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p-Aminobenzoic Acid Derivatives. Mode of Action and Structure–Activity Relationships in a Cell-Free System (*Escherichia coli*)

J. K. Seydel* and W. Butte[1]

Department of Pharmaceutical Chemistry, Borstel Research Institute, 2061 Borstel, West Germany. *Received June 7, 1976*

The agonistic and antagonistic effects of nuclearly substituted *p*-aminobenzoic acids (PABA) on the folate-synthesizing system of *E. coli* have been studied in whole cell and cell-free systems. All studied derivatives form dihydropteroic acid analogues in the presence of a cell-free folate-synthesizing enzyme system. A thin-layer chromatographic system has been elaborated to determine the rate of analogue formation in the cell-free system. Physicochemical parameters of the PABA derivatives, such as pK_a, π, and R_m values, have been determined. These values have been used in a structure–activity analysis which revealed that the rate of analogue formation in the absence of PABA is independent of the lipophilic properties. Ionization seems to be the decisive factor for the incorporation. As all studied PABA derivatives are totally ionized under the experimental conditions, the rates of analogue formation are very similar with the exception of compounds bearing bulky groups in the 2 position. The variance in inhibitory power may therefore either be due to differences in the ability of the analogues to serve as metabolites or to competition with PABA.

The general pathway of pteroic acid and folate synthesis, especially in *E. coli*, has been evaluated during the last decade by Brown,[2a] Jaenicke and Chan,[2b] and Shiota et al.[3] It has been shown that bacterial or plant cell-free folate-synthesizing extracts are inhibited by sulfonamides.[4,5] Bock et al.[6] have demonstrated that sulfonamides, in addition to competing with PABA for the active site of 7,8-dihydropteroate synthetase, also compete for the 7,8-dihydro-6-hydroxymethylpterin (H_2PtCH_2OH), the natural substrate, thereby forming a pteroic acid analogue that results in a diminished synthesis of pteroic acid per se (see Scheme I, A and B). More complicated and contradictory are the results reported on growth-promoting or inhibitory effects of PABA derivatives on different microorganisms. The complication may arise from the possibility that PABA derivatives incorporated into a folate analogue may or may not perform the function of the natural folate in various species,[7,8] whereas in case of the sulfonamides (SA) the reaction product of SA with phosphorylated 7,8-dihydro-6-hydroxymethylpterin (H_2PtCH_2OPP) cannot act as a folate metabolite.[6] Ariens and co-workers[9] reported the gradual change from growth factor to growth inhibitor for certain PABA derivatives for an *E. coli* strain which required PABA for growth. For 2-amino-5-carboxypyridine experimental results are published which make the incorporation of 2-amino-5-carboxypyridine into a folic acid analogue probable (for example, see ref 8). Wacker et al.[10] described the incorporation of *p*-aminosalicylic acid into a folate analogue by the folate-synthesizing enzyme system of enterococci (see Scheme I, C). The folate analogue could take over the function of the normal folate in this bacterial strain.

More recent work on the inhibition of folate-synthesizing enzyme extracts from *E. coli* by PABA derivatives has been published by Thijssen.[11,12] Using [^{14}C]-PABA the degree of inhibition of PABA incorporation into pteroic acid in the presence of PABA derivatives was determined by extraction of the [^{14}C]-PABA that had not reacted after a certain time interval. From these data Thijssen tried to derive some structure–activity correlations. With this technique, however, one is not able to decide whether the PABA derivatives are only competing with PABA for the active site of the enzyme or if there is also competition for the precursor H_2PtCH_2OH and resultant formation of dihydropteroic acid derivatives.

To decide this question we have developed techniques

Scheme I

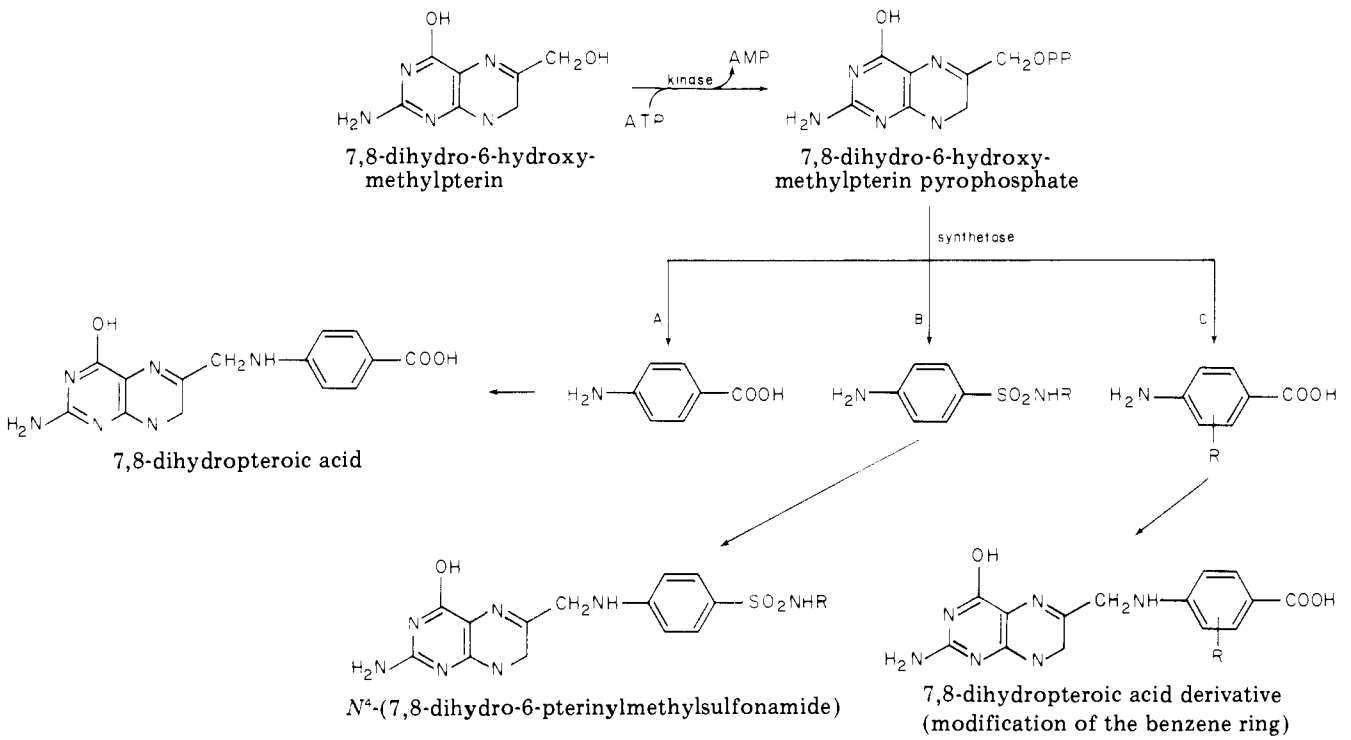


Table I. Biological Activities (ID_{50}) and Physicochemical Parameters (pK_a, π, R_m) and Protein Binding (Log BA) of Studied 2- and 3-Substituted 4-Aminobenzoic Acids

