Analogs of Tetrahydrofolic Acid XXV

2,4-Diamino-6-phenethylpteridine, an Inhibitor of Dihydrofolic Reductase

By B. R. BAKER and BENG-THONG HO

Condensation of 2,4,5,6-tetraaminopyrimidine with phenylethylglyoxal acetal (IX) gave 2,4-diamino-6-phenethylpteridine (VI); 2-amino-6-phenethyl-4-pteridinol (VII) was also synthesized from IX by condensation with 2,5,6-triamino-4-pyrimidinol. For comparative inhibition of dihydrofolic reductase, 2,4-diamino-6-methyl-5-(4-phenylbutyl)pyrimidine (XIII) was synthesized from the previously described 2-amino-6-methyl-5-(4-phenylbutyl)-4-pyrimidinol (I). As an inhibitor of dihydrofolic reductase, XIII was 150-fold more effective than the pteridine (VI). The greater effectiveness of XIII was traced to a large hydrophobic contribution of the *n*-butyl group of XIII to binding to dihydrofolic reductase. In fact, XIII was an excellent inhibitor of dihydrofolic reductase, being only thirtyfold less effective than aminopterin.

It has been previously reported that 2-amino-6-methyl-5-(4-phenylbutyl)-4-pyrimidinol (I) is a 27-fold better inhibitor of dihydrofolic reductase than the corresponding 5-(3-anilinopropyl)-4-pyrimidinol (II) (1). In fact, I binds to dihydrofolic reductase three times better than the prototype inhibitor (III) containing the intact carboxy-L-glutamate side chain (1, 2). If this increased binding observed between I and II could be carried over to 2,4-diamino-6-phenethylpteridine (VI), then VI should bind to dihydrofolic reductase even stronger than the potent inhibitors, aminopterin (IV) or amethopterin (V). The synthesis and enzymic evaluation of VI are the subjects of this paper.

DISCUSSION

One of the major routes to synthesis of 6-substituted pteridines is condensation of a 5,6-diaminopyrimidine such as X with 2-substituted glyoxals or methyl ketones substituted with a reactive group on the methyl (3). Three variants were investigated, the third being successful. Attempts to prepare the mono-phenylhydrazone or mono-*p*-nitrophenylhydrazone of phenethylglyoxal from sodium γ-benzylacetoacetate and phenyl or *p*-nitrophenyl diazonium chloride were unsuccessful; this method has been used for other substituted glyoxal mono-phenylhydrazones (4, 5). A methyl group attached to C=N of a heterocycle can sometimes be condensed with benzaldehyde in the presence of concentrated sulfuric acid or acetic anhydride to give a styryl heterocycle (6, 7) which can then be catalytically reduced to a phenethyl heterocycle (6); such reactions between 2,4-diacetamido-6-methylpteridine (8) and benzaldehyde were not promising.

Phenethylglyoxal diethyl acetal (IX) was obtained from VIII as described by Dakin and Dudley (9); the intermediate (VIII) could be obtained by benzylation of ethyl γ,γ-diethoxyacetoacetate (9) or more easily by Claisen condensation of ethyl hydrocinnamate with ethyl diethoxyacetate. Condensation of IX with the tetraaminopyrimidine (X) in 25% aqueous alcohol at pH 3 followed by further acidification, conditions used by Fairburn *et al.* (10) for a related pteridine, proceeded satisfactorily to the desired VI in 29% yield; VI was further characterized as its N^2,N^4-diacetyl derivative, m.p. 179–181°. The possibility that VI was contaminated with the 7-isomer (XIV) or was even all 7-isomer (XIV) had to be considered, although the ultraviolet comparisons with 6- and 7-methyl derivatives (Table I) indicated the 6-isomer structure.

