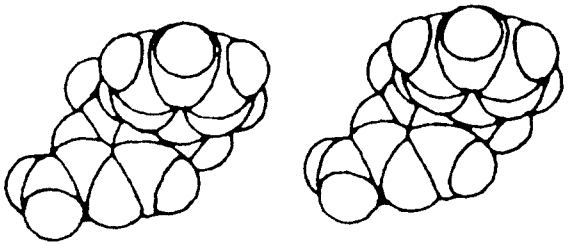

Figure 2. A space filling stereo molecular model of the active conformation for X = H.

receptor binding could certainly alter the conformation of a drug from any of the stable conformers realized in solution or free space. Nevertheless, the identification of a rigid active shape, in terms of θ (II) for the triazines and θ_1 and θ_2 (I) for the pyrimidines, leads to a very significant QSAR. In addition, for the triazines, quinazolines, and pyrmidines it is observed that one particular rotamer of a substituent, corresponding to a free-space minimum-energy conformer, correlates significantly better with observed activity than any other rotamer state of the substituent. The cis vs. trans amide substituents in the quinazoline and pyrimidine QSARs illustrate this point.

Thus, the hypothesis that one of the isolated stable conformers of a molecule corresponds to the active state is validated by the MSA QSARs developed for the triazine, quinazoline, and pyrimidine DHFR inhibitors. It is possible that some other conformer, probably "close" in shape to a postulated active conformer which is an isolated free-space relative minimum-energy state, could yield a better QSAR than presently found. This possibility can be tested using MSA. The hypohtesis of considering only stable intramolecular energy minima conformers in the development of a QSAR is only a working simplification and not a restriction of the MSA methodology. However, given the success we have enjoyed using this hypothesis, we plan to continue employing it to develop MSA QSARs on alternate data bases.

Acknowledgment. The theory and corresponding computer programs to calculate molecular shape descriptors have been developed under private funding. The application of MSA to the pyrimidines was funded under a contract from the National Cancer Institute (Contract NO1-CP-75927). The author appreciates helpful discussions with Dr. J. Y. Fukunaga of Schering-Plough Corp. concerning this work.

Effects of 1-Arylpyrroles and Naphthoflavones upon Cytochrome P-450 Dependent Monooxygenase Activities

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The inhibitions of cytochrome P-450 dependent monooxygenase activity in microsomes from rat liver by 1-phenylpyrrole, 1-(2-isopropylphenyl)pyrrole, 4(5)-phenylimidazole, and 1-(2-isopropylphenyl)imidazole have been compared. The results establish that the presence of an imidazole N-3 nitrogen substituent is not required to inhibit the monooxygenase activity measured by the deethylation of 7-ethoxycoumarin. The presence of an appropriately situated N-3 atom, however, as in 1-(2-isopropylphenyl)imidazole, significantly decreases both the K_i and αK_i of these mixed type inhibitors. The induction of 7-ethoxycoumarin deethylase activity in the microsomal fraction from rat liver by α-naphthoflavone, β-naphthoflavone, and 3-methylcholanthrene and the inhibition of these activities by flavone and α-, β-, and γ-naphthoflavone have also been examined. The results establish that α-naphthoflavone is the most effective in vitro inhibitor. The results also indicate that the microsomal monooxygenase activities induced in rat liver by α-naphthoflavone, β-naphthoflavone, and 3-methylcholanthrene are not equivalent. Based upon the observed results, it is concluded that differential effects of α- and β-naphthoflavone on aryl hydrocarbon skin tumorigenesis may be the result of differential enzyme induction rather than the result of differential enzyme inhibition.

In 1969, Johnson et al.[1] reported that 1-arylimidazoles were potent in vitro inhibitors of steroid 11β-hydroxylations catalyzed by the mitochondrial fraction of bovine adrenal cortex. Liebman and Ortiz[2] subsequently found that 1-(2-isopropylphenyl)imidazole and 1-(2-cyanophenyl)imidazole were inhibitors of the para-hydroxylation of aniline and acetanilide and of the N-demethylation of aminopyrine in rat liver microsomes. The 1-(2-isopropylphenyl)imidazole was also found to prolong and to intensify the in vivo action of hexobarbital in rats.[2] Other investigators,[3,4] especially Wilkinson and his co-workers,[5-9] have also investigated the inhibition of various cytochrome P-450 dependent monooxygenase activities by 1-arylimidazoles and related compounds.

Based upon the relative inhibition constants and the spectral dissociation constants of an extensive series of substituted imidazoles, Wilkinson and co-workers[6,7] proposed that the inhibition of cytochrome P-450 dependent monooxygenase activities by arylimidazoles is due to coordination of the nonbonded electrons of N-3 of the imidazole ring with the heme iron. Additional evidence for the binding of 1-arylimidazoles in the vicinity of the heme of cytochrome P-450 containing proteins has recently been provided by Swanson and Dus[10] who found that 1-(4-azidophenyl)imidazole can be used as a photoactive affinity probe of the heme site of the cytochrome P-450 dependent camphor hydroxylase of *Pseudomonas putida*.

(1) A. L. Johnson, J. C. Kauer, D. C. Sharma, and R. I. Dorfman, *J. Med. Chem.*, 12, 1024 (1969).
(2) K. C. Liebman and E. Ortiz, *Drug Metab. Dispos.*, 1, 775 (1973).
(3) E. D. Palmer and M. A. Cawthorne, *Xenobiotica*, 4, 209 (1974).
(4) G. M. Holder, P. J. Little, A. J. Ryan, and T. R. Watson, *Biochem. Pharmacol.*, 25, 2747 (1976).
(5) C. F. Wilkinson, K. Hetnarski, and T. O. Yellin, *Biochem. Pharmacol.*, 21, 3187 (1972).
(6) C. F. Wilkinson, K. Hetnarski, and L. J. Hicks, *Pestic. Biochem. Physiol.*, 4, 299 (1974).
(7) T. D. Rogerson, C. F. Wilkinson, and K. Hetnarski, *Biochem. Pharmacol.*, 26, 1039 (1977).
(8) L. R. Smith and C. F. Wilkinson, *Biochem. Pharmacol.*, 27, 1383 (1978).
(9) L. R. Smith and C. F. Wilkinson, *Biochem. Pharmacol.*, 27, 2466 (1978).
(10) R. A. Swanson and K. M. Dus, *J. Biol. Chem.*, 254, 7238 (1979).

0022-2623/81/1824-0822$01.25/0

Nevertheless, preliminary investigations indicated that 1-arylpyrroles, which lack the N-3 atom of the imidazoles, were inhibitors of microsomal monooxygenase activity.[11] Guengerich and Mitchell[12] also reported that certain pyrroles are activated by cytochrome P-450 dependent metabolism and produce type II or reverse type I difference spectra when added to microsomal suspensions. These observations prompted us to investigate the inhibition of cytochrome P-450 dependent monooxygenase activity by 1-arylpyrroles more fully. Since 1-(2-isopropylphenyl)-imidazole has been reported to be among the more potent 1-arylimidazole inhibitors,[6] we chose to synthesize the corresponding 1-(2-isopropylphenyl)pyrrole and to investigate its inhibitory properties. Experiments were also carried out with the commercially available 1-phenylpyrrole to ascertain the importance of the 2-isopropyl substituent upon the observed inhibitions. 4(5)-Phenylimidazole, whose biochemical activities have been reported to be comparable to the 1-arylimidazoles,[7] and 1-(2-isopropylphenyl)imidazole were utilized as reference arylimidazole inhibitors.