No.	Compound	ID_{50}	pK_a	pK_a[f]	π	R_m	Log BA[g]
0	4-Aminobenzoic acid (PABA)[a]			4.67	0.83[i]	1.21	
1	2-Fluoro-4-aminobenzoic acid (2-F-PABA)	125	4.13		0.46	1.62	1.27
2	2-Chloro-4-aminobenzoic acid (2-Cl-PABA)	45	3.88	3.93	0.50	2.17	1.36
3	2-Bromo-4-aminobenzoic acid hydrochloride (2-Br-PABA)	8	3.69	3.85	0.66	2.38	1.67
4	2-Iodo-4-aminobenzoic acid hydrochloride (2-I-PABA)	30	3.81		0.82	2.40	1.79
5	2-Hydroxy-4-aminobenzoic acid[b] (2-OH-PABA)	25	3.52	3.62	0.49	1.92	1.57
6	2-Nitro-4-aminobenzoic acid (2-NO_2-PABA)	100	2.78	3.22	0.38	2.30	1.33
7	2,4-Diaminobenzoic acid dihydrochloride (2-NH_2-PABA)	3.5	5.81		−1.14		1.00
8	4-Aminophthalic acid	120					0.45
9	2-Methoxy-4-aminobenzoic acid (2-OCH_3-PABA)	100	5.04	5.10	−0.38		1.26
10	2-Ethoxy-4-aminobenzoic acid	130	5.09		0.16	1.31	1.56
11	2-Propoxy-4-aminobenzoic acid (2-OC_3H_7-PABA)	140	4.96		0.70	1.75	1.69
12	2-Butoxy-4-aminobenzoic acid	125	5.25		1.24	2.40	1.69
13	2-*n*-Pentoxy-4-aminobenzoic acid	135	5.10		1.55	2.85	1.76
14	2-Isopentoxy-4-aminobenzoic acid	125	5.17		1.47[e]	2.70	
15	2-Hexoxy-4-aminobenzoic acid (2-OC_6H_{13}-PABA)	90	5.37		2.00[e]	3.25	
16	2-Heptoxy-4-aminobenzoic acid	90	5.3[c]		2.49[e]	3.77	
17	2-Octoxy-4-aminobenzoic acid	25[h]	5.4[c]		2.94[e]	4.25	
18	2-Allyloxy-4-aminobenzoic acid	125	5.01		0.42	1.68	1.63
19	2-Methyl-4-aminobenzoic acid (2-CH_3-PABA)	30	4.83		0.31	1.73	1.16
20	3-Fluoro-4-aminobenzoic acid (3-F-PABA)	13	4.35		0.58	1.66	1.52
21	3-Chloro-4-aminobenzoic acid	100	4.35		1.52	2.38	1.81
22	3-Bromo-4-aminobenzoic acid	110	4.24	4.31	1.40	2.85	1.89
23	3-Iodo-4-aminobenzoic acid	110	4.34		1.53	2.95	1.92
24	3-Hydroxy-4-aminobenzoic acid	85	4.59		−0.32		1.27
25	3-Nitro-4-aminobenzoic acid	130	3.96[d]		1.01	3.11	1.89
26	3,4-Diaminobenzoic acid dihydrochloride (3-NH_2-PABA)	40	4.84		−0.70		0.95
27	3-Rhodano-4-aminobenzoic acid	190	4.52		0.83		1.68
28	4-Aminoisophthalic acid	120					1.82
29	3-Hexyloxy-4-aminobenzoic acid	85	4.47				1.97
30	3-Methyl-4-aminobenzoic acid	120	4.62		0.54	1.6	1.37
31	3-Benzoyl-4-aminobenzoic acid	105	4.34				

[a] From E. Merck, Darmstadt (Germany). [b] From Fluka, Neu-Ulm (Germany). [c] Estimated from the correlation between the pK_a value and length of the carbon chain of 2-alkyloxy-PABA (Figure 2). [d] Using ρ_m from Taft[15] (3-NO_2-PABA) and White[17] (3-benzoyl-PABA) and regression analysis (eq 1b, Figure 1b). [e] Using the correlation between π and R_m (see eq 2, Figure 3). [f] Evaluated by Los.[18] [g] Protein binding, using a solution of 100 μmol/l. of PABA derivatives given as logarithm of percent bound to albumin. [h] Concentration >50 μmol/l. could not be evaluated because of the low solubility of this compound in water. [i] Log *P* value.

which are not subject to this limitation. The dependency of the obtained velocity constants for the formation of dihydropteroic acid analogues on structural parameters of the PABA derivatives is discussed and compared with the results from inhibitory experiments in whole cell cultures (*E. coli*).

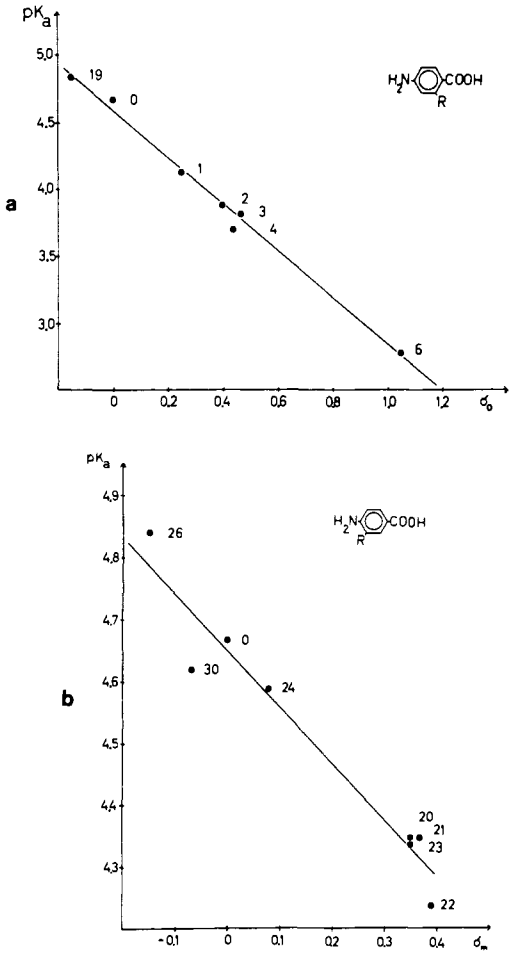


Figure 1. (a) Correlation between pK_a value of ortho-substituted PABA derivatives and σ_o.[14] (b) Correlation between pK_a value of meta-substituted PABA derivatives and σ_m.[15]

Results and Discussion

The PABA derivatives used in this study are listed in Table I. The Experimental Section includes synthetic procedures for those compounds not described in the literature. Dihydropteroic acid analogues expected as possible metabolites in the enzymatic reaction, which were needed for the identification and quantification of the assumed analogues, have been synthesized and characterized as described in the Experimental Section.

Electronic Substituent Effect. For acids and bases the dissociation constant can serve as an indicator for the electronic effects of the substituents. The acid dissociation constants for the amino and the carboxyl group of the substituted PABA derivatives are very close or overlapping. Therefore, a special UV spectroscopic procedure developed by Ang[13] has been used for the determination of the dissociation constant of the carboxyl group. The values are given in Table I. The pK_a values of the long carbon chain 2-alkoxy-PABA derivatives could not be determined spectrophotometrically because of their low solubility. Usually Hammett σ constants are successfully applied to describe the electronic influence of substituents. Figure 1 shows that this statement holds also for 2- and 3-substituted PABA derivatives. A correlation of high significance between pK_a and σ_o[14] and σ_m,[15] respectively, is obtained.

