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COMMENTARY

Mechanism of Action of Diazaborines

Clair Baldock, Gert-Jan de Boer,† John B. Rafferty,* Antoine R. Stuitje† and David W. Rice*‡*

*Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2TN, U.K.; and †Department of Genetics, Institute of Molecular Biological Studies (IMBW), Vrije Universiteit, Biocenter Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

ABSTRACT. The diazaborine family of compounds have antibacterial properties against a range of Gram-negative bacteria. Initially, this was thought to be due to the prevention of lipopolysaccharide synthesis. More recently, the molecular target of diazaborines has been identified as the NAD(P)H-dependent enoyl acyl carrier protein reductase (ENR), which catalyses the last reductive step of fatty acid synthase. ENR from *Mycobacterium tuberculosis* is the target for the front-line antituberculosis drug isoniazid. The emergence of isoniazid resistance strains of *M. tuberculosis*, a chronic infectious disease that already kills more people than any other infection, is currently causing great concern over the prospects for its future treatment, and it has reawakened interest in the mechanism of diazaborine action. Diazaborines only inhibit ENR in the presence of the nucleotide cofactor, and this has been explained through the analysis of the x-ray crystallographic structures of a number of *Escherichia coli* ENR–NAD^+–diazaborine complexes that showed the formation of a covalent bond between the boron atom in the diazaborines and the 2′-hydroxyl of the nicotinamide ribose moiety that generates a noncovalently bound bisubstrate analogue. The similarities in catalytic chemistry and in the conformation of the nucleotide cofactor across the wider family of NAD(P)-dependent oxidoreductases suggest that there are generic opportunities to mimic the interactions seen here in the rational design of bisubstrate analogue inhibitors for other NAD(P)H-dependent oxidoreductases. BIOCHEM PHARMACOL **55**;10:1541–1549, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. enoyl reductase; diazaborine; isoniazid; lipid biosynthesis; bisubstrate analogues

Diazaborines represent a group of antibacterial drugs of which the important structural element is a heterocyclic 1,2-diazine ring containing a boron as a third hetero atom (Fig. 1). Although the antimicrobial activity of these compounds was first described in the late 1960s, their biological target remained obscure until the 1980s. The first clue that diazaborines may interfere with membrane biosynthesis was obtained by Hogenauer and Woisetschlager [1]. Using specific mutant strains of *Escherichia coli* and *Salmonella typhimurium*, they showed that diazaborine inhibits the incorporation of radioactive galactose into the LPS§ of these bacteria. This result fitted nicely with earlier observations that the antibacterial activity of diazaborines is confined almost exclusively to Gram-negative bacteria, indicating that they specifically inhibit LPS synthesis, which is an integral part of the outer membrane of this group of bacteria. This notion triggered the search for new diazaborine derivatives and analogues in the treatment of bacterial infections, despite the inherent toxicity of boron-containing compounds [2].

‡ Corresponding author. Tel. 44-114-222-4242; FAX 44-114-272-8697.

§ *Abbreviations:* LPS, lipopolysaccharide; ACP, acyl carrier protein;

STRUCTURE–ACTIVITY RELATIONSHIPS

The schematic structure for compounds that are generally referred to as diazaborines is given in Fig. 1A. The more systematic name for these compounds is 1,2-dihydro-1-hydroxy-2-(organosulfonyl)-areno[*d*][1,2,3]diazaborines (arene = benzene, naphthalene, thiophene, furan, pyrrole). Systematic syntheses of these compounds, by a reaction of (organosulfonyl)hydrazones of arene aldehydes or ketones with tribromoborane in the presence of ferric chloride, were first described by Grassberger *et al.* [2]. In this study, the activities of approximately 80 different diazaborine derivatives against bacteria *in vitro* and *in vivo* (*E. coli* septicaemia) were determined. Although, in general, thieno-diazaborines were found to be the most potent inhibitors, followed by benzo-diazaborines and furo-diazaborines, whereas pyrrolo-diazaborines were totally inactive (Fig. 1B), this classification oversimplifies the extensive data of Grassberger *et al.* [2].

