3394 *J. Med. Chem.* **1996,** *39,* 3394−3400

Quantitative Structure−Activity Relationships for the *in Vitro* Antimycobacterial Activity of Pyrazinoic Acid Esters

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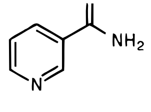
Received July 24, 1995[⊗]

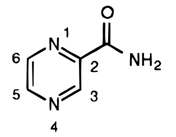
Substituted pyrazinoic acid esters have previously been reported to have *in vitro* activity against *Mycobacterium avium* and *Mycobacterium kansasii* as well as *Mycobacterium tuberculosis*. Modification of both the pyrazine nucleus and the ester functionality was successful in expanding the antimycobacterial activity associated with pyrazinamide to include *M. avium* and *M. kansasii*, organisms usually not susceptible to pyrazinamide. In an attempt to understand the relationship between the activity of the esters with the needed biostability, a quantitative structure−activity relationship has been developed. This derived relationship is consistent with the observation that *tert*-butyl 5-chloropyrazinoate (**13**) and 2′-(2′-methyldecyl) 5-chloropyrazinoate (**25**), compounds which are both 100-fold more active than pyrazinamide against *M. tuberculosis* and possess a serum stability 900−1000 times greater than the lead compounds in the series.

Introduction

Nicotinamide-derived compounds have been used in antituberculous therapy since the demonstration that nicotinamide was effective in the treatment of murine tuberculosis.[1] Pyrazinamide (PZA) was the most active of the nicotinamide analogs that were synthesized and evaluated for antituberculosis activity.[2] PZA, an unusual agent because of its narrow spectrum of activity, is inhibitory against most isolates of *Mycobacterium tuberculosis*, but *Mycobacterium bovis* (a closely related organism) and non-tuberculous mycobacteria are usually resistant. The literature contains conflicting reports regarding the activity of analogs of PZA against *M. tuberculosis* as a consequence of the fact that *in vivo* studies in mice were done without preliminary *in vitro* studies.[4] Both pyrazinoic acid and thiopyrazinamide were reported to be inactive in the murine tuberculosis model;[5] however later studies demonstrated that pyrazinoic acid is active *in vitro* against *M. tuberculosis* and *M. bovis*.[6] Esters of pyrazinoic acid and pyrazine-2,3-dicarboxylic acid have also been reported to have *in vitro* activity against *M. tuberculosis* H37Rv.[5,7]



nicotinamide



pyrazinamide (PZA)

The mechanism of action of PZA is unknown; however, it is known that *M. tuberculosis* isolates resistant to PZA have low levels of pyrazinamidase activity as do *M. bovis* isolates.[8] We postulated that PZA is a prodrug that is converted to pyrazinoic acid (the active agent) intracellularly by an amidase.[9] Pyrazinoic acid esters (PAE) which could be hydrolyzed by an esterase could equally well serve as prodrugs which would circumvent the requirement for activation by an amidase. A series of pyrazinoate esters was demonstrated to have substantially better *in vitro* activity than PZA against susceptible isolates of *M. tuberculosis*. These esters were also active against PZA-resistant *M. tuberculosis* isolates, *M. bovis* and *Mycobacterium kansasii*,[9] but were not active against *M. avium complex* isolates. Recently we have reported that modifications of the alkyl group of the ester function in addition to introduction of substituents on the pyrazine nucleus have been very successful in expanding the activity of pyrazinamide to include *Mycobacterium avium*.[10] (See Table 1.) It is also of note that many of these compounds demonstrate comparable activity against *M. tuberculosis* ATCC 35828,[11] a PZA-resistant organism and the two susceptible *M. tuberculosis* isolates (ATCC 27294 and 35801).[11] The *in vitro* activity of several of these compounds (5-chloro, 2′-octyl (**16**); 5-chloro, *n*-octyl (**8**); and 5-chloro, *n*-propyl (**3**)) are 100−1000-fold greater than that of pyrazinamide. In addition, a number of these esters have *in vitro* activity against the two *M. avium* isolates (organisms that are resistant to PZA, ATCC 49601[11] and MAC 101) that is better than that of pyrazinamide against PZA-susceptible *M. tuberculosis* isolates.

The use of quantitative structure−activity relationships (QSARs) to predict biological response and to aid in the design of effective agents has been carefully examined.[12] The parameters which relate chemical structure with biological activity include such physical properties as steric demand (E_s or υ), lipophilicity (log P, π) and electronic character (σ). Initially we applied this strategy of data analysis toward the problem of identifying the effect these physical parameters had on the rate of hydrolysis of substituted pyrazinoic acid esters in equine serum (1), the model we are employing for intrinsic biostability. The determined hydrolysis rate could reasonably be expected to reflect the sensitivity of the synthetic material to hydrolysis in the plasma and at the site of action. Subsequently we have applied our strategy to the *in vitro* biological response of various

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[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1996.

