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Mode of Action and Quantitative Structure–Activity Correlations of Tuberculostatic Drugs of the Isonicotinic Acid Hydrazide Type[1]

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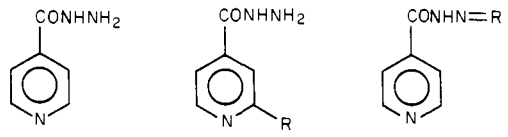
Quantitative structure–activity studies have been performed for a series of 2-substituted isonicotinic acid hydrazides by correlating electronic, steric, and lipophilic properties of the substituents with the biological activity data (MIC) from serial dilution tests with *Mycobacterium tuberculosis* (strain H 37 Rv). The reaction rates for the quaternization of 2-substituted pyridines with methyl iodide were also determined. The rate constants show a similar dependence on the steric and electronic effects of the substituents as the antibacterial activities of the corresponding pyridine-4-carboxylic acid hydrazides. The obtained correlations give evidence that the reactivity of the pyridine nitrogen atom is essential for the biological activity of 2-substituted isonicotinic acid hydrazides and seem to support the hypothesis that isonicotinic acid derivatives are incorporated into an NAD analogue.

Isonicotinic acid hydrazide (I) (INH, isoniazid) has been one of the most effective agents in tuberculosis therapy since 1952, when its action against *Mycobacterium tuberculosis* (*M. tbc*) was first discovered.[2] Although many derivatives of INH have been synthesized, none have demonstrated antibacterial activity greater than INH itself. Most derivatives involve ring substitution at position 2 and 6 of the pyridine moiety (II) or modification of the hydrazine moiety as in the hydrazone structure (III).



I II III

Isoniazid derivatives develop the typical high activity against *M. tbc* only if they can be transformed to INH (I) or to a derivative of type II. Substitution of electron-donating or -withdrawing groups at the position ortho to the ring nitrogen yields compounds with decreased antibacterial action. The hydrazones (III), on the other hand, have activities comparable to INH itself.

The mode of action of INH and its derivatives has not yet been established. The present state of knowledge has been summarized in several recent review articles.[3,4] Among the several proposed mechanisms of action, such as the formation of yellow pigments[5] and inhibition of mycolate synthesis,[6,7] only one hypothesis derives a detailed mechanism that may be tested in a quantitative structure–activity analysis. This is the "isonicotinic acid" hypothesis of Krüger-Thiemer[8–12] (Figure 1).

According to this hypothesis, isonicotinic acid (INA) is responsible for the inhibitory activity of INH against mycobacteria. INH, via passive diffusion, rapidly permeates the bacterial cell membrane, with the hydrazide function serving only as a carrier group. Once inside the cell, the INH is oxidized enzymatically to INA. At the predominant intracellular pH the INA is nearly completely ionized ($pK_a = 4.84$) and therefore cannot leave the cell.[13] This results in the accumulation of INA within the bacterial cell and accounts for the low MIC of INH (1 μM); special binding capacities on the part of the bacteria are therefore unnecessary. Subsequently the accumulated INA, instead of the natural metabolite nicotinic acid (NA), is quaternized and incorporated into an NAD analogue (or a precursor). The NAD analogue thusly produced can no longer function as the natural coenzyme. Disturbance of the normal metabolism (especially lipid metabolism) causes degeneration of bacterial cells (loss of acid fastness) and cell death.[14,15] The following observations, from this and other laboratories, strongly support the INA hypothesis.

1. The oxidative degradation of INH to INA can be performed in vitro by horseradish peroxidase and H_2O_2.[16,17] This is compatible with the observation that INH-resistant strains of *M. tbc* possess only small amounts of peroxidase (and catalase) as compared with INH-sensitive strains.[18,19]

2. Carboxylic acid hydrazides such as benzoic acid hydrazide, nicotinic acid hydrazide (NH), or picolinic acid hydrazide develop an antibacterial activity against *M. tbc* which is at least 200 times smaller than that of INH.[10]

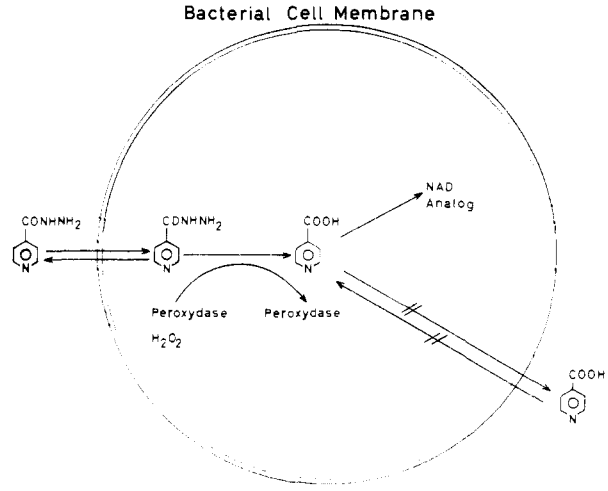


Figure 1. "Isonicotinic acid" mode of action hypothesis of INH action; schematic drawing according to Krüger-Thiemer.[8-12]

There is, however, a cross resistance between carboxylic acid hydrazides and pyridinecarboxylic acid hydrazides, indicating that one element of the mode of action is identical. This cross resistance concerns the oxidation of the carboxyl hydrazide group only.[10] One must conclude that both resistance and cross resistance are a consequence of the diminished peroxidase (catalase) system.

3. The proposed antagonism between INA and NA can be demonstrated indirectly with the observed antagonism between INH and NH.[20-22] The minimum inhibitory concentration (MIC) of INH increases from 0.5 to 1900 μM in the presence of 16 μM NH, which by itself has no antibacterial effect at that concentration (MIC_{NH} = 1900 μM). In a slightly acidic culture medium (pH 5.9) even INA inhibits the growth of mycobacteria. Under these conditions INA is partly un-ionized ($\approx$10%) and can permeate the cell wall. The observed MIC is 128 μM compared to MIC >4000 μM at pH 7.[10] It is interesting to note that INH-resistant strains are affected to the same degree as INH-sensitive strains. Further evidence for the importance of INA can be derived from the antagonistic effect of NA on growth inhibition of INA at pH 5.9.[10] This confirms the observation mentioned earlier that INH resistance is caused mainly by the lack of a catalase–peroxidase system which liberates INA from INH.

4. Since 1952 a number of papers have been published dealing with the biosynthesis of NAD and the interference of this synthesis exhibited by INH and INA.[23-28] In 1954 Goldman[29] and Zatman et al.[30,31] prepared the INH analogue of NAD (wherein the nicotinamide moiety of NAD was replaced by INH) by reacting INH with NAD in the presence of NAD-ase (NAD glycohydrolase). We have repeated this work in our laboratory and have further demonstrated that INA may also be incorporated into an analogue.[32] The preparation and properties of the 3-acetylpyridine analogue of NAD were reported in 1956.[33] Other papers dealing with the biosynthesis of NAD in various organisms and the disturbance of this synthesis by pyridine derivatives are cited in ref 34–37.

