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Porphyria-Inducing Activity of a Series of Pyridine and Dihydropyridine Compounds. Investigation in a Cell Culture System

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A series of analogs of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) was prepared and tested for porphyria-inducing activity in chick embryo liver cells. One of the analogs tested, viz. 3,5-di-*tert*-butoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine, was found to be highly active despite the absence of a 4-alkyl substituent. It was concluded that *tert*-butoxycarbonyl groups are resistant to enzymic hydrolysis and that compounds containing such groups are resistant to inactivation by chick embryo liver cells. Several analogs of DDC were found with considerably higher activity. These should be useful in inducing high levels of δ-aminolevulinic acid synthetase prior to undertaking the isolation of the enzyme.

The overproduction of porphyrins in liver cells induced by a variety of drugs results from an enhanced synthesis of the first enzyme in the porphyrin biosynthetic pathway, viz. δ-aminolevulinic acid (ALA) synthetase.[1-4] As a result of recent study it was concluded that for a chemical to induce porphyria it must remain in the liver for a period of at least several hours in order to induce and maintain high levels of ALA synthetase.[5] Consequently it is clear that a porphyria-inducing drug must possess chemical features that prevent it from being rapidly metabolized and inactivated by the liver. Previous studies on the relationship between chemical structure and porphyria-inducing activity of a series of compounds related to 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC, **1a**) and the related pyridine **2a** led to the suggestion that the underlying critical feature for activity was an ester group which is sterically hindered from hydrolysis.[6-9] Recently Parker and Weinstock[10] have studied the metabolism of 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester in the dog. Principle urinary metabolites were isolated and identified and showed that the above compound is aromatized and hydrolyzed during biotransformation. The two *o*-methyl substituents adjacent to each ethoxycarbonyl group of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine and related compounds were thought to protect the ethoxycarbonyl group from hydrolysis to yield the inactive free diacid. This explained the inactivity of 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (**1b**). If this interpretation was correct, it appeared possible that active analogs of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine might be obtained in which the ester group could be protected from enzymic hydrolysis by other means than two *o*-alkyl substituents. It was thought that replacement of the ethyl ester substituent by more bulky groups such as *tert*-butyl ester or benzyl ester groups might yield active analogs in compounds in which the 4-methyl substituent of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine was replaced by hydrogen. To test this idea the following four analogs of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine were synthesized: 3,5-di-*tert*-butoxycarbonyl-1,4-dihydro-2,6-dimethyl (**1h**) and the corresponding pyridine **2h,** 3,5-dibenzyloxycarbonyl-1,4-dihydro-2,6-dimethyl (**1f**) and the corresponding pyridine **2f** and their activity compared with that of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) and 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (OX-DDC, **2a**). For comparative purposes the analogs containing 4-methyl substituents were synthesized (**1g, 1i, 2g, 2i**).

For further progress in studies of the mechanism of ALA synthetase induction by drugs, it would be desirable to isolate this enzyme in pure form. This would enable an antibody to be prepared to this enzyme and would facilitate direct measurement of ALA synthetase. Before attempting the isolation of this enzyme it would be desirable to induce high levels of this enzyme. For this reason a study of DDC analogs was undertaken to find a drug of high activity. The following drugs were synthesized and their porphyrin-inducing activity measured: 3,5-dimethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (**1e**) and the corresponding pyridine **2e.**

In previous studies the porphyria-inducing activity of DDC analogs was assessed by a qualitative procedure which involved the observation of the fluorescence intensity of chick embryo liver cells 20 hr after incubation with a drug. In the present study the amount of porphyrin in chick embryo liver cells and growth medium was measured quantitatively.

Experimental Section

All melting points are uncorrected. The structures of all compounds are supported by their ir, uv, and NMR spectra. Spectra were recorded on Perkin-Elmer Model 173E, Unicam SP800 and a Varian A-60 spectrometer (Me_4Si). All compounds were analyzed for C, H, and N and the results were within ±0.4% of the theoretical value. Benzyl acetoacetate was obtained as a gift from Dr. S. F. MacDonald, N.R.C., Ottawa. *tert*-Butyl acetoacetate and methyl acetoacetate were purchased from Aldrich Chemical Co.