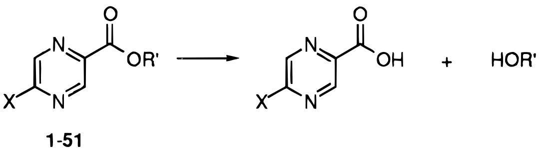
S0022-2623(95)00538-3 CCC: $12.00

Table 1. Physical Parameter Constants and Serum Half-Lives for 5-X-pyrazinoate Esters **1**−**51** (see Eq 1 for Structure)

							log (1/MMIC)[e]					
no.	X	R′	$\upsilon_{OR'}$[a]	log *P*[b]	σ[c]	*t* (min)[d]	MAC 101	ATCC 49601	SWK	ATCC 35801	ATCC 27294	ATCC 35828
1	Cl	Me	0.36	0.67	1.736	168	−0.171	0.431	1.936	1.334	1.635	1.033
2	Cl	Et	0.48	1.19	1.747	110	0.164	0.164	1.970	1.970	2.271	1.067
3	Cl	*n*-C3	0.56	1.67	1.751	18.7	1.098	1.098	2.904	3.825	3.524	2.904
4	Cl	*n*-C4	0.58	2.21	1.753	5.5	0.526	0.225	2.332	3.633	2.934	2.633
5	Cl	*n*-C5	0.58	2.73	1.753	3.2	0.854	0.854	3.882	2.961	3.581	3.262
6	Cl	*n*-C6	0.61	3.36	1.753	2.2	0.880	1.181	4.209	3.288	3.288	2.686
7	Cl	*n*-C7	0.61	3.87	1.753	3.2	1.506	1.205	3.932	3.932	3.631	3.932
8	Cl	*n*-C8	0.61	4.48	1.753	1.3	1.228	1.228	3.955	3.955	3.654	3.955
9	Cl	*n*-C9	0.61	4.98	1.753	2.3	0.949	1.250	3.358	2.455	3.977	3.977
10	Cl	*n*-C10	0.61	5.48	1.753	1.9	0.970	1.271	2.776	3.077	2.776	2.776
11	Cl	*n*-C11	0.61	5.98	1.753	6.0	1.291	1.592	3.717	2.796	3.097	2.796
12	Cl	*i*-Bu	0.62	1.97	1.755	11.8	0.526	0.526	2.332	2.934	2.031	2.031
13	Cl	*t*-Bu	1.22	1.70	1.764	1200	0.225	2.332	2.633	2.031	1.730	1.730
14	Cl	Benzyl	0.62	2.39	1.66	5.0	1.493	1.794	2.396	2.697	2.396	2.396
15	Cl	CH(Me)*n*-C5	0.96	3.00	1.765	16.2	0.904	0.904	3.313	2.711	3.631	3.631
16	Cl	CH(Me)*n*-C6	0.99	3.63	1.765	28.2	0.927	0.927	3.035	3.035	3.035	3.035
17	Cl	CH(Me)*n*-C7	0.99	4.14	1.765	30.0	0.949	0.949	3.977	2.756	2.455	2.153
18	Cl	CH(Me)*n*-C8	0.99	4.75	1.765	23.7	0.970	0.970	3.378	2.776	3.697	3.998
19	Cl	CH(Me)*n*-C9	0.99	5.25	1.765	29.6	1.291	1.291	3.097	3.398	3.087	2.495
20	Cl	CH(Me)*n*-C11	0.99	6.25	1.765	50.0	0.124	1.027	3.754	2.834	2.232	2.834
21	Cl	CH(Et)*n*-C5	1.10	4.24	1.776	7.0	0.626	0.927	3.955	3.035	2.734	3.035
22	Cl	CH(Et)*n*-C8	1.13	5.99	1.776	9.0	0.087	0.087	3.398	2.495	3.097	2.495
23	Cl	CH(*n*-C6)$_2$	1.26	7.04	1.782	60	1.329	0.726	2.834	2.834	3.135	2.834
24	Cl	C(Me)$_2$(*n*-C6)	1.44	4.39	1.781	510	0.648	0.648	2.455	1.852	3.057	3.057
25	Cl	C(Me)$_2$(*n*-C8)	1.44	5.51	1.781	957	0.388	0.388	2.194	2.796	1.592	1.592
26	Cl	C(Me)$_2$(*n*-C9)	1.44	6.01	1.781	902	0.106	0.106	2.815	2.514	1.611	1.310
27	Cl	C(Me)(Et)$_2$	1.52	2.74	1.786	1020	−0.023	−0.023	2.084	1.482	1.482	1.783
28	Cl	C(Me)(*n*-C5)$_2$	1.72	5.82	1.798	360	0.708	0.708	1.611	1.611	1.611	1.310
29	Cl	C(Me)(*n*-C6)$_2$	1.78	7.08	1.798	1080	0.744	1.346	2.249	2.851	3.152	2.851
30	Cl	C(Me)(*n*-C4)*n*-C5	1.72	5.30	1.798	390	0.087	0.087	1.291	1.592	1.893	1.291
31	Cl	C(Me)(*n*-C4)*n*-C6	1.75	5.93	1.798	648	0.106	0.106	1.912	1.611	1.912	1.310
32	Cl	C(Me)(*n*-C4)*n*-C7	1.75	6.44	1.798	600	0.124	0.124	1.329	1.931	1.630	1.329
33	Cl	C(Me)(*n*-C4)*n*-C8	1.75	7.05	1.798	1142	0.744	0.744	1.346	1.346	1.346	1.647
34	Cl	C(Me)(*n*-C5)*n*-C6	1.75	6.45	1.798	1410	0.726	0.726	1.630	1.630	2.232	1.329
35	Cl	C(Me)(*n*-C5)*n*-C7	1.75	6.96	1.798	3167	0.744	0.744	ND[f]	1.948	2.249	1.647
36	H	Me	0.36	−0.23	1.446	470	−0.268	−0.268	0.334	0.936	1.237	0.334
37	H	*i*-Bu	0.62	1.07	1.465	207	−0.152	−0.152	1.052	1.654	1.955	1.654
38	H	*s*-Bu	0.86	1.05	1.469	41.1	−0.152	−0.755	1.052	ND	1.353	1.052
39	H	*n*-C10	0.61	4.58	1.463	0.8	0.917	1.519	3.723	3.024	2.723	3.024
40	H	benzyl	0.62	1.49	1.370	4.3	0.525	0.525	2.331	2.331	2.331	2.030
41	F	Me	0.36	0.27	1.666	222	0.387	0.387	1.290	1.290	1.591	1.290
42	F	*n*-C6	0.61	2.96	1.683	2.8	0.548	0.548	1.452	1.753	ND	1.452
43	F	*n*-C10	0.61	5.08	1.683	4.0	0.946	0.946	2.451	1.849	2.150	1.849
44	F	CH(Me)*n*-C6	0.99	3.23	1.695	6.9	0.900	0.599	3.928	1.803	2.104	1.803
45	I	Me	0.36	0.91	1.716	200	0.013	0.013	1.519	1.218	1.218	1.218
46	Me	Me	0.36	0.15	1.246	1200	−0.226	−0.226	0.677	1.279	0.978	0.978
47	Me	*n*-C3	0.56	1.15	1.261	124	0.450	0.450	1.353	1.654	1.955	1.353
48	Me	*n*-C7	0.61	3.35	1.263	3.2	0.868	0.868	1.771	2.976	2.675	2.373
49	Me	*n*-C9	0.61	4.46	1.263	1.0	0.616	0.917	2.121	2.422	2.422	2.121
50	Me	CH(Et)*n*-C5	1.10	3.72	1.286	1.0	0.592	0.592	1.796	2.700	1.194	1.495
51	OMe	Me	0.36	0.74	1.586	300	−0.183	−0.183	0.420	ND	ND	ND