In a series of related experiments, we have investigated inhibition of cytochrome P-450 dependent monooxygenase activity in rat liver microsomal fractions by flavone and by 7,8-benzoflavone (α-naphthoflavone, αNF), 6,7-benzoflavone (γ-naphthoflavone, γNF), and 5,6-benzoflavone (β-naphthoflavone, βNF). αNF has long been recognized as an inhibitor of mixed-function monooxygenase activities,[13] and this synthetic flavanoid has been used to differentiate between different types of monooxygenase activities.[14,15] Nesnow has carried out some preliminary studies of structure–activity relationships of inhibition by αNF.[16] Although βNF has also been found to inhibit some types of monooxygenase activity,[17,18] this flavanoid isomer is known to be a potent inducer of mixed-function monooxygenase activity[19–24] and βNF has generally been utilized for this purpose in investigations of xenobiotic activation and metabolism.

Both αNF and βNF inhibit the tumorigenesis of certain aryl hydrocarbons.[25–27] Nevertheless, these flavanoid

Table I. Determined Kinetic Constants for 7-Ethoxycoumarin Deethylation in the Presence of Selected Arylpyrroles and Arylimidazoles[a]

		liver microsomes from rats exposed to βNF						liver microsomes from rats exposed to 3MC					
inhibitor added	concn, μM	K_M, μM	lower bound	upper bound	V_{max}, pmol min^{-1} mg^{-1}	lower bound	upper bound	K_M, μM	lower bound	upper bound	V_{max}, pmol min^{-1} mg^{-1}	lower bound	upper bound
none		16.7	14.2	18.3	860	836	890	10.6	9.4	12.0	189	173	199
1-(2-isopropylphenyl)-pyrrole	5							10.2	7.5	15.0	141	114	171
	10							17.9	15.0	21.3	156	136	170
	50	44.1	37.6	50.0	701	634	735	29.7	21.8	38.5	94	80	109
	100	52.2	44.3	67.9	652	569	718						
	200	91.3	83.3	100.2	493	458	530						
4(5)-phenylimidazole	1							14.4	13.6	15.0	199	194	204
	10	20.6	17.3	24.6	685	530	716	14.1	11.5	16.4	163	152	174
	20							18.6	16.9	21.0	149	142	158
	50	23.6	22.7	26.0	509	502	519						
	100	33.0	30.0	35.5	354	337	364						
none		16.7	14.5	17.6	879	869	889						
1-phenylpyrrole	50	28.4	27.5	29.1	819	806	829						
	100	41.2	36.6	48.8	760	741	794						
	150	64.3	56.0	95.0	669	631	794						
1-(2-isopropylphenyl)-imidazole	1	22.1	18.7	24.2	734	717	747						
	2.5	35.8	31.9	40.2	346	339	359						
	5.0	41.7	38.6	51.7	227	224	234						

[a] The in vitro 7-ethoxycoumarin deethylase activity in microsomal preparations obtained from the liver of male Sprague–Dawley rats that had been exposed to either βNF or 3MC was determined in the presence of selected arylpyrroles and arylimidazoles as described under Experimental Section. K_M and V_{max} values were established by the method of Eisenthal and Cornish-Bowden.[34] The indicated lower and upper bounds, corresponding to the 95% confidence limits, were calculated with the aid of a computer program, obtained from Cornish-Bowden, that uses distribution-free confidence limits as described by Cornish-Bowden et al.[35]

(11) W. L. Alworth, V. S. Ganu, S. W. Fingerman, and N. A. Branyas, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **37**, 1543, abstract (1978).
(12) F. P. Guengerich and M. B. Mitchell, *Drug Metab. Dispos.*, 8, 34 (1980).
(13) L. W. Diamond and H. V. Gelboin, *Science*, **166**, 1023 (1969).
(14) F. J. Wiebel and H. V. Gelboin, *Biochem. Pharmacol.*, **24**, 1511 (1975).
(15) C. R. Wolf, B. R. Smith, L. M. Ball, C. S. Singh, J. R. Bend, and R. M. Philpot, *J. Biol. Chem.*, **254**, 3658 (1979).
(16) S. Nesnow, *J. Med. Chem.*, **22**, 1244 (1979).
(17) F. J. Wiebel, J. C. Leutz, L. Diamond, and H. V. Gelboin, *Arch. Biochem. Biophys.*, **144**, 78 (1971).
(18) L. Diamond, R. McFall, J. Miller, and H. V. Gelboin, *Cancer Res.*, **32**, 731 (1972).
(19) L. W. Wattenberg, M. A. Page, and J. L. Leong, *Cancer Res.*, **28**, 934 (1968).
(20) L. W. Wattenberg and J. L. Leong, *Cancer Res.*, **30**, 1922 (1970).
(21) K. R. Cutroneo, R. A. Seibert, and E. Bresnick, *Biochem. Pharmacol.*, **21**, 937 (1972).
(22) I. S. Owens and D. W. Nebert, *Mol. Pharmacol.*, 11, 94 (1975).
(23) A. R. Boobis, D. W. Nebert, and J. S. Felton, *Mol. Pharmacol.*, **13**, 259 (1977).
(24) D. A. Haugen and M. J. Coon, *J. Biol. Chem.*, **251**, 7929 (1976).
(25) W. G. Pyerin and E. Hecker, *J. Cancer Res. Clin. Oncol.*, **93**, 7 (1979).
(26) L. W. Wattenberg and J. L. Leong, *Proc. Soc. Exp. Biol. Med.*, **128**, 940 (1968).
(27) N. Kinoshita and H. V. Gelboin, *Cancer Res.*, **32**, 1329 (1972).

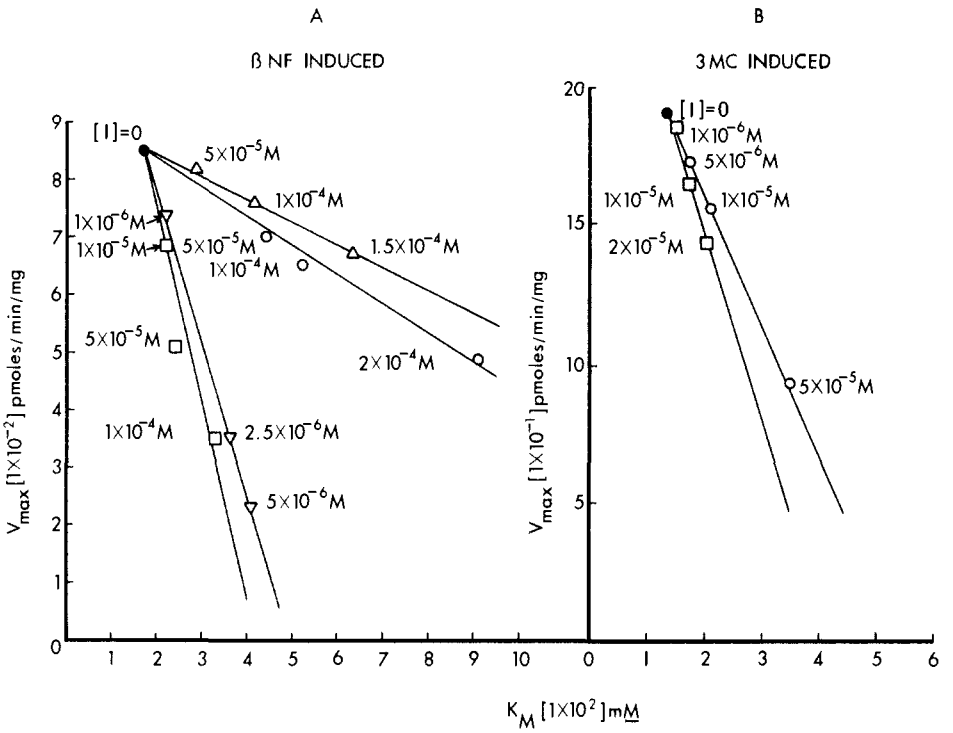

Figure 1. Changes in the kinetic parameters, K_M and V_{max}, as summarized in Table I for the deethylation of 7-ethoxycoumarin in the presence of varying concentrations of 4(5)-phenylimidazole (□), 1-(2-isopropylphenyl)imidazole (▽), 1-(2-isopropylphenyl)pyrrole (○), or 1-phenylpyrrole (△). Section A shows results obtained with microsomes from rats exposed to βNF and section B shows the results with microsomes from rats exposed to 3MC. The experiments were carried out as described under Experimental Section; the in vitro concentrations of the inhibitors used to determine each set of kinetic parameters are noted on the figure.

