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STERIC FACTORS IN THE INHIBITORY INTERACTION OF IMIDAZOLES WITH MICROSOMAL ENZYMES

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Abstract—The ability of substituted imidazoles to bind to cytochrome P-450 (type II) and to inhibit microsomal drug-metabolizing enzyme activity is highly dependent on the position of the substituent in the imidazole ring. Compounds containing substituents in the 1-, 4(5)- and 1,5-positions are effective inhibitors of the epoxidation of aldrin in rat liver microsomes and armyworm (*Spodoptera eridania*) gut preparations and exhibit low spectral dissociation constants (K_s). In contrast, compounds containing 2- and/or 4-substitutents are essentially inactive as inhibitors and exhibit high K_s values (low binding). It appears that both binding and inhibition depend on the accessibility of the non-bonded electrons on the nitrogen atom at position – 3 of the ring.

Studies in several laboratories have now clearly established that many 1-[1–5] and 4(5)-arylimidazoles [5, 6] and 1-alkylimidazoles [7, 8] are among the most potent inhibitors of microsomal drug oxidations yet described. *In vivo*, many are remarkably active potentiators of barbiturate sleeping time in mammals [4, 6–8] and several of the 1-substituted compounds are effective insecticide synergists to houseflies [5, 7].

Inhibitory activity of the 1- and 4(5)-substituted imidazoles appears to be closely related to their capacity to bind to cytochrome P-450 as measured by the spectral dissociation constants (K_s) obtained from the type II optical difference spectra they exhibit. In a homologous series of 1-alkylimidazoles, both inhibitory activities and K_s values are closely correlated with lipophilic character and are optimal in compounds containing an 8–10 carbon alkyl chain [7].

Although lipophilic character is clearly an extremely important determinant of inhibitory activity, the previously reported low activity of two 2-substituted imidazoles relative to the corresponding 4(5)-derivatives [5] suggested that steric parameters might also play a role. This paper is concerned with the steric factors which determine the ability of imidazoles to inhibit epoxidase activity in enzyme preparations from rat liver and the gut tissue of southern armyworm (*Spodoptera eridania*) larvae.

MATERIALS AND METHODS

Chemicals. The imidazoles employed in the study are shown in Table 1 (I–XV). Of these, compounds I, III and IV were purchased from Pfaltz & Bauer, Inc., Flushing, NY, VI and IX from ICN—K & K Laboratories, Plainview, NY, and XV from the Aldrich Chemical Co., Cedar Knolls, NJ, all were recrystallized prior to use. The remainder were prepared by a variety of synthetic procedures. 1-Methylimidazole (II), b.p. 199–200°, was obtained by the reaction of imidazole (I) with methyliodide [2, 9], and 4(5)-phenylimidazole (VII), m.p. 128–129° (lit. m.p. 128°) [10], was prepared by the method of Bredereck and Theilig [10] in which phenacyl bromide is heated under reflux with formamide. The same general procedure was also used to obtain 4,5-dimethylimidazole (VIII), m.p. HCl salt 305° (lit. m.p. 305°) [10] from acetoin and 4,5-diphenylimidazole (IX), m.p. 230–233° (lit. m.p. 231°) [10], from benzoin. Compound V, 1-phenylimidazole, b.p. 112–113°, 1.0 mm (lit. 100°, 0.5 mm) [11], was prepared by the Marckwald synthesis from phenylisothiocyanate and aminoacetaldehyde diethylacetal and subsequent oxidation of the intermediate 1-phenyl-2-mercaptoimidazole with nitric acid. The similar oxidation of 1,5-diphenyl-2-mercaptoimidazole produced by the reaction of phenacylamine hydrochloride and phenyl isothiocyanate [12] yielded 1,5-diphenylimidazole (XII), m.p. 204–205° (mol. wt of 220 confirmed by mass spectrometry). This procedure was also employed in the preparation of 1-methyl-5-phenylimidazole (XI), m.p. 96–98° (lit. m.p. 96–97°) [13], starting from phenacylamine hydrochloride and methylisothiocyanate. Methylation of VII with methyl sulfate yielded 1-methyl-4-phenylimidazole (X), m.p. 110–112° (lit. m.p. 110–111°) [13], and similar methylation of IX gave 1-methyl-4,5-diphenylimidazole (XIII), m.p. 147–148.5° (lit. 147°) [14]. The reaction between benzil, benzaldehyde and ammonium acetate provided 2,4,5,-triphenyl-imidazole (XIV), m.p. 271–273° (lit. m.p. 275°) [15], in good yield as described by Davidson *et al.* [15].

Analytical grade samples of aldrin (1,2,3,4, 10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-*endo*-*exo*-5,8-dimethanonaphthalene) and its 6,7-epoxide, dieldrin, were kindly provided by the Shell Development Co., Modesto, CA.

Biochemicals were purchased from CalBiochem, San Diego, CA, and all other chemicals and solvents employed were of analytical reagent grade.

