol). The I_{50} values for all studied benzylpyrimidines have been calculated by non-linear regression and are given in *table II*. The generation rate under the test conditions is approximately 45 min. Besides the effect of increasing concentrations, leading to a decline in the slope of the generation rate curves, it can also be seen that the onset of inhibition is constant and depends neither on the drug concentration nor on the mutant tested. The generation rates follow first order kinetics, i.e. the compounds are not subject to varying degrees of active efflux or other events which would lead to a decrease in concentration and thus to non-linear kinetics. Therefore it can be concluded that the on-set of inhibition is not diffusion controlled for the wild type *E. coli* and for the mutants with cell wall defects.

Typical bacterial generation curves are depicted in *figure 2*. To achieve similar inhibitory activity, 10 times the concentration of K-130 is needed in the case of the *E. coli* mutant F614 compared to the mutant F515 (I_{50} = 1.2 and 13.9 µmol/L, respectively), while TMP shows no large differences in the I_{50} values for the two mutants (I_{50} = 0.7 and 1.1 µmol/L, respectively) (*table II*). Therefore, for the small and hydrophilic TMP no barrier in permeation of the cell wall seems to exist in both *E. coli* mutants, while for K-130 such problems could be suggested. Indeed for K-130 the diffusion through porins can be hindered because of its high molecular weight (MW = 564). The exclusion size of the porins of *E. coli* is about 600 daltons, depending on the shape of the drug. Because in this homologous series of compounds, changes in molecular shape are reflected by changes in molecular weight, this can be used instead.

It can be assumed that differences in the outer core composition are responsible for the increase in I_{50} against F614 as long as simultaneous changes in the affinity to DHFR can be excluded (see next chapter). Inspection of the I_{50} values confirms the assumption made (*table II*). With the exception of the small hydrophilic TMP molecule where no significant differences in I_{50} for the tested mutants are observed, an increase in antibacterial activity is found for all other studied benzylpyrimidines in the sequence F614/F612, F558, F515. The derivative KC-1308 was found to be unstable in buffer solution, therefore the given I_{50} value relates to its unknown decomposition product.

The increase in inhibitory activity is paralleled by an increase in the defects of the core oligosaccharide region. The latter decreases the drug membrane interaction and facilitates the cell wall passage. Thus, the drug reaches the target enzyme without significant loss in concentration due to the binding to membrane components. The ranking of the I_{50} values points to the barrier function of the outer membrane and particularly to the composition of LPS as the decisive factor.

2.4. I_{50} values of 2,4-diamino-5-benzylpyrimidines against DHFR derived from the E. coli *mutants*

DHFR from the various mutants has been extracted and I_{50} values determined to exclude the possible influence of changes in affinity of the benzylpyrimidines to the different target DHFRs on the I_{50} values derived from the bacterial growth kinetics. In all cases nearly identical I_{50} values, with small differences only, were observed for the inhibition of the various DHFRs. Therefore it can be concluded that the mutations are essentially restricted to changes in the outer membrane.

The ratios of the I_{50} values determined against the *E. coli* strains (whole cell) and the corresponding cell-free DHFR extracts are summarized in *table III*. A loss of activity is observed comparing the ratios of the rough (F614) to those of the deep rough mutant (F515). Again, only for the small hydrophilic TMP no significant difference is recorded. For the other derivatives the quotients are up to 20 times larger. The drug–membrane interaction leads to an increase in the whole cell I_{50} so that for example K-130, KC-1303 and KC-1311 with high affinity towards DHFR are less active against the bacteria.

2.5. Interaction of 2,4-diamino-5-benzylpyrimidines with components of the outer membrane of E. coli *mutants*

LPS has been extracted from the *E. coli* mutants and its interaction with the compounds was determined using various techniques to estimate the significance of LPS–drug interactions for their antibacterial activity.

2.5.1. Binding measurements using an ultracentrifugation technique (UC)

The results of the UC-binding study are presented in *table IV*. They show a significant percent of binding of K-130 to different LPS types, most intensively towards LPS derived from the *E. coli* mutant F612. This is in agreement with the ratio of the I_{50} values comparing rough and deep rough mutants. If cell debris of the wild