Culture of Chick Embryo Cells on Petri Dishes. The procedure of Granick[4] was used with the following modifications. The enzyme solution for preparing the cell suspension, from four chick embryo livers, consisted of 5 ml of 2.5% trypsin in saline (Microbiological Associates) and 5 ml of magnesium and calcium free Earle's solution. The suspension was centrifuged at low speed and the supernatant discarded. The sediment was resuspended in culture medium (Eagle basal medium, Microbiological Associates,

Table I. Structure and Physical Properties of Porphyria-Inducing Dihydropyridines and Pyridines

$$R_1CHO + 2CH_3COCH_2CO_2R + NH_3 \longrightarrow \textbf{1a–i} \xrightarrow{[O]} \textbf{2a–i}$$

(1a–i: 4-R_1-3,5-bis(CO_2R)-2,6-dimethyl-1,4-dihydropyridine; 2a–i: 4-R_1-3,5-bis(CO_2R)-2,6-dimethylpyridine)

No.	R	R_1	Mp or bp (mm), °C	Crystn solvent	Yield, %	Formula	Analyses
1a	C_2H_5	CH_3	130–131[a]	MeOH	78	$C_{14}H_{21}NO_4$	
1b	C_2H_5	H	178–181[b]	EtOH	80	$C_{13}H_{19}NO_4$	
1c	C_2H_5	C_2H_5	108–110[c]	EtOH	70	$C_{15}H_{23}NO_4$	
1d	CH_3	H	225–227[d]	MeOH	68	$C_{11}H_{15}NO_4$	C, H, N
1e	CH_3	CH_3	155–157[e]	MeOH	72	$C_{12}H_{17}NO_4$	C, H, N
1f	$CH_2C_6H_5$	H	119–121	EtOH	60	$C_{23}H_{23}NO_4$	C, H, N
1g	$CH_2C_6H_5$	CH_3	125–127	EtOH	63	$C_{24}H_{25}NO_4$	C, H, N
1h	$C(CH_3)_3$	H	142–144	EtOH	66	$C_{17}H_{27}NO_4$	C, H, N
1i	$C(CH_3)_3$	CH_3	166–168	EtOH	65	$C_{18}H_{29}NO_4$	C, H, N
2a	C_2H_5	CH_3	138 (0.4)[f]		75	$C_{14}H_{19}NO_4$	
2b	C_2H_5	H	71–72[g]	EtOH	62	$C_{13}H_{17}NO_4$	
2c	C_2H_5	C_2H_5	130 (0.45)[h]		65	$C_{15}H_{21}NO_4$	
2d	CH_3	H	102–104[i]	Ether–*n*-pentane	73	$C_{11}H_{13}NO_4$	C, H, N
2e	CH_3	CH_3	78–80[e]	Ether–*n*-pentane	78	$C_{12}H_{15}NO_4$	C, H, N
2f	$CH_2C_6H_5$	H	88–90	EtOH	50	$C_{23}H_{21}NO_4$	C, H, N
2g	$CH_2C_6H_5$	CH_3	50–52	Ether–*n*-pentane	53	$C_{24}H_{23}NO_4$	C, H, N
2h	$C(CH_3)_3$	H	108–110	MeOH	35	$C_{17}H_{25}NO_4$	C, H, N
2i	$C(CH_3)_3$	CH_3	53–55	Ether–*n*-pentane	40	$C_{18}H_{27}NO_4$	C, H, N

[a]Lit. mp 131°: A. Hantzsch, *Justus Liebigs Ann. Chem.*, **215**, 1 (1882). [b]Lit.[13] mp 175–180°. [c]Lit. mp 110°: F. Engelmann, *Justus Liebigs Ann. Chem.*, **231**, 37 (1885). [d]Lit. mp 219–220°: T. B. H. McMurry and M. T. Richardson, *J. Chem. Soc. C*, 1804 (1967). [e] T. J. vanBergen and R. K. Kellogg, *J. Am. Chem. Soc.*, **94**, 8451 (1972). [f]Lit.[6] 138° (0.4 mm). [g]Lit.[14] 130° (0.45 mm). [i]Lit. mp 100–102°: T. J. van Bergen and R. M. Kellogg, *J. Org. Chem.*, **36**, 978 (1971).

Inc.). Bovine serum (Pentax Inc., Winley-Morris Co. Ltd.) was used throughout our experiments.