isomers show differential effects upon aryl hydrocarbon induced skin tumors.[28] Applications of αNF will decrease skin tumor initiation by 7,12-dimethylbenz[a]-anthracene[25,28] and by 3-methylcholanthrene[28] but can increase skin tumorigenesis by benzo[a]pyrene and by dibenz[a,h]anthracene.[28-30] Although less effective than αNF in preventing 3-methylcholanthrene-induced skin tumors,[28] βNF, in direct contrast to αNF, also inhibits benzo[a]pyrene- and dibenz[a,h]anthracene-induced skin tumors.[29,31] Recent reviews summarize the effects of αNF and βNF upon cytochrome P-450 dependent monooxygenases, upon tumorigenesis and cell transformations, and upon the binding of aryl hydrocarbons to DNA.[32,33] An analysis of the relative inhibition of monooxygenase activity produced by αNF, βNF, and γNF in liver microsomes from rats was carried out to determine if the observed inhibition constants could help explain the differential in vivo effects of αNF and βNF.

Results

The results of our investigations are summarized in Tables I–V. The kinetic parameters for the deethylation of 7-ethoxycoumarin by liver microsomal fractions from rats administered βNF or 3MC are listed in Tables I and II. Table I summarizes the observed effects of 1-(2-isopropylphenyl)imidazole, 4(5)-phenylimidazole, 1-phenylpyrrole, and 1-(2-isoprophyphenyl)imidazole on the observed kinetic parameters, while Table II summarizes the observed effects of αNF, βNF, γNF, and flavone. The values in Tables I and II were determined from the experimental data by the direct linear method of Eisenthal and Cornish-Bowden.[34] The indicated lower and upper limits, corresponding to the 95% confidence limits, were calculated with the aid of a computer program obtained from Cornish-Bowden that makes use of distribution-free confidence limits as described by Cornish-Bowden et al.[35] The K_M and V_{max} values from Tables I and II are presented graphically in Figures 1 and 2 in a manner that emphasizes the effects of the various inhibitors tested upon kinetic parameters. Figures 1 and 2 emphasize that, in general, the compounds tested show mixed-type inhibition with respect to 7-ethoxycoumarin.

The median values of K_M and V_{max} from Tables I and II were used to prepare replots of $1/V_{max}$ and K_M/V_{max} vs. inhibitor concentration. Least-squares fits of the data points were used to establish the inhibition constants for linear mixed-type inhibition, K_i (or K_{is}, the "competitive" inhibition constant that affects the slope of the customary double-reciprocal plots), and αK_i (or K_{ii}, the "uncompetitive" inhibition constant that affects the intercepts of the customary double-reciprocal plots). The determined inhibition constants are summarized in Table III.

Eadie-Scatchard plots (V/S vs. V) of the data obtained with microsomal preparations from the rats exposed to

(28) T. J. Slaga, D. L. Berry, M. R. Juchau, S. Thompson, S. G. Buty, and A. Vlaje, in "Carcinogenesis", Vol. 1, R. I. Freudenthal and P. W. Jones, Eds., Raven Press, New York, 1976, pp 127–137.
(29) G. T. Bowden, T. J. Slaga, B. G. Shapas, and R. K. Boutwell, *Cancer Res.*, **34**, 2634 (1974).
(30) N. Kinoshita and H. V. Gelboin, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 824 (1972).
(31) L. W. Wattenberg and J. L. Leong, *Cancer* Res., **30**, 1922 (1970).
(32) F. J. Wiebel, in "Carcinogenesis", Vol. 5, T. J. Slaga, Ed., Raven Press, New York, 1980, pp 57–84.
(33) J. DiGiovanni, T. J. Slaga, D. L. Berry, and M. R. Juchau, in ref 32, pp 145–168.
(34) E. Eisenthal and A. Cornish-Bowden, *Biochem. J.*, **139**, 715 (1974).
(35) A. Cornish-Bowden, W. R. Porter, and W. F. Trager, *J. Theor. Biol.*, **74**, 163 (1978).

Table II. Determined Kinetic Constants for 7-Ethoxycoumarin Deethylation in the Presence of αNF, βNF, γNF, and Flavone[a]

inhibitor added	concn, nM	liver microsomes from rats exposed to βNF: K_M, μM	lower bound	upper bound	V_{max}, pmol min^{-1} mg^{-1}	lower bound	upper bound	liver microsomes from rats exposed to 3MC: K_M, μM	lower bound	upper bound	V_{max}, pmol min^{-1} mg^{-1}	lower bound	upper bound
none		18.0	10.0	22.1	1380	1076	1436	15.0	14.2	17.0	168	166	179
αNF	5	18.5	11.2	26.2	1217	843	1404						
	10							19.6	17.0	20.8	137	134	140
	25							17.3	16.7	17.9	108	106	109
	27.5	17.0	16.0	19.7	904	891	918						
	50	25.7	22.4	29.8	665	606	711	23.4	22.1	24.6	69	68	70
βNF	25							19.2	18.5	19.6	159	158	160
	50							26.6	23.1	29.9	143	132	146
	75							39.7	36.0	41.2	134	131	136
	500	33.9	33.6	34.4	1200	1196	1207						
	1000	60	53.9	66.6	1102	1062	1170						
	1500	93.2	89.3	98.4	1000	984	1049						
γNF	50							19.2	18.1	19.5	150	146	151
	100							16.7	12.8	19.1	114	103	119
	200							31.5	28.9	33.3	88	86	90
	500	36.9	35.9	37.6	1302	1298	1314						
	1000	66.3	60.2	72.7	1242	1216	1303						
	1500	93.1	90.0	96.9	1214	1087	1257						
flavone	50							22.3	20.0	24.0	153	146	157
	100							35.8	34.6	37.5	149	146	150
	225							62.6	60.3	66.1	120	118	121
	500	50.3	40.8	65.7	1338	1262	1502						
	1000	114.9	95.0	132.2	1368	1264	1458						
	1500	135.9	121.6	204.5	1329	1224	1814						

[a] The in vitro 7-ethoxycoumarin deethylase activity in microsomal preparations obtained from the liver of male Sprague–Dawley rats that had been exposed to either βNF or 3MC was determined in the presence of αNF, βNF, γNF, and flavone as described under Experimental Section. K_M and V_{max} values were established by the method of Eisenthal and Cornish-Bowden.[34] The indicated lower and upper bounds, corresponding to the 95% confidence limits, were calculated with the aid of a computer program, obtained from Cornish-Bowden, that uses distribution-free confidence limits as described by Cornish-Bowden et al.[35]