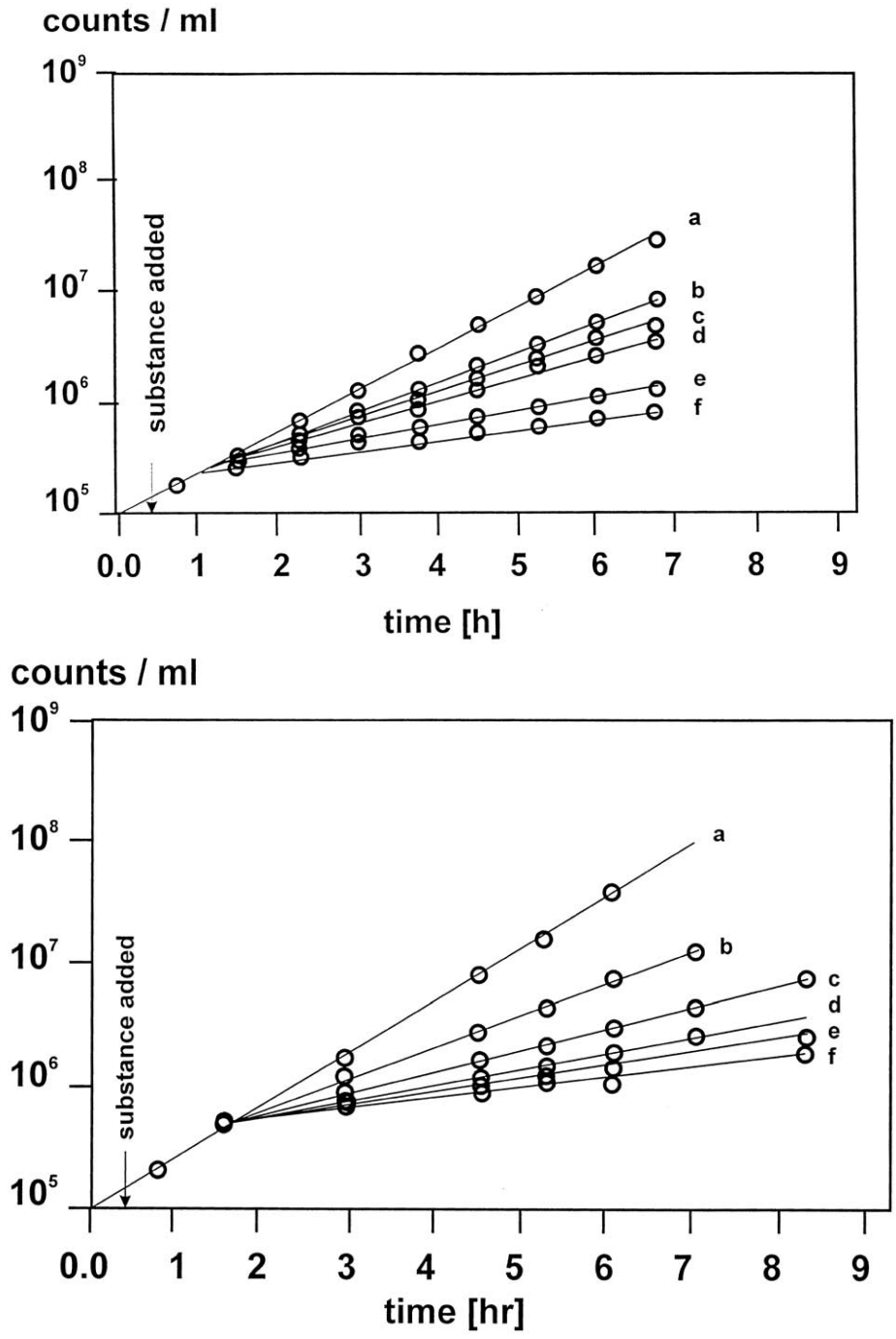


Figure 2. **A**: Bacterial growth kinetics of *E. coli* F515 in the absence (a) and presence (b–f) of 0.5, 0.75, 1.0, 2.5 and 5.0 µmol/L K-130. **B**: Bacterial growth kinetics of *E. coli* F614 in the absence (a) and presence (b–f) of 10, 20, 30, 40 and 50 µmol/L K-130.

type *E. coli* ATCC 11775 is used, the difference in binding of TMP compared to K-130 is even larger (12–16% for TMP, 80–84% for K-130). If the molar ratio is considered for the calculation of the percent binding to

Table III. I_{50} values [µmol/L] determined in cell free and whole cell systems of *E. coli* mutants F515 and F614 and the ratios of the I_{50} values.

Compound	*E. coli* F515			*E. coli* F614			Ratio F615/F515 w.c/w.c.
	I_{50} cell free (c.f.)	I_{50} whole cell (w.c.)	I_{50} w.c./c.f.	I_{50} cell free (c.f.)	I_{50} whole cell (w.c.)	I_{50} w.c./c.f.	
TMP	0.00218	0.80	367	0.00192	1.31	682	1.64
K-107	0.00082	3.60	4 390	0.00078	–	–	–
K-130	0.00056	1.22	2 179	0.0006	13.73	22 880	11.25
K-150	0.0011	84	764	0.0013	16.22	12 477	19.31
KC-1303	0.00057	39	684	0.00062	2.83	4 565	7.25
KC-1307	0.00078	1.94	2 487	0.00078	13.44	17 230	6.92
KC-1311	0.00055	75	1 364	0.00049	22.10	45 100	29.46
GH-101	0.018	3.02	167	0.027	35.36	1 310	11.72
GH-306	0.024	3.03	149	0.0213	42.74	2 007	14.10
Ro-118958	0.00039	32	821	0.0003	5.28	17 600	16.50
A-44733	0.084	11.69	139	0.085	73.74	867	6.30

F612 derived LPS the difference is even larger because of the larger molecular weight of F612-LPS compared to F515-LPS.

2.5.2. Photometric method for the determination of the interaction of benzylpyrimidines with LPS

Keler and Nowotny have described a spectrophotometric method for quantitative determination of bacterial endotoxins [16] which, in principal, could also be used for the determination of LPS–drug interactions. The basis of this method is the metachromatic reaction of the cationic phenothiazin dye, 1,9-dimethylmethylenblue, DMMB [17] with a polyanionic chromotrop, described already in 1970 by Toepfer [18]. According to Toepfer the normal orthochromatic absorption maximum of pure DMMB in watery solution (7×10^{-7} mol/L) is at 650 nm. This is the so called α band of the monomeric form of the dye. Increasing the concentration (2.8×10^{-5} mol/L) leads to self-association of DMMB. The dimeric form has an additional β band at 600 nm. Upon addition of heparin-Na, an anionic chromotrop, a metachromatic shift of DMMB is observed (µ band) with an isosbestic point at 575 nm [18].

According to Scheuner [19] the metachromatosy is a result of the self-association of dye molecules being induced by high dye concentrations but can also be induced by addition of chromotropic compounds. Chromotrops are macromolecules which possess anionic substructures such as carboxy, phosphate, sulfate or sulfonic acid groups [20]. LPS possesses such negatively charged functional groups and should also be able to induce a metachromatic shift in the absorption spectrum of DMMB. *Figure 3* shows the effect of LPS derived from different mutants. The new µ band appears at 550 nm. The strongest effect results from the addition of *E. coli* F470 derived LPS, the smallest by the LPS of the deep rough mutant F515 with the lowest content of phosphate groups. The induction of the µ band by LPS is concentration dependent (data not shown).

Table IV. Binding of K130 (in % of drug) to LPS. LPS content 50 µg/mL. Ultracentrifugation technique.

Type of LPS	% bound at total concentration of K130	
	5 µmol/L	20 µmol/L
F 515	0	10
F 588	10	20
F 612	15	25

The interaction of the cationic amphiphile polymyxin B (PMB) with the anionic LPS has been described by several authors [21–24] and was used as a control in our studies. For this purpose a constant amount of LPS (11.9 µg/mL) and increasing amounts of PMB were added to the DMMB solution and the corresponding spectra recorded. The LPS-F470 induced metachromatic shift was almost completely reversed by mixtures with LPS containing 10 µM PMB. The reversal of the metachromatic shift caused by the association of LPS and PMB can therefore be used for the determination of LPS–drug interactions. PMB and the benzylpyrimidines themselves produced no change in the absorption spectrum of DMMB. The effect of LPS-F515 and LPS-F470 mixtures with K-130 on the metachromatic shift produced by the pure LPSs (11.9 µg/mL) is shown in *figure 4*. It demonstrates that K-130 is able to interact with LPS, but weaker than PMB. The metachromatic shift is reversed approximately 25% (LPS-F470) and 50% (LPS-F515) by 25 µM K-130. This interaction of K-130 and other benzylpyrimidines

