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COMMENTARY

Mechanism of Action of Diazaborines

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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].

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 Table 1.

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§ *Abbreviations:* LPS, lipopolysaccharide; ACP, acyl carrier protein; ENR, enoyl ACP reductase; and FAS, fatty acid synthetase.

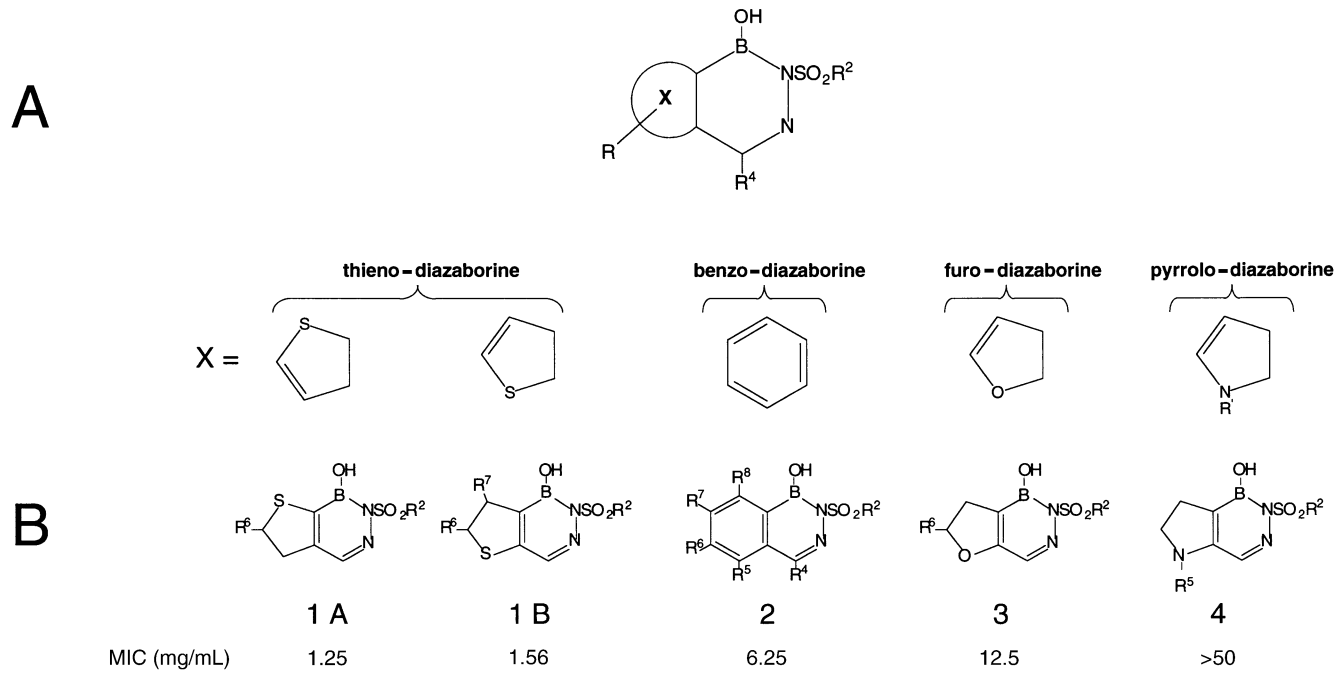


FIG. 1. (A) Structural formulae of a number of different classes of diazaborine; and (B) the MIC values for the best inhibitors in each class of diazaborine 1A, 1B, 2, 3 and 4 in an *in vivo* antibacterial assay against *E. coli* [2]. MIC = minimum inhibitory concentration.

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).