(a) Compounds **5**, **7**, and **9**–**18** (Table I) could not be included in the correlation (eq 1a) as no σ_o values determined by the same author were available. σ_o values from other sources[16] were also not complete for the series

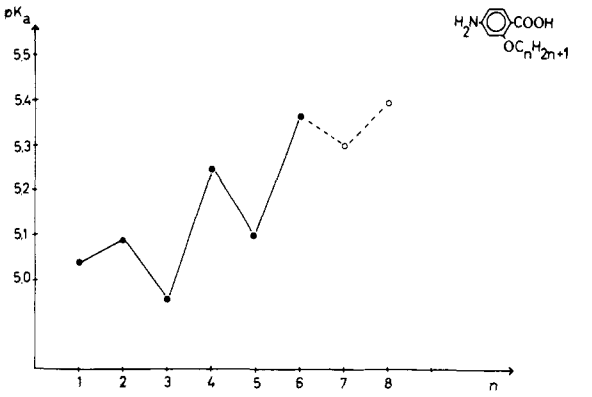


Figure 2. pK_a value dependence of the chain length of 2-alkoxy-PABA derivatives.

$$pK_a = (-1.75 \pm 0.08)\sigma_o + 4.58 \pm 0.04 \quad (1a)$$

n	r	s	F	S
7	0.995	0.07	535	>99.9

$$pK_a = (-0.91 \pm 0.09)\sigma_m + 4.64 \pm 0.02 \quad (1b)$$

n	r	s	F	S
8	0.972	0.05	101.7	>99.9

studied and differ from the values of Smith[14] significantly.

(b) In the meta-substituted series (eq 1b) compounds **25**, **28**, **29**, and **31** are not included as the pK_a values of compounds **25**, **29**, and **31** could not be determined due to their low solubility. The pK_a of compound **28** could not be determined as it is a dicarbonic acid.

In these equations 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, and S indicates the significance of the contribution of a parameter. The linear regression equation (1b) was used for the calculation of the pK_a values of 3-NO_2-PABA and 3-benzoyl-PABA using σ_m constants given by Taft[15] and White.[17]

Some of the pK_a values have already been reported in the literature[18] (see Table I). In general, they are in good agreement with our data. We have obtained, via the spectroscopic method, a pK_a value of 2.78 for the 2-NO_2 derivatives, whereas Los has reported a value of 3.22. Since our value is in complete agreement with eq 1a, we shall assume it to be the correct value. The dissociation constants for 2-OC_7H_{15}- and 2-OC_8H_{17}-PABA were estimated (low solubility) from Figure 2.

Hydrophobic Effects. π values, introduced by Hansch[19] and calculated from partition coefficients, describe the lipophilic influence of the substituent on the parent compound. (They have been determined for the PABA derivatives in the system octanol–water relative to PABA and are listed in Table I.) The π values for the long-chain 2-alkoxy-PABA derivatives could not be determined by the partition technique because of their extremely high lipophilicity. Therefore R_m values on polyamide layer, as proposed by Draber,[20] were measured (see Experimental Section). R_m values obtained in this manner do not only represent the changes in lipophilic properties as a function of substituents; besides these changes other effects such as hydrogen bonding or charge transfer can play a role. However, in a series of closely related compounds these factors can be considered to be constant. Therefore a linear correlation between π values and R_m values is obtained for a series of 2-alkoxy-PABA derivatives (Figure 3, eq 2). This equation has been used to calculate the π values of 2-*i*-C_5H_{11}-, 2-OC_6H_{13}-, 2-OC_7H_{15}-, and 2-OC_8H_{18}-PABA (Table I, footnote *g*).

Inhibitory Activity of PABA Derivatives on *E. coli*

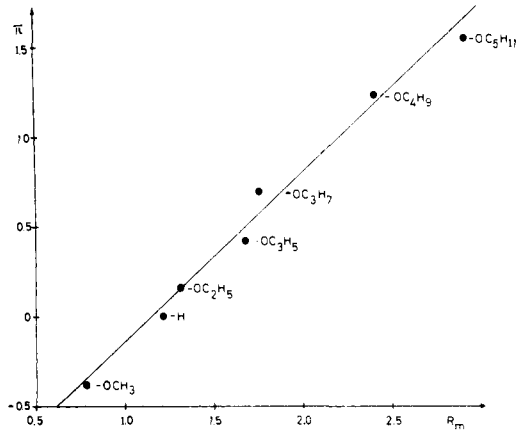


Figure 3. π-values dependence on the R_m values on polyamide for 2-alkoxy-PABA derivatives.

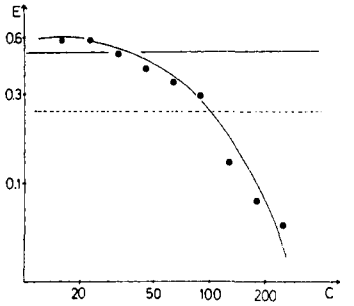


Figure 4. Growth of *E. coli* in the presence of various concentrations of 2-OCH_3-PABA; double logarithmic system.

$$\pi = 0.95R_m{}^0 - 1.11 \quad (2)$$
$$n = 7, r = 0.994, S > 99.9$$

(Whole Cell System). In contrast to the sulfonamides, MIC values for PABA derivatives could not be determined in a serial dilution test because no clear-cut end point determination was possible. Turbidity measurements were therefore performed and the inhibitory activity was recorded as ID_{50}. The turbidity of the culture after 24 h of incubation (37 °C) at 500 nm is plotted against the inhibitor concentration in a double logarithmic system. The concentration of PABA derivatives causing 50% inhibition of *E. coli* growth (turbidity), ID_{50}, was determined from such plots (Figure 4, Table I). In spite of a rather large variance in inhibitory activity, no significant correlation to electronic (pK_a Hammett σ), lipophilic (π, R_m), or steric (E_s) substituent parameters was obtained. For the correlation between log ID_{50} and pK_a for all ortho-substituted PABA derivatives only a regression coefficient of $r = 0.5$ was obtained. No further improvement was reached by stepwise multiple regression analysis including π, π^2, and E_s. The result of the present investigation, together with the observation of inhibitory effects for some derivatives at rather small concentrations, but growth-promoting effects at larger concentrations, may again hint at a complicated mechanism and at the fact that these compounds may be incorporated into a folic acid analogue as a function of the substituents in the PABA moiety. On the other hand, these folic acid analogues may serve as folate metabolites; their efficiency may be some other function of the substituent, i.e., both reactions may have different dependencies upon structural changes (parameters).

To throw more light onto this problem, the ability of synthetically prepared dihydropteroic acid analogues to reverse the inhibitory activity of sulfonamides on *E. coli* cultures has been studied; the results are given in Table II. Besides dihydropteroic acid the 3′-fluoro- and 2′-chlorodihydropteroic acid can serve as substrates for folic acid synthesis. Similar results have been obtained by Sirks[7] for 2′-fluoro-, 2′-chloro-, 2′-methyl-, 3′-methyl-, 2′-methoxy-, and 3′-methoxyfolic acid analogues using *S. faecalis* as test organisms.