2,5,6-Triamino-4-pyrimidinol (XI) was condensed with the acetal (IX) in glacial acetic acid under conditions used by Fairburn *et al.* (10) that afforded them mainly a 6-isomer. The comparative ultraviolet spectra were again more compatible with a 6-isomer (VII) than a 7-isomer (XV). When the condensation of IX and XI was performed in 3.5 *N* hydrochloric acid in 12% aqueous ethanol, conditions which usually increase the amount of 7-isomer (XV) (11), the product contained more of the 7-isomer (XV) than previously obtained; this could be concluded by the shift of the longer wavelength peaks to a lower wavelength as expected from the data in Table I on the 6- and 7-methyl isomers, XVIII and XIX, respectively. Attempts to separate this supposed mixture of VII and XV into two spots in a number of paper chromatographic systems were unsuccessful (Scheme I).

The usual way of determining 6 to 7 isomer ratios with 2-amino-4-pteridinols is to oxidize the side chain to the carboxylic acid with alkaline potassium permanganate, then compare the radically different ultraviolet spectra of the 2-amino-4-hydroxypteridine-6-carboxylic acid and 7-carboxylic acid (11). Unfortunately, with the saturated hydrocarbon-like phenethyl side chain of VII, no oxidation took place under the conditions used for the more easily oxidizable anilinomethyl type of side chain in folic acid

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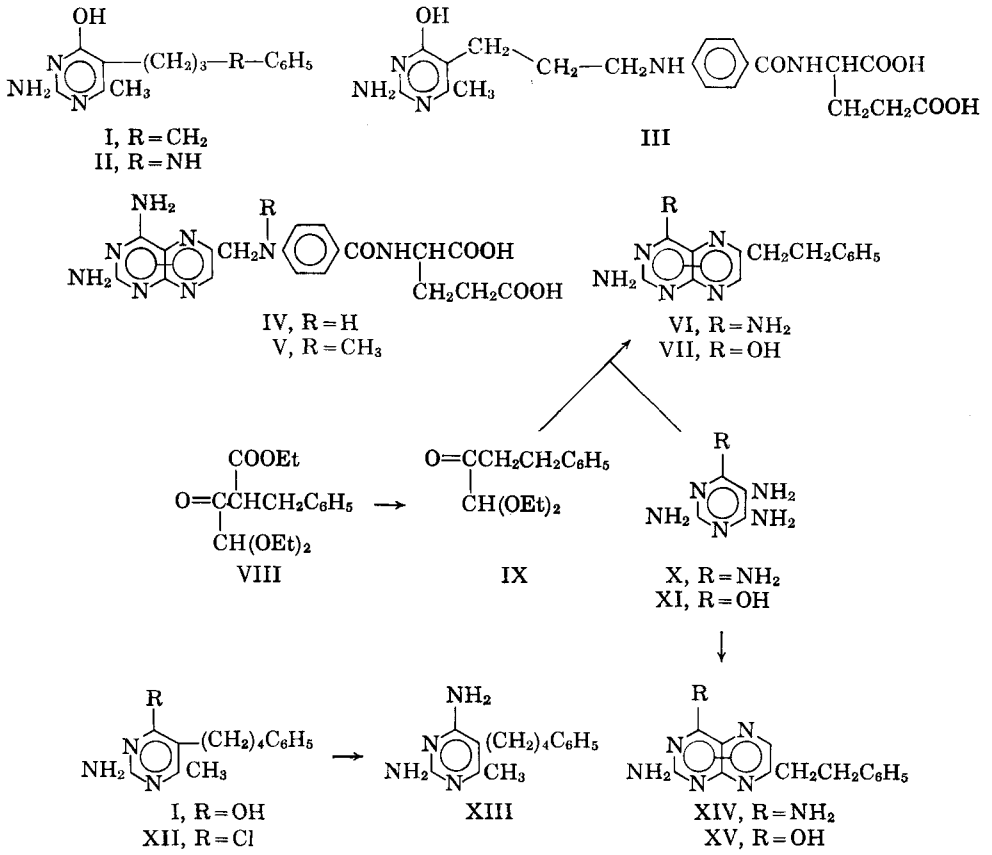


Scheme I

(11); under forcing conditions, over-oxidation took place as shown by loss of the ultraviolet absorption in the solution.