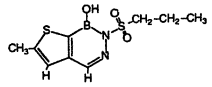
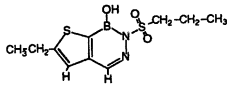
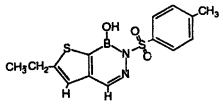
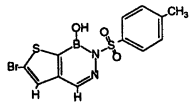
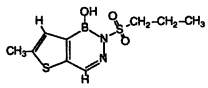
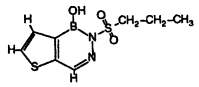
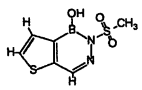
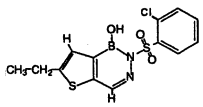
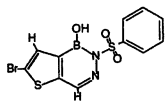
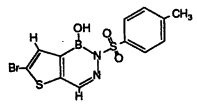
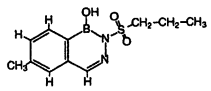
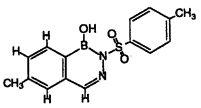
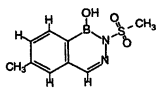
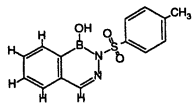
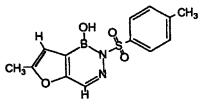
Benzo-diazaborines are generally less active than thieno-diazaborines, and substitutions by methyl or halogen (F, Cl, Br) on the benzene ring have no marked influence on the bacterial activities *in vitro*. In general, benzo-diazaborine derivatives with methyl or halogen in position 5 or 7 (Fig. 1) are 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, the stable *N*-methyl derivative (Fig. 2B), which served as a

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.

†MIC = minimum inhibitory concentration that inhibited visible growth of bacteria in trypticase soy broth (in μg/mL). Genus and species not specified in text: *E. aerogenes*, *Enterobacter aerogenes*; *K. pneumoniae*, *Klebsiella pneumoniae*; and *P. mirabilis*, *Proteus mirabilis*.

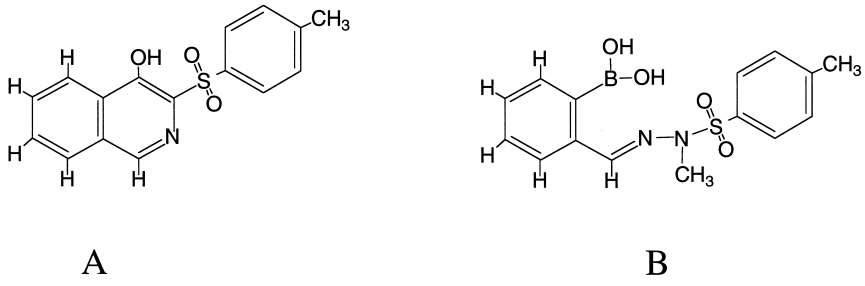


FIG. 2. Structural formulae of: (A) the carbacyclic analogue of benzo-diazaborine; and (B) the ring-opened derivative of benzo-diazaborine.

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].

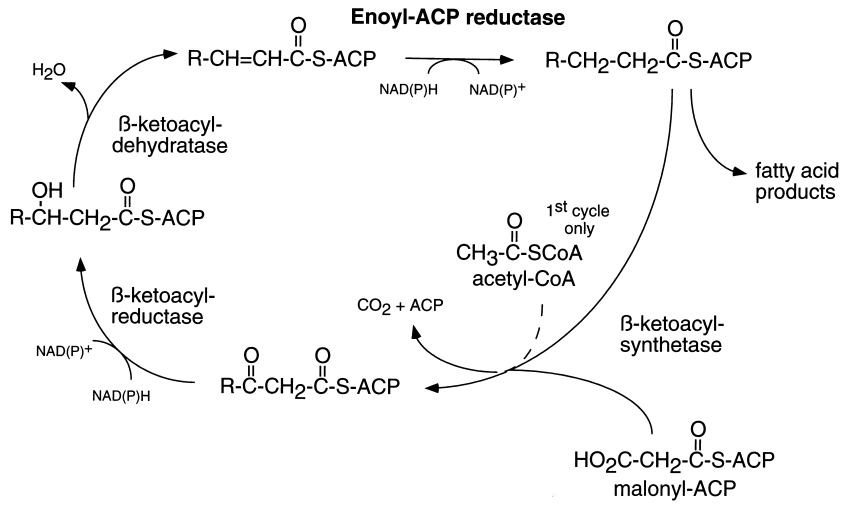


FIG. 3. Schematic representation of the fatty acid synthetase cycle.