Table II. MIC of Sulfabenzene and Sulfapyridine in the Presence of Various Concentrations of Pteroic Acids

Sulfapyridine	1[a]	>4[b]	⩾4[c]	1[d]	2[e]	1[f]	>4[g]
Sulfabenzene	11[a]	⩾64[b]	⩾64[c]	11[d]	32[e]	16[f]	>64[g]

[a] Without pteroic acids. [b] With 4 μmol/l. of dihydropteroic acid. [c] With 400 μmol/l. of dihydropteroic acid. [d] With 4 μmol/l. of 2′-chlorodihydropteroic acid. [e] With 400 μmol/l. of 2′-chlorodihydropteroic acid. [f] With 4 μmol/l. of 3′-fluorodihydropteroic acid. [g] With 400 μmol/l. of 3′-fluorodihydropteroic acid.

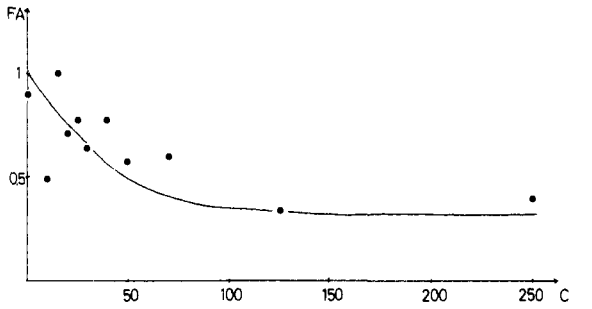


Figure 5. Inhibition of the pteroate synthesizing enzyme system (*E. coli*) by 3-F-PABA; folic acid equivalents (FA) as a function of the concentration, C (μmol/l.), of 3-F-PABA.

Inhibitory Activity of PABA Derivatives on Dihydropteroic Acid Synthesizing Enzymes (Cell-Free System). In addition to the complications discussed above, other difficulties, which may prevent a successful application of quantitative structure–activity correlations for these compounds, may arise from differences in their permeation ability relative to the bacterial cell wall. To exclude this parameter from the system, some PABA analogues have been studied in a cell-free folate-synthesizing extract. The degree of folate synthesis inhibition was determined microbiologically using *S. faecalis* as the test organism. A typical result given in Figure 5 shows the inhibition of folate production as a function of increasing 3-F-PABA concentrations. Even at higher inhibitor concentrations the obtained inhibition is small. It is noticeable to point to the fact that after a decrease of folate production to half of the control value no further effect is obtained by increasing the inhibitor concentration from about 100 to 250 μmol/l. For other tested derivatives (2-OH-, 2-Cl-, and 3-OC_6H_{13}-PABA) the inhibitory effect was even smaller and even at very high concentrations the ID_{50} could not be obtained. There are at least two possible explanations.

1. The inhibitory activity of PABA derivatives on the pteroic acid synthesizing enzyme system is extremely weak. This, however, is not very likely, considering the fact that some of the tested PABA derivatives (Table I) show rather strong inhibitory effects in the whole cell system on *E. coli*.

2. Competing with PABA, the analogues are incorporated into dihydropteroic acid analogues which can be used as metabolites by *S. faecalis*. Figure 6 underlines that this assumption is justified. Besides folic acid and pteroic acid, 2′-fluoropteroic acid (to a certain degree) can serve as a growth factor for *S. faecalis*.

Rate of Formation of Pteroic Acid Analogues in a Cell-Free System Followed by Quantitative Thin-

	n	r	s	F	S	
$area_{H_2PA} = (1320 \pm 0.05)C + 214 \pm 0.09$	9	0.99	0.13	673	>99.9	(3)
$area_{3'\text{-}FH_2PA} = (1197 \pm 0.07)C + 150 \pm 0.10$	6	0.99	0.14	249	>99.9	(4)
$area_{3'\text{-}OH\text{-}H_2Pt} = (1057 \pm 0.06)C + 186 \pm 0.06$	8	0.99	0.08	349	>99.9	(5)
$area_{2'\text{-}Cl\text{-}H_2Pt} = (1139 \pm 0.07)C + 6 \pm 0.12$	9	0.98	0.18	223	>99.9	(6)
$area_{\Sigma} = (1265 \pm 0.06)C + 58$	32	0.97	0.24	505	>99.9	(7)

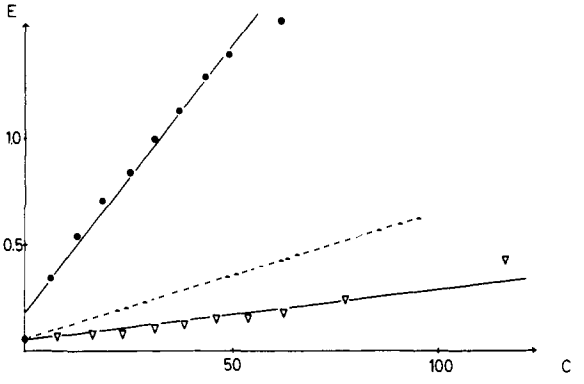


Figure 6. Growth of *S. faecalis* (E) as a function of various concentrations (C) of folic acid (●), 2′-fluoropteroic acid (▽), and dihydropteroic acid or pteroic acid (- - -).

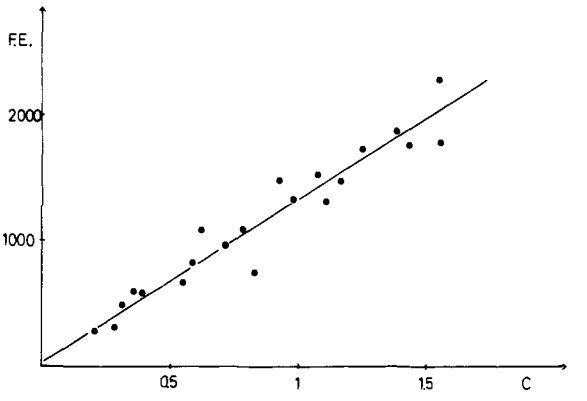


Figure 7. Dependence of fluorescence (given in area units of fluorescence equivalents, FE, mm²) on $H_2PtCOOH$ concentration, C (10^{-9} mol/5 μl).

Layer Chromatography (TLC). Evaluation of the Reaction Conditions. As discussed in the preceeding sections it is very probable that PABA analogues are incorporated into dihydropteroic acid analogues which can serve as metabolites for folic acid synthesis, especially for *S. faecalis*, to a different degree thus falsifying structure–activity correlations in the inhibitory reaction. The system used by Thijssen[11,12] has the disadvantage that only competition for the active site is considered; whether an analogue synthesis is performed or not cannot be differentiated. If different structural preconditions are operative for the competition mechanism at the receptor site and for the sequential reaction to products, i.e., change in the rate-determining step, then meaningful structure–activity correlations cannot be expected from the complex inhibitory data. To avoid these limitations an analytical procedure has been developed to determine the rate of dihydropteroic acid analogue production of PABA analogues in a cell-free pteroic acid synthesizing enzyme system in the absence of competing PABA. The advantage that several pteridine derivatives are strongly fluorescing agents was used to follow the enzymatic reaction by quantitative TLC (see Experimental Section). The intensity of the fluorescence of the various pteridine compounds was proportional to the concentration in the range applied for the enzymatic reaction (see also Henze[21]). The relation between fluorescence and concentration for dihydropteroic acid is given in eq 3. Nearly identical relations were observed for 2′-chloro-, 2′-hexoxy-, 3′-fluoro-, and dihydropteroic acid (eq 4–6). Therefore a general calibration curve including all data points and all derivatives was used (eq 7) (Figure 7).

