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Inhibition of microsomal oxidation by some bis(imidazolyl) derivatives

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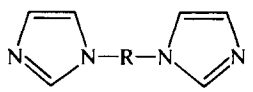
Several recent reports have described the inhibitory effects of a variety of imidazole derivatives on microsomal mixed-function oxidation in enzyme preparations from mammals and insects [1–8]. In one of these studies [5], 1,4-bis-(imidazolyl)benzene was found to exhibit an unusually high level of selectivity, being a potent inhibitor of microsomal oxidation in southern armyworm (*Spodoptera eridania*) midgut preparations while having little effect on mixed-function oxidation in rat liver microsomes. In view of the possibility that this apparent selectivity might reflect a more general steric or other difference between the microsomal oxidases of the two species, a variety of other bis-imidazolyl compounds have been synthesized and tested *in vitro* for binding and inhibitory activity toward aldrin epoxidation and *p*-chloro-*N*-methylaniline-*N*-demethylation by rat liver microsomes and southern armyworm midgut preparations.

Of the eleven imidazoles tested (Table 1), compound II was kindly supplied by Dr. A. L. Johnson, Central Research Department, E. I. DuPont de Nemours & Co., Wilmington, DE; I, III and IV were prepared by the Marckwald synthesis [9] from the corresponding diamines; and the remainder were obtained by the direct reaction of imidazole with the appropriate dibromide followed by neutralization and extraction [4, 9]. Compounds V–VII were purified by recrystallization from chloroform/hexane or methylene chloride/hexane. Compounds VIII, X and XI were purified by short path distillation at *ca.* 0.1 Torr and isolated as viscous oils, and IX was characterized as its crystalline dihydrate after recrystallization from damp acetone. The high water solubility of VIII and IX makes extraction difficult, but use of minimal water in the workup as well as the generous addition of solid sodium hydroxide to salt out the product allows their separation. It should be noted that the reaction of imidazole with 1,3-dibromopropane becomes very exothermic after a short warming period and caution is accordingly advised. Elemental analyses were provided by Galbraith Laboratories, Inc., Knoxville, TN, and in addition satisfactory spectroscopic data were obtained on all fractions employed for testing.

Rat liver microsomes were prepared as previously described [6] and resuspended in 1.15% KCl to a protein concentration of about 3 mg/ml (epoxidase assay) or 5 mg/ml (demethylase assay). The crude midgut homogenate of 2- to 3-day-old sixth instar southern armyworm larvae was also prepared as before [6] and used at a protein concentration of about 4 mg/ml (epoxidase) or 6 mg/ml (demethylase). The aldrin epoxidation and *p*-chloro-*N*-methylaniline demethylation assays have been described in earlier reports [10–12]. Incubations were carried out aerobically at 32° for either 10 min (epoxidation) or 20 min (demethylation); in both cases the reaction was initiated by the addition of substrate. Imidazoles were added to the incubation mixtures in 20 μl ethanol, and I_{50} values were determined graphically from data of duplicate incubations at each of several inhibitor concentrations.

Optical difference spectra were recorded with an Aminco DW-2 spectrophotometer with suspensions (4–5 mg protein/ml) of rat liver microsomes and armyworm midgut microsomes (prepared from larvae induced with pentamethylbenzene [13]) in 67 mM sodium phosphate

Table 1. Physical properties of bis-imidazoles

General structure: 

Compound	R	m.p. (°)	Elemental analysis	
			Calc	Found
I	*m*-phenyl	143.5–145.5	C:68.56 H: 4.79	C:68.58 H: 4.90
II	*p*-phenyl			
III	1,5-naphthyl	260–262	C:73.83 H: 4.65	C:73.74 H: 4.60
IV	2,7-naphthyl	199–200.5	C:73.83 H: 4.65	C:73.72 H: 4.73
V	α,α'-*o*-xylyl	154–156	C:70.57 H: 5.92	C:70.51 H: 5.86
VI	α,α'-*m*-xylyl	85.5–87	C:70.57 H: 5.92	C:70.35 H: 6.05
VII	α,α'-*p*-xylyl	132–134	C:70.57 H: 5.92	C:70.50 H: 6.06
VIII	$-(CH_2)_3-$	liq.	C:61.34 H: 6.86	C:61.60 H: 6.94
IX	$-(CH_2)_4-$(dihydrate)	84–86	C:53.08 H: 8.02	C:52.93 H: 8.09
X	$-(CH_2)_5-$	liq.	C:64.68 H: 7.89	C:64.99 H: 7.69
XI	$-(CH_2)_6-$	visc. oil	C:66.02 H: 8.31	C:66.25 H: 8.40