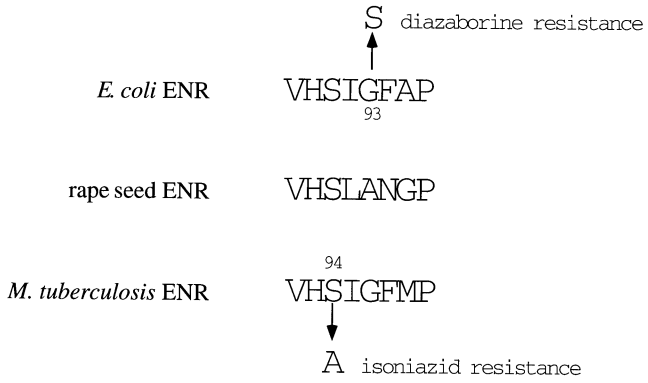


FIG. 4. Protein sequence alignment of *E. coli*, *B. napus* (rape seed) and *M. tuberculosis* ENRs in the region of the Gly93Ser and Ser94Ala mutations, associated with diazaborine and isoniazid resistance in *E. coli* and *M. tuberculosis* ENR, respectively.

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

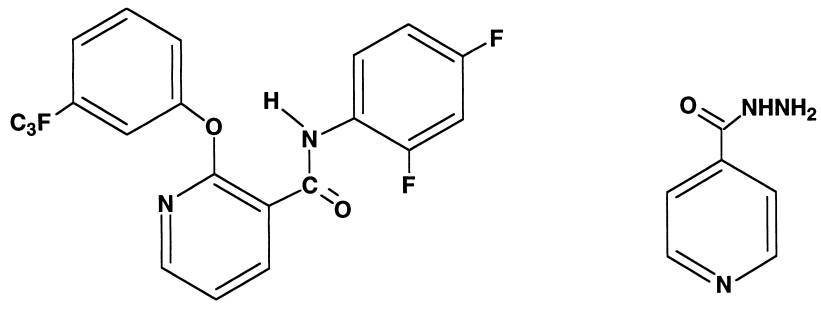


FIG. 5. Structural formulae of diflufenican and isoniazid.