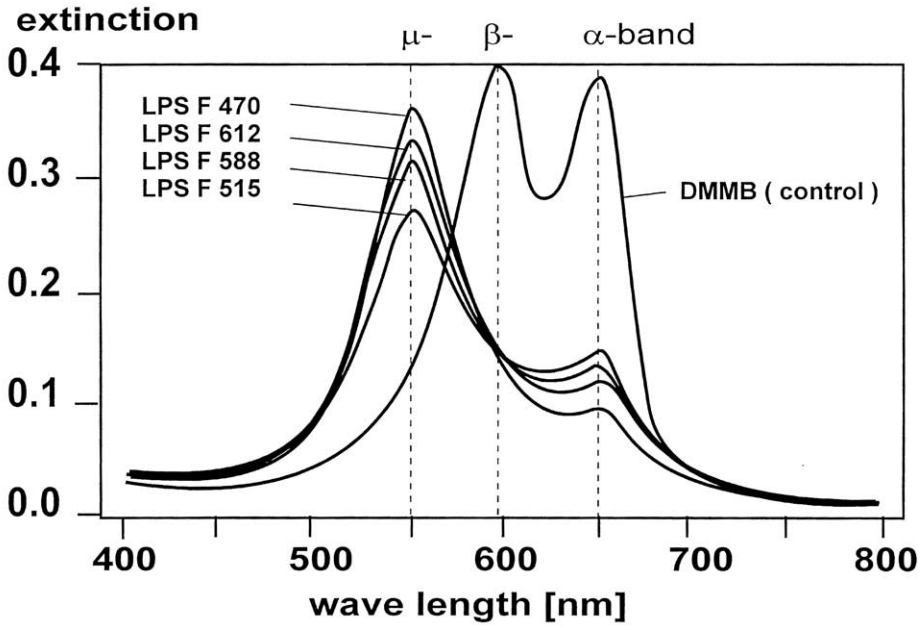


Figure 3. Metachromatic shift of the DMMB spectrum in the presence of LPS derived from the *E.coli* mutants F470, F612, F588 and F515. Final concentration was 47.6 μg/mL; pH 10.3.

possessing long hydrophobic substituents with LPS can explain the observed decrease in the inhibitory activity comparing cell-free and whole cell data. In contrast, no such reversal of LPS-F515 and LPS-F470 induced metachromatic shift is observed for TMP (data not shown).

2.5.3. Binding measurements using a nuclear magnetic resonance (NMR) technique

NMR spectra can generally be characterized by the following parameters: the magnetic field at which resonance occurs, the degree of spin–spin coupling produced by neighbouring effects, the spin-lattice relaxation rate $1/T_1$, and the spin–spin relaxation rate $1/T_2$, expressed as line width of the resonance signal at half peak height. One or several of these parameters may change upon interactions of small molecules with 'receptors'. We have observed such changes in $1/T_2$ and in chemical shift of the resonance signals of the benzylpyrimidines in the presence of increasing concentrations of LPS from various sources. The change in $1/T_2$ is related to a decrease in rotational freedom of the benzylpyrimidines in the presence of LPS.

The degree of interaction depends on the structure of the benzylpyrimidines and LPS. No interaction can be observed for both TMP and K-130 in the case of LPS-F515 which possesses the minimal LPS configuration (KDO only and no phosphate, data not shown). In contrast, increasing interactions occur between K-130, GH-306 and LPS derived from the deep rough (F588) and rough (F470) *E. coli* mutants, respectively. These compounds also show large differences in their antibacterial effect against these mutants (*table II*). The interactions with LPS-F588 are significant upon addition of 0.2 mg/mL LPS (*figure 5*).

The interaction with LPS-F470 is even stronger and the complex starts to precipitate during the NMR experiment, indicated by a decrease in the area under the resonance signals. In addition, a change in chemical shift is observed, especially for the aromatic and heteroaromatic protons of K-130 (*figure 6*).

If LPS derived from the wild type *E. coli* (serotype 055:B5; Sigma L2880) is used instead, the LPS remains in solution upon addition of K-130 and GH-306 because of its long hydrophilic O-specific side chain leading to improved water solubility. A broadening of the resonance signals of K-130 is already observed at very small LPS concentrations (0.05 mg/mL) (*figure 7*). The even larger broadening of the resonance signals of GH-306 under identical conditions corresponds to the observed larger ratio of the I_{50} values when the inhibition of the *E. coli* mutants F614 and F515 is compared (*figure 8*). The involvement of all spin systems of the benzylpyrimidines in the interaction with LPS supports the assumption that