Table III. Inhibition Constants Determined by Least-Squares Fit of K_M/V_{max} vs. I and $1/V_{max}$ vs. I[a]

inhibitor	microsomes from βNF-exposed rats: K_i, M	αK_i, M	microsomes from 3MC-exposed rats: K_i, M	αK_i, M
1-phenylpyrrole	2.7×10^{-5}	4.3×10^{-4}		
1-(2-isopropylphenyl)pyrrole	1.9×10^{-5}	2.7×10^{-4}	1.1×10^{-5}	6.0×10^{-5}
4(5)-phenylimidazole	2.6×10^{-5}	7.5×10^{-5}	2.0×10^{-5}	6.4×10^{-5}
1-(2-isopropylphenyl)imidazole	3.0×10^{-7}	1.4×10^{-6}		
αNF	2.4×10^{-8}	4.8×10^{-8}	1.7×10^{-8}	3.3×10^{-8}
βNF	1.3×10^{-7}	4.0×10^{-6}	2.6×10^{-8}	2.8×10^{-7}
γNF	2.4×10^{-7}	1.1×10^{-5}	4.9×10^{-8}	2.1×10^{-7}
flavone	1.9×10^{-7}	∞ (compet inhibn)	3.3×10^{-8}	5.7×10^{-7}

[a] The values of K_M and V_{max} that are summarized in Tables I and II were used to prepare plots of K_M/V_{max} and of $1/V_{max}$ vs. the tested inhibitor concentrations. A least-squares program was used to calculate the best linear fit of the data points. The calculated intercepts on the X axis of the K_M/V_{max} plots for each of the listed inhibitors ($-K_i$) were used to establish the indicated K_i values, and the X axis intercepts of the $1/V_{max}$ plots ($-\alpha K_i$) were used to establish the indicated αK_i values.

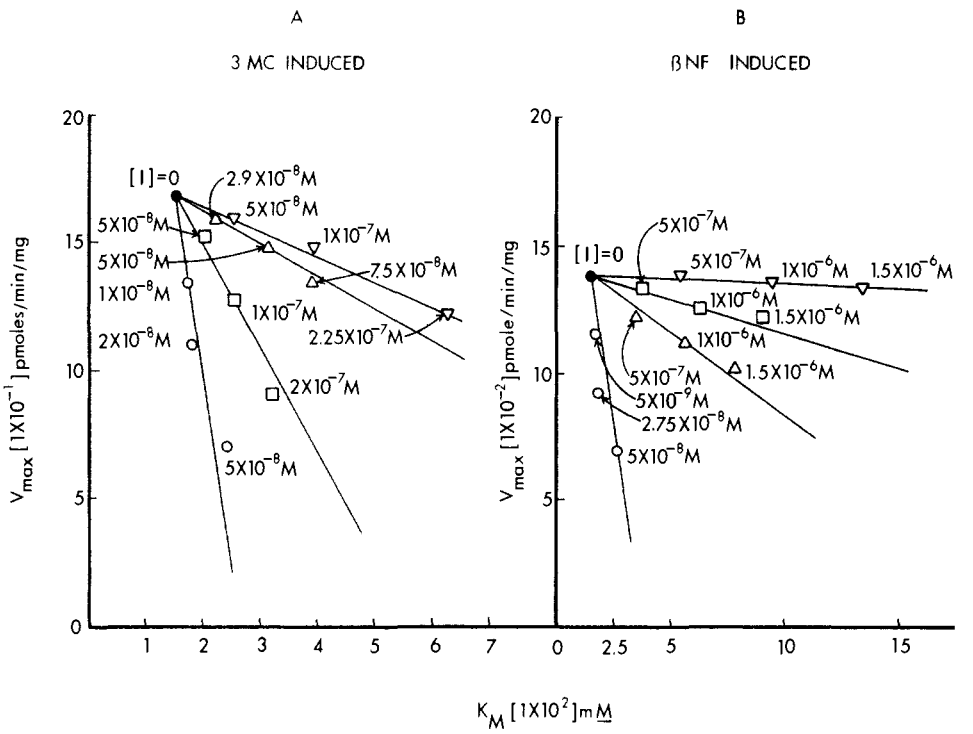

Figure 2. Changes in the kinetic parameters, K_M and V_{max}, as summarized in Table II for the deethylation of 7-ethoxycoumarin in the presence of varying concentrations of flavone (▽), αNF (○), βNF (△), or γNF (□). Section A shows the results obtained with microsomes from rats exposed to 3MC and section B shows the results with microsomes from rats exposed to βNF. The experiments were carried out as described under Experimental Section; the in vitro concentrations of the inhibitors used to determine each set of kinetic parameters are noted on the figure.

Table IV. Estimated Kinetic Parameters and Inhibition Constants Determined from Eadie–Scatchard Plots of Data from αNF-Exposed Rats[a]

inhibitor	K_{i_1}, M	αK_{i_1}, M	K_{i_2}, M	αK_{i_2}, M
none	$K_{M_1} = 3.4 \times 10^{-6}$ M	$V_{max_1} = 71$ pmol min^{-1} mg^{-1}	$K_{M_2} = 2.9 \times 10^{-4}$ M	$V_{max_2} = 400$ pmol min^{-1} mg^{-1}
αNF	1.2×10^{-8}	2.0×10^{-8}	4.7×10^{-7}	5.0×10^{-7}
βNF	1.6×10^{-8}	9.0×10^{-9}	3.7×10^{-7}	1.4×10^{-7}
γNF	1.2×10^{-8}	1.6×10^{-8}	3.7×10^{-7}	1.4×10^{-7}
flavone	2.8×10^{-8}	1.0×10^{-8}	3.7×10^{-7}	1.4×10^{-7}

[a] In vitro rates of 7-ethoxycoumarin deethylation by microsomal preparations from the liver of male Sprague–Dawley rats that had been exposed to αNF were determined as described under Experimental Section. These rates were determined in the presence of 1×10^{-8}, 5×10^{-8}, and 5×10^{-7} M αNF, βNF, γNF, or flavone at substrate concentrations that varied from 1.0 μM to 1.0 mM. At least three determinations of the rates at each substrate and inhibitor concentration were performed. The average velocity values were then used to prepare Eadie–Scatchard plots (V/S vs. S) as illustrated in Figure 3. Estimates of K_{M_1} and V_{max_1} values for the high-affinity form of the deethylase activity and of the K_{M_2} and V_{max_2} values for the low-affinity form were derived from the Eadie–Scatchard plots as described by Spears et al.[36] These estimates of K_M and V_{max} were used to prepare plots of K_M/V_{max} and of $1/V_{max}$ vs. the tested inhibitor concentrations. A least-squares program was used to calculate the best linear fit of the data points and the calculated X axis intercepts of the plots used to estimate K_i and αK_i values for each of the inhibitors toward both indicated forms of the enzymatic activity. The values designated by subscript 1 refer to the inhibition constants with respect to the high-affinity form; values designated by subscript 2 refer to the inhibition constants for the low-affinity form.

3MC and to βNF showed a single linear relationship. In contrast, the data obtained from the control microsomal preparations and from rats exposed to αNF showed marked deviation from linearity. As shown in Figure 3, the Eadie–Scatchard plot of the data from the αNF-exposed rats indicate multiple monooxygenase activities, a high V_{max}, high K_M form and a low V_{max}, low K_M form. The Eadie-Scatchard plot was analyzed to obtain estimates of V_{max_1}, K_{M_1} and of V_{max_2}, K_{M_2} by method described by Spears et al.[36] The monooxygenase activity in the liver microsomal fractions of control and of αNF-treated rats also displays multiple forms in the presence of the tested flavone and naphthoflavone inhibitors. Analysis of Eadie-Scatchard plots was used to obtain sets of apparent V_{max_1}, K_{M_1} and V_{max_2}, K_{M_2} values in the presence of three different concentrations of each of the flavone and naphthoflavone inhibitors. These apparent kinetic parameters were then used to calculate estimated K_i and αK_i values for each inhibitor tested in this system. The kinetic parameters estimated by analysis of Eadie–Scatchard plots of the experimental data obtained with the microsomes from αNF-exposed rats are summarized in Table IV and from the control rats in Table V.