[a] See ref 16. [b] See refs 18b and 19. [c] See refs 24 and 27. [d] Half-life represents time of 50% conversion to the corresponding pyrazinoic acid. [e] MMIC is the minimum inhibitory concentration in mmol/L. [f] ND = not determined.

strains of mycobacteria to the PAE's to optimize the balance between activity and biostability. *tert*-Butyl 5-chloropyrazinoate (**13**) and 2′-(2′-methyldecyl) 5-chloropyrazinoate (**25**) are illustrative examples which demonstrate this relationship. Both compounds are 100-fold more active than pyrazinamide against *M. tuberculosis* and have activity against a pyrazinamide-resistant strain of *M. tuberculosis* (ATCC 35828) but also possess a stability 900−1000 times greater than the lead compounds in the series.



1-51

(1)

Results and Discussion

Effect of Substitution on *in Vitro* Stability. We have modeled intrinsic biostability of pyrazinoate esters toward hydrolysis by screening those compounds with promising *in vitro* activity for their stability in equine serum. Horse serum is employed as a surrogate for human serum as it is available in quantity and represents a suitable biological challenge to the synthetic materials to assess their sensitivity to endogenous enzymatic action.

Steric Demand. The role of steric effects in controlling the rates of reactions has been useful in both chemical and biochemical transformations such as in understanding organic reaction mechanisms or in exploring host−guest relations. The introduction of steric demand and how it effects reaction rates was first presented in the 1950s by Taft.[13] Taft defined the steric

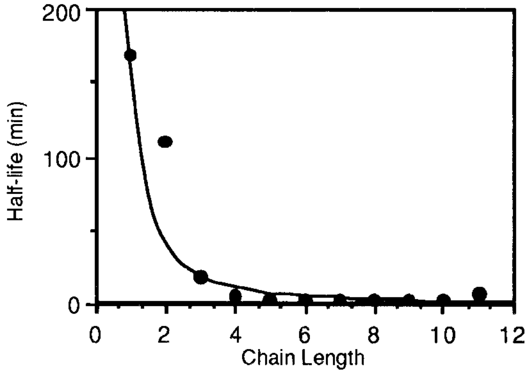


Figure 1. Plot of carbon chain length (R′, see **1**) vs half-life (t, min) of alkyl 5-chloropyrazinoates. The curve represents an exponential decrease as chain length increases.

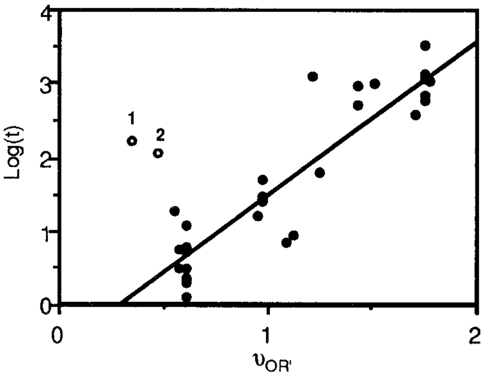


Figure 2. Plot of log t vs steric demand ($\upsilon_{OR'}$) for 5-chloropyrazinoate esters (**1**−**35**) (Table 2, line 4). The calculated line which best fits the data is log $t = 2.154\upsilon_{OR'} - 0.687$ ($r^2 = 0.843$, $n = 33$). The two points furthest from the line (not included in the calculations) are where R′ = Me (**1**) and where R′ = Et (**2**).