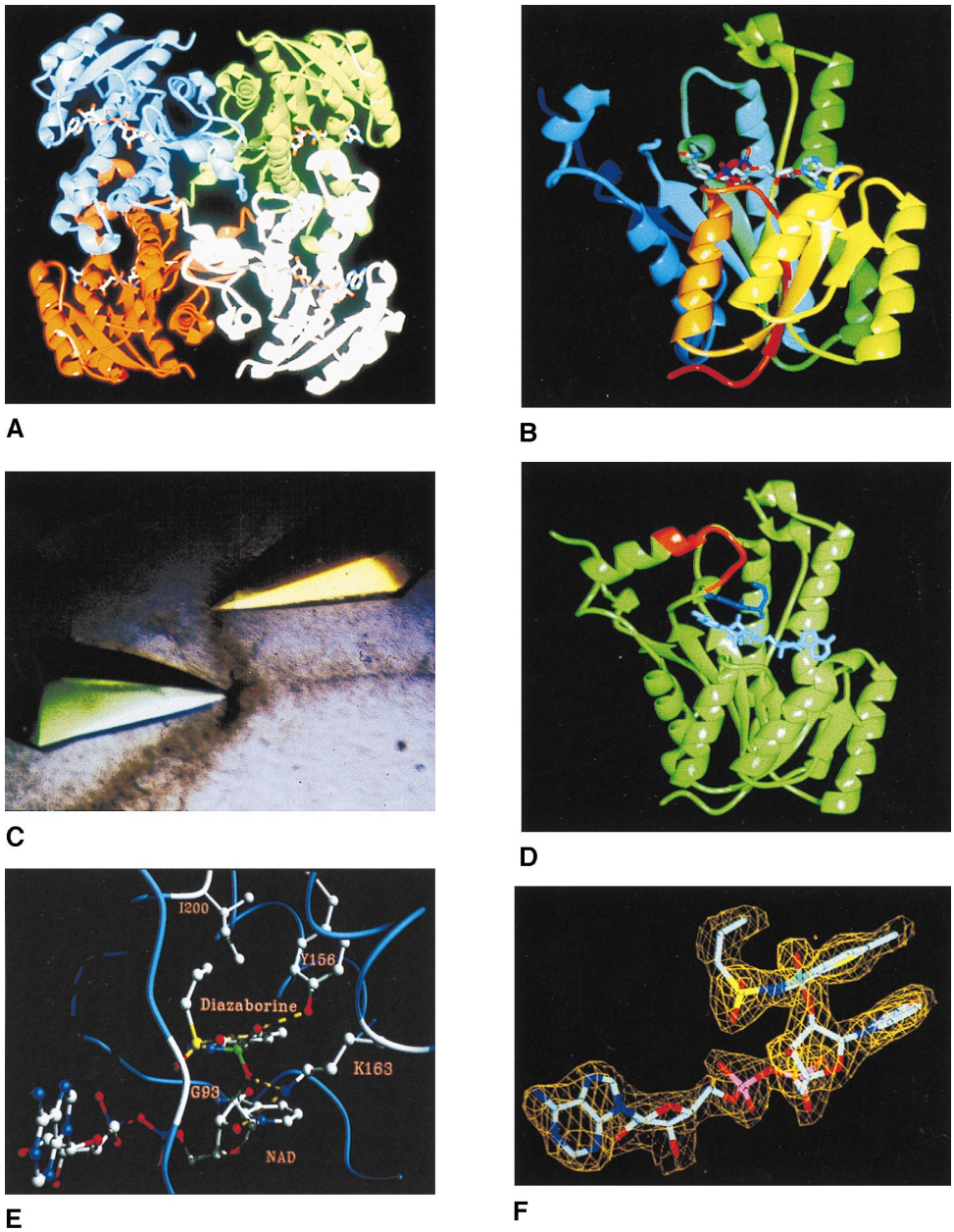


FIG. 6. (A) Schematic diagram of the ENR tetramer with the cofactor bound. The ribbon trace of *E. coli* ENR is shown coloured in red, white, green, and cyan for each subunit, and the NAD^+ is shown in an all-atom representation, coloured by atom (produced using MIDAS [14]). (B) Schematic diagram of a single subunit of the ENR–NAD^+ complex. The ribbon trace of *E. coli* ENR is shown coloured in rainbow colours from red at the N-terminus to blue at the C-terminus, and the NAD^+ is shown in an all-atom representation, coloured by atom (produced using MIDAS [14]). (C) Photograph of an ENR–NAD^+–diazaborine crystal. (D) Schematic diagram of a single subunit of the ENR–NAD^+–thieno-diazaborine complex. The ribbon trace of *E. coli* ENR is shown in green; the NAD^+ and diazaborine are shown in an all-atom representation and coloured cyan and blue, respectively. The loop that orders on diazaborine binding is highlighted in red (produced using MIDAS [14]). (E) Active site of the ENR–NAD^+–thieno-diazaborine complex. The Cα backbone trace is shown in cyan, with the NAD^+ and diazaborine shown in an all-atom representation and coloured by atom (produced using MIDAS [14]). (F) Fourier map of the NAD^+–thieno-diazaborine complex at 2.2 Å resolution with the final refined structure superimposed (produced using BOBSCRIPT [15]).

unknown species. This activation process is thought to involve the catalase-peroxidase KatG [12].

MOLECULAR BASIS OF DIAZABORINE ACTION

Given the importance of ENR as a drug target, recent crystallographic studies have sought to understand the molecular basis by which diazaborine inhibits the *E. coli* enzyme.

Structure and Mechanism of ENR

E. coli ENR is a homo-tetramer of subunit M_r of approximately 28,000 and, following crystallization [13], its structure was solved to 2.1 Å resolution (Fig. 6A), by a