Measurement of the Porphyria-Inducing Effect of Drugs. The culture medium was removed from each petri dish after 24 hr of incubation and replaced with 4 ml of fresh medium. Porphyria-inducing drugs were dissolved in 95% redistilled ethanol (5 μl) and added to the liver cells by means of disposable microliter pipets. After the addition of porphyria-inducing drugs the petri dishes were returned to the incubator for approximately 24 hr. The porphyrin content of cells and media was determined. After determining the protein contents of cells[11] the results were expressed as μg of porphyrin/mg of protein. Each drug was tested in triplicate at a particular concentration.

Preparation of Dihydropyridines. Dihydropyridines were synthesized according to the procedure of Loev and Snader[12] by condensation of a β-oxo ester (0.1 mol) with an aldehyde (0.05 mol) and concentrated ammonium hydroxide (14.3 *N*, 10 ml) in boiling ethanol (20 ml) for 2 hr. In some cases when only small quantities of the β-oxo ester were available the reactions were carried out on a smaller scale. Upon cooling, the solution was poured into cold water (150 ml) whereupon the dihydropyridine separated from solution. The dihydropyridine was extracted with ether. The ether solution washed successively with 10% sodium hydroxide, water, 5% hydrochloric acid, and water. The ether solution was dried (sodium sulfate) and evaporated and the residue crystallized twice. In the case of 3,5-dimethoxycarbonyl-1,4-dihydro-2,4-dimethylpyridine, the crude product separated from the ethanol solution after cooling and was removed by filtration and crystallized. The properties of the dihydropyridines are reported in Table I.

Conversion of Dihydropyridine to Pyridines. 3,5-Dimethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine and 3,5-dimethoxy-1,4-dihydro-2,4,6-trimethylpyridine were oxidized to the corresponding pyridines with a mixture of glacial acetic acid and sodium nitrite by the method of Loev and Snader.[12] 3,5-Dibenzyloxycarbonyl-1,4-dihydro-2,6-dimethylpyridine and 3,5-dibenzyloxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine were oxidized to the corresponding pyridine with a mixture of nitric acid, sulfuric acid, and water by the procedure of Singer and McElvain.[13]

3,5-Di-*tert*-butoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine and 3,5-di-*tert*-butoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridines were oxidized to the corresponding pyridines with chloranil according to the procedure of Braude.[14] The properties of the pyridines are reported in Table I.

Results and Discussion

The first analogs investigated for porphyria-inducing activity were 3,5-di-*tert*-butoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (**1i**), 3,5-di-*tert*-butoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (**1h**), 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC, **1a**), and 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (**1c**). The results which are shown in Figure 1 reveal that the *tert*-butoxycarbonyl compounds are considerably more potent than DDC (**1a**) and its closely related 4-ethyl analog **1c**. The high activity of the dihydropyridine (**1h**) which lacks a 4-methyl substituent was of particular interest in view of the fact that all previous aromatic compounds which we have shown to be active contained ethyl ether groups protected by two *o*-alkyl substituents. It would thus appear that the *tert*-butoxycarbonyl groups are resistant to enzymic hydrolysis and that compounds containing these groups resist inactivation by liver cells. In our next series of experiments (Figure 2) the activity of the corresponding pyridines (**2i, 2h, 2a,** and **2c**) was investigated. All compounds showed marked activity and it was of considerable interest that the *tert*-butoxycarbonyl derivative **2h** which lacked a 4-methyl substituent showed marked activity.

Replacement of the ethoxycarbonyl substituents of DDC (**1a**) and the corresponding pyridine derivative **2e** with benzyloxycarbonyl substituents (1 and **2g**) led to decreased activity (Figure 3). When the 4-methyl substituents of benzylcarbonyl compounds (1 and **2g**) were replaced by hydrogen, there was virtually no retention of activity in these analogs (1 and **2f**). It thus appears likely that benzylcarbonyl