constant E_s which is derived from the rate constant of acid-catalyzed hydrolysis of aliphatic esters as

$$\log[k(\mathrm{RCO_2Et})/k(\mathrm{CH_3CO_2Et})] = E_s \qquad (2)$$

or more generally as

$$\log(k/k_0) = aE_s + b \qquad (3)$$

where k_0 is the rate of reaction for a reference compound within a series of compounds with rates k. Similarly, the half-life (t) of an ester can be expressed as a function of steric effects where

$$\log t = aE_s + b \qquad (4)$$

Over the past 25 years, Taft's E_s parameters have been reexamined and modified replacing E_s with other sets such as E_s^c, E_s', or by υ or θ_2.[14−17] Charton's steric substituent constant, υ,[13] is based on statistical calculations using van der Waals volumes. These υ values have been shown to correlate very well with Taft's E_s parameters ($r^2 = 0.954$).[15] In general, the reaction rate for the hydrolysis of substituted esters RCOOR′ depends on the steric demands of both R and OR′.[16,17] In the serum-promoted hydrolysis of pyrazinoate esters, only the steric effects due to the alkoxy group (i.e., $\upsilon_{OR'}$) are considered as the effects of the aryl group R remain constant.

In a preliminary study, we examined the half-life of the pyrazinoate ester vs the alkyl group R′ carbon chain length. The results show an exponential decrease in half-life with increasing chain length (Figure 1). Since hydrolysis rates generally decrease with steric bulk around the ester carbonyl, we determined the steric parameter $\upsilon_{OR'}$ for the esters and found that the rate of hydrolysis behaves linearly as a function of steric demand (Figure 2; Table 2, line 4), where in the case of the 5-chloropyrazinoates

$$\log t = 2.154\upsilon_{OR'} - 0.687 \quad (r^2 = 0.843) \qquad (5)$$

The positive coefficient for $\upsilon_{OR'}$ confirms the prediction that a more sterically hindered ester should have a longer half-life. The longer half-lives may also be understood in terms of the degree of substitution at the α-carbon since there is very little difference for the values of $\upsilon_{OR'}$ among entries **3**−**12**, primary alkyl groups, among entries **15**−**20**, secondary alkyl residues, or among entries **30**−**35**, tertiary centers. The esters which have longer half-lives than that predicted by the associated $\upsilon_{OR'}$ values are R′ = Me (**1**) and Et (**2**). This greater stability may be a consequence of the fact that the hydrolysis of the esters in serum is no doubt an enzymatically assisted process, where the enzymes simply do not accommodate these substrates well.

Lipophilicity. Hydrophobic parameters,[18] such as log P (octanol−water partition coefficients) or π (=log P_X − log P_H),[19] are important in correlating structure to drug−receptor interactions or transport to the site of action.[12c] Some of these interactions have a linear correlation with the lipophilic character (log P)[12d] of a compound as in

$$\log(k/k_0) = a(\log P) + b \qquad (6)$$

while other responses require a higher order equation such as the parabolic relationships

$$\log(k/k_0) = -a(\log P)^2 + b(\log P) + c \qquad (7)$$

The negative coefficient for the squared term signifies that molecules which are highly hydrophilic will not penetrate lipophilic barriers readily. Molecules having a very high log P value will be strongly held by the first lipophilic material they encounter.[12d] Thus, at either extreme of the log P value, a longer half-life would be observed reflecting poor compound−enzyme interactions with both highly lipophilic and hydrophilic compounds. The lipophilic parameter, log P, of the pyrazinoate esters was determined by the sum of the measured log P of methyl pyrazinoate (−0.23)[20] and the π values for the alkyl substituent R′ and the 5-X substituent. A plot of eq 7 is shown in Figure 3 with moderate correlation (Table 2, line 5, $r^2 = 0.593$) with respect to (log P)^2 and no linear correlation with respect to log P (r^2= 0.0092).

Electronic Character. The rate of ester hydrolysis of RCOOR′ will be greatly influenced by the electronic character of R and/or of OR′. The largest electronic influence in the case of the pyrazinoate esters comes from the aromatic substituent (R) as opposed to the alkyl group (R′).[21] Nuclear substitution of the ring (e.g., carbon−nitrogen substitution) as well as the aromatic substituent (X) will have a profound effect on the overall electronic character of the aromatic ring and therefore on the site of attack, the ester carbonyl. The influence of modification of electronic character is most often approached through linear free energy relationships (LFERs) employing an appropriate electronic substitu-

Table 2. Correlation Coefficients[a] of Serum Half-Life as $\log t = a\sigma_I + b\upsilon_{OR'} + c(\log P) + d$

line	parameter	set of compds	a	b	c	d	n	s	r^2
1	σ	all (**1**−**51**)	1.484			−0.855	51	1.047	0.057
2		5-Cl (**1**−**35**)	25.317			−43.001	35	0.815	0.411
3	$\upsilon_{OR'}$	(**1**−**51**)		1.360		0.317	49	0.889	0.341
4		(**1**−**35**)		2.154		−0.687	33	0.432	0.843
5	log *P*	(**1**−**51**)			0.193(log *P*) − 1.361	3.029	48	0.682	0.593
6		(**1**−**35**)			0.170(log *P*) − 1.176	2.841	32	0.742	0.510
7	$\sigma + \upsilon_{OR'}$	(**1**−**51**)	−0.449	1.441		0.989	49	0.896	0.346
8		(**1**−**35**)	−4.064	2.339		6.296	33	0.433	0.846
9	σ + log *P*	(**1**−**51**)	1.702		−0.041	−1.052	49	1.067	0.060
10		(**1**−**35**)	60.228		−0.200	−104.014	33	0.574	0.724
11	$\upsilon_{OR'}$ + log *P*	(**1**−**51**)		2.432	−0.339	0.619	49	0.735	0.560
12		(**1**−**35**)		2.470	−0.139	−0.379	33	0.394	0.874
13	$\sigma + \upsilon_{OR'}$ + log *P*	(**1**−**51**)	0.514	2.384	−0.353	−0.139	49	0.740	0.564
14		(**1**−**35**)	−1.492	2.529	−0.135	2.175	33	0.400	0.874

[a] *n* = number of points. *s* = standard error of estimates. r^2 = the fraction of total variance in the data which is explained by the regression.