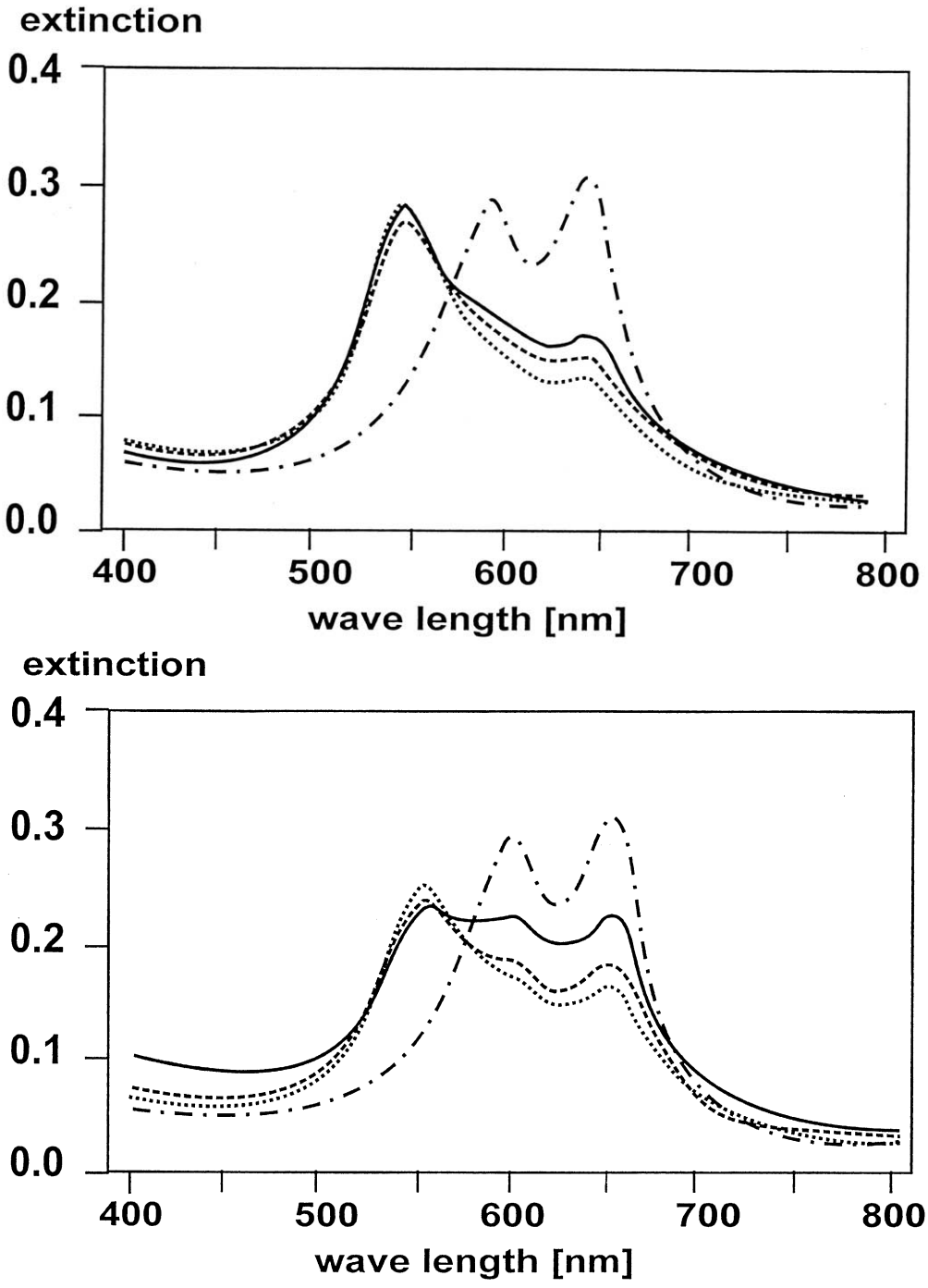


Figure 4. Change in the LPS induced metachromatic shift (dotted line) of the DMMB spectrum (dashed dot) in the presence of 13 (dashed) and 25 µM K-130 (solid line), pH 7.3; **A**: LPS-470 (11.9 µg/mL); **B**: LPS-F515 (11.9 µg/mL).

the primary amino groups of the benzylpyrimidines interact with the charged phosphate groups and that this interaction is reinforced by hydrophobic interaction forces of the substituents at the benzene ring, provided they are

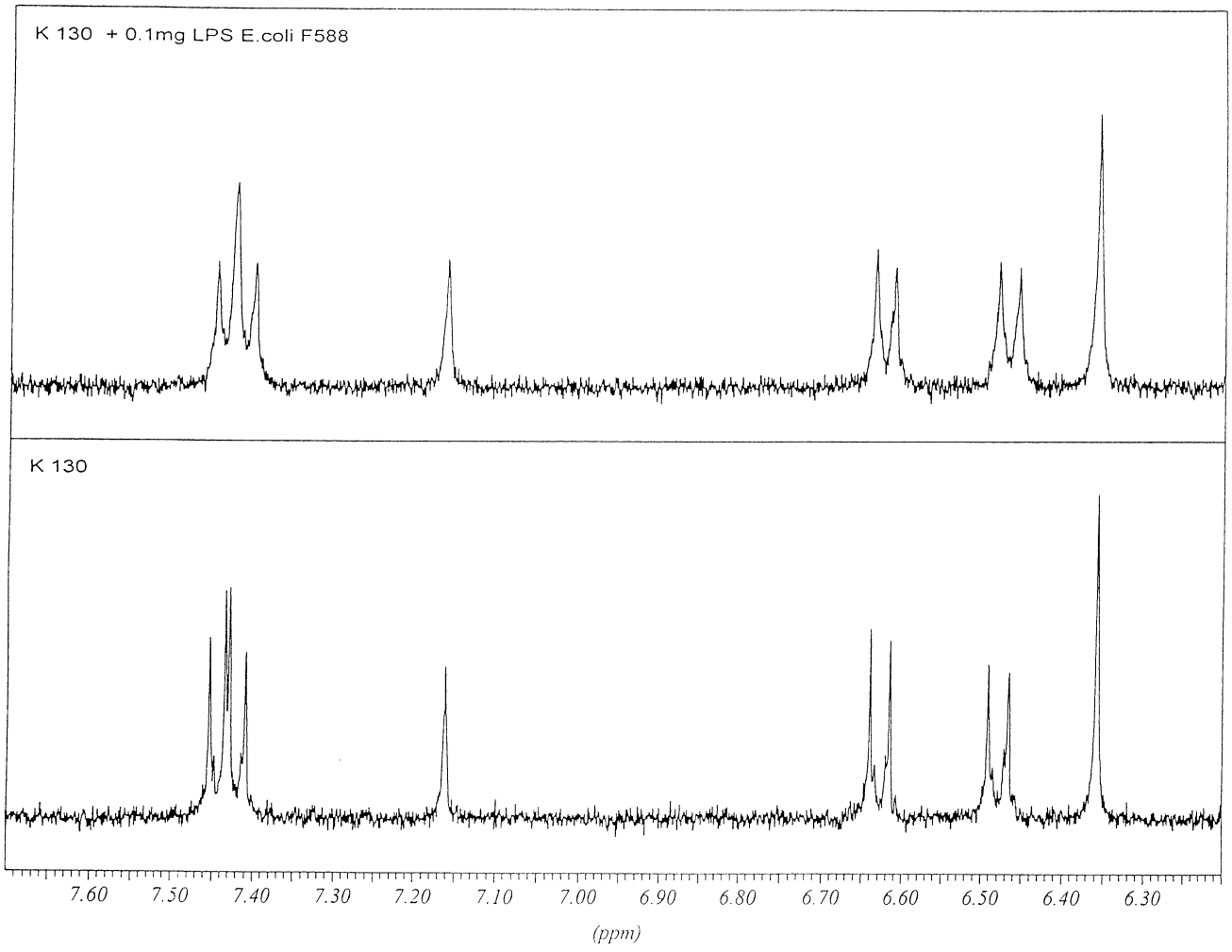


Figure 5. NMR spectrum of K-130 (0.5 μM) in the absence (below) and presence of 100 μg *E. coli* F588 derived LPS.

extended enough to reach down to lipid A, that is not possible for example for the derivative Ro-18958, despite its relatively high lipophilicity or for TMP without an extended substituent at the benzene ring.

2.6. Quantitative structure–binding relationships

The tested compounds are catamphiphiles. Under the conditions of the bacterial growth experiments, (pH 6.9) the 4-amino group of the pyrimidine moiety is more than 50% ionized (pK_a of TMP = 7.3). It could, therefore, be speculated that the major factor in the binding of the benzylpyrimidines to LPS is the interaction of the positively charged amino group at the pyrimidine ring and the negatively charged phosphate or carboxy groups of LPS. The number of phosphate groups decreases from smooth to deep rough mutants (*table I*). The charge interaction could be reinforced by hydrophobic interaction of the substituents in the 4-position of the benzyl moiety and the hydrophobic area of lipid A of LPS. This would mean that the overall lipophilicity of the substituents would not be important for the strength of interaction but the area of the lipophilic substituents reaching down to Lipid A.