Hydrolysis of the presumed 2,4-diamino-6-phenethylpteridine (VI) with hot 6 *N* hydrochloric acid (12) gave a product with the same ultraviolet spectra as the presumed 2-amino-6-phenethyl-4-pteridinol (VII), prepared at pH 3 (Table I); both materials had the same mobility in several paper chromatographic systems.

Although it cannot be unequivocally stated that VI is a pure 6-isomer, it seems even less plausible that it is a pure 7-isomer (XIV) from the method of preparation (3); at worst, the 6-isomer (VI) is not likely to be contaminated with more than 50% of the 7-isomer (XIV), meaning that the enzyme inhibition observed (Table II) is unlikely to be in error by more than a factor of 2.

2,4-Diamino-6-phenethylpteridine (VI) showed 50% inhibition of dihydrofolic reductase at a level of 4.2 μM when 6 μM dihydrofolate was used as substrate (Table II). Aminopterin (IV) showed 50% inhibition at a concentration of 8×10^{-4} μM under the same conditions (*cf.* 13); thus, VI is only 2×10^{-4} as effective as aminopterin (IV). From the relative inhibition by the phenylbutyl pyrimidinol (I) and the pyrimidinol analog of tetrahydrofolic acid (III), it was anticipated at the start of this project that the phenethylpteridine (VI) should have the same order of activity as aminopterin (IV). It was therefore obvious that the relationship between I and III did not carry over to the relationship between IV and VI. Therefore, additional inhibitor studies were performed to try to resolve this enigma.

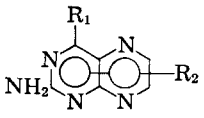
2,4 - Diamino - 6 - methyl - 5 - (4 - phenylbutyl)-pyrimidine (XIII) was a strong inhibitor of dihydrofolic reductase (Table II); XIII showed 50% inhibition of dihydrofolic reductase at 0.027 μM which was only thirtyfold less effective than aminopterin (IV), but 150-fold more effective than the phenethylpteridine (VI). Furthermore, 2,4-diamino - 6 - methyl - 5 - (4 - phenylbutyl)pyrimidine (XIII) was eightyfold more effective than the corresponding 5-(3-anilinopropyl)pyrimidine (XXII) (14), as anticipated from the same magnitude (27-fold) previously observed (1) with the 4-pyrimidinols, I and II.

2,4-Diamino-6-methylpteridine (XVI) has been reported to be a fiftyfold better inhibitor of folic reductase than 2,4-diamino-6-methylpyrimidine (XX) when assayed at pH 6 with folic acid as substrate (15). With the dihydrofolic reductase system (pH 7.4) used in Table II, the pteridine (XVI) was only fivefold more potent than the pyrimidine (XX).

The attachment of the phenylbutyl side chain (as in XIII) to 2,4-diamino-6-methylpyrimidine (XVI) enhanced activity by a factor of 40,000; but, in contrast, introduction of the benzyl group (VI) on 2,4-diamino-6-methylpteridine (XVI) enhanced activity only 52-fold, a nearly 1000-fold difference from the factor of 40,000 that was anticipated.

The discrepancy in binding of the phenethyl-

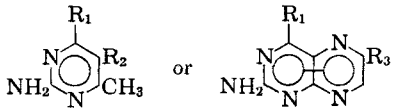
TABLE I.—ULTRAVIOLET SPECTRA OF



Compd.	R_1	R_2	$\lambda_{max.}$ in mμ, pH 1	$\lambda_{max.}$ in mμ, pH 13
VI[a]	NH_2	6—$C_6H_5CH_2CH_2$—	244, 285, 340	257, 372
XVI[b]	NH_2	6—CH_3—	242, 281, 337	255, 369
XVII[b]	NH_2	7—CH_3—	242, 281, 332	254, 361
XVIII[c]	OH	6—CH_3—	246, 325	251, 367
XIX[c]	OH	7—CH_3—	247, 317	250, 358
VII*A*[d]	OH	6—$C_6H_5CH_2CH_2$—	251, 327	254, 364
VII*B*[e]	OH	6—$C_6H_5CH_2CH_2$—	243–248, 325	254, 365
XV + VII[f]	OH	6(7)—$C_6H_5CH_2CH_2$—	244–248, 323	253, 363
XV[d,g]	OH	7—$C_6H_5CH_2CH_2$		255, 355

[a] See *Experimental* for preparation. [b] Data from *Reference 8*. [c] Data from *Reference 11*. [d] *Preparation A* under *Experimental*. [e] *Preparation B* under *Experimental*. [f] *Preparation C* under *Experimental*. [g] The material was not fully characterized.