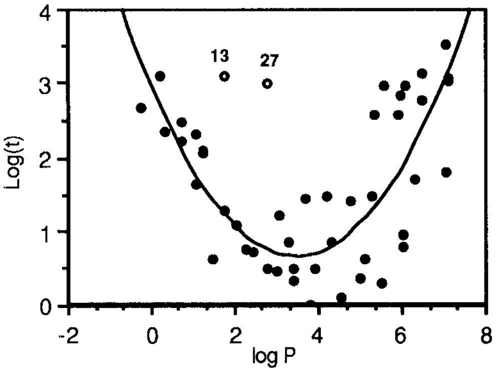


Figure 3. Plot of log *t* as a function of the lipophilicity (log *P*) for alkyl 5-chloropyrazinoates (**1**−**35**) (Table 2, line 6). The data best fits a curve derived from a π^2 relation. Two points which fall in the center of the curve are R′ = *t*-Bu (**13**) and R′ = C(Me)(Et)$_2$ (**27**), two compounds with high steric demand but comparatively low lipophilicity.

ent constant, σ.[21] Calculation of substituent effects of substituted heterocycles has been shown for the most part to be additive,[22] although only pyrazines will be examined in this report ($\sigma = \sigma_{2N} + \sigma_{3N} + \sigma_{para}$). When OR′ is held constant and only the electronic influence of the pyrazine substituents are considered, the effect of substitution at carbon 5 can be correlated with the rate of enzymatic hydrolysis. When the alkyl group R′ is methyl, the rate of hydrolysis with varied substitution at carbon 5 follows the Hammett relation:[23]

$$\log(k/k_0) = \sigma\rho + c \tag{8}$$

or

$$\log t = \sigma\rho + c \tag{9}$$

where *t* is the half-life in serum and σ is the parameter of Taft ($\sigma_p{}^0$)[24] (Figure 4). A good correlation is observed ($r^2 = 0.992$) with a ρ value of −1.67 for five compounds (not including X = OMe). σ values determined for X = OMe of nonheterocyclic compounds often give inconsistent results for pyrazinoates presumably due to the resonance stabilization of the α_N substituent.[25] Since $\rho < 0$, electron-donating substituents, but not those stabilized through resonance, would decrease the rate of hydrolysis and thus would increase the half-life of the compound in serum. More methyl pyrazinoate derivatives are being prepared to see if this relation holds.

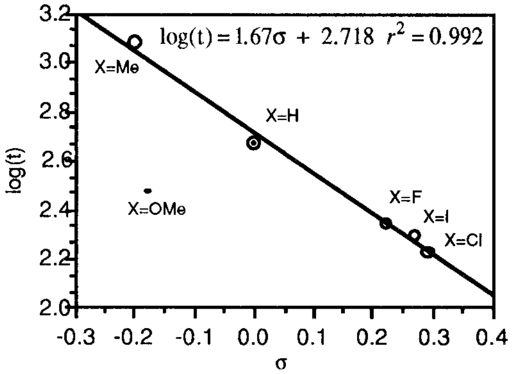


Figure 4. Hammett plot of $\sigma_p{}^0$ (ref 23) vs log *t* for methyl 5-X-pyrazinoates. The failure of the compound that does not readily fall on the line (X = OCH$_3$ (**51**)) may be a consequence of the resonance stabilization effect of the α-nitrogen.

Thus far, we have only examined hydrolysis of pyrazine derivatives. The effects of the number and position of nuclear nitrogen substitution on rates of reaction has been examined in only a few systems. The most extensively studied heterocycles are substituted pyridines.[22] The general trend is that σ values are positive ($\gamma > \alpha > \beta$) and are additive with respect to the branched substituents.[26] Pyrazine, pyridazine, and pyrimidine systems have only been briefly discussed.[22,27] Substituent effects of nicotinoate derivatives are under investigation in our laboratory.

The absolute electronic effect of the hydrocarbon alkoxy substituent OR′ is minimal[28] compared with the overall effect of the aromatic ring. However, there is a linear correlation observed for 5-chloropyrazinoate esters with varied OR′ groups ($r^2 = 0.411$, Table 2, line 2). The coefficient for σ is largely due to the small range of $\Sigma\sigma$ values ($1.736 < \sigma < 1.798$) and positive signifying a longer half-life for a more highly substituted ester (though correlation may be influenced strongly by steric effects; see Table 2, line 4).