Discussion

The results of the experiments involving the inhibition of 7-ethoxycoumarin deethylase activity by 1-(2-isopropylphenyl)pyrrole that are summarized in Tables I and III and in Figures 1 and 2 establish that this pyrrole is very comparable to 4(5)-phenylimidazole in its activity. The calculated inhibition constants for both compounds are very similar. We have also found[11,37] that 1-(2-iso-

(36) G. Spears, J. G. T. Sneyd, and E. G. Loten, *Biochem. J.*, **125**, 1149 (1971).

Table V. Estimated Kinetic Parameters and Inhibition Constants Determined from Eadie–Scatchard Plots of Data from Control Animals[a]

inhibitor	K_{i_1}, M	αK_{i_1}, M	K_{i_2}, M	αK_{i_2}, M
none	$K_{M_1} = 1.7 \times 10^{-6}$ M	$V_{max_1} = 6.0$ pmol min^{-1} mg^{-1}	$K_{M_2} = 4.13 \times 10^{-4}$ M	$V_{max_2} = 370$ pmol min^{-1} mg^{-1}
αNF	2.7×10^{-8}	3.5×10^{-7}	7.2×10^{-8}	5.8×10^{-7}
βNF	1.3×10^{-8}	2.6×10^{-7}	5.2×10^{-8}	3.0×10^{-7}

[a] In vitro rates of 7-ethoxycoumarin deethylation by control microsomal preparations from the liver of male Sprague–Dawley rats that had been exposed only to corn oil were determined as described under Experimental Section. The rates were determined in the presence of 1×10^{-8}, 5×10^{-8}, and 5×10^{-7} M αNF or βNF at substrate concentrations that varied from 1.0 μM to 1.0 mM. At least three determinations of the rates at each substrate and inhibitor concentration were performed. The K_M and V_{max} values and the K_i and αK_i values were then determined as described in Table IV.

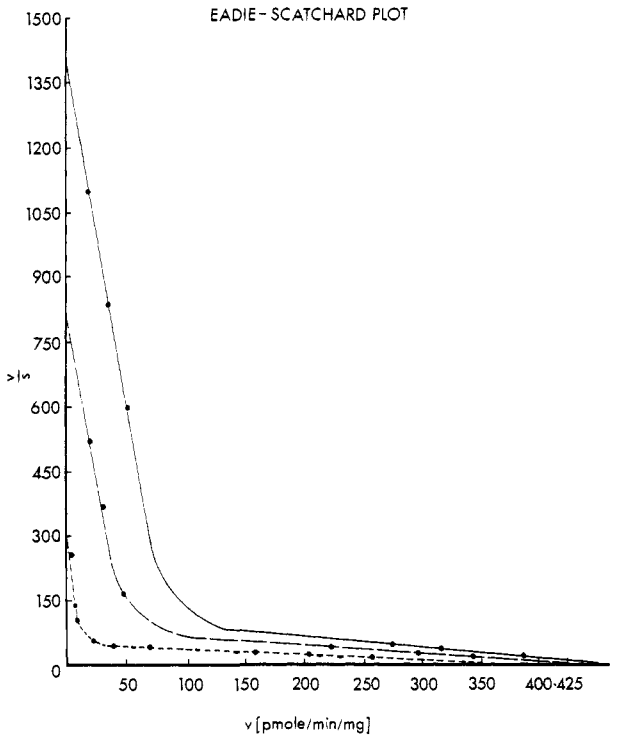

Figure 3. The in vitro rates of 7-ethoxycoumarin deethylation were determined as described under Experimental Section. This Eadie–Scatchard plot shows the variation of V/S vs. V for this activity in microsomes from control animals (male Sprague–Dawley rats injected intraperitoneally with corn oil, --), from αNF-exposed animals (—), and from αNF-exposed animals when assayed in the presence of 1×10^{-8} M αNF (– –). Assays were performed in triplicate; the concentration of microsomal protein in each assay was 0.195 mg/mL and the substrate concentration was varied over a range from 1.0 μM to 1.0 mM.

propylphenyl)pyrrole will inhibit the monooxygenase activity in microsomal preparations when assayed with [4′-^{3}H]benzenesulfonanilide[38] or with benzo[*a*]pyrene[39] as substrates. While detailed analyses of the inhibition constants were not performed using these alternative measures of monooxygenase activity, it is important to note that the inhibition by 1-(2-isopropylphenyl)pyrrole described here is not limited to the 7-ethoxycoumarin deethylase assay of cytochrome P-450 dependent monooxygenase activities.

The experiments with 1-phenylpyrrole which were carried out with the βNF-induced microsomes (Table I and Figure 1) also establish that this 1-arylpyrrole is an inhibitor of the assayed monooxygenase activity. The determined inhibition constants (Table III) are very comparable to those determined for 4(5)-phenylimidazole and for 1-(2-isopropylphenyl)pyrrole. It appears that the 2-isopropyl substituent has negligible effect upon the observed inhibition by 1-arylpyrroles. Since the tested 1-arylpyrroles lack the N-3 atom of the arylimidazoles, we conclude that the inhibition of monooxygenase activities by arylimidazoles is not absolutely dependent upon coordination of the nonbonded electrons of N-3 of the imidazole ring with the heme iron. Wilkinson et al.[40] have demonstrated that relative inhibitions of microsomal monooxygenase by 1-alkylimidazoles can be satisfactorily explained in terms of relative hydrophobic character, and Estabrook et al.[41] have proposed that the high affinity of cytochrome P-450 for benzo[*a*]pyrene might be due to interactions between the π-electrons of the heme macrocycle and the π-electrons of the aryl hydrocarbon. Such hydrophobic and/or π-electronic interactions may account for the inhibition by the 1-arylpyrroles described above.

The observed inhibition by 1-(2-isopropylphenyl)-imidazole (Tables I and III) emphasized the potential additional effect of an imidazole N-3 atom in favorable cases. The calculated inhibition constants summarized in Table III indicate that 1-(2-isopropylphenyl)imidazole is about two orders of magnitude more effective at inhibiting 7-ethoxycoumarin deethylase activity than is 1-(2-isopropylphenyl)pyrrole, 1-phenylpyrrole, or 4(5)-phenylimidazole. We propose that the exceptional effectiveness of 1-(2-isopropylphenyl)imidazole as an inhibitor is the result of inherent structural features that not only permit maximum hydrophobic and π-electronic interactions but also the coordination of N-3 with the heme iron.[6,7]

The data in Figure 1 emphasize another difference between the arylpyrrole and arylimidazole inhibitors. Although the four tested compounds show mixed-type inhibition, the 1-arylpyrroles are more nearly competitive with respect to 7-ethoxycoumarin ($K_i < \alpha K_i$) while the arylimidazoles are more nearly noncompetitive ($K_i \simeq \alpha K_i$). Thus, the 1-arylpyrroles preferentially bind to the free enzyme, while the arylimidazoles bind about equally well to the free enzyme and the enzyme–7-ethoxycoumarin complex. This may reflect the fact that the arylimidazoles can bind to both the hydrophobic 7-ethoxycoumarin substrate site and, through the N-3 nitrogen, to the heme iron (O_2 substrate site). In terms of the model recently described by Guenthner et al.,[42] the arylpyrroles would act primarily as type I inhibitors of the cytochrome P-450 dependent monooxygenases, while the arylimidazoles, because of the additional presence of the N-3 nitrogen, could act as both type I and type II inhibitors. Coordination of the imidazole N-3 with the low-spin ferric iron

(37) W. L. Alworth and M. Lelcht, unpublished observations.
(38) J. W. Daly, *Anal. Biochem.*, **33**, 286 (1979).
(39) D. W. Nebert and H. V. Gelboin, *J. Biol. Chem.*, **243**, 6242 (1968).
(40) C. F. Wilkinson, K. Hetnarski, G. P. Cantwell, and F. J. Dicarlo, *Biochem. Pharmacol.*, **23**, 2377 (1974).
(41) R. W. Estabrook, J. Werringloer, J. Capdevila, and R. A. Prough, *Polycyclic Hydrocarbons Cancer*, 1, 308 (1978).
(42) T. M. Guenthner, G. F. Kahl, and D. W. Nebert, *Biochem. Pharmacol.*, **29**, 89 (1980).