To facilitate a more comprehensive understanding of the significance of the organosulfonyl side chain, the arene group, and various substitutions on this group in relation to antibacterial activity of diazaborines, we have summarized the most relevant data, in this respect, in

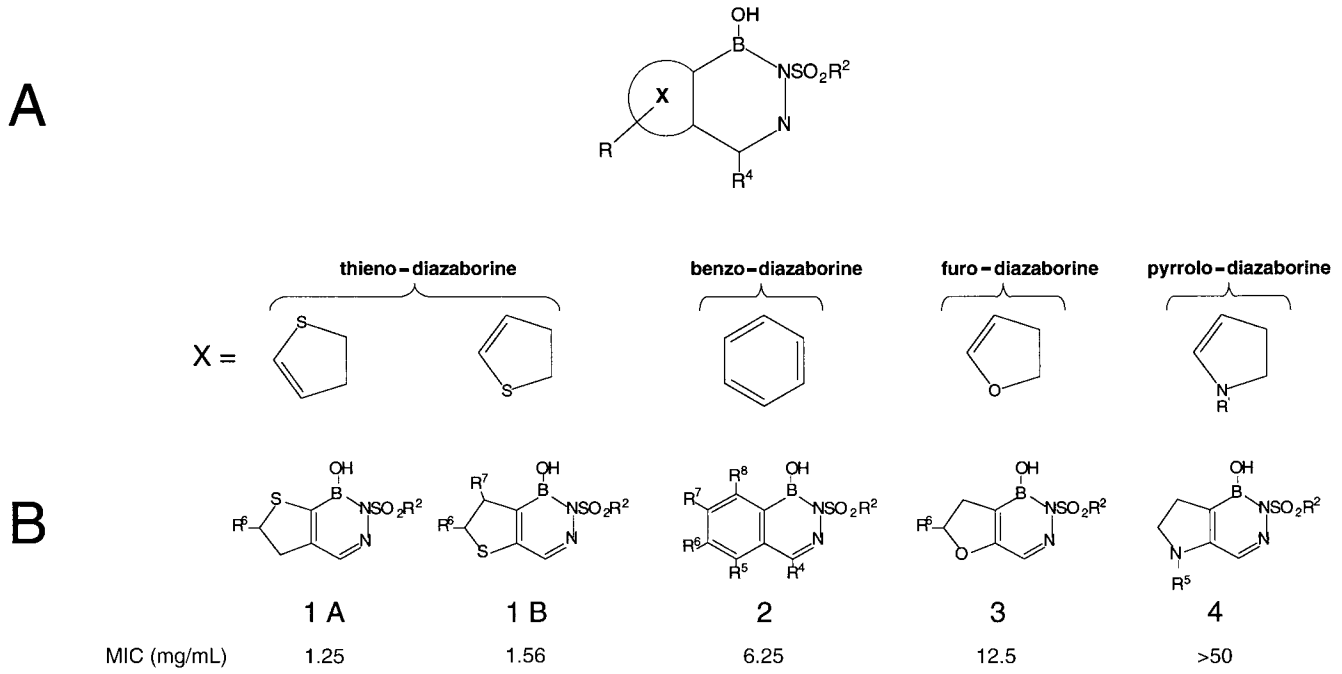


FIG. 1. (A) Structural formulae of a number of different classes of diazaborine; and (B) the MIC values for the best inhibitors in each class of diazaborine 1A, 1B, 2, 3 and 4 in an *in vivo* antibacterial assay against *E. coli* [2]. MIC = minimum inhibitory concentration.

Importance of the Organosulfonyl Side Chain (SO_2R^2)

As depicted in Table 1, irrespective of the nature of the arene group, diazaborines with propylsulfonyl side chains show the highest antibacterial activity. Decreasing the chain length of the alkyl group greatly reduces activity (Table 1, No. 6 vs No. 7 or No. 11 vs No. 13). Derivatives with benzylsulfonyl side chains show considerable activity, whereas substitutions on the benzene ring are generally not favourable (No. 9 vs No. 8 and 10).