Combination of Parameters. The Taft−Ingold equation[13] incorporates both steric and electronic parameters for reactions which depend on both of these factors.

$$\log(k/k_0) = \rho\sigma + \delta E_s \tag{10}$$

The results of a linear combination of any two or the combined set of parameters with half-life are presented in Table 2 (lines 7−12). The highest correlations are

Table 3. Correlation Coefficients[a] of Biological Activity as $\log(1/\text{MMIC}) = a\sigma + b\upsilon_{\text{OR}'} + c(\log P) + d \log t + e$

line	Mycobacteria	set of compds	a	b	c	d	e	n	s	r^2
1	M. avium	all (**1**−**51**)	0.415	−0.770	0.224		−0.226	48	0.320	0.560
2	101		0.581	−0.261	0.140	−0.215	−0.310	48	0.281	0.669
3		5−Cl (**1**−**35**)	10.839	−1.219	0.189		−18.014	32	0.324	0.510
4			−15.003	0.426	0.147	−0.287	26.654	32	0.302	0.587
5	M. avium	(**1**−**51**)	0.339	−0.968	0.273		−0.079	48	0.355	0.615
6	ATCC		0.485	−0.546	0.205	−0.182	−0.158	48	0.310	0.679
7	49601	(**1**−**35**)	1.364	−0.950	0.207		−1.574	32	0.320	0.526
8			−31.359	0.931	0.166	−0.268	54.990	32	0.304	0.588
9	M. kansasii	(**1**−**51**)	2.473	−1.618	0.322		−1.432	48	0.744	0.483
10	SWK		2.902	−0.330	0.114	−0.554	−1.669	48	0.640	0.626
11		(**1**−**35**)	6.465	−1.884	0.203		−7.560	34	0.670	0.464
12			7.327	−0.549	0.640	−0.576	−8.905	34	0.611	0.569
13	M. tuberculosis	(**1**−**51**)	0.875	−1.226	0.252		1.084	49	0.666	0.312
14	ATCC		1.179	−0.050	0.070	−0.485	0.959	49	0.571	0.504
15	35801	(**1**−**35**)	2.647	−1.479	0.161		−1.238	35	0.579	0.452
16			3.298	−0.352	0.051	−0.476	−2.276	35	0.532	0.552
17	M. tuberculosis	(**1**−**51**)	1.744	−1.430	0.245		−0.121	47	0.585	0.477
18	ATCC		1.966	−0.629	0.129	−0.327	−0.252	47	0.542	0.561
19	27294	(**1**−**35**)	5.747	−1.648	0.207		−6.735	34	0.602	0.449
20			6.712	−0.359	0.074	−0.544	−8.274	34	0.540	0.570
21	M. tuberculosis	(**1**−**51**)	1.358	−1.474	0.316		0.025	49	0.712	0.408
22	ATCC		1.722	−0.388	0.148	−0.456	−0.216	49	0.638	0.536
23	35828	(**1**−**35**)	2.022	−1.651	0.257		−0.593	34	0.734	0.392
24			3.358	0.134	0.074	−0.753	−2.722	34	0.632	0.564

[a] For footnotes, see Table 2. MMIC is the biological response measured as the minimum inhibitory concentration (mmol/L).

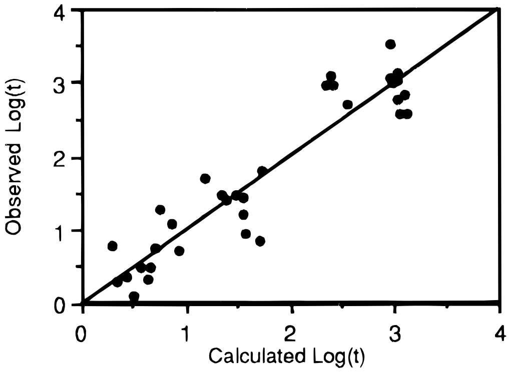


Figure 5. Plot of calculated vs observed log *t* of 5-chloropyrazinoates (**1**−**35**) (Table 2, line 14). The calculated line corresponds to $-1.492\sigma + 2.529\upsilon_{\text{OR}'} - 0.135 \log P + 2.175 = \log t$ ($r^2 = 0.874$, $n = 33$).

observed in expressions where the steric parameter is employed. The combination of steric demand with lipophilicity or electronic character shows little improvement over steric demand alone. A lower correlation is observed between half-life and log $P + \sigma$ (Table 2, line 10, $r^2 = 0.724$) and would possibly be improved if $(\log P)^2$ were incorporated. When all three parameters were considered (Figure 5, Table 2, line 14, $r^2 = 0.874$), a similar or slightly better correlation was observed than the equations derived from the dual parameters, in lines 8, 10, and 12 ($r^2 = 0.846$, 0.724, and 0.874, respectively). The coefficient for $\upsilon_{\text{OR}'}$ was the only positive term reemphasizing the influence of steric demand on half-life.