(type II inhibition) would block transfer of the first electron from the cytochrome reductase and thus render the P-450 complex incapable of binding molecular oxygen.[42]

The concentrations of tested compounds necessary to produce 50% inhibition (I_{50} values) have been frequently used to compare the potency of various inhibitors of monooxygenase activity (cf., for example, ref 5, 6, and 16). In addition, an observed correspondence between the magnitude of the dissociation constants as determined by changes in the cytochrome P-450 visible spectra in the presence of various inhibitors and the I_{50} values for these inhibitors has been used to support the concept that the observed inhibition of monooxygenase activity was due to ligand binding of the inhibitor to the heme of the cytochrome P-450 (see ref 5 and 6). When I_{50} values are used in such a fashion it is assumed that the I_{50} values are directly related to inhibition constants which are equilibrium constants for the formation of enzyme–inhibitor (EI) complexes. Because our results indicate that mixed-type inhibition of microsomal monooxygenase activity by arylimidazoles and flavones is common, we believe it important to emphasize that in the case of linear mixed-type inhibition the I_{50} value is a function of both K_i and of αK_i, that is, of the dissociation constants of both the EI and ESI complexes. Additional complications arise in the case of hyperbolic inhibition, where the ESI complex shows some catalytic activity, or if more than one inhibitor molecule can bind to a single enzyme molecule. Unless the kinetic parameters for a particular set of inhibitors have been carefully analyzed, therefore, it cannot be assumed that I_{50} values can be used as quantitative measures for the dissociation of possible EI complexes. The relationships between K_i and I_{50} in enzymatic reactions have been analyzed in detail by Chou[43] and by Cheng and Prusoff.[44]

A series of recent investigations has established that the cytochrome P-450 dependent monooxygenase activity in liver microsomal fractions is due to a family of discrete proteins with broad, overlapping substrate specificities.[45-53] It has also been demonstrated that different inducers of monooxygenase activity tend to increase the levels of individual members of this group of enzymes.[45-51] It is not surprising, therefore, that the results summarized in Figure 3 indicate the presence of different enzymatic activities. Greenlee and Poland[54] have demonstrated that Eadie–Hofstee plots of the 7-ethoxycoumarin deethylase activity in liver microsomes from control, phenobarbital-treated and 3MC-treated mice show at least two components, a high-affinity and a low-affinity form. As shown in Figure 3, the Eadie–Scatchard plots show two components in the liver microsomes from αNF-treated rats. These results were obtained in induction studies carried out with two separate groups of animals. Similar results were obtained with liver microsomes from control animals. In contrast, Eadie–Scatchard plots of the data from both 3MC- and βNF-treated rats were linear.

In a detailed investigation of the induction of benzo[a]pyrene hydroxylase activity in mouse and rat liver, Boobis et al.[23] concluded that 3MC and βNF acted via a common mechanism of induction. They also found that, at least under optimum conditions for induction, the extent of the induction by these two substances was very comparable. The observed differences were explained in terms of a more rapid mobilization and metabolism of intraperitoneal βNF. Preliminary reports in the literature also suggest that the cytochrome P-450 in liver microsomes treated with 3MC or with βNF are indistinguishable[55] and it has been found that one of the purified cytochrome P-450 dependent monooxygenases from 3MC-treated rats (cyt P-450c) preferentially catalyzes the deethylation of 7-ethoxycoumarin, the hydroxylation of benzo[a]pyrene, and the hydroxylation of zoxazolamine.[48]

Nevertheless, the results of our studies indicate that the monooxygenase activities in the microsomal fractions from the liver of rats exposed to 3MC or to βNF differ. Although each fraction gave a linear Eadie–Scatchard plot, Table II shows that the 7-ethoxycoumarin deethylase activity in the microsomal fraction from βNF-treated animals was several-fold greater than that from 3MC-treated animals. This result was observed in two separate induction studies with different groups of animals. The data in Table III and Figure 3 also suggest that the observed difference in deethylase activity between the βNF- and 3MC-derived microsomes is not just due to differing amounts of a single form of monooxygenase, caused by differing degrees of induction. It can be seen that γNF and flavone are better inhibitors of the 3MC-induced activity than of the βNF-induced activity and that these two compounds also give different patterns of inhibition with the two types of microsomes. (Compare the patterns produced by βNF with the two types of microsomes.) We conclude that differences in the 7-ethoxycoumarin deethylase activity exist in the liver microsomal fractions derived from αNF-, βNF-, and 3MC-treated rats. It should be noted that Saito and Strobel recently isolated a cytochrome "P-446" from the liver microsomes of βNF-pretreated rats which appears to be distinct from the major benzo[a]pyrene-metabolizing cytochrome induced by 3MC.[56]

The data in Tables III and IV indicate that αNF is a more potent inhibitor of the 7-ethoxycoumarin deethylase activity in microsomes from rats exposed to 3MC, βNF, or αNF than is βNF. In addition, in these cases, αNF tends to be more of a "pure" noncompetitive inhibitor with αK_i being nearly equal to K_i ($\alpha \simeq 1$). The inhibitions by αNF and βNF of the deethylase activity in microsomes from control animals were found to be comparable (Table V).

It is frequently stated that αNF is a potent inhibitor of those forms of the cytochrome P-450 dependent monooxygenase that are inducible by 3MC (frequently designated cytochrome P-448, P-450c, or P_1-450) but is not an inhibitor of the cytochrome P-450 dependent monooxygenases in control or phenobarbital-induced animals (designated cytochrome P-450b). Indeed, it has been reported that the cytochrome P-450 monooxygenase activities in control or phenobarbital-exposed animals are

(43) T.-C. Chou, *Mol. Pharmacol.*, **10**, 235 (1974).
(44) Y.-C. Cheng and W. H. Prusoff, *Biochem. Pharmacol.*, **22**, 3099 (1973).
(45) K. Comai and J. L. Gaylor, *J. Biol. Chem.*, **248**, 4947 (1973).
(46) D. A. Haugen and M. J. Coon, *J. Biol. Chem.*, **251**, 7929 (1976).
(47) F. P. Guengerich, *J. Biol. Chem.*, **253**, 7931 (1978).
(48) D. E. Ryan, P. E. Thomas, D. Korzeniowski, and W. Levin, *J. Biol. Chem.*, **254**, 1365 (1979).
(49) L. H. Botelho, D. E. Ryan, and W. Levin, *J. Biol. Chem.*, **254**, 5635 (1979).
(50) P. E. Thomas, A. Y. H. Lu, D. Ryan, S. B. West, J. Kawalek, and W. Levin, *Mol. Pharmacol.*, **12**, 746 (1976).
(51) M. Negishi and D. W. Nebert, *J. Biol. Chem.*, **254**, 11015 (1979).
(52) L. S. Kaminsky, M. J. Fasco, and F. P. Guengerich, *J. Biol. Chem.*, **254**, 9657 (1979).
(53) M. Agosin, A. Morello, R. White, Y. Repetto, and J. Pedemonte, *J. Biol. Chem.*, **254**, 9915 (1979).
(54) W. F. Greenlee and A. Poland, *J. Pharmacol. Exp. Ther.*, **205**, 596 (1978).
(55) L. S. Kaminsky, M. J. Fasco, and F. P. Guengerich, *J. Biol. Chem.*, **255**, 85 (1980).
(56) T. Saito and H. W. Strobel, *J. Biol. Chem.*, **256**, 984 (1981).