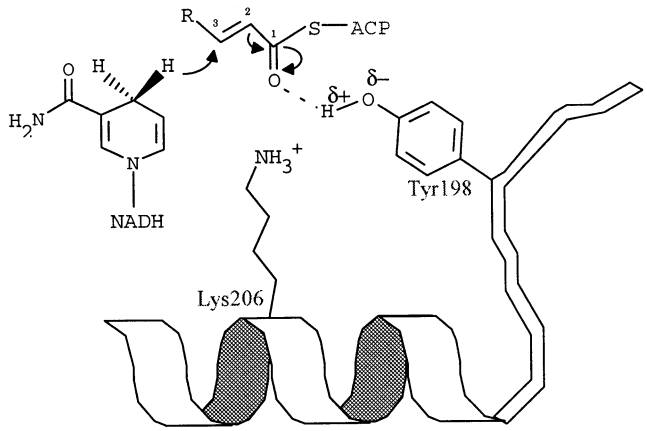


FIG. 7. Proposed catalytic mechanism of reduction of the double bond in an enoyl substrate by ENR.

combination of isomorphous replacement and molecular replacement using the *B. napus* ENR structure (Protein Data Bank entry 1ENO; [16]) as a search model [17]. A notable feature in the structure of this enzyme is a region of 10 amino acid residues from Leu195 to Met206 that forms a disordered loop between strand β6 and helix α6, which borders the nucleotide binding site (Fig. 6B). In the *B. napus* structure, residues in the equivalent region (residues Ala240 to Thr249) have high temperature factors and have been implicated in substrate binding [16]. Therefore, this disorder may reflect the fact that the acyl substrate is not present in the crystals of the *E. coli* enzyme.

The ENR subunit comprises a single domain of dimensions 45 × 48 × 50 Å and is formed from seven β-strands (β1–β7), creating a parallel β-sheet with seven flanking α-helices (α1–α7). The β-sheet is flanked on one side by helices α1, α2, and α7 and on the other by helices α3, α4, and α5 with α6 sitting along the "top edge" of the β-sheet above the COOH-terminal ends of strands β6 and β7. This fold is highly reminiscent of the Rossmann fold commonly found in dinucleotide binding enzymes [18]. The cofactor is bound in a similar and extended conformation to that observed in other dehydrogenases at the COOH-terminal end of the β sheet with the nicotinamide ring lying deep in a pocket on the enzyme surface.

A plausible mechanism for the catalytic activity of ENR has been described previously [16]. This involves the hydride transfer from the C-4 position of the NADH to the C-3 position at the double bond in the enoyl substrate. This leads to the formation of an enolate anion intermediate that can then be protonated on the oxygen to form an enol; subsequent tautomerization of the enol would then lead to the production of the reduced acyl product. Residues implicated in this mechanism include Lys163 whose role is thought to be to stabilize the positive charge of the transition state and Tyr156, thought to be the proton donor to the enolate anion (Fig. 7). Both of these residues are conserved in the sequences of the ENRs from *E. coli*, *M. tuberculosis*, *B. napus*, *Anabaena spheroides*, and *Haemophilus influenzae*.

Structural Studies on the E. coli *ENR–NAD^+–Diazaborine Complexes*

To understand the mode of diazaborine action, crystals of two *E. coli* ENR–NAD^+–diazaborine complexes were prepared (Fig. 6C) [in the presence of a thieno (No. 5) and a benzo-diazaborine (No. 14)], and the structures were solved independently by molecular replacement using the structure of *E. coli* ENR–NAD^+ as a search model to 2.2 and 2.6 Å, respectively [17]. Unlike the situation for the ENR–NAD^+ complex, in both the diazaborine complexes the loop between Leu195 and Met206 is well defined (Fig. 6D) and provides two residues, Ile200 and Phe203, whose side chains are in van der Waals' contact with the non-boron-containing 5- and 6-membered rings of the thieno- and benzo-diazaborines, respectively.