As the charge interaction can be considered as constant within this series (no change in pK_a as a function of the substituents in 4-position of the benzyl moiety) the interaction strength will depend primarily on lipophilicity

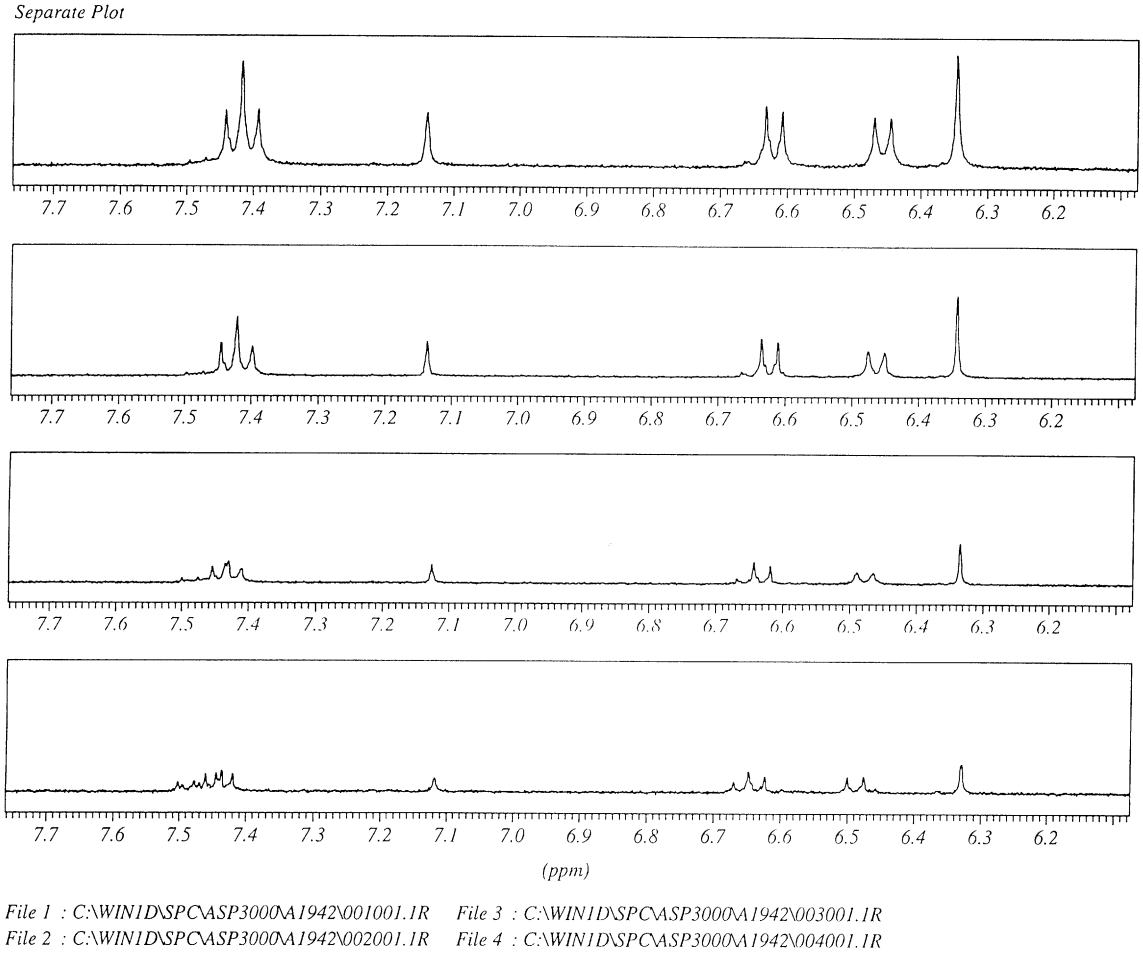


Figure 6. NMR spectrum of K-130 (0.5 µM) in the absence (top) and in the presence of increasing concentrations of *E. coli* F470 derived LPS (0.1, 0.4 and 0.6 mg LPS added to 500 µL volume). No change in relaxation rate ($1/T_2$) of the internal standard DMSO at 2.5 ppm occurs.

of the substituents and/or their molecular weights. A first inspection of structures, lipophilicity and strength of interaction seems to support this argument.

A maximum of 11 derivatives could be included in the analysis of structure–binding relations because I_{50} values against F614 and/or F612 could not be determined for some compounds due to low solubility and activity and the instability of KC-1308. The loss of these derivatives made the set more heterogeneous than originally planned.

First, a PCA was performed to compare the ratios in the I_{50} values obtained for the mutants in various test systems (F614/F515; F612/F515; F588/F515; $I_{50\ F614}$ whole cell/cell-free and $I_{50\ F515}$ whole cell/cell-free). As expected only one principal component can be extracted, explaining 88% of the observed total variance and being highly correlated with the lipophilicity of the compounds. If the I_{50} values obtained for the cell-free system (DHFR-enzyme) are included, a second PC is extracted mainly loaded with the original I_{50} values from the cell-free system and the molecular weight of the derivatives.

Regression analysis was performed with the logarithm of the ratio of the I_{50} values found for the inhibition of the *E. coli* mutants F614/F515. It revealed a significant correlation with the lipophilicity of the derivatives. The exception was Ro-118958 where both I_{50} values are significantly lower than observed for the other derivatives. But the ratio is still large, showing that the compound interacts with membrane constituents. An