Importance of the Arene Group (X)

Irrespective of the nature of the organosulfonyl group, thieno-diazaborines are more potent than benzo-diazaborines (No. 1 vs No. 11, No. 12 vs No. 3 and 10). In the thieno-diazaborine series, however, the thieno[2,3-*d*]diazaborines (No. 1–4) are generally slightly more active than their thieno[3,2-*d*] counterparts (No. 5–10).

Substitution of a methyl for a hydrogen in position 6 (Fig. 1) of thieno-diazaborine significantly increases biological activity (e.g. No. 6 vs No. 5), whereas replacement of a methyl by bromine has little effect, with the exception of the 6-bromo derivative of thieno[2,3-*d*]diazaborine, which is totally inactive (e.g. No. 4 vs No. 9 and 10). Substitution of hydrogen in position 7 is probably not advisable, since replacement with bromine resulted in a complete loss of biological activity (not shown).

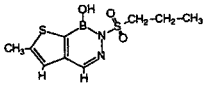
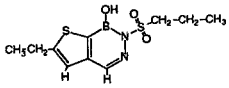
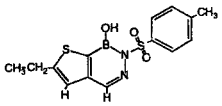
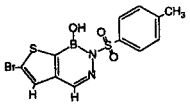
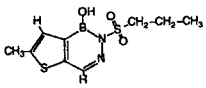
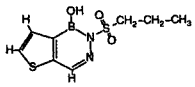
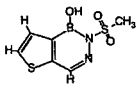
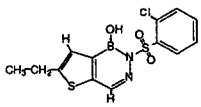
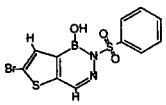
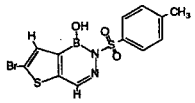
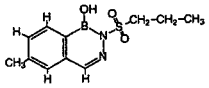
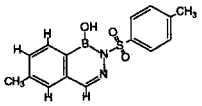
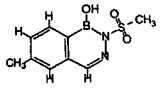
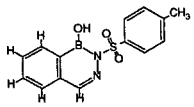
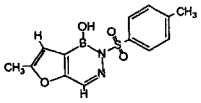
Benzo-diazaborines are generally less active than thieno-diazaborines, and substitutions by methyl or halogen (F, Cl, Br) on the benzene ring have no marked influence on the bacterial activities *in vitro*. In general, benzo-diazaborine 1) are less active than the unsubstituted parent compound (No. 14; not shown). Substitution (methyl, halogen) in position 6 slightly increases antibacterial activity (No. 14 vs No. 12). Substitution with polar groups (OH, NH_2, NR_2, $NHCOCH_3$, COOH) in position 6 or 7 generally leads to a complete loss of activity (not shown).

Only a few furo- and pyrrolo-diazaborines (Fig. 1) were analysed in the work of Grassberger *et al.* [2]. While pyrrolo-diazaborines were found to be completely inactive, 2 out of the 3 furo-diazaborines tested showed substantial antibacterial activity. Both biologically active furo-diazaborines have a tosyl group attached to the sulfonyl moiety (Table 1, No. 15), and a methyl or bromine substitution in position 6. Although alkylsulfonyl derivatives were not included in the study, it seems very likely that substitution of a propyl moiety instead of a tosyl moiety will further enhance the biological activity of furo-diazaborines.

BORON-FREE ANALOGUES

A major problem with diazaborines is their inherent toxic potential, which is probably due to the arenoboronic acid amide moiety [2]. To help design boron-free analogues, Grassberger *et al.* [2] tried to address the question as to whether the bicyclic areno-diazaborines themselves are the active species or whether hydrolytic cleavage at the BN bond to give the corresponding (dihydroxy)arenes is essential for biological activity of the compounds. For this purpose they synthesized the carbacyclic analogue of benzo-diazaborine (No. 14). This isoquinoline (Fig. 2A), however, was inactive in all test systems. On the other hand,