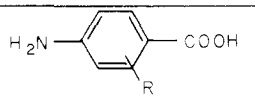
Determination of the Reaction Rate for PABA and PABA Derivatives. The velocity of the formation of dihydropteroic acid and dihydropteroic acid analogues was studied under optimal conditions of substrate, enzyme concentrations and ratios and without preincubation for H_2PtCH_2OPP (see Experimental Section). For the PABA derivatives the reaction was performed in the absence of PABA. The experiments have been performed under conditions where the substrate concentration [S] was five times the concentration found to give v_{max} for pteroic acid formation in the presence of PABA at constant enzyme concentration (67 μg/300 μl). Assuming that the substrate concentration [S] to obtain v_{max} is probably smaller for all PABA derivatives, we have used this concentration of S for all derivatives tested. This means that the experimental conditions are such that the Haldane–Briggs equation $v = k_2E_t$ can be applied. Further experimental details will be published elsewhere.[36]

The specific reaction rate for synthetase (catalyzing the production of dihydropteroic acid from H_2PtCH_2OPP in the presence of PABA) was 5.2×10^{-9} mol/min/mg of protein, i.e., 5.2×10^{-9} mol of dihydropteroic acid was synthesized by 1 mg of protein in 1 min. This reaction rate is ten times the rate reported by Ho[22] and 100 times the reaction rate obtained by Thijssen.[12] This high activity of the enzyme preparation was the most important supposition for a successful application of quantitative TLC for the measurement of the velocity of the enzymatic synthesis for various pteroic acid derivatives. The velocity of the formation of dihydropteroic acid analogues from PABA derivatives was studied in the absense of PABA. The reaction rates for three or four derivatives were determined at the same time in one experiment together with PABA as a standard under constant and identical experimental conditions. The average rate constant for the synthesis of dihydropteroic acid obtained from three different experiments was $3.34 \pm 0.34 \times 10^{-10}$ mol/min (Table III).

It is astonishing that for most of the compounds studied a nearly constant reaction rate is observed in spite of the fact that these derivatives show a large variation in lipophilic and electronic properties (Table III, pK_a and π). The unimportance of the lipophilicity of the compounds for the reaction rate is strikingly demonstrated on the example of the 2-F-, 2-Br-, and 2-I-PABA, where the pK_a is nearly constant and the π value varies from 0.46 to 0.82 without any influence on the reaction rate of the compounds in the enzymatically catalyzed reaction. Assuming hydrophobic binding to the enzyme plays a role for the enzymatic reaction, then variation in π values should cause variations in the (observed) reaction rates.

	n	r	s	F	S	
$\log \%\ \text{bound}_m = (0.41 \pm 0.05)\pi + 1.30 \pm 0.05$	9	0.95	0.11	70.2	>99.9	(8)
$\log \%\ \text{bound}_o = (0.30 \pm 0.06)\pi + 1.35 \pm 0.05$	14	0.79	0.16	19.9	>99.9	(9)
$\log \%\ \text{bound}_{o+m} = (0.35 \pm 0.04)\pi + 1.33 \pm 0.04$	23	0.88	0.14	69.7	>99.9	(10)

Table III. Rate of Incorporation of PABA Derivatives into Dihydropteroic Acid Analogues



R	Rate of incorp × 10^{10}, mol/min	pK_a	E_s[c,d]	π	σ_m[b,d]	σ_p[b,d]
H	3.34 ± 0.34	4.67	1.24	0	0	0.0
2-F	3.31 ± 0.26	4.13	0.78	0.46	0.34	0.0
2-Cl	4.92 ± 0.56[a]	3.88	0.27	0.50	0.37	0.0
2-Br	3.75 ± 0.56	3.69	0.08	0.66	0.39	0.0
2-I	3.28 ± 0.26	3.81	−0.16	0.82	0.35	0.0
2-OH	2.94 ± 0.24	3.52	0.69	0.49	0.12	0.0
2-NO_2	3.35 ± 0.27	2.78	0.23	0.38	0.71	0.0
2-NH_2	4.45 ± 0.56[a]	5.81	0.63	−1.14	−0.16	0.0
2-CH_3	3.76 ± 0.35	4.83	0.0	0.31	−0.07	0.0
3-F	2.95 ± 0.46	4.35		0.58		
3-NH_2	1.84 ± 0.23	4.84		−0.70		
2-OCH_3	3.03 ± 0.27	5.04	0.69	−0.38	0.12	0.0
2-OC_3H_7	1.31 ± 0.07	5.25		1.24	0.10	0.0
2-OC_6H_{13}	0.92 ± 0.14	5.37		2.00		0.0
PABA ethyl ester, no reaction						0.45

[a] A precise analysis by fluorescence intensity measurements was difficult in this experiment, due to incomplete separation on the chromatograms. [b] σ values with respect to the NH_2 group. [c] E_s values with respect to the COOH group. [d] See ref 37.

That the generally observed dependency of protein binding on π observed for different classes of compounds holds also for the studied PABA derivatives is demonstrated with the results of binding measurements performed with human serum albumin. As the solubility of the long carbon chain alkoxy derivatives in water is limited, the dissociation constant of the drug albumin complex could not be determined. Therefore, only the percentage of free drug in the presence of 4% albumin solution at a constant total drug concentration was determined. The correlation obtained between the logarithm of the degree of protein binding (log percent bound) and the lipophilic properties of the derivatives (π) is given in eq 8 for the nine meta derivatives. The correlation obtained for the ortho-substituted derivatives is not satisfying but still significant (eq 9). As the coefficients with π in both equations are comparable, the two series of compounds have been combined. The correlation equation for 23 compounds is given in eq 10. The degree of binding to serum albumin increases with increasing π values.

Compounds **28**, **29**, and **31** have been excluded in eq 8 and 10—**28** because the pK_a could not be determined and

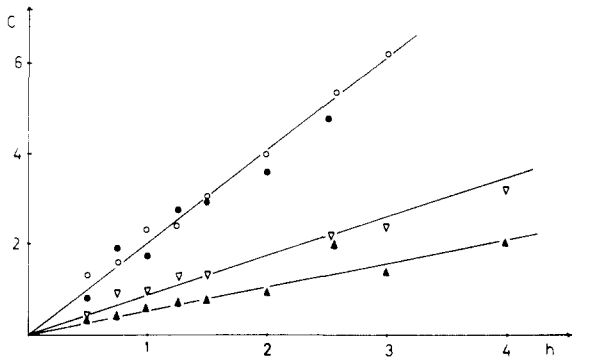


Figure 8. Rate of incorporation of PABA (○), 2-OCH_3-PABA, (●), 2-OC_3H_7-PABA (▽), and 2-OC_6H_{13}-PABA (▲) into a dihydropteroic acid analogue.

therefore π could not be corrected for ionization. In case of compounds **29** and **31**, π could not be obtained because of the unsufficient concentration in the water phase at the partition coefficient determination. Compounds **8** and **14–17** have been excluded in eq 9 and 10—compound **8** because the pK_a could not be determined (see compound **28**) and compounds **14–17** were not sufficiently soluble for protein binding measurements.