Enzyme preparation. Livers from male Sprague–Dawley rats, purchased from Blue Spruce Farms, Altamont, NY, were homogenized in ice-cold 1.15%

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KCl (1:4, w/v) and microsomes were sedimented from the post-mitochondrial supernatant (20,000 g_{max} for 20 min) by centrifugation at 100,000 g_{max} for 1 hr in an International Equipment Co. (IEC) B-60 preparative ultracentifuge equipped with an IEC A-321 angle-head rotor. For enzyme assay the microsomal pellet was resuspended in 1.15% KCl to a concentration of 0.6 to 1.0 mg/ml.

The insect preparation was as previously described [16]. It consisted of a crude homogenate of cleaned midguts from the sixth instar southern armyworm larvae (2 guts/ml) in ice-cold 1.15% KCl and had a protein concentration of approximately 1 mg/ml.

Protein concentrations were determined by a modified Biuret method [17] using bovine serum albumin as a standard.

Aldrin epoxidase. Incubations were carried out aerobically in 25-ml Erlenmeyer flasks shaken in a water bath at 30°. The standard 5-ml incubation mixture consisted of 0.5 ml of the appropriate enzyme suspension and the following components (final concentration): Tris–HCl buffer (5×10^{-2} M), pH 7.4 (rat) or 7.8 (armyworm); G-6-P (2.4×10^{-3} M); KCl (27.6×10^{-3} M); NADP (5.2×10^{-5} M); G-6-P dehydrogenase (1.6 units) and 100 μg aldrin in 25 μl ethanol. The reactions were initiated by addition of the enzyme and incubations were carried out for 15 min before being terminated by the addition of acetone. Extraction and gas chromatographic assay of aldrin and dieldrin were as previously described [18].

The imidazoles were added to the incubations in 10 μl ethanol and I_{50} values were determined from the means of duplicate incubations with at least four different inhibitor concentrations.

Binding studies. Optical difference spectra were recorded with a Norelco Unicam SP-800 spectrophotometer equipped with a scale expander and accessory recorder. Suspensions of rat liver microsomes containing 1–2 mg protein/ml in 67 mM phosphate buffer, pH 7.4, were employed. Spectral dissociation constants (K_s) were determined from the abscissal intercepts of double reciprocal plots of $\Delta O.D._{430-390\,nm}$ vs imidazole concentration. The means of duplicate determinations with five or six different imidazole concentrations were employed.

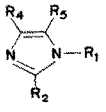
RESULTS

The molar I_{50} values of 15 imidazoles toward epoxidase activity in enzyme preparations from rat liver and armyworm gut are shown in Table 1; spectral dissociation constants (K_s, M) for their binding to rat liver cytochrome P-450 are also shown. These data clearly demonstrate the dramatic changes in inhibitory potency and P-450 binding capacity which result from the incorporation of substituents in different positions of the imidazole ring.

As previously reported [5–7], imidazole (I) itself is a relatively poor inhibitor of epoxidase activity and exhibits a K_s value of 2.1×10^{-4} M. Substitution with a methyl group in the 1-position of the ring causes little or no change in either inhibition or binding, but similar substitution in the 2-position essentially eliminates both types of activity. The inclusion of ethyl (IV) and phenyl (VI) groups at carbon-2 similarly yields compounds devoid of any significant inhibitory activity or P-450 binding capacity. The monophenyl substituted compounds (V, VI and VII) dramatically illustrate the effects on activity associated with the position of substitution in the ring, the 2-substituted derivative (VI) being three or four orders of magnitude less active than either the 1- or 4(5)-phenylimidazoles.

Table 1. Biological activity of substituted imidazoles*

General structure:



Compound	R_1	R_2	R_4	R_5	I_{50} (M) aldrin epoxidation RLM†	I_{50} (M) aldrin epoxidation AG‡	Spectral dissociation constant (K_s) (M), RLM*
I	H	H	H	H	3.6×10^{-3}	2.9×10^{-3}	2.10×10^{-4}
II	CH_3	H	H	H	2.9×10^{-3}	1.3×10^{-3}	2.0×10^{-4}
III	H	CH_3	H	H	$> 10^{-2}$	$> 10^{-2}$	$> 10^{-2}$
IV	H	C_2H_5	H	H	$> 10^{-2}$	$> 10^{-2}$	$> 10^{-2}$
V	C_6H_5	H	H	H	1.5×10^{-6}	3.4×10^{-6}	7.1×10^{-6}
VI	H	C_6H_5	H	H	$> 10^{-2}$	5.0×10^{-3}	3.5×10^{-3}
VII§	H	H	(H)	(C_6H_5)	4.6×10^{-6}	1.3×10^{-5}	5.2×10^{-6}
VIII	H	H	CH_3	CH_3	$> 10^{-2}$	$> 10^{-2}$	$> 10^{-2}$
IX	H	H	C_6H_5	C_6H_5	3.5×10^{-3}	1.4×10^{-3}	$> 10^{-3}$
X	CH_3	H	C_6H_5	H	$> 10^{-2}$	2.1×10^{-3}	$> 10^{-3}$
XI	CH_3	H	H	C_6H_5	4.2×10^{-6}	1.6×10^{-7}	1.2×10^{-6}
XII	C_6H_5	H	H	C_6H_5	6.6×10^{-6}	3.3×10^{-7}	9.1×10^{-7}
XIII	CH_3	H	C_6H_5	C_6H_5	1.7×10^{-4}	9.0×10^{-5}	$> 10^{-3}$
XIV	H	C_6H_5	C_6H_5	C_6H_5	$> 10^{-1}$	$> 10^{-1}$	$> 10^{-2}$
XV	Benzimidazole				$> 10^{-2}$	$> 10^{-2}$	$> 10^{-2}$