stimulated in vitro by αNF. (See the recent review by Wiebel,[32] for a detailed summary of the observed effects of αNF upon different monooxygenase systems.) It must be emphasized, however, that these differential effects of αNF upon different classes of cytochrome P-450 dependent monooxygenases have been generally observed only in liver preparations.[17] In other tissues, both αNF and βNF have been found to inhibit the monooxygenase activities of control and of 3MC-exposed animals. For example, αNF and βNF strongly inhibit the in vitro epidermal aryl hydrocarbon hydroxylase activities of both control and 3MC-treated mice[57] and both the basal and 3MC-induced aryl hydrocarbon hydroxylase activities of human β-lymphocytes.[58] Variable effects of αNF upon monooxygenases have been reported even in liver preparations, depending upon the particular species and assay utilized,[57-62] and Wiebel and Gelboin[14] found that the effects of αNF upon the benzo[a]pyrene hydroxylase activity in Sprague–Dawley rat liver are strongly influenced by the age, sex, and nutritional status of the animals.

Our observation that both αNF and βNF inhibit the 7-ethoxycoumarin deethylase activity in liver microsomes from control and from 3MC-, βNF-, or αNF-pretreated male Sprague–Dawley rats (100–150 g), while unanticipated, is, nevertheless, not without precedent. We conclude that this experimental observation reflects the influence of αNF and βNF on the particular set of cytochrome P-450 dependent monooxygenase isozymes present in these liver microsomes that are detected with the 7-ethoxycoumarin assay. Recent investigations emphasize the fact that the responses of rat liver microsomal fractions to administered xenobiotics are complex.[63,64] The view that there is a single form of hepatic cytochrome P-448 dependent monooxygenase that may be uniquely inhibited by αNF appears to be an oversimplification. (See, for example, the recent characterization of cytochromes P-450a, P-450b, and P-450c in rat liver microsomes by Thomas et al.[65])

The effects of αNF or of βNF upon aryl hydrocarbon induced skin tumors have generally involved simultaneous applications of the naphthoflavones and the carcinogenic hydrocarbon (for reviews, see ref 32 and 33). Kinoshita and Gelboin[27] and Pyerin and Hecker,[25] however, have examined features of the time course of αNF inhibition of skin tumor initiation by 7,12-dimethylbenz[a]-anthracene. It was found that the tumorigenesis was significantly reduced when αNF was topically applied 6 h before, simultaneously with, or up to 6 h after the 7,12-dimethylbenz[a]anthracene.[25,27] These workers concluded that the inhibition of 7,12-dimethylbenz[a]-anthracene by αNF was due to its ability to inhibit cytochrome P-488 dependent monooxygenase activities.

There are inherent dangers in extrapolating the experimental results obtained in liver preparations to an epidermal system. Nevertheless, we note that the observations reported here, that αNF and βNF are both potent inhibitors of the 7-ethoxycoumarin deethylase activities in a variety of liver microsomal preparations, including preparations from control rats and rats exposed to some inducers of microsomal enzymes, are consistent with the experimental results obtained in mouse skin preparations.[57] In contrast to the similar inhibition of monooxygenase activity produced by αNF and βNF, we find that the exposure of rats to αNF or to βNF produces distinctly different levels and patterns of monooxygenase activities in the liver microsomes subsequently isolated. Based upon these observations, we conclude, therefore, that the differential effects of αNF and βNF toward various types of aryl hydrocarbon induced tumors[32,33] may be the result of differential induction of monooxygenase activities rather than the result of differential inhibition of monooxygenase activities.

Experimental Section

4(5)-Phenylimidazole, 1-phenylpyrrole, flavone, and α- and β-naphthoflavone (αNF and βNF) were obtained from Aldrich Chemical Co. 3-Methylcholanthrene (3MC) was obtained from Eastman Kodak Co. The γ-naphthoflavone (γNF) was synthesized via published procedures.[66] The sample used in these studies was recrystallized from EtOH before use, mp 171 °C (lit. mp 171 °C). Anal. ($C_{19}H_{12}O_2$) C, H. 1-(2-Isopropylphenyl)pyrrole was synthesized by the method of Josey[67] and purified by column chromatography on silica gel using hexane as eluent. Anal. ($C_{13}H_{15}N$) C, H, N. 1-(2-Isopropylphenyl)imidazole was synthesized by the method of Johnson et al.[1] and purified by crystallization from ethyl acetate, mp 72 °C (lit. mp 68 °C). Anal. ($C,1,2H_{14}N_2$) C, H, N. Analyses were performed by Spang Microanalytical Laboratories.

Microsomal preparations were obtained as previously described from young male Sprague–Dawley rats grown on rat chow.[68] In the experiments to determine how prior exposure to βNF, αNF, or 3MC modified monooxygenase activity, separate groups of young male rats (75–125 g) were injected interperitoneally on 3 successive days with βNF, αNF, or 3MC (50 mg/kg of body weight) dissolved in corn oil and then sacrificed, and the microsomes were isolated on the 4th day. Animals injected intraperitoneally with corn oil alone served as the control animals. The protein concentration of the microsomal preparations were determined by the method of Bradford[69] using the commercially available (Bio-Rad Laboratories) reagent. Bovine serum albumin, dissolved in Tris buffer, pH 7.4, was used as a protein standard.

The fluorometric assay for mixed-function monooxygenase activity using 7-ethoxycoumarin as substrate developed by Aitio[70] was used. The incubation mixture for the ethoxycoumarin deethylation assay contained 1.39 mL of a 0.1 M Tris-HCl buffer, pH 7.4, and 1.11 mL of an NADPH-regenerating system containing 1.3 μmol of NADPH, 13.6 μmol of glucose 6-phosphate, 62.1 μmol of KCl, 13.7 μmol of $MgCl_2$, 13.5 μmol of $MnCl_2$, and 0.135 units of glucose-6-phosphate dehydrogenase (Sigma Chemical Co.) dissolved in 0.18 M Tris-HCl buffer, pH 7.4. Substrate was added (2.5 to 300 μM final concentration) in 50-μL aliquots of a 75% methanol solution. The reaction was initiated by adding microsomal protein (0.375–0.56 mg) in 25–50 μL of Tris buffer, pH 7.4, and the reaction mixture was incubated at 37 °C for 4 min. One milliliter of the reaction mixture was then added to 0.5 mL of 0.3 M trichloroacetic acid, and the fluorescence of this solution was measured after adding 4 mL of 1.6 M NaOH–glycine buffer, pH 10.3. The readings for velocity determinations were corrected for the fluorescence measured in the complete