The observed constancy of the reaction rates for the studied substituted PABA derivatives may therefore justify the assumption that ionic forces are the decisive parameter for the reaction. As the carboxyl group of all compounds studied in these experiments is completely ionized under the conditions of the experiment (pH 8) no striking variance in the degree of ionization occurs and a constant reaction rate is expected. A "steric hinderance" of the ionized center, however, should lead to a decrease in reaction speed as observed for the 2-OC_3H_7- and OC_6H_{13}-PABA derivatives. The dependency of the reaction rate on the carbon chain length for 2-alkoxy-PABA derivatives is shown in Figure 8 and Table III for the 2-OCH_3-, 2-OC_3H_7-, and 2-OC_6H_{13}-PABA. Here the rate seems to decrease with increasing lipophilicity. However, considering the invariance of the observed rate as a function of changes in π for all the other compounds studied, one may argue that the decrease in rate for the long-chain alkoxy derivatives might be due to a "steric effect" of these substituents rather than to the lipophilicity. This is especially true as the π values are not extremely high and the bypass of a maximum seems very unlikely. This steric effect is not described by E_s which is certainly a parameter concerning only intramolecular steric effects. That E_s is not the decisive parameter is underlined by the observed constancy in rate for the studied series with the exception of the long-chain alkoxy groups in spite of a large variation in E_s (Table III). We suppose that the striking change in rate for the long-chain alkoxy groups might be explained by a "steric hinderance" of the substituent on the approach of the molecule, especially of the ionized carboxy group, to the enzyme surface, i.e., a shielding effect of the extended hydrophobic substituent. Further support for the importance of the ionized carboxy group comes from the results obtained with the ethyl ester of PABA. In this case ionization is not possible and no formation of dihydropteroic acid ethyl ester or consumption of H_2PtCH_2OPP

in the enzymatic reaction was observed. An electronic effect on the primary amino, i.e., by change of σ_p from 0.00 for COO^- to 0.45 for $CO_2CH_2CH_3$, can be excluded as a reason for this. Even stronger effects by 3-nitro (σ_m = 0.71) or by 2-amino substitution (σ_m = -0.16) (Table III) do not alter the rate of incorporation. Another exception from the observed constancy of the rate of dihydropteroic acid formation is 3-NH_2-PABA, where the incorporation might be hindered by the additional amino group in the neighborhood of the reaction center.

These observations which did not lead to a correlation between rate of product formation and physicochemical parameters are contrasted by the results published by Thijssen[12] where a "significant" correlation between cell-free inhibitory activities of PABA derivatives and certain substituent constants, such as π, E_s, and σ, is reported. Close inspection of the data reveals, however, that the statistics applied are not sound.

In the final correlation equation, three independent variables are correlated with six data points (compounds). Some other compounds studied were even omitted. The regression coefficients in the stepwise regression show insignificant values (Student's t test) for the single parameters.

Our results on PABA derivatives resemble those evaluated in our laboratory on the example of the sulfonamides.[5] For this class of compounds, which are also competitive inhibitors of dihydropteroic acid synthesis, it was shown that the degree of dihydropteroic acid synthesis inhibition is strongly correlated with the concentration of the ionized fraction of the inhibitor molecule. As the sulfonamides are weaker acids the ionized fraction varies with certain substituents at the pH condition of the reaction or culture medium, whereas the pK_a of the PABA derivatives is such that nearly total ionization occurs for all studied PABA derivatives under experimental conditions, i.e., the ionized fraction is constant. As a consequence the inhibitory activity of sulfonamides varies as a function of the pK_a. For PABA derivatives, however, no dependency is found, as long as the ionized group is not "sterically" screened. The variance observed in inhibitory activities in whole cell systems and in the degree of dihydropteroic acid formation in a cell-free system in the presence of PABA derivatives must therefore be due to other factors. Among these factors we can include (1) differences in the ability of synthesized analogues to serve as folate metabolites, or (2) the possibility that the rate-determining step is *competition* with PABA for the active site of the synthetase (as opposed to formation of the product as the rate-limiting factor), this then being a function of the group characteristics at positions 2 or 3.

Experimental Section

General. Melting points were taken with a Leitz melting point microscope and are uncorrected. IR spectra were taken with a Leitz spectrograph. The NMR spectra were determined using a Varian HA 100 spectrometer with Me_4Si as internal standard. A Zeiss PMQ II and a Cary 14 spectrometer was used to obtain ultraviolet spectra. The quantitative evaluation of TLC plates was carried out with a spectrodensitometer, Model SD 3000 of Schoeffel-Instruments. For the TLC silica gel plates without a fluorescence indicator (Merck, Germany) were used. Statistical evaluations were carried out with a Wang Computer 700 B.

(1) **Synthesis of the PABA Derivatives.** Most PABA derivatives were synthesized according to known procedures (melting points of known derivatives are in accordance with the literature).

2-Alkoxy-4-aminobenzoic Acids. For the synthesis of the 2-alkoxy-4-aminobenzoic acids a method described by Collins[23] for 2-octoxy-4-aminobenzoic acid was used. The potassium salt of 4-acetylamino-2-hydroxybenzoic acid methyl ester was condensed with the corresponding alkyl bromide, the condensation product being hydrolyzed to the alkoxy-PABA derivative. Recrystallization was from EtOH–H_2O and MeOH–CCl_4. 2-*n*-Pentoxy-4-aminobenzoic acid: yield, 40%; mp 122.5–123.5 °C. Anal. ($C_{12}H_{17}NO_3$) C, H, N. 2-Isopentoxy-4-aminobenzoic acid: yield, 35%; mp 202–204 °C. Anal. ($C_{12}H_{17}NO_3$) C, H, N. 2-*n*-Hexoxy-4-aminobenzoic acid: yield, 40%; mp 138–139 °C. Anal. ($C_{13}H_{19}NO_3$) C, H, N. 2-*n*-Heptoxy-4-aminobenzoic acid: yield, 30%; mp 86–87.5 °C. Anal. ($C_{14}H_{21}NO_3$) C, H, N.

3-Benzoyl-4-aminobenzoic acid was made from 3-benzoyl-4-aminobenzoic acid ester (Sternbach[24]) by boiling 6 h in MeOH–2 N NaOH (1 + 1). Recrystallization was from acetone: yield, 80%; mp 265–267 °C. Anal. ($C_{14}H_{11}NO_3$) C, H, N.

3-Hexoxy-4-aminobenzoic Acid. 3-Hydroxy-4-nitrotoluene was condensed with hexyl bromide in acetone using K_2CO_3 to form 3-hexoxy-4-nitrotoluene (Allen[25]): yield, 36%; mp 150–154 °C.

3-Hexoxy-4-nitrotoluene was oxidized in pyridine–H_2O (1 + 1) with threefold of the theoretical amount of $KMnO_4$ to form 3-hexoxy-4-nitrobenzoic acid: yield, 45%; mp 176–177 °C. Recrystallization was from acetone–H_2O.

3-Hexoxy-4-aminobenzoic acid was prepared from 3-hexoxy-4-nitrobenzoic acid by hydrogenation with H_2 (Pd/C) in EtOH. Recrystallization was from CCl_4: yield, 75%; mp 114–116 °C. Anal. ($C_{13}H_{19}NO_3$) C, H, N.