5. As mentioned earlier, the production of the INA analogue of NAD disturbs the normal cellular metabolism. The analogue is probably incapable of participating in redox reactions. Goldman has demonstrated that the INH analogue of NAD is not reduced by dithionite or in several enzymatic assay systems and does not form a dihydropyridine with cyanide ion.[29] In contrast to this, the 3-acetylpyridine analogue described by Kaplan et al.[33] is reduced by $S_2O_4^{2-}$, CN^-, and yeast alcohol dehydrogenase. The disturbance of metabolism by INH or INA apparently leads to a breakdown of mycolic acid synthesis[6,7] and to damage of the cell wall structure,[38] the latter evidenced by a loss of "acid fastness". The decrease in mycobacterial NAD^+, as measured by Bekierkunst[39] and Winder and Collins,[6] occurs during the same time interval and, to a similar degree, as the decrease in mycolic acid content. From the data presented to date it is not possible to make a definitive statement concerning the sequence within the inhibition mechanism.

In 1957 Dunbar[40] observed the loss of "acid fastness" in *M. tbc* treated with INH. Utilizing the Dunbar method we have demonstrated in our laboratory[10,41] that as a general rule tuberculostatic drugs containing a 4-substituted pyridine ring cause a loss of acid fastness (the benzoic acid hydrazide inhibitor does not elicit this effect). The substituent must be either a carbonyl function or a carboxylic acid or a group that may easily be converted to such a function. This does not preclude the possibility that a loss of acid fastness may arise as a consequence of inhibitory mechanisms exerted by other agents with different modes of action. Additional substitution in the 2 position of the pyridine ring with groups of increasing size decreases the rate of the process (presumably the formation of the analogue) that results in the loss of acid fastness. From the preceding discussion it follows that INA, under the proper conditions (pH 5.9), should also cause the loss of acid fastness. This, in fact, has been observed.[41]

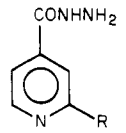
Quantitative Structure–Activity Relationships (QSAR). If we assume that the liberation of INA by the oxidizing system is not rate determining in analogue formation and if we limit our studies to INH-sensitive strains (with complete oxidizing enzyme system), the biological activity of INH derivatives should depend on the reactivity of the pyridine nitrogen atom (the possible disturbance of the functions of the bacterial peroxidase in response to different substituents is presently being investigated and will be published elsewhere). Our interest therefore was focused on structural parameters which could help to quantify the relationship between differences in the biological effect and changes in the reactivity of the pyridine ring.

Assuming that the synthesis of an analogue of NAD occurs within the mycobacteria, the quaternization reaction at the pyridine nitrogen atom could be the decisive rate-determining step. We can expect that ligands in the position ortho to the reaction center will influence the reactivity and that electronic and steric effects would be of major importance. In order to test this assumption a series of 2-substituted isonicotinic acid hydrazides was synthesized with substituents that possess a wide range of electronic, steric, and hydrophobic properties. The biological activity was tested in a liquid culture medium by serial dilution tests. Using *M. tbc* strain H 37 Rv as the test organism we obtained activity data for the hydrazides in the form of minimum inhibitory concentrations (MIC, in μM). The MIC given (Table I) is an average of several determinations. INH was used as a standard in each experiment. The antibacterial activity is expressed as 1/MIC.

Protein binding in the culture medium should be without consequence for QSAR because of the low protein content (0.7%) of the test medium and the assumed accumulating effect of oxidases on INH derivatives within the bacterial cells.

Electronic Effects. Usually σ Hammett constants are

Table I



Compd	R	MIC_{obsd}, μmol/l.	π^a	R_m	Log $1/MIC_{obsd}$	Log 1/MIC (calcd by eq 12)	Log 1/MIC (calcd by eq 29)
1a	H	1.1	0.0	0.372	−0.041	−0.255	−0.297
2a	CH_3	5.2	0.769	0.697	−0.716	−0.901	−1.168
3a	C_2H_5	21.1	1.253	0.862	−1.324	−1.413	−1.582
4a	n-C_3H_7	55.2	1.765	1.168	−1.742	−1.963	−1.83
5a	i-C_4H_9	450.0	2.162	1.366	−2.653	−2.389	−2.00
6a	CH_3O	153.0	1.04	0.783	−2.185	−1.860	n
7a	C_2H_5O	450.0	1.62	0.954	−2.655	−2.387	−2.452
8a	NH_2	14.5	0.16	0.32	−1.161	−0.795[i]	−0.736
			0.837[b]	0.595[c]			
9a	CH_3CONH	2150.0	−0.11	0.266	−3.332	−2.474[j]	−3.402
			1.835[b]	1.195[c]			
10a	$CH_3CONHCH_2$	243.0	−0.439	0.34	−2.386	−2.281[k]	−2.109
			1.685[d]	1.105[e]			
11a	$(C_2H_5)_2N$	717.0	2.254	1.383	−2.856	−2.599[l]	−4.307
			2.65[b]	1.685[c]			
12a	F	260.0	1.03	0.42	−2.415	−2.661	−5.257
13a	Cl	392.0	1.25	0.63	−2.593	−2.628	−3.487
14a	Br	616.0	1.39	0.866	−2.79	−2.737	−4.042
15a	I	254.0	1.652	0.931	−2.404	−2.804	−2.852
16a	NO_2	371.0	0.378	0.577	−2.569	−2.370	−4.932
17a	C_6H_5	50.0	2.492	1.643	−1.699	−1.627[m]	−2.74
			1.13[f]	0.779[g]			
18a	$C_6H_5CH_2$	38.5	2.145	1.365	−1.585	−2.565	−1.551
19a	CH_2=CH	35.0	1.53[h]	1.02	−1.544	−1.511[m]	−2.519

[a] Log P_{1NH} = −1.137 (octanol). [b] Calculated from V_w[52] by eq 3. [c] Calculated from V_w[52] by eq 6. [d] Calculated from $V_{w(cor)}$ by eq 3; see text. [e] Calculated from $V_{w(cor)}$ by eq 6; see text. [f] Calculated from $E_{s(C_6H_5\ perp)}$[49] by eq 2. [g] Calculated from $E_{s(C_6H_5\ perp)}$[49] by eq 5. [h] Calculated from R_m = 1.02 by eq 4. [i] Calculated with π = 0.837. [j] Calculated with π = 1.835. [k] Calculated with π = 1.685. [l] Calculated with π = 2.65. [m] Calculated with π = 1.13. [n] No k_{rel} available; see text.

successfully applied to describe the electronic influence of substituents. In the case of 3- and 4-substituted pyridine compounds, however, a change in the electronic influence is directly expressed by a change in basicity.[42,43] Recently a paper on this subject has been published by Chakrabarty et al.[44] The pK_a value therefore can be used as a specific indicator of the electron-attracting or -releasing effects of the substituents. Figure 2 shows that this statement also holds for 2-substituted pyridines. A correlation of high significance between pK_a[45,46] and σ_I[47] is obtained.

$$pK_a = -10.46\ (19.8)\ \sigma_I + 5.65 \qquad (1)$$

n	r	s	F	S
25	0.97	0.6	392	>99.9

In this equation n is the number of data points used in the analysis, r is the correlation coefficient, s is the standard error of estimate, the F value is the decision statistic of the F test of significance, S indicates the significance (%) of the contribution of a parameter to the activity in a series of compounds, and the value in parentheses behind the coefficient is the t test.

A nearly identical equation has been obtained by Charton[43] for a restricted series of 13 2-substituted pyridines.

Assuming that the additional electronic effect of the carboxyhydrazide group is approximately constant within the series of compounds studied, we have used the pK_a values of the corresponding 2-substituted pyridines (Table II) to characterize the electronic influence of the substituents.