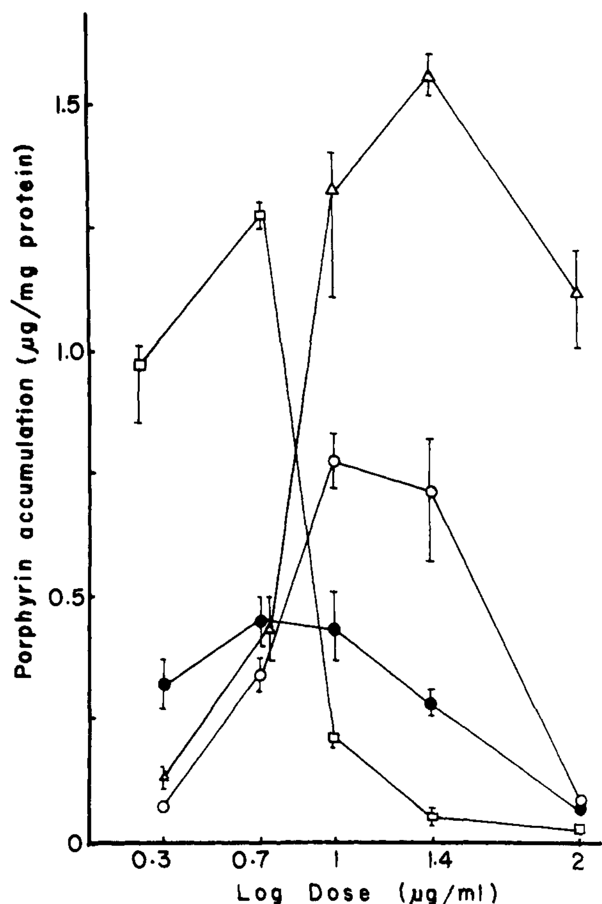

Figure 1. Dose-response curve for 3,5-di-*tert*-butoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (**1h**, △—△); 3,5-di-*tert*-butoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (**1i**, □—□); 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (**1a**, ○—○); and 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (**1c**, ●—●).

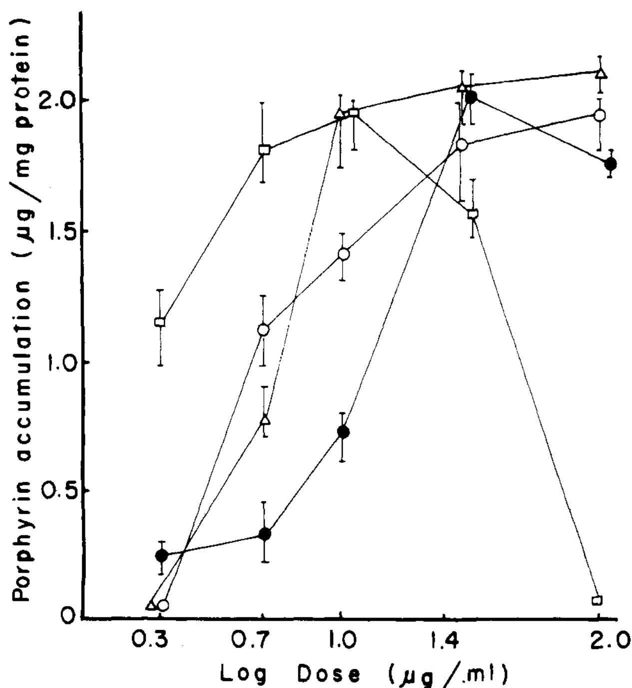

Figure 2. Dose-response curves for 3,5-di-*tert*-butoxycarbonyl-2,6-dimethylpyridine (**2h**, △—△); 3,5-di-*tert*-butoxycarbonyl-2,4,6-trimethylpyridine (**2i**, □—□); 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (**2a**, ○—○); and 3,5-diethoxycarbonyl-2,6-dimethyl-4-ethylpyridine (**2c**, ●—●).