Effect of Substitution on Biological Activity. Quantitative−structure activity relationships are often based upon a combination of one or more of the three extra thermodynamic parameters ($\upsilon_{\text{OR}'}$, log *P*, and σ) as well as the rate of ester to carboxylic acid conversion (*t*) in our case. These relationships can lead to valuable predictions of biological response helpful in drug design. Biological response (BR), defined as the reciprocal of the molar concentration of compound effective in inhibiting bacterial growth, C_x, can be expressed as a function of some or all of these three parameters[29] in equations such as the parabolic function

$$\log(1/C_x) = -a(\log P)^2 + b(\log P) + \rho\sigma + c \tag{11}$$

or the linear function

$$\log(1/C_x) = b(\log P) + \rho\sigma + c \tag{12}$$

or as a linear combination of all three parameters

$$\log \text{BR} = aE_s + b(\log P) + c\sigma + d \tag{13}$$

Since the variables are not linearly independent, their role cannot be quantitatively discerned, nonetheless using eq 13, the biological response was investigated through linear regression analysis for *M. avium* complex (101 and ATCC 49601), *M. kansasii* (SWK), and *M. tuberculosis* (ATCC 27294, 35801, and 35828), and the coefficients of correlation are presented in Table 3. The data was divided into two sets, all compounds (**1**−**51**) and those consisting of only 5-chloropyrazinoate esters (**1**−**35**). A better correlation for the calculation of biological response is observed when the half-life of the ester is factored into the equation (Table 3, even lines vs odd). Figures 6 and 7 (Table 3, lines 6 and 22, respectively) are two representative examples where calculated biological response for *M. avium* ATCC 49601 and *M. tuberculosis* ATCC 35828 were plotted against the observed activity data. A good correlation is observed in both instances ($r^2 = 0.679$ and 0.536, respectively).

Conclusion

There are many obstacles an antituberculous agent must overcome in order to be effective *in vivo.* To mention a few: A compound must be hydrophilic enough to permit the development of plasma concentrations sufficient to deliver the drug to the site of infection, yet the compound must be lipophilic enough to penetrate the mycobacterial cell. Simultaneously the compound

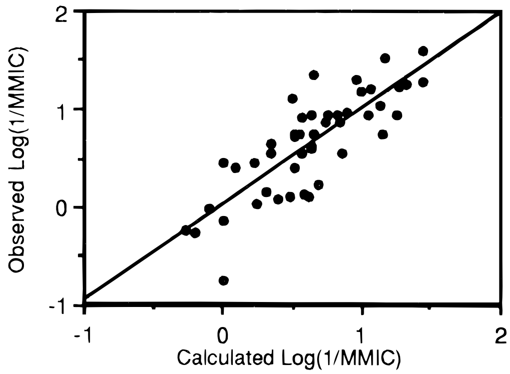


Figure 6. Plot of calculated vs observed biological response for *M. avium* complex ATCC 49601 for all compounds **1**−**51** (Table 3, line 6), where log(1/BR) = 0.485σ − 0.546$v_{OR'}$ + 0.205(log *P*) − 0.182 log *t* − 0.158 ($r^2 = 0.679$, $n = 48$).

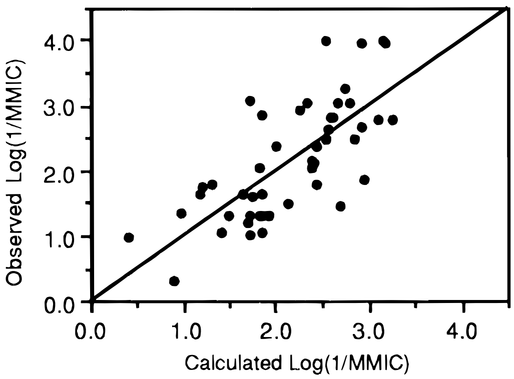


Figure 7. Plot of calculated vs observed biological response for *M. tuberculosis* ATCC 35828, for all compounds **1**−**51** (Table 3, line 22), where log(1/BR) = 1.722σ − 0.388$v_{OR'}$ + 0.148(log *P*) − 0.456 log *t* − 0.216 ($r^2 = 0.536$, $n = 49$).

must be relatively susceptible to hydrolysis such that the prodrug is uneffected by the "extracellular" enzymes but will be readily hydrolyzed at the site of action.

The factors which influence the above criteria include the extra thermodynamic parameters $v_{OR'}$ (steric demand), log *P* (lipophilicity), and σ (electronic influence). From Tables 2 and 3 it is possible to draw a few conclusions about the importance $v_{OR'}$, log *P*, and σ on the serum stability and *in vitro* activity of pyrazinoate esters. Not surprisingly stability is proportional to steric demand (Figure 2). Electron-releasing pyrazine substituents increase the stability of methyl esters (Figure 4) but tend to decrease the biostability of the PAE when considered overall (Table 2, line 31). The *in vitro* activity of the esters against *M. kansasii* is strongly effected by increases in lipophilicity (Table 3, line 9) but activity against *M. tuberculosis* and *M. avium* less so (Table 3, lines 13 and 1). *In vitro* activity against *M. kansasii* is also inversely effected by an increase in half-life (*t*) but activity against *M. tuberculosis* and *M. avium* is affected less (Table 3, lines 10, 6 and 22). We have found that compounds such as the *tert*-butyl 5-chloropyrazinoate (**13**) and 2′-(2′-methyldecyl) 5-chloropyrazinoate (**25**), compounds with a high steric demand and low to moderate lipophilicity, possess the stability and *in vitro* activity desired in candidates for further study in *in vivo* animal models of infection.

Experimental Section

Linear Regression Calculations. Calculations were performed using standard statistical software.