TABLE 1. Antibacterial activity of selected diazaborines*

Structure	No.	ED_{50} (mg/kg)	MIC† values (μg/mL)					
			E. coli	*E. aerogenes*	*S. typhimurium*	*K. pneumoniae*	*P. mirabilis*	*N. gonorrhoeae*
	1	2	1.25	1.56	0.19	0.31	1.56	0.5
	2	4	2.5	5	0.31	0.31	1.25	0.5
	3	9	6.25	25	1.56	1.25	1.25	0.25
	4	>300	>50	>50	>50	>50	>50	>8
	5	4.5	1.56	3.12	0.78	0.39	0.78	1
	6	28	6.25	25	3.12	1.56	25	8
	7	113	>50	>50	>50	>50	>50	>8
	8	49	3.12	12.5	0.78	0.31	0.39	0.5
	9	~20	3.12	12.5	0.78	0.78	1.56	1
	10	~15	6.25	25	3.12	1.56	1.56	0.5
	11	73	6.25	10	2.5	1.25	1.56	2
	12	~15	12.5	50	3.12	1.56	3.12	1
	13	42	25	>50	12.5	6.25	25	>8
	14	~25	25	>50	6.25	3.12	12.5	2
	15	~10	12.5	25	3.12	1.56	3.12	1

*Data excerpted from Ref. 2. Reprinted with permission from *J Med Chem* **27:** 947–953, 1984. © 1984 American Chemical Society.

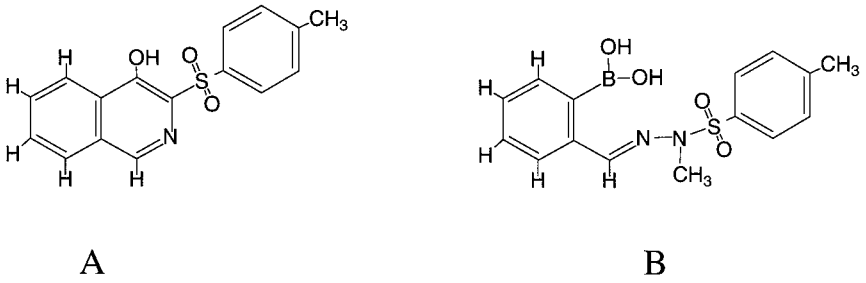


FIG. 2. Structural formulae of: (A) the carbacyclic analogue of benzo-diazaborine; and (B) the ring-opened derivative of benzo-diazaborine.

stable ring-opened analogue of benzo-diazaborine (No. 14), showed some biological activity on *E. coli*, but was at least ten times less active than the corresponding diazaborine. Although these results seem to support to some extent the hypothesis that ring opening is essential for biological activity, no firm conclusions could be made.

CELLULAR TARGET OF DIAZABORINES

The antibacterial activity of diazaborines is confined almost exclusively to Gram-negative bacteria. A rationale behind this observation was first presented by Hogenauer and Woisetschlager [1], who demonstrated that a specific thieno-diazaborine (Table 1, No. 5) inhibited LPS biosynthesis in both *E. coli* and *Salmonella*. In a later paper of Turnowsky *et al.* [3], it was shown that the primary target of inhibition was not LPS biosynthesis itself, but rather an earlier step in lipid A precursor biosynthesis, such as *de novo* fatty acid biosynthesis or the acyl transfer to the UDP-*N*-acetylglucosamine moiety. In addition, these authors carried out a thorough molecular genetic analysis of diazaborine resistance in both *E. coli* and *Salmonella* and demonstrated that resistance results from a point mutation in the *envM* genes of these bacteria. In an earlier study, the *envM* gene of *E. coli* was identified as an essential gene, since an allelic mutant form (*envM392*) results in a temperature-sensitive growth phenotype [4]. In fact, the similarity of the effects on *E. coli* cells seen after treatment with diazaborine or by incubating a temperature-sensitive *envM* mutant at 42° strongly suggested that the envM protein was the actual target of the drug.