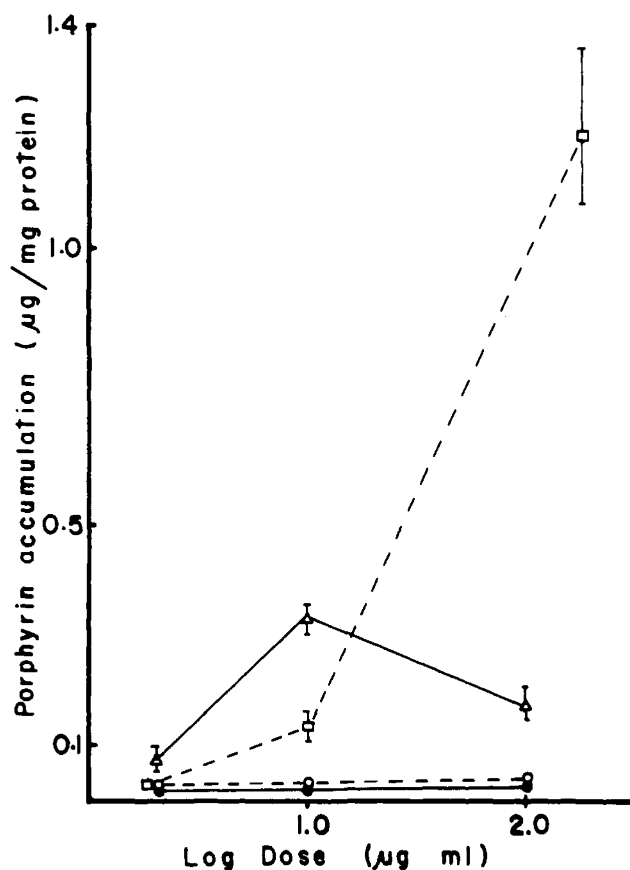

Figure 3. Dose-response curves for 3,5-dibenzyloxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (**1f**, ●—●); 3,5-dibenzyloxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (**1g**, △—△); 3,5-dibenzylcarbonyl-2,6-dimethylpyridine (**2f**, ○- - -○); and 3,5-dibenzyloxycarbonyl-2,4,6-trimethylpyridine (**2g**, □- - -□).

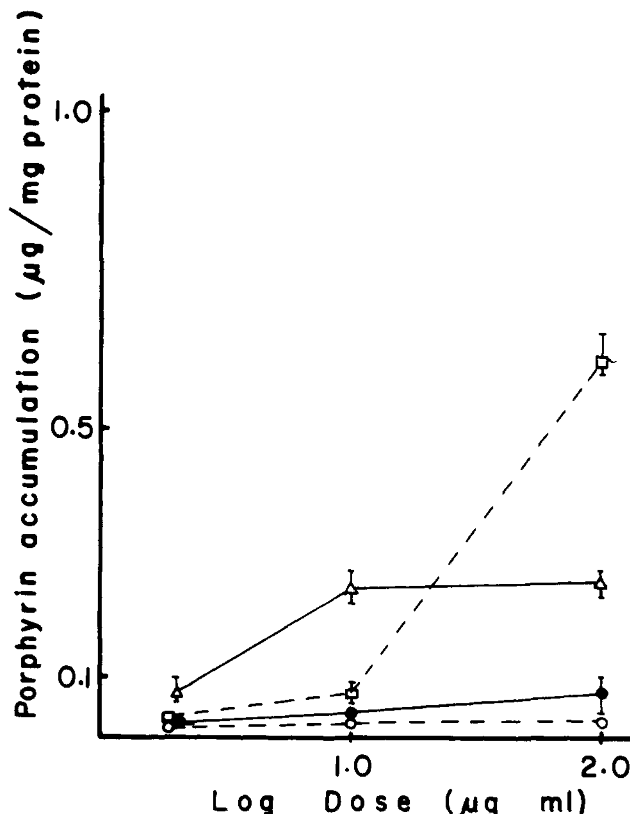

Figure 4. Dose-response curves for 3,5-dimethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (**1d**, ●—●); 3,5-dimethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (**1e**, △—△); 3,5-dimethoxycarbonyl-2,6-dimethylpyridine (**2d**, ○- - -○); and 3,5-dimethoxycarbonyl-2,4,6-trimethylpyridine (**2e**, □- - -□).

groups of compounds (1 and **2f**) are hydrolyzed by liver enzymes to inactive diacids. The difference in activity of pyridines and dihydropyridines containing benzyl and *tert*-butyl ester groups is probably due to their different hydrolysis mechanism and rate.[15]

Replacement of the ethoxycarbonyl substituents of DDC (**1a**) and the corresponding pyridine derivative **2a** with the methoxycarbonyl substituent (1 and **2e**) resulted in decreased activity (Figure 4). When the 4-methyl substituent of methoxycarbonyl compounds was replaced by hydrogen (1 and **2d**) the compounds showed no activity (Figure 4). This inactivity was similar to that previously found when the 4-methyl substituents of DDC (**1a**) and OX-DDC (**2a**) were replaced by hydrogen.[3]

It was concluded that the *tert*-butoxycarbonyl compounds would induce higher levels of ALA synthetase in chick embryo than DDC (**1a**). For this reason these compounds should be valuable in inducing higher levels of ALA synthetase in chick embryo liver prior to attempting to isolate this enzyme.