(2) **Synthesis of Pterin Derivatives. (a) 7,8-Dihydro-6-hydroxymethylpterin and 7,8-Dihydro-6-hydroxymethylpterin Pyrophosphate.** Folic acid (Merck, Darmstadt, Germany) was cleaved with HBr to form 6-pterinaldehyde.[26] It was reduced to 6-hydroxymethylpterin with $NaBH_4$[27] and then with $Na_2S_2O_4$ to 7,8-dihydro-6-hydroxymethylpterin (H_2PtCH_2OH).[28] Heating 6-hydroxymethylpterin with pyrophosphoric acid adsorbing the reaction product onto active carbon and eluting with 3 N NH_4OH gave 6-hydroxymethylpterin pyrophosphate.[22] It was reduced to 7,8-dihydro-6-hydroxymethylpterin pyrophosphate (H_2PtCH_2-OPP) with $Na_2S_2O_4$. The NMR and UV spectra of the synthesized products were the same as previously reported.[27,28]

(b) Pteroic Acid and Pteroic Acid Derivatives. A method described by Plante[29] was used for the synthesis of the benzene-substituted pteroic acid derivatives, condensing N^2-acetylpterin-6-aldehyde with PABA and PABA derivatives [NMR signals >10 ppm (of $-NH_2$ and $-OH$ groups) are not reported]. Pteroic acid: yield 38%; NMR 8.99 (s, 1 H, C_7), 5.25 (s, 2 H, C_9), 8.41 (d, 2 H), 7.88 ppm (d, 2 H). 2′-Fluoropteroic acid: yield 77%; NMR 8.99 (s, 1 H), 5.20 (s, 2 H), 8.30 (m, 1 H), 7.55 ppm (m, 2 H). Anal. ($C_{14}H_{11}FN_6O_3 \cdot H_2O$) C, H; N: calcd, 24.1; found, 23.0. 3′-Fluoropteroic acid: yield 56%; NMR 9.02 (s, 1 H), 5.25 (s, 2 H), 8.12 (m, 2 H), 7.80 ppm (m, 1 H). Anal. ($C_{14}H_{11}FN_6O_3 \cdot 2H_2O$) C, H, N. 2′-Chloropteroic acid: yield 43%; NMR 9.02 (s, 1 H), 5.28 (s, 2 H), 8.26 (d, 1 H), 7.98 (d, 1 H), 7.81 ppm (dd, 1 H). Anal. ($C_{14}H_{11}ClN_6O_3 \cdot 2H_2O$) H; C: calcd, 43.9; found, 45.2; N: calcd, 21.9; found, 21.2. 2′-Hexoxypteroic acid: yield 32%; NMR 9.03 (s, 1 H), 5.26 (s, 2 H), 8.41 (d, 1 H), 7.65 (d, 1 H), 7.81 (dd, 1 H), 4.42 (t, 2 H), 2.01 (m, 2 H), 1.47 (m 6 H), 0.96 ppm (t, 3 H). Anal. ($C_{20}H_{24}N_6O_4 \cdot 2H_2O$) C, N; H: calcd, 6.3; found 5.4. None of the described pteroic acid derivatives possess a defined melting point as already known from the literature.[29]

(c) 7,8-Dihydropteroic Acid and Its Derivatives. Pteroic acid was reduced with $Na_2S_2O_4$ to dihydropteroic acid according to a procedure given by Pfleiderer[28] for pteridin alcohol. The obtained product was a colorless powder. Dihydropteroic acid derivatives were prepared from pteroic acid derivatives in the same manner. In general, the yields were about 50%. All the dihydro compounds were unstable against air, light, and temperature; they became dark brown. Solutions in alkali were oxidized quickly, becoming yellow. Therefore no elemental analysis was performed. This has, however, been done for the oxidized form 1 (see above) [UV wavelength of maxima in nm in 0.1 NaOH (ϵ)]. Dihydropteroic acid: UV 280 (21 500), 330 sh (7500). 2′-Fluorodihydropteroic acid: UV 240 sh (12 300), 280 (17 000), 325 sh (6500). 3′-Fluorodihydropteroic acid: UV 275 (16 800), 330 sh (5500). 2′-Chlorodihydropteroic acid: UV 285 (15 500), 320 sh (7200). 2-Hexoxydihydropteroic acid: UV 270 (12 700), 325 sh (5000). Melting points could not be given, because all the pterin derivatives do not have a defined melting point. The UV and NMR spectra of pteroic acid and dihydropteroic acid were identical with the spectra described previously.[3,29,30] An appropriate shift in

the UV spectrum as observed for the reduction of pteroic acid to dihydropteroic acid was also found for the pteroic acid derivatives when reduced to their dihydro analogues. All compounds were checked for purity by TLC using the solvent system *n*-BuOH–HOAc–H_2O (20 + 5 + 10). The dihydropteroic acid derivatives show identical R_f values (R_f = 0.45) clearly separated from the unhydrogenated starting compounds (R_f = 0.0). In addition, the hydrogenated derivatives show a strong fluorescence on irridation with uv light (360 nm), whereas the pteroic acid derivatives do not show fluorescence.

(3) Evaluation of pK_a Values. pK_a values were measured spectrophotometrically according to the procedure described by Ang[13] using citrate buffers of various pH.

(4) Evaluation of the Lipophilic Properties. (a) π Values. π values were determined with citrate buffer as aqueous phase and octanol as organic layer according to Hansch.[19] The values are corrected for the undissociated forms of the PABA derivatives (see Table I). The partition coefficient of PABA was used as reference (π = 0); log *P* for PABA was 0.83 (Leo[31] gives 0.68–0.89). It was proven that the concentration of PABA derivatives had no influence on the partition coefficients. This finding was in accordance to experiments of Terada.[32]

(b) R_m Values. Compounds were dissolved in acetone and 5 μl of solution was spotted on a polyamide coated TLC plate. An aqueous citrate buffer (Sörensen) with various proportions of acetone was used as the mobile phase. The experimental R_m values were calculated by regression analysis as R_m with buffer alone as the mobile phase (see Table I).

(5) Binding to Serum Albumin (Log BA). Binding of the PABA derivatives to serum albumin (RHA 20, Behring, Marburg, Germany) was studied by equilibrium dialysis using a three-chamber dialysis cell. Concentration of bound drug was obtained by UV spectroscopy after equilibrium was obtained. Concentration of albumin was 4 g/100 ml of a phosphate buffer (0.2 M) + 0.15 M NaCl, pH 7.4, temperature 22 ± 1 °C. Listed are the logarithms of percent bound drug (log BA). The drug concentration used was 100 μmol/l. (see Table I). The accuracy of repeated protein binding determination was within the range of 3–5%.

(6) ID_{50} of PABA Derivatives for *E. coli* (Whole Cell System). Conditions were the same as described previously for the MIC of sulfonamides.[33] Turbidity was measured with a Zeiss PMQ II spectrophotometer using λ = 500 nm. The extinction of the solutions was used as a measure for the growth of the bacteria. ID_{50} determinations were repeated at least twice. The variation was in the limits of 10–20%.

(7) MIC of Sulfonamides with Various Concentrations of Dihydropteroic Acid Derivatives for *E. coli* (Whole Cell System). Conditions were the same as described previously.[33] The MIC of sulfabenzene and 2-sulfapyridine was taken without and with various concentrations of dihydropteroic acid, 3′-fluorodihydropteroic acid, and 2′-chlorodihydropteroic acid.