BIOCHEMICAL TARGET OF DIAZABORINES

Despite the demonstration that both inhibition of wild-type *E. coli* cells by diazaborine and shifting the *envM392* mutant to the nonpermissive temperature result in immediate cessation of fatty acid biosynthesis, the actual function of the envM protein in fatty acid biosynthesis remained obscure until more recently. The notion that the envM protein might actually be the ENR component of the bacterial fatty acid synthetase (FAS II) was only recognized a few years ago on the basis of amino acid sequence homology with the purified ENR protein and the corresponding cDNA sequence from oilseed rape (*Brassica napus*) [5, 6]. ENR catalyses the last reductive step in the cyclic process of fatty acid elongation, as depicted in Fig. 3. Direct evidence that the *E. coli envM* gene encodes a diazaborine-sensitive ENR was provided independently by two laboratories in 1994 [7, 8]. In addition, it was established that the *envM392* (ts) allele encoded an extremely temperature-sensitive ENR and that diazaborine is a specific inhibitor of this *E. coli* enzyme [8]. The enzyme studies also showed that NAD^+ is required as a cofactor for both the inhibition and the binding of diazaborine to the ENR enzyme [8]. Based on the fact that the *envM* gene encodes the ENR component of the *E. coli* fatty acid synthetase, this gene was recently renamed *fabI* [7].

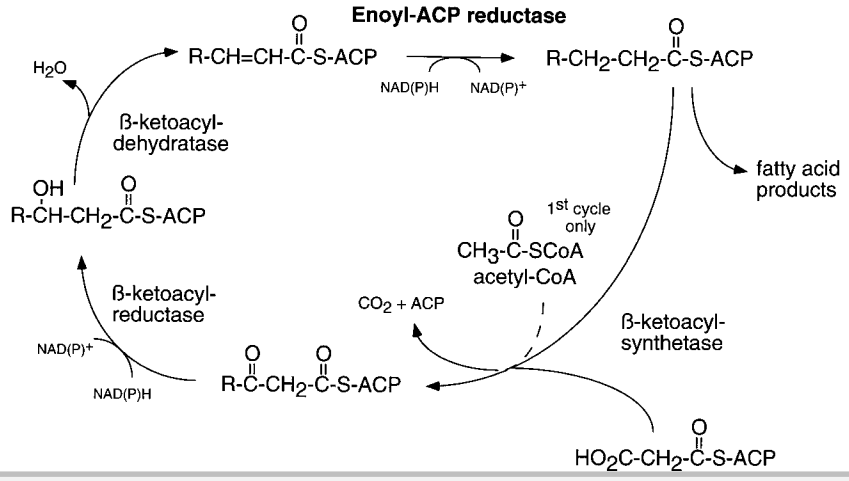


FIG. 3. Schematic representation of the fatty acid synthetase cycle.

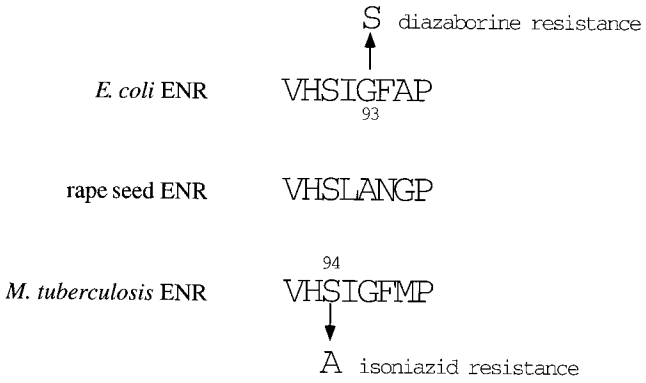


FIG. 4. Protein sequence alignment of *E. coli*, *B. napus* (rape seed) and *M. tuberculosis* ENRs in the region of the Gly93Ser and Ser94Ala mutations, associated with diazaborine and isoniazid resistance in *E. coli* and *M. tuberculosis* ENR, respectively.