Steric Effects. In order to describe the steric effects of the substituents, primarily the literature values for E_s given by Taft[48] and Kutter and Hansch[49] have been used. These compilations are not complete, however, and E_s values for several substituents are not available. During our investigation we observed that the partition coefficients

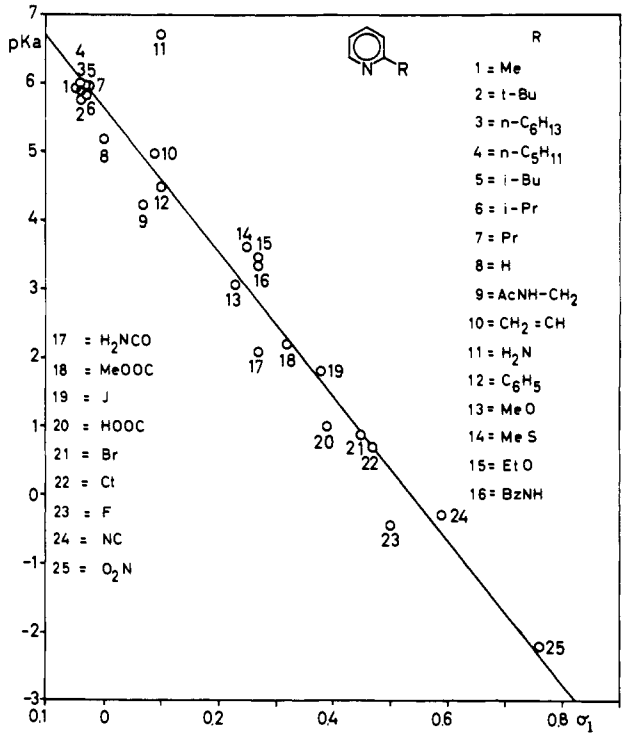
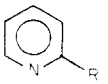


Figure 2. Hammett plot of pK_a's of 2-substituted pyridines.

Table II



Compd	R	k_{rel} (obsd)[a]	Log k_{rel} (obsd)	Log k_{rel} (calcd by eq 23)	pK_a[b]	E_s[l,m]	V_w[n]
1b	H	1.0	0.0	−0.338	5.17	1.24	3.45
2b	CH_3	0.324	−0.489	−0.389	5.94	0	13.67
3b	C_2H_5	0.189	−0.723	−0.551	5.97	−0.07	23.9
4b	n-C_3H_7	0.137	−0.863	−0.732	5.97	−0.37	34.13
5b	i-C_4H_9	0.11	−0.958	−0.872	5.97[d]	−0.93	44.35
6b	CH_3O	[c]			3.06	0.69	16.87
7b	C_2H_5O	0.06	−1.222	−1.398	3.47[e]		27.1
8b	NH_2	0.574	−0.241	−0.192[f]	6.71		10.54
9b	CH_3CONH	0.0179	−1.747	−1.296[g]	4.09		33.45
10b	$CH_3CONHCH_2$	0.0957	−1.019	−1.203[h]	4.23[e]		43.68
11b	$(C_2H_5)_2N$	0.00554	−2.256	−0.657[i]	7.32[e]		52.13
12b	F	0.00162	−2.790	−2.312	−0.44	0.78	5.8
13b	Cl	0.016	−1.796	−2.057	0.72	0.27	12.0
14b	Br	0.0078	−2.108	−2.054	0.9	0.08	15.12
15b	I	0.0365	−1.438	−1.883	1.82	−0.16	19.64
16b	NO_2	0.00247	−2.607	−2.587	−2.2[e]	0.23	16.8
17b	C_6H_5	0.0423	−1.373	−1.416[j]	4.48	−2.58 (+0.23)[l]	45.84
18b	$C_6H_5CH_2$	0.1967	−0.706	−1.107	5.13	−0.38	56.07
19b	CH_2=CH	0.0563	−1.249	−0.933[k]	4.98		20.41

[a] $k_{pyridine} = 2.94 \times 10^{-5}$ l. sec^{-1} mol^{-1} (25 °C ±0.05, CH_3OH). [b] See ref 45 and 46. [c] No k_{rel} available; see text. [d] Estimated value. [e] See Experimental Section. [f] Calculated with $\pi_{2\text{-R-INH}} = 0.837$. [g] Calculated with $\pi_{2\text{-R-INH}} = 1.835$. [h] Calculated with $\pi_{2\text{-R-INH}} = 1.685$. [i] Calculated with $\pi_{2\text{-R-INH}} = 2.65$. [j] Calculated with $\pi_{2\text{-R-INH}} = 2.492$. [k] Calculated with $\pi_{2\text{-R-INH}} = 1.53$. [l] See ref 49. [m] See ref 48. [n] See ref 52.

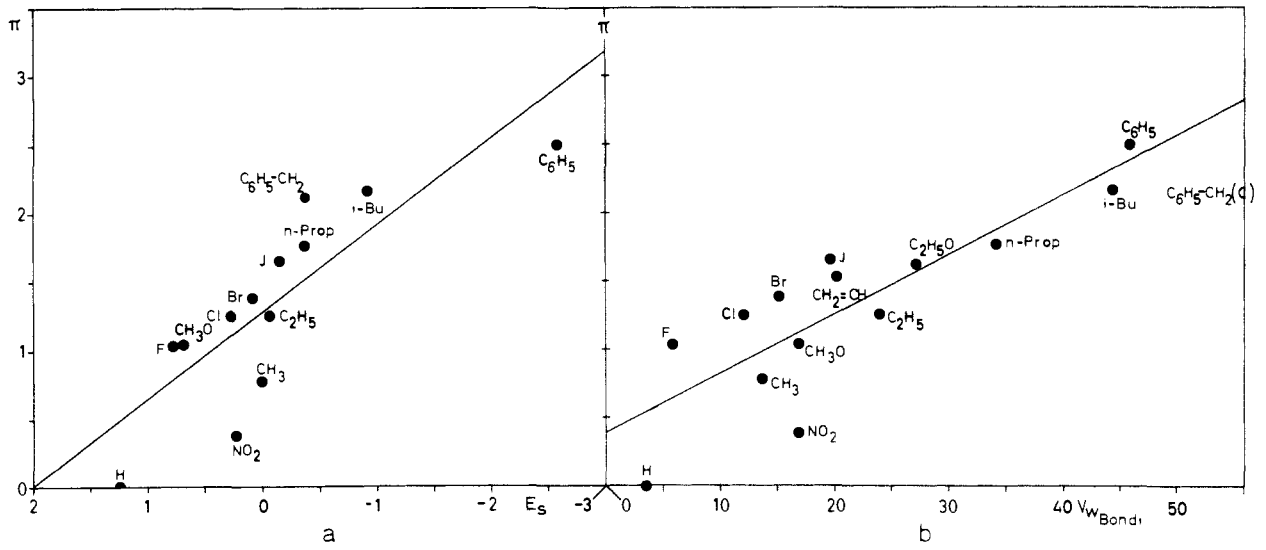


Figure 3. Correlation of π values of 2-R-INH with steric parameters: (a) E_s values of Taft[48] and Kutter;[49] (b) van der Waal's volumes of the substituents R given by Bondi.[52]

determined in the octanol–water system and the resulting substituent constants π[50] (Table I) which express the lipophilic properties of the ligands correlate significantly with the steric parameter E_s (eq 2, Figure 3a, Table II).

$$\pi = -0.63\,(4.77)\,E_s + 1.275 \quad (2)$$

n	r	s	F	S
13	0.822	0.428	22.7	>99.9

$$\pi = 0.0436\,(5.53)\,V_w + 0.377 \quad (3)$$

n	r	s	F	S
14	0.847	0.36	30.5	>99.9

An equally significant correlation is found between π (2-R-INH) and the van der Waals volumes V_w of the substituents given by Bondi[51,52] (eq 3, Figure 3b, Table II). For the calculation of this correlation the benzyl group has been excluded, since V_w ($C_6H_5CH_2$) is not available. In this special case V_w ($C_6H_5CH_2$) cannot be calculated by addition of the fragmental values V_w (C_6H_5) and V_w (CH_2). This procedure would lead to V_w ($C_6H_5CH_2$) > V_w (C_6H_5), whereas in reality the steric effect of the phenyl group (upon the ring nitrogen) should be diminished by the methylene group introduced between the two rings.