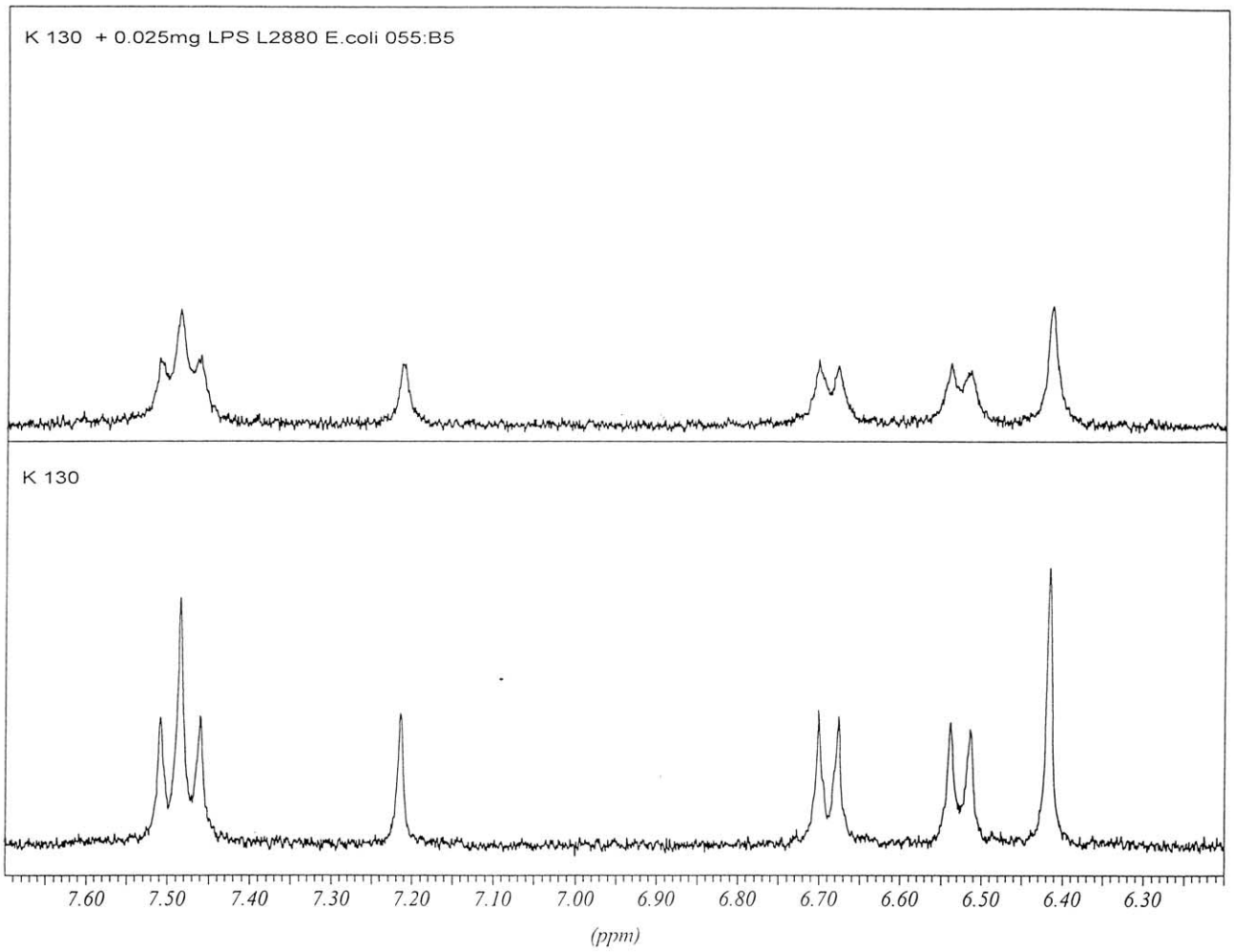


Figure 7. NMR spectrum of K-130 (0.5 µM) in the absence (below) and presence of 25 µg *E. coli* 055:B5 L2880 derived LPS.

explanation for the exception of Ro-118958 could be that it possesses only a short and bulky substituent as compared to the other derivatives and so it may not be able to reach down to lipid A to the same extent. KC-1307 did also not optimally fit into the derived correlations. Here the sterical orientation of the naphthyl ring may play a role or the calculated log k′ might be not correct as the calculated log P values by the two programs differ significantly.

If the two aforementioned derivatives are excluded, statistically significant equations are derived with almost identical regression coefficients (slope), where log k′ is the descriptor of the lipophilicity of the tested derivatives, n is the number of derivatives, r the regression coefficient, s the standard deviation, F the ratio of explained to unexplained variance and Q^2 is the squared correlation coefficient of the plot $y_{obs.}$ versus $y_{pred.}$ by the leave-one-out procedure.

$$\log I_{50\,F614}/I_{50\,F515} = 0.362\ (\pm 0.142)\ \log k'_{calc.} + 0.09\ (\pm 0.152)$$

$$n = 8;\ r^2 = 0.86;\ s = 0.149;\ F = 38.9;\ Q^2 = 0.77 \quad (1)$$

$$\log I_{50\,F612}/I_{50\,F515} = 0.323\ (0.171)\ \log k'_{calc.} + 0.151\ (\pm 0.449)$$

$$n = 8;\ r^2 = 0.78;\ s = 0.180;\ F = 21.4;\ Q^2 = 0.68 \quad (2)$$

inclusion of KC-1307 led to the following eqs.:

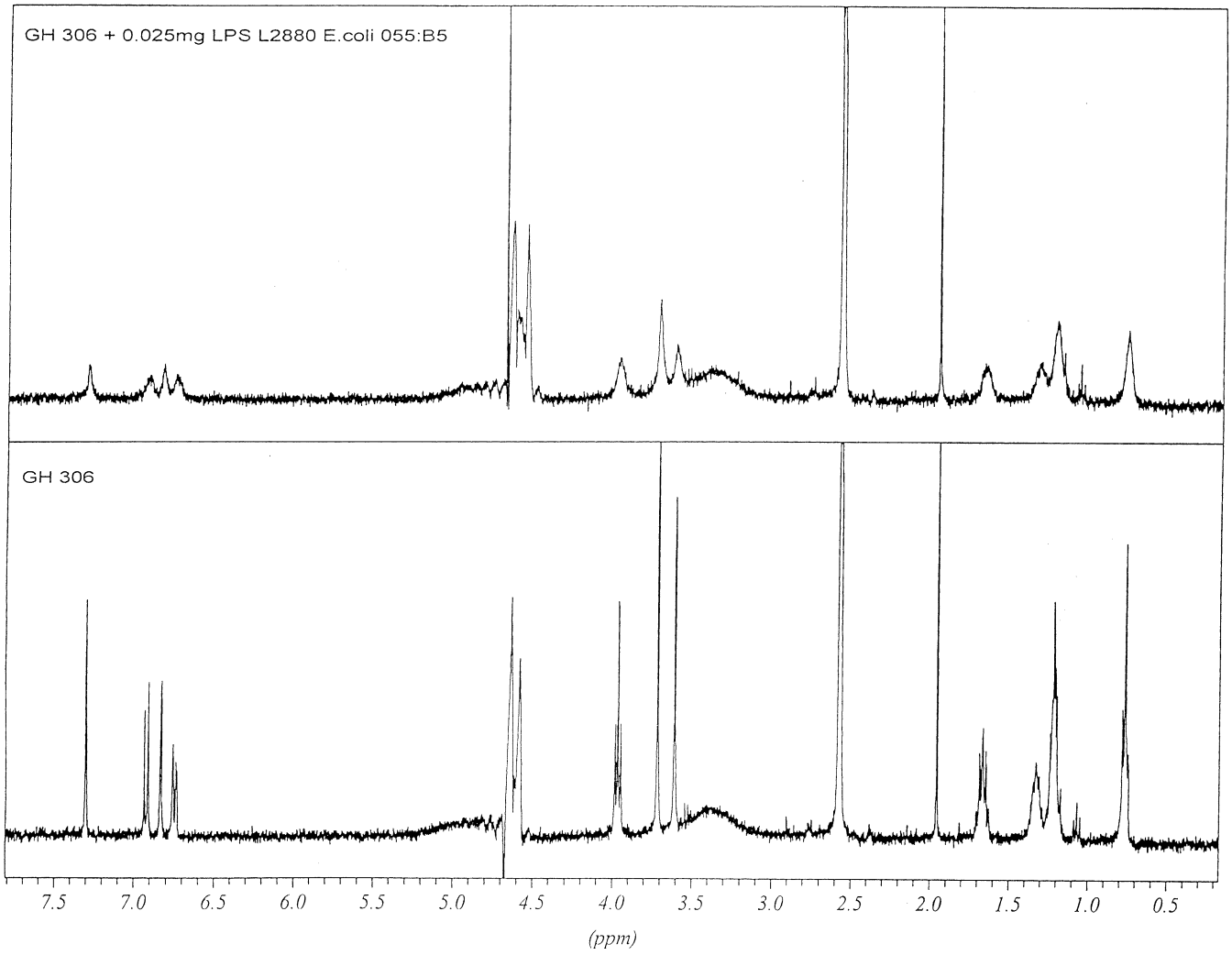


Figure 8. NMR spectrum of GH-306 (0.5 µM) in the absence (below) and presence of 25 µg *E. coli* 055:B5 L2880 derived LPS. The relaxation rate of the internal standards DMSO and CH_3CN remain unchanged as in *figure 6*.

$$\log I_{50\,F614}/I_{50\,F515} = 0.322\ (\pm 0.175)\log k'_{calc.} + 0.149\ (\pm 0.489)$$

$$n = 9;\ r^2 = 0.72;\ s = 0.20;\ F = 18.3;\ Q^2 = 0.52 \quad (3)$$

$$\log I_{50\,F612}/I_{50\,F515} = 0.296\ (\pm 0.169)\log k'_{calc.} + 0.188\ (\pm 0.458)$$

$$n = 9;\ r^2 = 0.71;\ s = 0.192;\ F = 17.1;\ Q^2 = 0.56 \quad (4)$$

If the molecular weight is used as a second parameter the following equations are derived:

$$\log I_{50\,F614}/I_{50\,F515} = 0.287\ (0.185)\log k'_{calc.} + 9.35 \times 10^{-4}\ (\pm 1.69 \times 10^{-3})\,MW + 0.039\ (\pm 0.511)$$

$$n = 9;\ r^2 = 0.88;\ s = 0.19;\ F = 11.12;\ Q^2 = 0.62 \quad (5)$$

$$\log I_{50\,F612}/I_{50\,F515} = 0.241\ (\pm 0.118)\log k'_{calc.} + 1.49 \times 10^{-3}\ (\pm 1.08 \times 10^{-3})\,MW + 0.012\ (\pm 0.328)$$

$$n = 9;\ r^2 = 0.899;\ s = 0.12;\ F = 26.8;\ Q^2 = 0.83 \quad (6)$$

In equation 5 the MW term is not significant on the 95% level but it improves the predictive power, Q^2. The inter-correlation between log k′ and MW is negligible (r^2 = 0.115).

Equations 7–9 show that the whole cell inhibitory activity can be described by the respective cell-free activities and the lipophilicity of the derivatives:

$$\log I_{50w.c\ F614} = 0.392\ (\pm 0.287)\ \log I_{50c.f.\ F614} + 0.388\ (\pm 0.255)\ \log k'_{calc.} + 1.17\ (\pm 1.02)$$

$$n = 9;\ r^2 = 0.81;\ s = 0.28;\ F = 12.6;\ Q^2 = 0.50 \quad (7)$$

(strongest deviation by derivative K-130, s = 0.44)

$$\log I_{50w.c.\ F612} = 0.332\ (\pm 0.270)\ \log I_{50c.f.\ F612} + 0.357\ (\pm 0.243)\ \log k'_{calc.} + 1.06\ (\pm 0.961)$$

$$n = 9;\ r^2 = 0.79;\ s = 0.27;\ F = 11.3;\ Q^2 = 0.50 \quad (8)$$

$$\log I_{50w.c.\ F515} = 0.501\ (\pm 0.201)\ \log I_{50c.f.\ F515} + 1.43\ (\pm 0.554)$$

$$n = 11;\ r^2 = 0.78;\ s = 0.22;\ F = 31.5;\ Q^2 = 0.66 \quad (9)$$

As expected, the I_{50} values of the deep rough mutant with minimal LPS configuration (no phosphate groups) only correlate with the I_{50} values obtained from the cell-free system. The lipophilicity of the derivatives (log k′) drops out of the regression equation in agreement with the results of the binding experiments where no interaction was observed in the case of LPS-F515. Other parameters like molecular weight, van der Waals volume etc. were not significant on the 90% level and the cell-free I_{50} values in all equations were highly intercorrelated with MW.

The correlation described by equation 9 is in agreement with the observations made when an *E. coli* mutant defective in the biosynthesis of the lipid A part of LPS has been studied [25]. These authors found a significant increase in susceptibility compared to the parent cell line against lipophilic antibiotics, especially for those with large molecular weight, pointing to a loss of the barrier function of lipid A.

The results show the importance of lipophilicity and drug–membrane interactions for the whole cell activity of this type of compound. They also underline that early studies of drug–membrane interactions in vitro are indicated in the drug development process of antibacterials.

3. Experimental protocol

3.1. Chemistry

TMP and Ro-118958 were a gift from Hoffman LaRoche, Basel, A-44733 from the Abbott Laboratories. All other benzylpyrimidines used in this study were synthesized in our laboratories and the details are given in the cited literature [11–13]. Other chemicals were purchased from Merck, Germany or Aldrich at the highest purity available.

3.1.1. Binding measurements

a) Ultracentrifugation technique:

LPS extracted from different mutants was suspended in phosphate buffer. The final LPS concentration was 50 µg/mL. K-130 was solved in 3 mL DMSO and phosphate buffer was added to obtain 10 mL of solution. The concentration range of the K-130 preparations was 5–20 µmol/L (DMSO concentration 2.5% (v/v)). The samples were incubated for 30 min at room temperature, ultracentrifuged at 150 000 *g* for 5 h (Beckman Spinco L2/50B, titan rotor 50/2T). Concentration of free K-130 was determined in the supernatant by UV (Zeiss PMQ 3) at 300 nm.