MOLECULAR GENETICS OF DIAZABORINE RESISTANCE

The demonstration that ENR is the target of diazaborine and the observation that all diazaborine-resistant mutants isolated thus far have the same Gly93Ser (see Fig. 4) amino acid substitution in this gene justify the conclusion that the primary effect of diazaborine is on core fatty acid biosynthesis, whereas the effects on, for example, membrane integrity and LPS biosynthesis are of a pleiotropic nature [8]. It was also shown that both the rape seed ENR and the equivalent *E. coli* enzyme encoded by the diazaborine-resistant allele are insensitive to the drug [8]. In this respect, it is important to realize that both rape seed plantlets and *E. coli* strains with a resistant *fabI* allele are still sensitive to diazaborine at concentrations above 20 and 200 μg/mL, respectively (Stuitje AR, unpublished observation). This observation suggests that other targets for diazaborine may exist in these organisms.

The insensitivity of the plant ENR towards diazaborine has facilitated gene replacement experiments, demonstrating that the coding sequence of the essential *E. coli fabI* gene can be replaced by a cDNA sequence encoding rape seed ENR. Although the resulting *E. coli* strain shows slightly different growth characteristics and membrane fatty acid composition, it is viable under laboratory conditions, demonstrating that plant ENR can functionally replace its counterpart in the bacterial multi-enzyme FAS system [8].

SENSITIVITY OF ENR TO OTHER DRUGS

Recently, it has become evident that besides diazaborines, ENR is a potential target for at least two other drugs (see Fig. 5), the bleaching herbicide diflufenican [*N*-(2,4-difluorophenyl)-2-[3-(trifluoromethyl)phenoxy]-3-pyridinecarboxyamide] and the antituberculosis drug isoniazid [INH; isonicotinic acid hydrazide].

Although the primary mode of action of diflufenican is on carotenoid biosynthesis, with phytoene synthase being the main target, it was also reported to inhibit plant fatty acid synthetase *in vitro* [9]. In fact, Ashton *et al.* [10] demonstrated that both plant and *E. coli* ENR are also a target for diflufenican. Although it has structural similarities, in part, with pyridine nucleotides, the mode of action of diflufenican is still obscure, since it is clearly not a general inhibitor of pyridine nucleotide-dependent enzymes. The NADPH-dependent β-keto reductase component of FAS, for example, is not inhibited by diflufenican [10].

Isoniazid has been used since 1952 as one of the most effective drugs for the treatment and prophylaxis of tuberculosis. A single missense mutation in the *inhA* gene of *Mycobacterium tuberculosis* can confer resistance to this drug. Based on the similarities of the corresponding inhA protein to *E. coli* (40%) and rape seed ENR (37%), it was demonstrated recently that this gene also encodes an NADH-specific ENR [11]. Despite the fact that the crystal structure of the inhA protein is solved and that the mutation that leads to resistance to isoniazid, Ser94Ala (Fig. 4), maps to a region close to the nucleotide binding site [11], little is known about the mode of action of isoniazid at the molecular level. Further mechanistic studies on enzyme inhibition by isoniazid have been seriously hampered, because it is now recognized that isoniazid is a prodrug that is activated by mycobacteria to an as yet

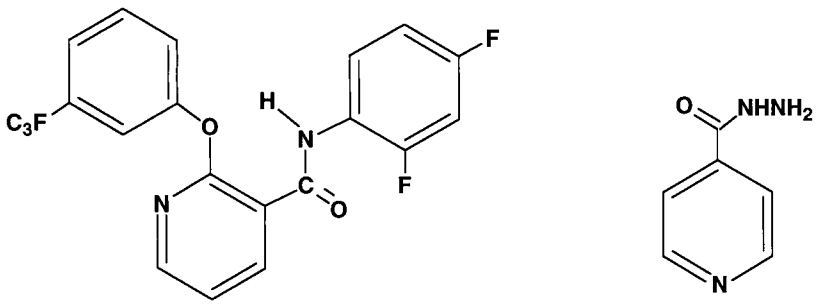


FIG. 5. Structural formulae of diflufenican and isoniazid.

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