(57) T. J. Slaga, S. Thompson, D. L. Berry, J. DiGiovanni, M. J. Juchau, and A. Viaje, *Chem.-Biol. Interact.*, **17**, 297 (1977).
(58) H. L. Guertoo, N. B. Parker, B. Paigen, M. B. Havens, J. Minowada, and H. J. Freedman, *Cancer Res.*, **39**, 4620 (1979).
(59) J. J. Stegeman and B. R. Woodin, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **39**, 1752, abstract (1980).
(60) M.-T. Huang, R. L. Chang, and A. H. Cooney, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **39**, 2053, abstract (1980).
(61) J. Kapltulnik, P. J. Poppers, M. K. Buening, J. G. Fortner, and A. H. Cooney, *Clin. Pharmacol. Ther.*, **22**, 475 (1977).
(62) P. Lesca, E. Rafidinarivo, P. Lecointe, and D. Mansuy *Chem.-Biol. Interact.*, **24**, 189 (1979).
(63) M. Warner and A. H. Neims, *Drug Metab. Dispos.*, 1, 188 (1979).
(64) R. N. Sharma, R. G. Cameron, E. Farber, M. J. Griffin, *J.-G.* Joly, and R. K. Murray, *Biochem. J.*, **182**, 317 (1979).
(65) P. E. Thomas, L. M. Reik, D. E. Ryan, and W. Levin, *J. Biol. Chem.*, **256**, 1044 (1981).
(66) V. V. Virkar and T. S. Wheeler, *J. Chem. Soc.*, 1681 (1939).
(67) A. D. Josey, *Org. Synth.*, **47**, 81 (1967).
(68) V. S. Ganu and W. L. Alworth, *Biochemistry*, **17**, 2876 (1978).
(69) M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
(70) A. Aitio, *Anal. Biochem.*, **85**, 488 (1978).

incubation mixture at zero incubation time. The fluorescence was measured with a Perkin-Elmer 650-10S instrument with excitation at 390 nm, emission at 440 nm, and excitation and emission slit widths of 2 nm. The instrument was standardized with authentic 7-hydroxycoumarin (Aldrich Chemical Co.) in 1.6 M NaOH–glycine buffer, pH 10.3.

Inhibitors were added in 20-μL aliquots from concentrated stock solutions in methanol. At least four assays at each substrate concentration in the presence and in the absence of inhibitors were performed.

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Novel Immunosuppressive Agents. Potent Immunological Activity of Some Benzothiopyrano[4,3-*c*]pyrazol-3-ones

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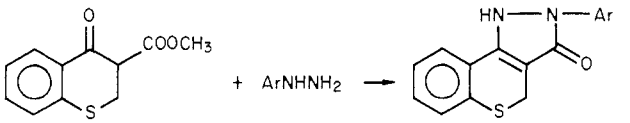
A unique class of immunosuppressant agents has been found by the use of a mouse model of immunity. A number of benzothiopyrano[4,3-*c*]pyrazol-3-ones are potent inhibitors of both the humoral and cellular immune response developed in mice in response to El_4 tumor cells. These compounds exhibit a selectivity for inhibiting the humoral response. Structure–activity relationships and Hansch analyses of data from the mouse model are discussed. One of the most potent compounds, 2-(4′-chlorophenyl)benzothiopyrano[4,3-*c*]pyrazol-3-one (1, CP-17 193), exhibits ED_{50} values for inhibiting the humoral and cellular responses in the range of 0.5–2 mg/kg, po, in the mouse model and also inhibits antibody production to several other antigens and prolongs skin graft survival in mice. These compounds are shown in several ways to be distinct from the lymphocytotoxic drugs cyclophosphamide and azathioprine.

The pathological involvement of the immune response has been implicated in a number of diseases, such as rheumatoid arthritis, systemic lupus erythematosis, and certain forms of nephritis, arteritis, and anemia.[1,2] Therapy for such diseases could logically be directed toward controlling the aberrant immune response. However, most of the clinical agents presently known to affect immune function[3] are lymphocytotoxic due to their effects on the DNA of rapidly dividing cells. These drugs, for example, alkylating agents such as cyclophosphamide and antimetabolites such as azathioprine, were originally developed as agents to inhibit proliferation of malignant cells. Only secondarily were they found to inhibit the immune response. In view of their mechanisms of action and their effects not just on lymphocytes but on many other proliferating cells, it is not surprising that such agents have a limited therapeutic ratio and a number of toxic effects[4,5] on rapidly proliferating tissues such as the gastrointestinal mucosa, bone marrow, and hair follicles. The frequency and severity of clinical side effects has prevented widespread acceptance of these immunosuppressive agents[6] in spite of the fact that significant therapeutic effects have been noted in several disease states.[7-9]

In the present work, compounds with the obvious potential for acting as alkylating agents or as antimetabolites were deliberately avoided. Instead, novel heterocyclic structures capable of regulating the immune response were sought. In the absence of structural prototypes, this study began by examining a large number of structurally novel organic compounds in an assay of cellular and humoral immunity carried out in the mouse.[10] This screening technique has uncovered a number of benzothiopyrano[4,3-*c*]pyrazol-3-ones which potently inhibit the immune response and form the basis for this report.

Chemistry. Although some benzothiopyrano[4,3-*c*]pyrazoles are known,[11] no 3-oxo derivatives of this ring system appear to be known. Almost all of the benzothiopyrano[4,3-*c*]pyrazol-3-ones (compounds **1–21**) shown in Table I were prepared by essentially one synthetic technique. Thus, combination of a hydrazine with 3-carbomethoxy-4-thiochromanone in the presence of acetic acid at elevated temperature gave fair yields of the title compounds (I).



I

The Experimental Section provides details for the preparation of a representative compound, **1**. Variations in reaction time or in equivalents of a particular hydrazine were made as appropriate to complete the reaction; Table I lists these specific conditions.

Data supporting the structural assignment for one representative compound (**1**, CP-17 193) are detailed under Experimental Section. All analogues were analyzed (C, H, and N) and were compared to **1** by infrared and mass spectral data. Several compounds, titrated with NaOH in 2:1 dioxane–H_2O solvent, were shown to be of intermediate acid strength with pK_a values in the range of 7.1–7.7 (Table I). Thus, there appears to be only small effects on acidity resulting from changing the aryl substituent (Ar) in I from 4-methoxyphenyl (compound **8**) to

(1) C. G. Cochrane and D. Koffler, *Adv. Immunol.*, **16**, 185 (1973).
(2) N. J. Zvaifler, *Adv. Immunol.*, **16**, 265 (1973).
(3) G. H. Hitchings and G. B. Elion, *Pharmacol. Rev.*, **15**, 365 (1963).
(4) M. C. Berenbaum and I. N. Brown, *Immunology*, **7**, 65 (1964).
(5) J. F. Bach, "The Mode of Action of Immunosuppressive Agents", Elsevier, New York, 1975.
(6) S. R. Kaplan and P. Calabresi, *N. Engl. J. Med.*, **289**, 952 and 1234 (1973).
(7) Cooperating Clinics Committee of the American Rheumatism Association, *N. Engl. J. Med.*, **283**, 883 (1970).
(8) A. D. Steinberg, H. B. Kaltreider, P. J. Staples, E. J. Goetzl, N. Talal, and J. L. Decker, *Ann. Intern. Med.*, **75**, 165 (1971).
(9) S. N. Novak and C. M. Pearson, *N. Engl. J. Med.*, **284**, 938 (1971).
(10) I. G. Otterness and Y.-H. Chang, *J. Clin. Immunol.*, **26**, 346 (1976).
(11) For a review of benzothiopyrans, including certain benzothiopyrano[4,3-c]pyrazoles, see S. W. Schneller, *Adv. Heterocycl. Chem.*, 18, 88 (1975).

0022-2623/81/1824-0830$01.25/0