That π indeed describes a steric substituent effect in this series of compounds can be demonstrated on the above example. The experimental π value of the benzyl group was determined to be 2.14 compared to the value of 2.49 observed for the 2-phenyl group. Purely geometrical calculation should lead to the reverse result.

An overlap between π–2 and E_s–2 (r = 0.98) was also reported by Hansch for a series of 9-(X-phenyl)guanines.[53] In another paper Hansch[50] stated that "Steric effects can be quite varied in nature. The shielding of lone-pair electrons by inert alkyl groups produces a significant increase in π-values". Similar molecular conditions can be assumed for these 2-substituted pyridines.

Being aware of the original meaning of π as a parameter expressing lipophilicity it seems justified, therefore, to take

π, with certain limitations, as a parameter for the steric effects. These restrictions appear when the ligand at the 2 position contains an amino function. In that case the additional formation of hydrogen bonds strongly influences the partitioning in the octanol–water system. Thus, the π values of these substituents are about 0.4–2 units too small for the characterization of the steric effect corresponding to the ligands. To circumvent this difficulty a "steric" π value was calculated from van der Waals volumes V_w by eq 3 and introduced into the regression analyses.

Another method by which the lipophilic character of compounds may be estimated is via the determination of R_m values (which are related to π) utilizing reversed phase TLC (see ref 54 for a short survey). The relationship between R_m (Table I) and π is shown in eq 4. The correlation of R_m with E_s and V_w is given by eq 5 and 6.

$$\pi = 1.967\ (11.03)\ R_m - 0.477 \quad (4)$$

n	r	s	F	S
18	0.94	0.31	121.7	>99.9

$$R_m = -0.371\ (6.67)\ E_s + 0.864 \quad (5)$$

n	r	s	F	S
13	0.895	0.18	44.4	>99.9

$$R_m = 0.0262\ (11.13)\ V_w + 0.319 \quad (6)$$

n	r	s	F	S
14	0.955	0.11	123.9	>99.9

"Steric" R_m values were calculated by eq 6 for the INH derivatives containing an amino function in the 2 position. There was no significant correlation between pK_a and π (or R_m) in this series of compounds.

A more comprehensive paper about the relationship between the steric effects of substituents and the π or R_m value of 2-substituted INH and pyridine derivatives is in preparation and will be published elsewhere.

Multiparameter Regression Analysis. To quantify the connection between the antibacterial action and the electronic and steric effects of substituents, a multiparameter regression analysis was performed and eq 7–15 have been derived (compounds **1a–7a**, **12a–19a**, Table I).

$$\log 1/MIC = 0.186\ (2.81)\ pK_a - 2.558 \quad (7)$$

n	r	s	F	S
15	0.615	0.65	7.92	>97.5

$$\log 1/MIC = -0.64\ (1.94)\ \pi - 1.129 \quad (8)$$

n	r	s	F	S
15	0.474	0.73	3.77	>90.0

$$\log 1/MIC = 0.247\ (6.82)\ pK_a - 0.959\ (5.91)\ \pi - 1.57 \quad (9)$$

n	r	s	F'	S
15	0.917	0.34	31.77	>99.9

The first data set includes only those compounds whose π values and steric parameters are in agreement. Compounds with amino functions are excluded. Stepwise regression analysis shows a significant contribution of electronic and steric effects (expressed as π values). The combination of the two parameters results in a strong increase in significance. The INH derivatives become more active with increasing basicity of the heterocycle and with decreasing π value, i.e., decreasing steric hindrance of the reaction center. Usually in QSAR studies, increases in biological activity parallel an increase in π. The parabolic dependency of biologic activity on π is observed only with compounds that are highly lipophilic; i.e., once a maximum π value ($\log P_0 \approx 2$) is achieved, further increases in lipophilicity result in diminished biologic activity. In the present study, however, the maximum activity is observed in the parent compound with $\log P = -1.14$. Introduction of a π^2 term does not improve the correlation. In a recent study by Silipo and Hansch[53] involving a similar correlation with negative coefficients for π (indicating that large groups lower the activity) the authors suggest that steric effects are most likely involved.

The next data set includes the derivatives with amino function (compounds **8a–11a**) and for these ligands the π values calculated from V_w (eq 3) are used.

$$\log 1/MIC = 0.137\ (1.07)\ pK_a - 2.563 \quad (10)$$

n	r	s	F	S
19	0.431	0.77	3.87	>90.0

$$\log 1/MIC = -0.732\ (2.82)\ \pi - 1.027 \quad (11)$$

n	r	s	F	S
19	0.564	0.70	7.94	>97.5

$$\log 1/MIC = 0.232\ (5.79)\ pK_a - 1.073\ (6.57)\ \pi - 1.454 \quad (12)$$

n	r	s	F	S
19	0.883	0.41	28.3	>99.9

The significance of eq 12 is decreased (compared to eq 9) because the π values calculated from V_w are probably not exact. If the observed π values instead of the calculated values are used for the four derivatives containing amino groups in the 2 position, the significance of this correlation becomes quite low.

$$\log 1/MIC = -0.227\ (0.92)\ \pi - 1.789 \quad (13)$$

n	r	s	F	S
19	0.217	0.832	0.832	<90.0

$$\log 1/MIC = 0.152\ (2.20)\ pK_a - 0.304\ (1.34)\ \pi - 2.289 \quad (14)$$

n	r	s	F	S
19	0.518	0.75	2.93	>90.0

This result strongly supports the assumption that the biological activity is a function of the pK_a and the steric effects of the substituents but not of the hydrophobic properties of the ligands. Further on, this assumption is supported by eq 15 where instead of π the van der Waals volumes, V_w, are applied (compounds **1a–9a**, **11a–16a**) (for selection criteria leading to only 15 compounds in eq 15, see remarks below).

$$\log 1/MIC = 0.264\ (5.60)\ pK_a - 0.0584\ (5.97)\ V_w - 1.773 \quad (15)$$

n	r	s	F	S
15	0.89	0.45	22.77	>99.9

The coefficient with V_w (eq 15) is very small, but one has to take into consideration that the V_w values are about ten times higher than the pK_a values (see also t test).

Similar correlations of antibacterial activity with pK_a and R_m (instead of π or V_w) are shown in eq 16 and 17 (compare with eq 11 and 12).

In summary, QSAR's given in eq 7–17 seem to be in agreement with the discussed hypothesis about the mode of action of INH derivatives according to which a correlation between N reactivity and MIC can be expected.