Binding of Diazaborine

Analysis of the diazaborine binding sites showed that both the diazaborine compounds bind in a closely related manner, adjacent to the nicotinamide ring of the cofactor, in a pocket formed by the side chains of Tyr146, Tyr156, Met159, Ile200, Phe203, Leu100, Lys163, and the main chain peptide between Gly93 and Ala95. The bicyclic rings of the diazaborines form a face-to-face interaction with the nicotinamide ring, allowing the formation of extensive π–π stacking interactions with additional van der Waals' interactions between the rings and the side chains of Tyr156, Tyr146, Phe203, and Ile200. The major difference between the binding of the two diazaborines is that their respective tosyl and propyl groups occupy subtly modified positions. The tosyl moiety lies perpendicular to the bicyclic ring and interacts with the main chain peptide between Gly93 and Ala95 and the side chain of Leu100, whereas the propyl moiety folds back onto the planar bicyclic ring system in a manner reminiscent of a scorpion's tail and forms interactions with the side chain of Met159 and Ile200 and the main chain peptide of both Gly93 and Phe94 (Fig. 6E). Additional interactions made by both drugs include hydrogen bonds between the boron hydroxyl and the phenolic hydroxyl of Tyr156 and between a nitrogen in the boron-containing ring and an ordered solvent molecule.

MECHANISM OF DIAZABORINE RESISTANCE

Previous studies have shown that a Gly93Ser mutation in *E. coli* ENR leads to diazaborine resistance [3, 8] and analysis of the structure shows that the alpha carbon of Gly93 lies close to the sulphonyl group of the diazaborine. Modelling studies show that in the absence of changes to the main-chain torsion angles in the Gly93Ser mutant, the Cβ atom of the serine side-chain would be unacceptably close to the two oxygens of the sulphonyl group of the diazaborine (2.1 and 2.6 Å, respectively). Therefore, resistance to diazaborine can be explained by the serine side-

chain of the mutant encroaching into the drug-binding site and causing severe steric hindrance. In *B. napus* ENR, the equivalent residue to Gly93 is an alanine, and the *B. napus* enzyme is insensitive to diazaborine, presumably because a steric clash with the methyl group of the alanine prevents diazaborine from binding. In *M. tuberculosis* ENR, the Ser94Ala mutation, which leads to resistance to isoniazid [19], maps to a similar region of the structure as Gly93 in *E. coli* ENR. However, in contrast to the evidence supporting the steric mechanism responsible for diazaborine resistance in *E. coli* ENR, Dessen and coworkers [11] proposed that the serine to alanine mutation in *M. tuberculosis* ENR causes the disruption of a hydrogen-bonding network associated with the cofactor binding, which leads to a lower affinity for the cofactor and a consequent reduction in the binding of the isoniazid-derived inhibitor.

MECHANISM OF DIAZABORINE ACTION

Analysis of the drug complex showed that the distance between the boron atom of the diazaborine and the 2′OH of the nicotinamide ribose was approximately 1.7 Å, comparable with a B—O covalent bond length of 1.6 Å. The quality of the electron density map (particularly for the thieno-diazaborine complex at 2.2 Å) implies that the errors in coordinates are very small, and thus the interaction between these two atoms is covalent. This is further supported by continuous electron density between the 2′OH of the nicotinamide ribose and the boron and the unambiguous identification of the position of the hydroxyl oxygen to which the boron is linked, which can be seen to form part of a tetrahedral, rather than a trigonal, arrangement as required if the boron forms four covalent bonds (Fig. 6F). This finding provides a clear explanation for the strong inhibitory properties of the diazaborines and for the requirement of NAD^+ for diazaborine binding. The formation of a covalent bond by the boron atom in diazaborines is similar to the mechanism of the boronic acid inhibitors of serine proteases, which chemically modify the active site serine to give a tetrahedral adduct [20].

The position of the aromatic bicyclic ring of the diazaborine above the nicotinamide ring strongly resembles the proposed model for the binding of the enoyl substrate suggested from studies on *B. napus* ENR [16] with the proposed position for the negatively charged oxygen of the enolate anion of the substrate close to that of the boron atom in the drug. The amino group of the putative catalytic lysine (Lys163) of ENR is only 4.1 Å from the boron atom, and, therefore, this residue may afford partial stabilization of the negatively charged boron, in a manner similar to its proposed role in the stabilization of the enolate anion during catalysis. Thus, it is clear that the inhibitor action of the diazaborines derives, in part, from their structural resemblance to the substrate of the enzyme.