(8) Inhibitory Effect in Cell-Free Pteroate Synthesizing System (*S. faecalis*). Reaction conditions were the same as described previously.[5] The amount of dihydropteroic acid synthesized was expressed as folic acid equivalents (F.A.). Folic acid equivalents of dihydropteroic acid, pteroic acid, 2′-fluoropteroic acid, and 2′-chloropteroic acid were measured directly by their growth-promoting effect for *S. faecalis* (2′-chloropteroic acid had no growth-promoting effect but no growth-inhibiting effects as well) (see Figure 6).

(9) Quantitative TLC Calibrating Curves. An accurately weighed quantity of dihydropteroic acid derivative (~0.5 mg) was dissolved in 200 μl of 0.1 N NaOH and diluted with 3 ml of H_2O adding 20 μl of mercaptoethanol (to protect against oxidation). From this solution a sequential dilution with 10, 20, 30, 40, 50, 60, 80, and 100% was made and from each probe exactly 5 μl was spotted on silica gel plates using microcaps. Chromatograms were developed for 10 cm using 1-butanol–acetic acid–water (20 + 5 + 10). After drying they were scanned with a TLC densitometer. Plates not evaluated at once were kept under argon.

Instrument setting: single beam, remission; uv lamp, λ = 365 nm, gain 750 m; split width, 1.5 mm; filter, λ = 430 nm. The obtained peaks were measured by the method "height times width in half-height"[34] and expressed as area units (1 F.A. = 7.9×10^{-13} mol of dihydropteroic acid (or dihydropteroic acid derivative). The area obtained was a linear function of the quantity spotted on the plate.

(10) Enzymatic Reaction Mixture for the Determination of Reaction Rates for PABA and PABA Derivatives Followed by TLC. Hydroxymethyldihydropterin pyrophosphokinase (kinase) and dihydropteroic acid synthetase (synthetase) were prepared by methods reported previously.[5,6] Protein concentrations were determined by the method of Folin and Lowry,[35] using bovine serum albumin as a standard. Solutions of enzymes were kept at 4 °C and used within 1 week. A reaction mixture with a total volume of 300 μl was prepared. The compounds were dissolved in 0.2 M Tris buffer, pH 7.95 (20 °C): H_2PtCH_2OH, 5.2×10^{-4} mol/l.; ATP, 2.7×10^{-3} mol/l.; $MgCl_2$, 6×10^{-3} mol/l.; PABA, 5×10^{-4} mol/l.; kinase, 18.6 μg of protein; synthetase, 67 μg of protein, mercaptoethanol, 6.7×10^{-2} mol/l. This mixture was incubated at 37 °C and kept under argon atmosphere. Every 30 min 5-μl samples were taken and spotted directly on silica gel plates (plates were also kept under argon atmosphere). After the reaction was finished (~5 h) the TLC plates were developed and scanned as described in the section on "quantitative TLC calibration curves".[36]

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Notes

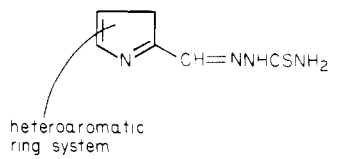
Elucidation of the Structure of the Antineoplastic Agents, 2-Formylpyridine and 1-Formylisoquinoline Thiosemicarbazones

Ippolito Antonini, Francesco Claudi, Palmarisa Franchetti, Mario Grifantini,* and Sante Martelli

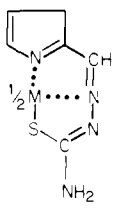
Institute of Pharmaceutical and Organic Chemistry, University of Camerino, Camerino, Italy. *Received March 10, 1976*

The geometrical isomers of the antineoplastic agents 2-formylpyridine and 1-formylisoquinoline thiosemicarbazones were synthesized and their structure was studied by spectroscopic methods. It was found that the compounds previously described in the literature and tested for carcinostatic activity were isomers with *E* configuration which probably contained minor amounts of *Z* isomers.

α-*N*-Formyl heterocyclic thiosemicarbazones are potential antineoplastic agents, particularly thiosemicarbazones of 1-formylisoquinoline and 2-formylpyridine, and a multitude of their derivatives have been examined for activity against a variety of transplanted animal tumors.[1,2] These compounds primarily block DNA synthesis in mammalian cells by inhibiting the enzyme ribonucleoside-diphosphate reductase, presumably either via chelation with an iron ion required by the enzyme or because a preformed metal chelate of the inhibitor interacts with the target enzyme.[3-7] French and Freedlander[8] proposed a sterically noncommittal ligand model for these compounds. Then French and Blanz[9] postulated the general formula **1** as a model for this series of antineoplastic agents.



1



2

This formula can be rewritten in the S–H tautomeric form. Formula **2** illustrates the functioning of the α-*N*-formyl heterocyclic thiosemicarbazones as tridentate ligands. A requirement for this was, according to French and Blanz, that the thiosemicarbazone is in the *Z* (syn) form. This statement was rectified by Mathew and Palenik[10] who performed a x-ray diffraction study and a complete structural formulation on bis(isoquinoline-1-carboxaldehyde thiosemicarbazanato)nickel(II) monohydrate. They clearly established that in the transition metal chelates the α-*N*-formyl heterocyclic thiosemicarbazones act as tridentate ligands in the thiol form. Besides, their work ascertained that, in the chelate form, the aldimine bond is *E* and that the geometry of the other double bond (pseudothiourea) is *Z*.

In order to verify if the ligand also has a structure similar to that of the chelate, we synthesized the geometrical isomers of the 2-formylpyridine and 1-formylisoquinoline thiosemicarbazones, two known tumor-inhibitory agents,[4,11,12] studying their structure by spectroscopic methods.

Chemistry and Spectral Studies. 2-Formylpyridine and 1-formylisoquinoline thiosemicarbazones (PT and IQ-1) were synthesized from 2-formylpyridine or 1-formylisoquinoline and thiosemicarbazide in hot ethanol according to the methods described in the literature.[13-15] In both cases, the reaction between the aldehyde and thiosemicarbazide gave pale yellow crystals which consist of a mixture of geometrical isomers [(*E*)- and (*Z*)-PT, (*E*)- and (*Z*)-IQ-1], as was checked by TLC, in which the slower eluted isomers largely prevail. When these crystals were purified by crystallization from EtOH, the slower eluted pure isomers of PT and IQ-1 were obtained. The faster eluted isomers were separated by column chromatography on silica gel. As the yields of faster eluted isomers were very low, we experimented with several isomerization methods with the aim of isomerizing the slowly eluted isomers. We found that the most convenient method consists of heating the slowly eluted isomers with SiO_2 in methanol. In this way the faster eluted isomers in about 30% yields were obtained. In solution, in several protic and aprotic solvents, they are labile and within some time are converted to a mixture of the geometrical isomers; however, in the dry crystalline state they are stable for several days.

Qualitative tests in methanol show that with iron(II) the slowly eluted isomers of PT and IQ-1 form intensely colored complexes (red and dark green, respectively); the faster eluted isomer of PT gives a light green color which on standing changes into red, whereas the faster eluted isomer of IQ-1 gives no coloring within 10 min; after this time a green color appears. We did not verify if this transition metal acts as a catalyst in the isomerization process of the faster eluted isomers or if only the solvent is responsible for that.

Comparison of the NMR spectra in dimethyl-d_6 sulfoxide at 60 MHz revealed that in the PT and IQ-1 slower eluted isomers, the proton H_a linked to the carbon of the