Remarks. 1. For the calculation of eq 8, 9, and 11–14, π values derived from eq 2 have been used in the case of the phenyl and vinyl group. The observed π values for

$$\log 1/\mathrm{MIC} = -1.035\ (1.96)\ R_m - 1.088 \quad (16)$$

n	r	s	F	S
19	0.429	0.77	3.83	>90.0

$$\log 1/\mathrm{MIC} = 0.272\ (5.75)\ \mathrm{p}K_a - 2.061\ (5.75)\ R_m - 1.20 \quad (17)$$

n	r	s	F	S
19	0.857	0.45	22.09	>99.9

these ligands [2.492 (phenyl) and 1.53 (vinyl) (calculated from R_m by eq 4)] correspond to functions coplanar to the reaction center (or to freely rotating functions). With these π_{obsd} values, very high MIC values are calculated (from eq 12, MIC (phenyl) = 1226 μM, MIC (vinyl) = 87.2 μM). From MIC determination the values 50 (phenyl) and 35 μM (vinyl) are obtained. In enzyme-catalyzed reactions such as the quaternization of INA derivatives by the ribosyl moiety, the possibility exists that a phenyl group in the 2 position is perpendicular, rather than coplanar, to the reaction center. Thus the effective π value should be much lower. Kutter and Hansch[49] calculated E_s values for the phenyl group both coplanar (E_s = −2.58) and perpendicular (E_s = 0.23) to the reaction center. Introducing E_s = 0.23 into eq 2 one obtains π (phenyl-perp) = 1.13. With this π value an MIC of 42.4 μM is calculated (eq 12). Assuming that a vinyl group in perpendicular arrangement has about the same E_s or π (perp) value, the calculated MIC value (eq 12) for 2-vinyl-INH is 32.4 μM [for the computation of eq 12 2-phenyl-INH and 2-vinyl-INH are included with π (perp) = 1.13; if these two compounds are excluded a nearly identical QSAR is obtained]. For the interpretation of the unexpected high antibacterial activity of 2-phenyl- and 2-vinyl-INH one can of course assume some additional, yet unknown mechanism of action. This problem is presently being further investigated.

2. The π values of the ligands NH_2, AcNH, $AcNHCH_2$, and Et_2N have been calculated from van der Waals volumes V_w by eq 3. In the case of the $AcNHCH_2$ ligand a corrected V_w value was used. Just as with the benzyl group, the methylene group adjacent to the pyridine ring diminishes the steric effect of the AcNH ligand, whereas V_w ($AcNHCH_2$) > V_w (AcNH) is obtained by addition of fragmental values. For correction the following procedure was chosen. From Figure 3b it can be taken that V_w ($C_6H_5CH_2$) is about 15 units of V_w too high for the representation of the steric effect. Assuming that the methylene group has about the same effect for $AcNHCH_2$ as for $C_6H_5CH_2$, V_w ($AcNHCH_2$) was corrected from 43.68 to the value $V_w(AcNHCH_2)_{cor}$ = 30. This value was used for the calculation of the "steric" π ($AcNHCH_2$) by eq 3. The same arguments hold for all correlations containing R_m instead of π.

3. For the calculation of eq 15 the INH derivatives with R = C_6H_5, vinyl, $C_6H_5CH_2$, and $AcNHCH_2$ have been excluded. V_w (C_6H_5) and V_w (vinyl) represent the steric effect of coplanar functions; the values V_w ($C_6H_5CH_2$) and V_w ($AcNHCH_2$) do not consider the influence of the CH_2 group (see above) and were excluded for these reasons.

Quaternization of Pyridines by Methyl Iodide as a Model Reaction. In an attempt to facilitate the interpretation of the SAR presented above and to have a more reliable model, the quaternization of 2-substituted pyridines (corresponding to the 2-substituted INH derivatives) by CH_3I in methanol was studied. The rate constants were determined partly by NMR technique following the procedure of Deady and Zoltewicz[55,56] and partly by conventional methods (titration of developing I⁻).

The Menschutkin reaction has been studied in many laboratories[56-64] and it has been shown that the rates of quaternization of 3- and 4-substituted pyridines in various solvents may be correlated by both the Hammett and Bronstedt equations.[43,58,60,61] The rates of N-alkylation of 2-substituted pyridines showed deviations in these plots (log k vs. σ or pK_a). The differences were qualitatively explained by steric effects of the substituents.[56,60,61] Gallo et al.[63,64] demonstrated for a restricted series of 2-alkylpyridines (with $pK_a \approx$ constant) that quaternization rates are correlated with E_s (Taft). Obviously the same holds for the entropies of activation, $\Delta S^{\ddagger}$, determined by Brown and Cahn.[57]

Taking these results into consideration it may be expected that the reactivity of 2-substituted pyridines toward quaternization can be described on a quantitative level by a multiparameter equation containing electronic and steric parameters, as has been anticipated by Deady and Zoltewicz.[56]

The results of our investigation of the Menschutkin reaction of ortho-substituted pyridines are summarized in Table II. The reaction rate is given relative to the quaternization rate of the unsubstituted pyridine which was set equal to one. These findings, to our knowledge, provide the most extensive set of rate data yet reported for this group of compounds. For the characterization of electronic and steric substituent effects in this series $pK_{a\ (2\text{-R-pyr})}$ and $\pi_{(2\text{-R-INH})}$ (or $R_{m\ (2\text{-R-INH})}$) values have been used and introduced into the computation of QSAR. Selecting groups of compounds analogous to eq 7–17, the following correlations have been derived (2-Et_2N- and 2-MeO-pyridine have been excluded, see Remarks; compounds **1b–5b**, **7b**, **12b–19b**, Table II).

$$\log k_{rel} = 0.26\ (7.10)\ \mathrm{p}K_a - 2.2 \quad (18)$$

n	r	s	F	S
14	0.899	0.36	50.37	>99.9

$$\log k_{rel} = 0.053\ (0.16)\ \pi - 1.38 \quad (19)$$

n	r	s	F	S
14	0.046	0.83	0.025	<90.0

$$\log k_{rel} = 0.289\ (9.12)\ \mathrm{p}K_a - 0.337\ (2.68)\ \pi - 1.827 \quad (20)$$

n	r	s	F	S
14	0.94	0.29	41.7	>99.9

Comparative F statistics for eq 20 over eq 18 demonstrate the superiority of eq 20 (S >97.5).

For the calculation of these equations the ligands NH_2, AcNH, and $AcNHCH_2$ were excluded for the same reason as in eq 7–9. If these substituents are included with a π value calculated from V_w (eq 3) we get

$$\log k_{rel} = 0.264\ (7.54)\ \mathrm{p}K_a - 2.232 \quad (21)$$

n	r	s	F	S
17	0.89	0.364	56.88	>99.9

$$\log k_{rel} = -0.057\ (0.19)\ \pi - 1.17 \quad (22)$$

n	r	s	F	S
17	0.05	0.8	0.03	<90.0

$$\log k_{rel} = 0.287\ (9.82)\ \mathrm{p}K_a - 0.353\ (3.01)\ \pi - 1.822 \quad (23)$$

n	r	s	F	S
17	0.935	0.29	48.3	>99.9

Comparative F statistics for eq 23 over eq 21 demonstrate the superiority of eq 23 (S >99.0).

Using R_m instead of π, eq 24 and 25 are obtained.

$$\log k_{rel} = 0.254\ (0.45)\ R_m - 1.49 \quad (24)$$

n	r	s	F	S
17	0.12	0.8	0.21	<90.0

$$\log k_{rel} = 0.295\ (8.98)\ pK_a - 0.593\ (2.45)\ R_m - 1.798 \quad (25)$$

n	r	s	F	S
17	0.924	0.32	41.0	>99.9

Comparative F statistics for eq 25 over eq 21 demonstrate the superiority of eq 25 (S >95.0).