Table 2. Activity of compounds studied *in vitro*

Compound	I_{50} (M) N-demethylation RLM*	I_{50} (M) N-demethylation AW†	I_{50} (M) Epoxidation RLM	I_{50} (M) Epoxidation AW	Spectral dissociation constant (K_s) RLM	Spectral dissociation constant (K_s) AWM‡
I	$> 10^{-4}$ (45)	1.8×10^{-6}	1.1×10^{-4}	2.3×10^{-5}	$3.56 \pm 0.44 \times 10^{-6}$	$1.27 \pm 0.12 \times 10^{-6}$
II	$> 10^{-4}$ (35)	5.0×10^{-7}	$> 10^{-4}$ (31)	6.4×10^{-6}	$2.60 \pm 0.56 \times 10^{-6}$	$1.26 \pm 0.15 \times 10^{-6}$
III	2.2×10^{-5}	1.1×10^{-7}	2.1×10^{-5}	2.0×10^{-7}	$1.14 \pm 0.58 \times 10^{-5}$	$1.27 \pm 0.18 \times 10^{-6}$
IV	1.0×10^{-5}	2.1×10^{-7}	2.3×10^{-5}	1.7×10^{-6}	$3.11 \pm 0.46 \times 10^{-6}$	$1.52 \pm 0.19 \times 10^{-6}$
V	$> 10^{-4}$ (35)	1.3×10^{-6}	4.5×10^{-6}	3.5×10^{-6}	$2.66 \pm 0.26 \times 10^{-6}$	$5.18 \pm 0.52 \times 10^{-6}$
VI	$> 10^{-4}$ (42)	1.4×10^{-6}	1.6×10^{-5}	1.1×10^{-5}	$6.7 \pm 1.2 \times 10^{-6}$	$4.75 \pm 0.42 \times 10^{-6}$
VII	$> 10^{-4}$ (38)	1.0×10^{-6}	4.5×10^{-5}	1.3×10^{-5}	$1.73 \pm 0.73 \times 10^{-5}$	$3.15 \pm 0.27 \times 10^{-6}$
VIII	$> 10^{-4}$ (16)	2.8×10^{-5}	$> 10^{-4}$ (7)	5.4×10^{-5}	$6.84 \pm 0.55 \times 10^{-6}$	$1.26 \pm 0.12 \times 10^{-6}$
IX	$> 10^{-4}$ (25)	3.8×10^{-6}	$> 10^{-4}$ (0)	5.2×10^{-5}	$2.14 \pm 0.21 \times 10^{-6}$	$1.67 \pm 0.15 \times 10^{-6}$
X	$> 10^{-4}$ (38)	1.2×10^{-6}	$> 10^{-4}$ (27)	1.9×10^{-5}	$2.45 \pm 0.27 \times 10^{-6}$	$1.59 \pm 0.08 \times 10^{-6}$
XI	5.5×10^{-5}	4.5×10^{-7}	6.5×10^{-5}	8.5×10^{-6}	$3.58 \pm 0.52 \times 10^{-6}$	$3.35 \pm 0.48 \times 10^{-6}$

* Rat liver microsomes. Numbers in parentheses indicate per cent inhibition at 10^{-4} M.
† Armyworm midgut preparation.
‡ Armyworm midgut microsomes

buffer, pH 7.4 (rat liver) or 7.8 (armyworm gut). Spectral dissociation constants (K_s) were determined from the intercepts of the abscissae of double reciprocal plots of ΔO.D. 430–390 nm vs inhibitor concentration using the Wilkinson program [14] and the IBM-370 computer at Cornell University. Duplicate determinations were made with five or six different inhibitor concentrations.

The bis-imidazoles selected for study consisted of several sets of compounds related by well-defined changes in structure. In the structurally rigid series (I–IV), for example, the dual aspects of hydrocarbon moiety size (phenyl vs naphthyl) and the distance between the imidazole rings (I vs II and III vs IV) came into play. The series V–VII offers differing placements of the imidazole groups in combination with greater, though still constrained, flexibility. Finally, the set of VIII–XI provides the greatest potential variation with a series of very flexible structures differing markedly in water solubility and potential separation of the functional groups. The data in Table 2 indicate that the armyworm gut preparation is generally 10- to 100-fold more sensitive to inhibition than rat liver microsomes. This is much more pronounced with *N*-demethylation than with epoxidation, the former reaction being notably insensitive to inhibition in rat liver microsomes, as has been noted with other imidazoles [5]. The species difference is more variable in the case of epoxidation and indeed compounds V–VII are equally active toward the rat and armyworm enzymes.

Previous studies with a series of 1-alkylimidazoles [4] showed a good correlation between inhibitory activity and microsomal binding, as indicated by the spectral (type II) dissociation constants (K_s). This relationship appears to break down with the present series of bis-imidazoles, since despite the relatively large variations in inhibitory activity K_s values remain uniformly low. Evidently the ability of these ligands to reach and bind to the heme moiety of cytochrome P450 is not, of itself, sufficient for inhibitory activity of all reactions. Similar results have been reported by others in some cases [7, 15]. It is not clear whether these data reflect any basic differences between the various forms of cytochrome P450 of insects and mammals or whether they are indicative of selective binding of the imidazoles to specific forms of cytochrome P450, each exhibiting a different spectrum of substrate specificity.

The inhibitory potency of the most active bis-imidazoles toward the armyworm oxidases is similar to that previously reported for other 1- and 4(5)-imidazole derivatives [4, 5]. Consequently there appears to be no additional inhibition associated with the presence of two imidazole groups in the molecule, and in all probability only one of these groups participates in the inhibitory process. In accordance with this, the distance between the imidazole groups in the bis-imidazoles appears to play no significant role in determining inhibitory activity. Thus, although the greater inhibitory activity of III and IV compared with I and II could be explained on the basis of the greater distance between the imidazole groups, this conclusion is not supported by compounds V–VII in which V, with the smallest separation between the groups, is the most active. It is more probable that compounds III and IV owe their high activity to their relatively higher lipophilic character, a factor previously established to be of primary importance in determining imidazole inhibition [4] and further emphasized by the increasing activity of compounds VII–XI. In general, a high degree of steric specificity (aside from the known sensitivity to hindrance in the imidazole ring itself [6, 7]) does not appear necessary for inhibitory action.

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