* Data for compounds I, II, V and VII have been reported in previous publications from this laboratory [5–7].
† Rat liver microsomes.
‡ Armyworm gut preparation.
§ Tautomeric structure.

Substitution in the 4-position of the ring also leads to compounds with little or no significant activity. This can be seen clearly with the compounds containing 4,5-disubstituents (VIII, IX, XIII and XIV) and may account for the observed inactivity of benzimidazole (XV). That the low activity of these compounds is associated mainly with the 4-substituent is well demonstrated by the fact that the 1-methyl-5-phenyl derivative (XI) is approximately four orders of magnitude more active than the 1-methyl-4-phenyl (X) isomer. The, 1,5-disubstituted compounds (XI and XII) were the most active compounds evaluated.

DISCUSSION

Combined with previous data [1–8], those reported here allow a fairly precise qualitative understanding of the structural features which determine the ability of imidazoles to bind to cytochrome P-450 and to inhibit microsomal drug oxidation.

In the case of monosubstituted compounds, a large number of 1-aryl-, 1-alkyl- and 4(5)-arylimidazoles show high inhibitory potency toward a variety of different types of drug oxidation [1–8]. The usually close agreement between the I_{50} and K_s values strongly suggests that inhibition results from the capacity of the imidazoles to bind to the microsomal cytochrome P-450 complex [6, 7]. Multiple regression analysis of binding and inhibition data from a homologous series of 1-alkylimidazoles has established that the major factor determining activity is the hydrophobic binding constant (π) and that optimal biological activity occurs with 1-alkyl substituents of 8–10 carbon atoms. It is probable that lipophilic character is also of dominant importance with 1- and 4(5)-substituents other than alkyl groups.

In contrast, imidazoles containing substituents in either the 2- or 4-positions of the ring show little propensity for binding and these compounds exhibit little or no inhibitory activity toward drug oxidation irrespective of their lipophilic character. With these compounds steric considerations are undoubtedly of paramount importance and indeed the remarkable 10^4-fold differences in the activities of the monophenyl imidazoles (V, VI and VII) and of the two methyl-phenyl isomers (X and XI) provide excellent examples of the importance of steric parameters in enzyme/substrate/inhibitor interactions. The relatively high inhibitory activity of 2-(2,4-dichlorophenyl)thiomethylimidazole (3.4×10^{-5} M) previously reported [5] is an exception to the usually low activity of the 2-substituted compounds. However, this compound is substantially less active than the corresponding 4(5)-derivative (almost 1000-fold in the case of the armyworm gut preparation), and it is possible that the structural flexibility provided by the thiomethyl linkage allows a relatively more favourable steric alignment of the bulky aromatic ring than with other more compact 2-substituents.

It is usually considered that the type II optical difference spectra such as those exhibited by the imidazoles and other nitrogen-containing compounds result from direct interaction between the non-bonded electrons of the nitrogen atom and the fifth or sixth ligand on the heme moiety of the cytochrome P-450 [19–22]. Presumably in the imidazole ring the non-bonded electrons associated with nitrogen – 3 are those responsible for ligand binding and consequently these must be freely accessible to approach the heme moiety. This appears to be the case with 1- and 4(5)-monosubstituted and 1,5-disubstituted derivatives but not with compounds containing 2- and/or 4-substituents. Even relatively small groups in these latter positions prevent or reduce ligand interaction by either directly affecting the accessibility of the non-bonded electrons (an intramolecular effect) or by preventing a sufficiently close approach of the imidazole molecule to the heme (an intermolecular effect). The latter possibility suggests that cytochrome P-450 might be situated at the bottom of a membrane "hole" with a diameter closely approximating that of the imidazole ring. An alternative explanation could be that binding of the 2- and/or 4-substituents to the hydrophobic patch, which aids the interaction of 1-, 4(5)- and 1,5-substituted compounds [5, 7] in some manner changes the spatial orientation of the imidazole nitrogen and prevents its interaction with cytochrome P-450. The nature of the hydrophobic site(s) with which the imidazoles interact is not known, although it has been suggested that they may be closely associated with the sites involved in substrate binding [5, 7]. This suggestion is supported by the fact that the degree of inhibition of drug oxidation by the imidazoles is to some extent dependent on the substrate employed and by the demonstration of both type I and type II characteristics in the binding of 1-geranylimidazole to rat liver microsomes [8].

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