TABLE II.—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R_1	R_2	R_3	μ*M* Concn. for 50% Inhibition
I[a]	OH	$C_6H_5(CH_2)_4$—	...	30
II[b]	OH	$C_6H_5NH(CH_2)_3$—	...	800
VI	NH_2	...	$C_6H_5CH_2CH_2$—	4.2
VII[c]	OH	...	$C_6H_5CH_2CH_2$—	>200
XIII	NH_2	$C_6H_5(CH_2)_4$—	...	0.027
XVI[d]	NH_2	...	CH_3	220
XX[e]	NH_2	H	...	1100
XXI[e]	OH	H	...	19,000
XXII[b]	NH_2	$C_6H_5NH(CH_2)_3$—	...	2.2
IV	NH_2	...	—CH_2—PABG[f]	0.00086

The dihydrofolic reductase was a 45–90% ammonium sulfate fraction from pigeon liver that was isolated and assayed with 6 μ*M* dihydrofolate and 12 μ*M* TPNH in Tris buffer at pH 7.4 as previously described (14). The technical assistance of Miss Shirley Herrmann, Miss Karen Smith, and Miss Gail Westley with these assays is acknowledged. [a] Previously reported in *Reference 1*. [b] Previously reported in *Reference 14*. [c] *Preparation A*; this compound, in the presence of 12 μ*M* TPNH, showed no substrate properties at 200 μ*M* when assayed in place of 6 μ*M* dihydrofolate. [d] Prepared according to *Reference 8*. [e] From Aldrich Chemical Co. [f] PAGB = *p*-aminobenzoyl-L-glutamic acid; from Sigma Chemical Co. and corrected for 75% purity.

pteridine (VI) compared to the phenylbutylpyrimidine (XIII) or aminopterin (IV) raised the serious doubt that the phenylbutyl side chain of XIII was complexed to the same locus on the enzyme that complexed the phenethyl group of VI, even if it is assumed that the phenylethyl group of VI and the *p*-aminobenzoyl moiety of aminopterin (IV) are complexed to the same enzyme locus. This doubt was further enhanced by the following fact.

The 27-fold difference in binding between the 5-(4-phenylbutyl)-4-pyrimidinol (I) and the 5-(3-anilinopropyl)-4-pyrimidinol (II) was previously attributed to the difference in σ-values (16) between NH and CH_2 effecting the binding of the phenyl ring in a charge–transfer complex (1); this theory was additionally supported by one series of compounds (2), but not in two other series (17, 18).

Part of the enigma has been resolved by the discovery that the butyl group of 5-(*n*-butyl)-2,4-diamino-6-methylpyrimidine increases binding by a factor of 550-fold compared to 2,4-diamino-6-methylpyrimidine (XX). The *n*-butyl group can only contribute to binding by hydrophobic bonding or van der Waals forces or both; the *n*-butyl group has been shown to contribute to the binding of other 2,4,6-trisubstituted pyrimidines to dihydrofolic reductase (19). Thus, the increment in binding observed with a 5-phenylbutyl pyrimidine compared to a 5-(3-anilinopropyl)pyrimidine, which is probably due to the butyl group, *a posteriori* cannot be expected to carry over to a 6-phenethyl pteridine. In fact, the 52-fold increment in binding observed when the benzyl group is placed on the 6-methyl of 2,4-diamino-6-methylpteridine (XVI) to give VI, is the same order observed when a benzyl group is placed on the terminal carbon of a 5-*n*-propyl-2,4,6-trisubstituted pyrimidine (19).