If π_{obsd} values are introduced into the calculation of QSAR's for the amino groups, the significance of the correlations decreases considerably.

$$\log k_{rel} = -0.051\ (0.22)\ \pi - 1.2 \quad (26)$$

n	r	s	F	S
17	0.057	0.797	0.049	<90.0

$$\log k_{rel} = 0.267\ (7.69)\ pK_a - 0.12\ (1.15)\ \pi - 2.11 \quad (27)$$

n	r	s	F	S
17	0.9	0.36	29.69	>99.9

The equation corresponding to eq 15 using V_w instead of π or R_m is

$$\log k_{rel} = 0.291\ (8.80)\ pK_a - 0.0186\ (2.66)\ V_w - 1.941 \quad (28)$$

n	r	s	F	S
15	0.931	0.32	38.8	>99.9

As can be seen from these equations, the log of the relative rate of reaction is correlated to the same electronic and steric effects operating with the INH derivatives substituted in the 2 position. In contrast to the equations derived for the biological effect, however, the main contribution comes from the electronic effect (pK_a), the contribution of the steric effect being small but still significant. The dependence of k_{rel} on steric effects becomes evident in the series of pyridines with approximately equal pK_a values but different E_s, V_w, π, or R_m values and different rates of quaternization (e.g., R = Me, Et, *n*-Pr, *i*-Bu with $pK_a \approx 5.97$ and k_{rel} from 32.4 to 11%; R = H, $C_6H_5CH_2$ with $pK_a \approx 5.15$ and k_{rel} from 100 to 19.7%).

We believe that the smaller contribution of steric effects in the quaternization reaction can be explained by the smaller volume of the approaching methyl group compared to the more bulky ribosyl moiety in the supposed biochemical reaction. This assumption is substantiated by the findings of Brown and Cahn,[57] who found that $k_{rel} = k_{Me}/k_H$ decreases from 0.47 to 0.054 (in $C_6H_5NO_2$) if the quaternizing agent is changed from iodomethane to 2-iodopropane. Thus the steric effect of the alkyl halide becomes evident. Certainly, the N-alkylation of 2-substituted pyridines by isopropyl iodide represents a better model reaction for the hypothetical processes within mycobacteria treated with INH; in this model system, however, the kinetics become complicated due to elimination side reactions;[57] these side reactions are impossible with CH_3I.

In summary, the reaction rate for quaternization increases with increasing pK_a (basicity) and with decreasing π values (steric influences of the substituents). It seems justified, therefore, to assume that there is a similar dependence upon steric and electronic influences of the substituents for the antibacterial activity of 2-substituted INH derivatives and for the reactivity of 2-substituted pyridines toward quaternizing agents.

Remarks. 1. No k_{rel} value was obtained for 2-methoxypyridine owing to product instability (formation of pyridone).[65]

2. In contrast to Deady and Zoltewicz,[56] who found k_{rel} = 1.23 for the methylation of 2-aminopyridine in Me_2SO, we obtained k_{rel} = 0.574 in methanol. Obviously this difference is due to interactions of the pyridine with the protic solvent caused by hydrogen bonding. The decrease of k_{rel} is reflected by the higher MIC value of 2-NH_2-INH compared to INH. Thus, methanol seems to be a better model for the medium within mycobacteria than Me_2SO.

3. 2-Diethylaminopyridine was excluded from the QSAR of 2-substituted pyridines for the following reason: by eq 20 k_{rel} = 0.25 is calculated, whereas k_{rel} = 0.0055 is observed. The reasons for this discrepancy are not clear. Obviously the observed pK_a value (7.32) introduced into the calculation is too high, compared to a pK_a of 4.5 calculated by eq 1 with $\sigma_I = 0.11$. The difference in pK_a is a result of the mesomeric electron-donating effect which is not described by eq 1. Assuming that the methyl group can approach the ring nitrogen atom only if the ortho substituent (Et_2N) is no longer coplanar with the pyridine ring and assuming that the effective pK_a is about 4.5 (instead of 7.32), then $k_{rel} \approx 0.038$ is calculated. This value compares better with the observed one. Another explanation of the low reactivity of 2-diethylaminopyridine could be that this compound undergoes amino group alkylation, as has been shown for 2-dimethylaminopyridine.[66,67]

Comparison of Biological and Chemical Reactivity. The similarity between biological and chemical reactivity is underlined by the dependence of the activities upon the same physicochemical parameters. If the logarithm of the reciprocal MIC is plotted vs. the logarithm of the relative rate constants, a linear correlation is obtained for those pairs of compounds (2-R-INH/2-R-Py) where the relative rate of quaternization is at least ≈2% of the rate of the unsubstituted compound (compounds **1a,b–5a,b**, **7a,b–10a,b**, **15a,b**, **18a,b**, Tables I and II).

$$\log 1/MIC = 1.778\ (7.60)\ \log k_{rel} - 0.297 \quad (29)$$

n	r	s	F	S
11	0.93	0.377	57.7	>99.9

Not included in the correlation eq 29 are derivatives with the substituents R = CH_3O, C_6H_5, vinyl, F, Cl, Br, Et_2N, and NO_2. 2-Methoxypyridine is excluded by reason of side reaction during quaternization[65] and the 2-phenyl and 2-vinyl derivatives by reason of (hypothetically) different steric substituent effects in the chemical and in the biochemical quaternization. The other five INH derivatives not included in the correlation possess a higher activity than can be calculated by eq 29. The absolute antibacterial activity for these five compounds is, however, very low compared to INH. We assume an effect connected with the carboxylic acid hydrazide group—as demonstrated for benzoic acid hydrazide—in addition to the very low activity caused by the ring quaternization. This additional effect is negligible for the compounds with relatively high reactivity at the pyridine nitrogen. For all these compounds we have parallel substituent effects in the biological activity and the quaternization reaction.

The result indicates that the differences in MIC are paralleled by differences in the quaternization reaction studied in an isolated homogeneous chemical system. Therefore, these differences in activity cannot be attributed to permeation factors but must be associated with the reaction at the receptor site. The occurrence of such a relationship as shown in eq 29 between biological activity and quaternization reactivity in an isolated system is good

evidence that the rate-determining step in both systems is similar. While there is no definite proof that these reactions are indeed one and the same, there is nothing in our data which contradicts such conclusions. If all compounds (with the exception of $-OCH_3$ and $-NEt_2$, where no unequivocal data could be obtained) are included in the regression analysis log 1/MIC vs. log k_{rel}, the quality of the correlation decreases as expected; however, the correlation is still significant.

$$\log 1/\mathrm{MIC} = 0.81\ (4.24) \log k_{rel} - 0.958 \qquad (30)$$

n	r	s	F	S
17	0.74	0.59	17.9	>99.9

Experimental Section

General. Melting points were taken with a Leitz melting point microscope and are uncorrected. The NMR spectra were determined using a Varian HA-100 spectrometer with Me_2SO-d_6 (or $CDCl_3$) as solvent and tetramethylsilane as internal standard. A Leitz ir spectrometer was used to obtain the ir spectra. For the computation of equations a Wang 700 B was used.