Because of the relatively large MW of K-130 there is the risk of sedimentation of the unbound drug during ultracentrifugation. The final values have therefore been corrected for this factor using appropriate controls which contained K-130 only.

b) DMMB-assay:

Because of the low solubility of some of the benzylpyrimidines all compounds were taken up in 10% DMSO, 20% polyethylenglycol 400 (v/v) to prepare the stock solutions. The stock solution of the different LPS consists of 1.0 or 0.5 mg/mL LPS in phosphate buffer pH 7.2. Mixtures of LPS, drug and controls were pre-incubated for 30 min at 35 °C (Eppendorf thermoshaker). One milliliter of DMMB stock solution (16 mg DMMB, 5 mL ethanol, 3.52 g KH_2PO_4, 7.26 g $Na_2HPO_4 \cdot 2H_2O$, 3.33 g NaCl, aqua bidest ad 1 000 mL, final pH 7.0) was placed in an Eppendorf tube and 50 µL of the pre-incubated mixture of LPS and drug added, mixed for 15 s and transferred to a cuvette in a UVICORD spectrophotometer. The absorbance was continuously measured in the range of 400–800 nm at 25 °C.

c) NMR technique:

Benzylpyrimidines were dissolved in DMSO so that the stock solution contained 1 µM in 100 µL. LPS was dissolved in phosphate buffer at a concentration of 5 µg/µL. Because of solubility problems the experiments have been performed at pH 4.9. To 450 µL phosphate buffer pH 4.9, 50 µL of the drug stock solution were

added and the NMR measurements run. Acetonitrile and/or DMSO were used as internal standard. Increasing amounts of LPS were added stepwise in 5 µL portions and the spectra recorded. The experiments were performed with an AM 360L spectrometer (Bruker, Darmstadt, Germany) at a probe temperature of 23 °C. Data acquisition included 32 scans, 32K FID, sweep width 4098 Hz, 0.25 Hz/Point and homonuclear pre-saturation to depress the H_2O signal. Peak broadening (change in $1/T_2$) in half peak height as a function of LPS concentration was determined.

Reproducibility was checked on selected examples and the significance of $1/T_2$ changes could be derived from the following observations: a) the resonance signals were symmetrical; b) a reduction in peak half-width is observed upon addition of drug at constant LPS concentration; c) the resonance signal of the internal standard remained unchanged upon addition of LPS.

3.1.2. Determination of log k′

Apparent partition coefficients, log k′ (octanol/buffer) at the pH of the biological experiments were determined by HPLC. Log k′-values were obtained using the method of Unger [26] with some modifications. An ODS-Hypersil® column (100 × 4.6 mm) was conditioned with octanol saturated water. Mobile phase was octanol saturated PBS buffer. The drugs were solved in 20–100 µL DMF and diluted with octanol saturated buffer so that the extinction at 254 nm or 280 nm after passage of the column was within 0.01 units. This avoids overload of the octanol film. Thiourea was used for the determination of the total dead volume. Log k′ was calculated according to:

$$\log k' = \log ((t_r - t_0)/t_0)$$

where t_r = retention time of the test substance and t_o = retention time of thiourea.

3.1.3. QSAR analysis

Linear and non-linear multiple regression, as well as principal component and partial least squares analyses, were carried out using locally written programs. The calculated log P values were obtained using the ClogP program [27] or the method of Viswanadhan and Ghose [28]. Both calculation methods gave high correlations with the experimental log k′ values, however, they showed significant deviations for different compounds. Therefore, PLS was used to generate the best estimate for the missing log k′ values leading to the following equation:

$$\log k' = 0.481\ \mathrm{ClogP} + 0.570\ \log \mathrm{P\_VG} - 0.373$$

$$n = 10;\ r^2 = 0.929;\ s = 0.240;\ F = 105.4$$

3.2. Biology

The *E. coli* mutants were supplied by G. Schmidt, Laborgruppe Bakteriengenetik, Forschungszentrum Borstel, D-23845 Borstel and the immunochemistry and analytical values of the core oligosaccharides were determined [29] and are summarized in *table I*.

3.2.2. Bacterial growth kinetics

Stock cultures of *E. coli* mutants were maintained on agar slants at room temperature. The culture broth was dextrose/salts/casamino acids (vitamin free), pH 6.9 [30]. The medium was sterilized by filtration through cellulose ester membranes (0.22 µ).

3.2.2.1. Culture sytems and conditions

A broth culture was inoculated from an agar culture and bacteria were allowed to grow for 12–16 h at 37 °C. Cultures were prepared from this broth by dilution with medium to obtain 10^4 cells/mL. Portions of 100 mL were transferred to 1 L Erlenmeyer flasks. Thirty minutes later when the bacteria were in logarithmic growth phase the drugs were added. Samples of the incubated cultures were taken every 45 min. Particle-free saline (0.85%) and formaldehyde (2%) solution were added to stop multiplication as well as to dilute the solution to about 1 000–10 000 organisms per count. Counting was achieved with a Coulter Counter Model ZB equipped with a 30 µm orifice; counts of 50 µL were collected. The instrument settings were: 1/aperture current, 1; 1/amplification, 1/2; matching switch, 40 K; gain, 10; lower threshold, 7; upper threshold, maximum.

The quotient of the bacterial generation rate, k, in the presence and, k_o, in the absence of drug describes the inhibitory effect. A non-linear fit to the following equation is used to determine the I_{50}:

$$k = k_o \times I_{50}^b / (I_{50}^b + c^b)$$

The exponent b is the Hill coefficient, for b = 1 a hyperbolic curve is obtained for b > 1 an asymmetric sigmoidal curve.

3.2.3. Extracts of DHFR from E. coli *ATCC 11775 and* E. coli *mutants F515 and F614*

The partially purified DHFR was derived from the test organisms by the procedure described by Bartels and Bock [31].

3.2.4. Inhibition of DHFR, determiantion of I_{50} values

The DHFR activity was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm as a function of time [32, 33]. The assay reaction mixture contained 100 mM Tris buffer pH 7.2, 0.1 mM NADPH, isolated DHFR and varying amounts of the inhibitor

benzylpyrimidines. After pre-incubation for 5 min at 25 °C the reaction was started by adding H_2 folate (final concentration 0.03 mM/L). The concentration of the benzylpyrimidines which cause 50% inhibition, I_{50}, of the synthesis of DHFR and bacterial generation rates, respectively, was calculated by non-linear regression analysis using locally written software.

3.2.5. Extraction of LPS from E. coli *mutants*

Extraction of LPS from *E. coli* mutants was performed according to Galonos [34]. The obtained fractions were free of impurities by nucleic acids, proteins, phospholipids and other constituents of the bacterial cell wall [35].

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