Although 2,4-diamino-6-phenethylpteridine (VI) did not inhibit as strongly as anticipated, this discrepancy has led to the finding of extremely potent hydrophobic bonding with dihydrofolic reductase, which will be the subject of another paper (19).

EXPERIMENTAL

Melting points were determined with a Fisher-Johns apparatus and those below 230° are corrected. Ultraviolet spectra were determined in water at the pH indicated with a Perkin-Elmer 202 spectrophotometer. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B spec-

trophotometer. Paper chromatograms were run on Whatman No. 1 paper by the ascending technique in isopropyl alcohol–concentrated ammonia water–water (7:1:2) (20) (solvent A) or in *n*-butanol–morpholine–water (3:1:3) (21) (solvent B); spots were detected under ultraviolet light.

2,4-Diamino-6-phenethylpteridine (VI).—To a stirred solution of 476 mg. (2 mmoles) of X sulfate in 15 ml. of water adjusted to pH 3–4 with 10% sodium hydroxide was added at 95–100° a solution of 472 mg. (2 mmoles) of phenethylglyoxal diethyl acetal (IX) (9) in 5 ml. of ethanol over a period of 90 min. After being heated with stirring for an additional 3 hr., the mixture was acidified with 0.1 ml. of 12 *N* hydrochloric acid, then heated 15 min. more. The hot solution was filtered, then cooled. A brown precipitate (60 mg.) of X sulfate separated on cooling. The filtrate was made basic with concentrated ammonia water. The yellow product was collected on a filter and washed with water; yield, 154 mg. (29%), m.p. 232–234°. The crude product was dissolved in 30 ml. of 10% acetic acid at room temperature by stirring for 40 min. After clarification by filtration, the solution was made slightly basic with ammonia water. The product was collected and washed with boiling water to give 122 mg. (23%) of yellow product, m.p. 236–238°; $\lambda_{max.}$ (pH 1) 244 (ϵ 14,900), 285 (ϵ 4700), 339 (ϵ 10,000), 350 mμ (sh, ϵ 8800); $\lambda_{max.}$ (pH 13) 257 (ϵ 21,200), 372 mμ (ϵ 6860); $\nu_{max.}$ 3500, 3350, 3200 (NH); 1670, 1640–1625, 1590, 1560, 1540 (NH, C=C, C=N); 765, 695 cm.$^{-1}$ (C_6H_5).

The compound moved as a single spot in solvent A with R_f 0.86.

Anal.—Calcd. for $C_{14}H_{14}N_6 \cdot {}^1/_2H_2O$: C, 61.2; H, 5.50; N, 30.6. Found: C, 61.1; H, 5.18; N, 30.9.

Treatment of VI with boiling acetic anhydride, then recrystallization from ethyl acetate, gave the N^2,N^4-diacetyl derivative as a beige-yellow solid, m.p. 179–181°; $\nu_{max.}$ 3400, 3250, 3150, 3050 (NH); 1705, 1690 (C=O); 1610, 1580, 1550, 1530, 1495 cm.$^{-1}$ (NH, C=C, C=N); $\lambda_{max.}$ ($CHCl_3$) 257, 350 mμ.

Anal.—Calcd. for $C_{18}H_{18}N_6O_2$: C, 61.7; H, 5.18; N, 24.0. Found: C, 61.6; H, 5.06; N, 23.8.

2-Amino-6(7)-phenethyl-4-pteridinol (VII, XV).—*Preparation A.*—A mixture of 239 mg. (1 mmole) of XI sulfate, 164 mg. (2 mmoles) of anhydrous sodium acetate, 236 mg. (1 mmole) of IX, and 6 ml. of glacial acetic acid was stirred at room temperature for 30 min. protected from light, then refluxed for 60 min. The mixture was centrifuged, and the product was washed with water (2 × 10 ml.), then acetone (2 × 10 ml.); yield, 82 mg. (31%), $\lambda_{max.}$ (pH 13) 254, 365 mμ. When the combined mother liquor and washings were adjusted to pH 6.5 with aqueous ammonia, 52 mg. of brownish-yellow solid separated that appeared to be mainly 2-amino-7-phenethyl-4-pteridinol (XV) with $\lambda_{max.}$ (pH 13) 255, 355 mμ.