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Adenosine Cyclic 3′,5′-Monophosphate Phosphodiesterase Inhibitors. 2. 3-Substituted 5,7-Dialkylpyrazolo[1,5-*a*]pyrimidines

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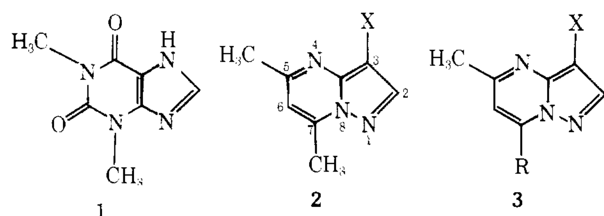
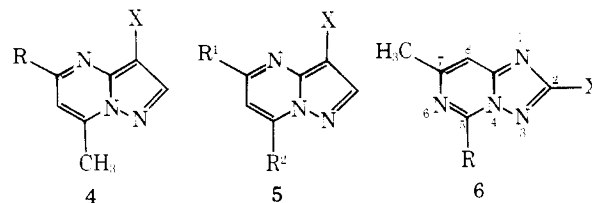
A number of 3-bromo-, 3-nitro-, and 3-ethoxycarbonyl-5,7-dialkylpyrazolo[1,5-*a*]pyrimidines were synthesized and screened as in vitro cAMP phosphodiesterase inhibitors. The condensation of 3-aminopyrazole with symmetrical β-diketones (acetylacetone, heptane-3,5-dione, etc.) afforded symmetrical dialkylpyrazolo[1,5-*a*]pyrimidines (**5**). The reaction of 3-aminopyrazole with unsymmetrical β-diketones (hexane-2,4-dione, heptane-3,5-dione, etc.) gave a mixture of 5-methyl-7-alkylpyrazolo[1,5-*a*]pyrimidine (**3**) and 5-alkyl-7-methylpyrazolo[1,5-*a*]pyrimidines (**4**). The technique for the separation of **3** from **4** is described. The inhibition constants, α (the ratio of the molar I_{50} of theophylline to the molar I_{50} of the test compounds), were subjected to a Hansch correlation analysis. The results indicated that PDE isolated from beef heart tissue was most sensitive to changes in the length of the alkyl group in the 5 position of the pyrazolo[1,5-*a*]pyrimidine ring, whereas the PDE isolated from rabbit lung tissue was more sensitive to changes in the length of the 7-alkyl group. Experimentally and theoretically, the *n*-propyl group was found to approximate the ideal size for the alkyl group in both the 5 and 7 positions; 5,7-di-*n*-propyl-3-ethoxycarbonylpyrazolo[1,5-*a*]pyrimidine (**5e**) was the most potent inhibitor of both lung and heart PDE.

It has been reported that the pharmacological effects of theophylline (**1**) might be due to the inhibition of 3′,5′-AMP phosphodiesterase (PDE) by this compound.[1] Rose and coworkers[2] demonstrated that certain dialkyl derivatives of the triazolo[2,3-*c*]pyrimidines (**6**) [which bear some structural resemblance to theophylline (**1**)] inhibited PDE from lung tissue to a greater extent than **1**. More importantly, derivatives of **6** effectively protected guinea pigs from histamine-induced bronchospasms. Bronchial constriction was known to be accompanied by changes in the intracellular concentrations of 3′,5′-AMP. Others have explored the inhibition of PDE by certain pyrazolo[3,4-*b*]pyridines which were also found to be effective as in vivo antidiabetic agents.[3-5]





We reported on the in vitro PDE inhibition of the dimethyl derivatives of the structurally similar pyrazolo[1,5-*a*]pyrimidine (**2**) in a recent publication.[6] It was found that one of the better PDE inhibitors, 3-bromo-5,7-dimethylpyrazolo[1,5-*a*]pyrimidine (**2d**, ICN 3009), produced an immediate and moderately prolonged increase in the cardiac output of anaesthetized dogs.[7] The observed lack of