The formation of a covalent bond between the enzyme-bound NAD^+ and diazaborine generates a tight, non-covalently bound bisubstrate analogue. In this respect, diazaborines are similar to inhibitors of pyridoxal phosphate-containing enzymes (e.g. gabaculine), which covalently modify the cofactor [21]. The best analogy is perhaps with 5-fluoro-2-deoxyuridylic acid, which acts as a potent inhibitor of thymidylate synthase [22]. This inhibitor also modifies one of the substrates (the deoxynucleoside) to covalently modify the other substrate (methylene tetrahydrofolate) to form a bisubstrate analogue. Potent bisubstrate inhibitors of other enzymes with nucleotide substrates have also been described (for example, the polyoxin inhibitors of chitin synthase [23] and various synthetic inhibitors of protein kinase C [24]). Hitherto, no such good bisubstrate inhibitors have been described for NAD(P)-dependent oxidoreductases. Examples of this type designed to inhibit 3-hydroxy-3-methyl glutaryl CoA reductase were only very weak inhibitors of cholesterol biosynthesis, possibly not only because of the lack of a moiety to mimic the adenosine diphosphoribose but also, quite probably, because of steric problems in the active site associated with the nature of the linkage that utilized the C4 atom of the nicotinamide ring [25]. Therefore, an important feature of the diazaborine study is that it indicates the type of linkage that might be used to create a bisubstrate analogue with the necessary geometry to occupy the active site cleft within a pyridine nucleotide-dependent enzyme. This may prove crucial in the design of a new generation of antibacterial agents against a range of drug-resistant organisms including *M. tuberculosis*. Furthermore, the broad spectrum antibacterial activity of diazaborines against organisms such as *Enterobacter*, *Neisseria gonorrhoeae*, *Proteus*, and *Salmonella* [2] suggests that bisubstrate analogues designed from the structure/activity profiles of the diazaborines may be effective as broad spectrum antibiotics and may even be targeted against organisms such as the multi-drug-resistant strains of staphylococcus that are proving to be a problem for the current range of antibiotics. However, given the inherent toxic potential of boron [2], it is obviously important to consider the possibility of designing a pre-formed bisubstrate analogue substituting the boron for another atom in order to develop a range of inhibitors with minimal side-effects.

GENERIC APPLICATIONS

The similarities in chemistry catalysed by the family of NAD(P)-dependent oxidoreductases give rise to generic opportunities for the creation of a series of novel enzyme inhibitors based on a related chemistry. Across the family of enzymes that belong to this class, the catalytic cycle of oxidation/reduction necessarily leads to a situation where the π electron system of a substrate approaches the face of the nicotinamide ring. Furthermore, the glycosidic bond between the nicotinamide ring and its associated ribose moiety generally adopts only one of two conformations that differ by a rotation of approximately 180° and that lead to the presentation of either the *pro*-4*R* or *pro*-4*S* hydrogen of the NADH to the active site. These conformations merely

result in a shift in the position of the carboxyamide moiety of the nicotinamide ring on the enzyme surface and do not affect the relative positions of the nicotinamide ring to its ribose group. Moreover, the structural analysis of members of this family has shown that the active site in these enzymes is consistently positioned in the same relative orientation to the nicotinamide ribose. Thus, for the subset of dehydrogenases where there is sufficient space in the structure around the 2′OH group of the nicotinamide ribose, there is an excellent opportunity to mimic the chemistry seen in the diazaborine–NAD^+ complex in the synthesis of new enzyme inhibitors. A number of NAD(P)-dependent oxidoreductases are known to be drug targets, including dihydrofolate reductase, the target for the anti-cancer agent methotrexate [26, 27], steroid 5α-reductase, the target for finasteride, used to treat benign prostatic hyperplasia [28], and inosine monophosphate dehydrogenase (IMPDH), the target for the immunosuppressant, mycophenolic acid (MPA) [28]. For example, there is considerable similarity between the ENR–NAD^+–diazaborine complex and the orientation of the inosine-5′-monophosphate thioimidate intermediate and the inhibitor MPA in IMPDH [29], suggesting that the 2′-hydroxyl of the inosine may be linked to the inhibitor in a manner similar to that seen in the diazaborine–NAD^+ complex. This suggests that the utilization of the ribose hydroxyl to create a bisubstrate analogue might find important applications in other areas of medicinal chemistry.

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