Of the 82 mg. of product (VII), 73 mg. was dissolved in 10 ml. of 1 *N* aqueous sodium hydroxide. The solution was clarified by centrifugation, then adjusted to pH 7 with 1 *N* hydrochloric acid. The yellow product was collected by centrifugation and washed with water (2 × 10 ml.), acetone (2 × 10 ml.), and ether (1 × 5 ml.); yield, 45 mg. (19%) of VII, m.p. greater than 300°; $\lambda_{max.}$ (pH 13) 254 (ϵ 23,200), 365 mμ (ϵ 7200); $\nu_{max.}$ 3480, 3300 (NH, OH); 1710, 1680, 1600, 1525 (C=O, NH, C=C, C=N); 750, 695 cm.$^{-1}$ (C_6H_5). The precipitate was dissolved in 20 ml. of 3 *N* hydrochloric acid by warming; the solution was clarified by centrifugation, then adjusted to pH 7 with aqueous sodium hydroxide. After being washed as before, the product weighed 34 mg. and had $\lambda_{max.}$ (pH 13) 254 (ϵ 23,600), 365 mμ (ϵ 7550). For analysis, precipitation from an acid solution was performed once more to give a yellow powder, the ultraviolet spectra of which remained unchanged.

The compound moved as a single spot in solvent B with R_f 0.89 and in solvent A with R_f 0.53 or 0.57 in two runs.

Anal.—Calcd. for $C_{14}H_{13}N_5O$: C, 62.9; H, 4.90; N, 26.2. Found: C, 62.7; H, 5.11; N, 26.0.

Preparation B.—A mixture of 130 mg. (0.49 mmole) of VI and 7 ml. of 6 *N* hydrochloric acid was refluxed for 30 min. The filtered mixture was adjusted to pH 7 with 30% aqueous sodium hydroxide. The precipitate was collected on a filter and washed well with water; yield, 75 mg. (58%). (See Table I for ultraviolet spectra.) The compound moved as a single spot with identical mobility to *Preparation A* in solvent A with R_f 0.57 or 0.56 in two runs.

Preparation C.—To a hot solution of 478 mg. of XI sulfate (2 mmoles) in 15 ml. of 4 *N* aqueous hydrochloric acid was added a solution of 427 mg. (2 mmoles) of IX in 2 ml. of 95% ethanol. The mixture was refluxed with magnetic stirring for 1 hr., filtered hot through a glass wool pad, then adjusted to pH 7 with concentrated ammonia water; yield, 276 mg. (52%). (See Table I for ultraviolet data.) The compound moved as a single spot with mobility identical to *Preparation A* in solvent A with R_f 0.54 or 0.58 in two runs.

2 - Amino - 4 - chloro - 5 - (4 - phenylbutyl) - 6-methylpyrimidine (XII).—A mixture of 1.5 Gm. (5.83 mmoles) of I and 4.5 ml. of phosphorus oxychloride was heated under a condenser protected from moisture in an oil bath preheated to 110°. After 45 min., the resulting solution was poured into a stirred mixture of 50 Gm. of crushed ice and 20 ml. of petroleum ether (b.p. 30–60°). The gummy product solidified upon trituration. This white solid was collected on a filter, then transferred to a mortar, ground with water, and filtered. This process was repeated 10 times until an aqueous washing was no longer acidic. After being dried over KOH for 15 hr., the crude product weighed 1.77 Gm. (110%), m.p. 105–110°, and was used for the following amination.