Minimum Inhibitory Concentrations. MIC values have been determined according to ref 10, except that dilution steps of $\sqrt{2}$ were chosen for the present study. The MIC values of Table I are the average of several (at least two) determinations. The standard error of the average values given is estimated at ±25%.

Quaternization of Pyridines. For the determination of (relative) rate constants two methods were employed. (a) k_{rel} values of the more reactive 2-alkylpyridines were evaluated by NMR measurements at 22 °C (solvent, CD_3OD; alkylating agent, CH_3I) following the procedure of Deady and Zoltewicz.[55,56] Pyridine was the rate standard. (b) For rate measurements of the other pyridine derivatives the argentometric determination of developing iodide was chosen (method of Volhard). Identical volumes of 1 N solutions of reactants in dry methanol were combined and held in the dark at a bath temperature of 25 °C (±0.05). The slope of a plot of $1/c_{pyr}$ vs. time, determined by the method of least squares, gave the second-order rate constants k_2. c_{pyr} is the concentration of unreacted pyridine at time t. k_{rel} (Table II) was calculated by $k_{rel} = k_{2\ (2\text{-R-pyr})}/k_{2\ (pyr)}$. Due to the low solubility of 1-methyl-2-iodopyridinium iodide in methanol, the rate constant of 2-iodopyridine was determined in a solution containing only 20% of the aforementioned concentrations of each reactant.

pK_a Values. The majority of pK_a values of 2-substituted pyridines have been taken from ref 45 and 46. Several pK_a values not available in the literature (Table II) were determined spectrophotometrically at 25 °C (±1) following the procedures given by Albert.[68] For the determination of the approximate pK_a of 2-nitropyridine the Hammett acidity function (H_0) has been used.[68]

Partition Coefficients. P values have been determined according to Fujita et al.[69] (1-octanol–phosphate buffer, pH 7.4).

R_m Values. R_m values were obtained by the procedure described by Biagi et al.[70] The test compounds were partitioned between a polar, mobile phase (acetone–H_2O) and a nonpolar, stationary phase obtained by impregnating a silica gel layer with a paraffin solution in n-hexane. The R_m values were calculated by $R_m = \log (1/R_f - 1)$.

Syntheses of Compounds. The identity and purity of all compounds were unequivocally proven by NMR and ir spectroscopy and by TLC.

Isonicotinic acid hydrazide (**1a**) was obtained from commercial sources. 2-Methylisonicotinic acid hydrazide (**2a**)[71,72] and 2-n-propylisonicotinic acid hydrazide (**4a**)[71,72] were a generous gift of the "Deutsche Hoffmann-La Roche".

2-Isobutylisonicotinic acid hydrazide (**5a**),[71,72] 2-phenylisonicotinic acid hydrazide (**17a**),[71] 2-fluoroisonicotinic acid hydrazide (**12a**),[73-75] 2-bromoisonicotinic acid hydrazide (**14a**),[73,76] 2-nitroisonicotinic acid hydrazide (**16a**),[77-79] and 2-aminoisonicotinic acid hydrazide (**8a**)[79] were prepared by literature procedures with slight modifications. The synthesis of ethyl 2-benzylisonicotinate is described in ref 72. This compound was transformed to 2-benzylisonicotinic acid hydrazide (**18a**) by treatment with $N_2H_4 \cdot H_2O$: mp 101–102 °C.

2-Ethylisonicotinic acid hydrazide (**3a**)[71] was prepared by standard procedures starting with commercially available 2-ethylisonicotinic acid thioamide.

2-Chloroisonicotinic acid hydrazide (**13a**) was prepared by the following standard procedures: (1) formation of the N-oxide of isonicotinic acid (30% H_2O_2, AcOH); (2) reaction of the N-oxide with PCl_5–$POCl_3$ to 2-chloroisonicotinic acid; (3) formation of the ester by reaction with (a) $SOCl_2$ and (b) EtOH, bp 130–132 °C (20 mm); (4) by treatment of the ester with $N_2H_4 \cdot H_2O$, **13a** was obtained, mp 176–177 °C (i-PrOH). Anal. ($C_6H_6N_3OCl$) C, H, N.

2-Iodoisonicotinic Acid Hydrazide (**15a**). 2-Iodoisonicotinic acid was obtained by a procedure described by Setliff and Price[80]. 2-Chloroisonicotinic acid (2.5 g), 8.5 g of NaI, 75 ml of ethyl methyl ketone, and 2 ml of hydroiodic acid ($d \approx 1.67$) were stirred under reflux for 6 h. The mixture was evaporated to dryness; the residue was dissolved in H_2O and made alkaline by diluted NaOH (some undissolved material was filtered). After addition of diluted Na_2SO_3 the mixture was acidified and the precipitate was filtered and dried to give 2-iodoisonicotinic acid (3 g, 76% yield), mp 246–248 °C dec (sublimation beginning at 150 °C). The acid was converted to **15a** by the method of Staab et al.:[81] mp 174–175 °C (i-PrOH). Anal. ($C_6H_6N_3OI$) C, H, N.

2-Methoxyisonicotinic Acid Hydrazide (**6a**). Methyl 2-chloroisonicotinate was transformed to the 2-methoxy derivative by refluxing it with $NaOCH_3$–CH_3OH. The ester subsequently was converted to **6a** as described before: mp 138–140 °C (EtOH–H_2O). Anal. ($C_7H_9N_3O_2$) C, H, N.

2-Ethoxyisonicotinic acid hydrazide (**7a**) was prepared in a manner analogous to **6a**: mp 155–156 °C (EtOH). Anal. ($C_8H_{11}N_3O_2$) C, H, N.

2-Acetylaminoisonicotinic acid hydrazide (**9a**) was prepared from 2-acetylaminoisonicotinic acid[82,83] as described for **15a**: mp 244–246 °C (MeOH).

2-Diethylaminoisonicotinic Acid Hydrazide (**11a**). 2-Diethylamino-4-cyanopyridine[10] was transformed to the ethyl ester by the Pinner synthesis and subsequent hydrolysis of the imidate salt:[84] bp 155–160 °C (12 mm). **11a** was obtained by treatment of the ester with $N_2H_4 \cdot H_2O$: mp 124–126 °C (CCl_4). Anal. ($C_{10}H_{16}N_4O$) C, H, N.

2-Acetylaminomethylisonicotinic Acid Hydrazide (**10a**). (a) Ethyl isonicotinate was transformed to the N-oxide by refluxing with H_2O_2 (30%)–AcOH. Work-up was as usual: mp 67–68 °C (toluene). (b) The N-oxide was converted to ethyl 2-cyanoisonicotinate analogous to a procedure described by Feely et al.:[85] mp 44–45 °C. (c) **10a** was prepared by catalytic hydrogenation (Pd/C–Ac_2O) of ethyl 2-cyanoisonicotinate to ethyl 2-acetylaminomethylisonicotinate, mp 62–64 °C, and subsequent reaction of the ester with $N_2H_4 \cdot H_2O$: mp 180–181 °C (i-PrOH). Anal. ($C_9H_{12}N_4O_2$) C, H, N.