Preparation of Pyrazinoates. The preparation of the pyrazinoate esters has been described previously.[10]

Determination of Physical Parameters. $v_{OR'}$ values not represented in other works[17] were calculated by substitution using available data, i.e., $v_{OCH(Me)C5} = v_{OCH(Me)C2} + (v_{OC5} - v_{OC2}) = 0.86 + 0.1 = 0.96$.

log *P* values for the pyrazinoate esters were calculated as the sum of log $P_{pyrazinoate} + \pi_X + \pi_{R'}$. The octanol−water partition coefficients for methyl pyrazinoate (−0.23) and for other substituted pyrazines were previously determined[20,30] by HPLC methods to give π constants for π_X ($\pi_F = 0.55$, $\pi_{Cl} = 0.96$, $\pi_I = 1.14$,[19b] $\pi_{Me} = 0.47$, $\pi_{OMe} = 0.95$). $\pi_{R'}$ was obtained from the corresponding alcohol ($\pi_{R'} = \pi_{R'OH} - \pi_{OH} = \pi_{R'OH} + 1.16$). Alcohols not represented in the tables[19b] were determined by substitution, i.e., $\pi_{CH(Me)C5} = \pi_{CH(Me)C3} + (\pi_{C5} - \pi_{C3}) = 1.77 + 1.06 = 2.83$.

σ values for the most part were considered to be additive. Therefore, σ(pyrazinoate) = σ(ring) + σ(R′) = $\sigma_{2N} + \sigma_{3N} + \sigma_X + \sigma(R') = 0.75 + 0.65 + \sigma_p^o(X) + \sigma_I(R')$ where σ_{2N} and σ_{3N} are the inductive effects of a nitrogen ring substitution[25] and $\sigma_p^o(X)$ are the para-σ values of Taft[24] except for σ_{OMe}. σ_{OMe} was adjusted to 0.14 to fit on the line (as opposed to often used values of −0.02 or −0.18).[21] $\sigma_I(R')$ values were determined by DeTar.[28] Those values not represented were calculated by substitution, i.e., $\sigma_{CH(Me)C5} = \sigma_{CH(Me)C2} + (\sigma_{C5} - \sigma_{C2}) = 0.69 + 0.06 = 0.75$.

Bioassay Procedures. Mycobacterial Isolates. *M. tuberculosis* ATCC 27294 (H37Rv), ATCC 35801 (Erdman), and ATCC 35828 (PZA-resistant) were obtained from the American Type Culture Collection, Rockville, MD. *M. avium* ATCC 49601 (serotype 1) is a clinical isolate from a patient with AIDS at State University of New York Health Science Center, Syracuse, NY. This isolate has been used previously in beige mouse studies in our laboratory. *M. avium* strain 101 (serotype 1) was provided by Dr. Lowell Young, Kuzell Institute for Arthritis and Infectious Diseases, San Francisco, CA. *M. kansasii* strain SWK was a clinical isolate from a patient at the Veterans Affairs Medical Center, Syracuse, NY.

Medium. The organisms were grown in modified Middlebrook 7H10 broth (7H10 agar formulation with agar and malachite green omitted) with 10% OADC enrichment (Difco Laboratories, Detroit, MI) and 0.05% Tween 80[31] on a rotary shaker at 37 °C for 3 days. The culture suspensions were diluted with 7H 10 broth to yield 1 Klett unit per milliliter of *M. tuberculosis* and 0.1 Klett unit per milliliter of *M. avium* complex or *M. kansasii* (Klett-Summerson colorimeter- Klett Manufacturing Brooklyn, NY) approximately 5 × 10^5 viable organisms/mL. The final concentration of mycobacteria used for susceptibility testing was approximately 2.5 × 10^4 viable organisms/mL.

Susceptibility Testing. Stock solutions of PZA, pyrazinoic acid, and the PAE were prepared by hydrating a known weight of agent in water or DMSO. The stock solutions were sterilized by passage through a 0.2 μm nylon membrane filter. Serial 2-fold dilutions of the compounds in 7H10 broth at pH 5.8 (testing at pH 5.6 would yield a lower MIC for PZA; however, many of the organisms grow poorly at this pH) were prepared. The tubes were incubated at 37 °C on a rotary shaker for 7−10 days. A control tube without any drug was included in each experiment. The MIC was defined as the lowest concentration of drug that yielded an absence of visible turbidity.

Determination of *t* (Half-Life) in Equine Serum. Equine serum (900 μL of a 5% aqueous solution) in 0.01 M KH_2PO_4 (pH 7.2) was added to 100 μL of a 1 mg/mL stock solution of the pyrazinoic acid ester and mixed in a microcentrifuge tube. At 0, 5, 10, 15, 30, 60, and 120 min intervals a 100 μL aliquot is removed and reaction stopped with an equivalent volume of acetonitrile. After mixing thoroughly, samples were then filtered through a microcentrifuge tube outfitted with a 0.2 μm nylon filter. The filtrate (50 μL) was injected onto a Vydac 201HS54C_{18} analytical column (250 mm × 4.6 mm) and eluted isocratically at 1.5 mL/min with a mobile phase of 80% acetonitrile:20% 0.1 M sodium acetate (pH 5.0). The pyrazinoic acid esters were detected by absorbance at 265 nm. A range of standards were made in a 50/50 acetonitrile/buffer solution prepared in the same manner as the sample. Serum levels of the esters were determined by comparison to a standard curve, with detection limits being ≥0.1 μg/mL in serum.

Acknowledgment. Financial support of this work by National Science Foundation Grants CHE-8901986 and CHE-9413004 and National Institute of Health Grant A133690 is gratefully acknowledged as is the generosity of the Biotec Division of LONZA, Ltd., in providing 5-chloropyrazinoyl chloride and 5-methylpyrazinoic acid. The authors also gratefully acknowledge the helpful comments and suggestions of Dr. John A. Lowe, III.

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JM950538T