2,4 - Diamino - 5 - (4 - phenylbutyl) - 6 - methylpyrimidine (XIII).—A mixture of 1.5 Gm. of the above crude chlorpyrimidine (XII) and 40 ml. of methanolic ammonia, prepared by saturating methanol with ammonia at 0°, was heated in a steel bomb in an oil bath at 150° for 24 hr. After clarification by filtration, the solution was spin-evaporated *in vacuo*. The residue was spin-evaporated with absolute ethanol (2 × 5 ml.), leaving a white hydrochloride; yield, 2.05 Gm., m.p. 202–205°. This solid was dissolved in 60 ml. of warm 10% aqueous acetic acid. The solution was clarified by filtration, then brought to about pH 12 with 25% sodium hydroxide solution. The white precipitate was collected on a filter and washed with water; yield, 0.985 Gm., m.p. 160–163° (78% based on the 4-pyrimidinol, I). Recrystallization from ethanol by

the addition of water gave 0.780 Gm. (62%), m.p. 167–169°. Another recrystallization from the same solvent system gave 0.704 Gm. (56%), m.p. 169–171°; $\nu_{max.}$ 3500, 3350, 3200 (NH); 1645, 1620, 1570 (NH, C=N, C=C); 750, 695 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}$ (pH 1) 277 mμ (ε 7600); $\lambda_{max.}$ (pH 7) 280 mμ (ε 5800); $\lambda_{max.}$ (pH 13) 289 mμ (ε 6800).

Anal.—Calcd. for $C_{15}H_{20}N_4$: C, 70.3; H, 7.86; N, 21.9. Found: C, 70.5; H, 7.80; N, 21.7.

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Synthesis and Anticonvulsant Activity of Some Alkyl Esters of 6-Chloro-2-sulfamoylbenzoic Acid

By GLENN H. HAMOR and NAIM FARRAJ

The application of the Rule of Six for the estimation of steric effects to a series of alkyl esters of 4-amino-2-sulfamoylbenzoic acid possessing pronounced anticonvulsant activity shows the highly hindered isopropyl and *sec*-butyl esters to be much more potent than the less hindered methyl, ethyl, and *n*-propyl compounds. In order to provide further information concerning steric factors and anticonvulsant properties, four alkyl 6-chloro-2-sulfamoylbenzoates, which contain the chlorine atom in the shielding position adjacent to the alkoxycarbonyl group, were synthesized: the methyl, ethyl, *n*-propyl, and isopropyl esters. Preliminary pharmacological results indicate that the isopropyl 6-chloro-2-sulfamoylbenzoate produces strong antielectroshock effects in mice which are quite specific, with no other CNS activity except at high doses in the gross observations.

RECENT work has shown that alkyl esters of 2-sulfamoylbenzoic acid and 4-amino-2-sulfamoylbenzoic acid (I) possess marked anticonvulsant activity as indicated by their prevention of the effect of strychnine or maximal electroshock in mice (1–3).

The results (heretofore unpublished) of pharmacological testing of a series of alkyl 4-amino-2-sulfamoylbenzoates are shown in Table I. The property of these compounds in preventing strychnine-induced convulsions is quite unusual. In fact, Horrom and Lynes in 1963, reporting the anticonvulsant potency of two benzamide compounds, stated: "to our knowledge no other compounds are known to antagonize strychnine-induced convulsions at doses which produce little or no neurological symptoms" (4). The methyl, ethyl, and *n*-propyl esters in Table I are considerably less potent than the isopropyl compound.

If the intact ester group is necessary for anticonvulsant activity, then steric effects in the

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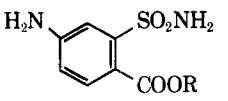
TABLE I.—ANTICONVULSANT ACTIVITIES OF ALKYL 4-AMINO-2-SULFAMOYLBENZOATES[a]



Compd.	R	Antielectroshock ED_{50}, Mice, mg./Kg.	Antistrychnine ED_{50}, Mice, mg./Kg.
XIII	CH_3	48	115
XIV	C_2H_5	34	80
XV	*n*-C_3H_7	45	125
II	*i*-C_3H_7	13	46
XVI	*n*-C_4H_9	70	130
XVII	*s*-C_4H_9	13.8	83
XVIII	*n*-C_5H_{11}	30	122
XIX	$CH(C_2H_5)_2$	82	142
XX	*n*-C_6H_{13}	95	175
XXI	Mephenesin	140	355

[a] The pharmacological testing was performed by Smith Kline & French Laboratories, Philadelphia, Pa.