2-Vinylisonicotinic Acid Hydrazide (**19a**). Methyl 2-methylisonicotinate[71] was transformed to methyl 2-vinylisonicotinate by a modification of the procedure described by Korytnyk et al.[86] (a) Methyl 2-methylisonicotinate was treated by 3-chloroperbenzoic acid in $CHCl_3$ for 3 days at 20 °C. Work-up was as described in ref 86: yield of N-oxide, 99.5%; mp 139–143 °C [sublimation, $CHCl_3$–Et_2O–petroleum ether (bp 50–70 °C)]. (b) The N-oxide was treated with $(CF_3CO)_2O$ and worked up as described in ref 86. Recrystallization of the oily residue after evaporation of $CHCl_3$ gave methyl 2-hydroxymethylisonicotinate: mp 69–72 °C ($CHCl_3$–n-hexane). Work-up of the concentrated mother liquor by column chromatography (SiO_2–AcOEt) gave additional product: total yield, 38%. (c) Methyl 2-hydroxymethylisonicotinate (1.4 g) was oxidized to the 2-formyl derivative by refluxing it with Seloxcette (CrO_3 intercalated into graphite, Alfa-Ventron)[87] (18 g added in several portions at intervals of time) in toluene (75 ml) for 40 h (N_2). The oxidant was filtered off and extracted two times with 50 ml of hot toluene. The combined filtrates were evaporated to dryness giving 1.35 g of raw product (brownish oil that slowly crystallized on standing). (d) The 2-CHO group of the raw product was converted by a Wittig reaction to the 2-vinyl group. Inverse addition of the phosphine ylide was chosen in order to prevent reaction of the ylide with the ester group[88]; $(C_6H_5)_3PCH_3^+Br^-$ (1.4 g, 4 mmol) was added under Ar to a stirred solution of 1.8 ml of 2.2 N BuLi–hexane (4 mmol) in 150 ml of dry Et_2O. The mixture was stirred at 20 °C for 4 h.

Then the yellow mixture was added under Ar in several portions to a stirred cool solution (0 °C) of (1) dry MeOH (10 mmol), (2) BuLi–hexane (10 mmol), and (3) raw aldehyde (650 mg, 4 mmol/25 ml of dry benzene) in 150 ml of dry Et_2O. The combined solutions were stirred for 1.5 h at 0 °C and 2 h at 20 °C. The ethereal solution was washed with $NaHCO_3$, dried (Na_2SO_4), and evaporated to dryness. The oily residue (1 g) was dissolved in AcOEt, some insoluble material [$(C_6H_5)_3PO$, mp 155–157 °C] was filtered off, and 2-vinylisonicotinic acid methyl ester was isolated from the filtrate by column chromatography (SiO_2–AcOEt; TLC, R_f 0.4): yield, 150 mg of oil (23%). (e) Methyl 2-vinylisonicotinate was converted to **19a** by reaction with $N_2H_4 \cdot H_2O$. The product was isolated by column chromatography [SiO_2–$CHCl_3$ (80), CH_3OH (15), v/v]: yield, 30 mg; mp 123–125 °C. Anal. ($C_8H_9N_3O$) C, H, N.

The following 2-substituted pyridines were obtained from commercial sources: **1b–4b**, **6b–8b**, **12b–14b**, and **17b–19b** (Table II). **18b** and **19b** were purified by distillation prior to use, whereas **17b** was purified by column chromatography [SiO_2–acetic acid (1), acetic ester (99), v/v].

2-Nitropyridine (**16b**),[89] 2-acetylaminopyridine (**9b**),[90] 2-diethylaminopyridine (**11b**),[91] and 2-iodopyridine (**15b**)[92] were prepared by literature procedures. 2-Acetylaminomethylpyridine (**10b**) was obtained as a yellow oil by acetylation of commercial picolylamine.

2-Isobutylpyridine (5b) was prepared by metalation of **2b** with BuLi–hexane and subsequent alkylation by 2-iodopropane analogous to the procedure described by Kaiser et al.[93] Work-up of the reaction mixture by (1) distillation, bp 165–175 °C, and (2) by column chromatography [SiO_2–CCl_4 (80), acetone (20), v/v] gave pure **5b** (10% yield). Anal. ($C_9H_{13}N$) C, H, N.

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References and Notes

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Structure–Activity Relationships in Luteinizing Hormone-Releasing Hormone

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Three analogs of luteinizing hormone-releasing hormone (LH-RH) of the structure <Glu-His-Trp-Ser-Tyr-Gly-Gly-Leu-Arg-Pro-Gly-NH_2, involving substitutions in positions 1, 3, and 8 with nonprotein amino acids, have been synthesized by the solid-phase method. They are [pyro-L-α-(1-aminoadipic)]-LH-RH, [3-(2-naphthyl)-L-Ala^3]-LH-RH, and [δ-*N*-*i*-Pr-L-Orn^8]-LH-RH. Their LH-RH activities in vivo were 12.5, 51.8, and 3.7% that of LH-RH, respectively, in the assay using ovariectomized, estrogen- and progesterone-treated rats. In a test based upon subcutaneous injection into immature male rats, [3-(2-naphthyl)-Ala^3]-LH-RH released 1.2 times as much LH and 0.8 times as much FSH as synthetic LH-RH.

The isolation and structural elucidation of the decapeptide <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, luteinizing hormone-releasing hormone,[1,2] has led to the synthesis and testing of many analogs for agonist and antagonist activity. Most of the analogs have involved the substitution of one of the residues by another of the amino acids that occurs in proteins or the deletion of one or more residues entirely. We report the synthesis and testing of three analogs having nonprotein amino acids: (1) pyro-L-aminoadipic acid (<Aad, pAad) in place of the N-terminal pyroglutamic (XI), (2) 3-(2-naphthyl)-L-alanine in place of tryptophan (XII), and (3) δ-*N*-isopropyl-L-ornithine in place of arginine (XIII).

Synthesis. Synthesis of the peptides was carried out essentially by the Merrifield method of solid-phase peptide synthesis[3] except for a few minor modifications. The following side-chain protecting groups were used in the synthesis: Arg, N^G-Tos, Tyr and Ser, *O*-Bzl, δ-*N*-*i*-Pr-Orn, N^δ-Tos; N^{im}-Bzl-His was used for synthesizing the peptides XI and XIII, and the N^{im}-Tos derivative was used in the synthesis of XII. When N^{im}-Bzl-His was used, the DCC coupling was carried out in the presence of *N*-1-hydroxybenzotriazole[4] in order to minimize racemization. <Aad and <Glu in the case of peptides XI and XIII were coupled to the peptide chain in the presence of DCC with DMF as the solvent, but pentachlorophenylpyroglutamate in DMF (reaction time 36 h) was used in the synthesis of peptide XII. To ensure the complete removal of the Boc group, the deblocking step was carried out twice, once with 1 N HCl–HOAc and secondly with 25% TFA in CH_2Cl_2.[5] The peptides were checked for their homogeneity by TLC in three different solvent systems, TLE, and amino acid analysis.

Bioassays. LH-RH activities were determined in vivo by stimulation of LH release at two dose levels in ovariectomized rats pretreated with estrogen and progesterone,[6] followed by radioimmunoassay for LH.[7] Serum LH levels after injection of samples are compared with those obtained after administration of saline and two doses of natural LH-RH. A four-point factorial assay[8] was used to calculate the LH-RH activity with 95% confidence limits. The LH/FSH-RH activities of [3-(2-naphthyl)-Ala^3]-LH-RH were also assayed against synthetic LH-RH by subcutaneous injection in immature male rats. Integrated levels of LH and FSH over a 6-h period after the injection of peptide were considered as a parameter of the LH/FSH-releasing activities.[9-11] Rat FSH was measured by a RIA.[12]

Biological Results and Discussion. The LH-releasing activities of the peptides are shown in Table I. The