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STUDIES OF THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND PORPHYRIA-INDUCING ACTIVITY—III*

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Abstract—A series of analogues of 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine has been prepared and tested for porphyria-inducing activity in chick embryo liver cells. These studies show that the critical feature for activity in these compounds is an ethoxycarbonyl substituent in a pyridine, dihydropyridine, or benzene ring with two *ortho*-alkyl substituents. The porphyria-inducing activity detected in 2,4,6-trimethylbenzamide suggests a relationship between this group of compounds and those of the allylisopropylacetamide group.

THE STUDIES of Granick and co-workers[1–4] showed that the overproduction of porphyrins in liver cells, induced by 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC; Fig. 1a) and a variety of other drugs, results from an enhanced synthesis of the first enzyme in the porphyrin biosynthetic pathway, viz. δ-aminolaevulic acid synthetase. A study of Fisher–Hirschfelder–Taylor models and the u.v.

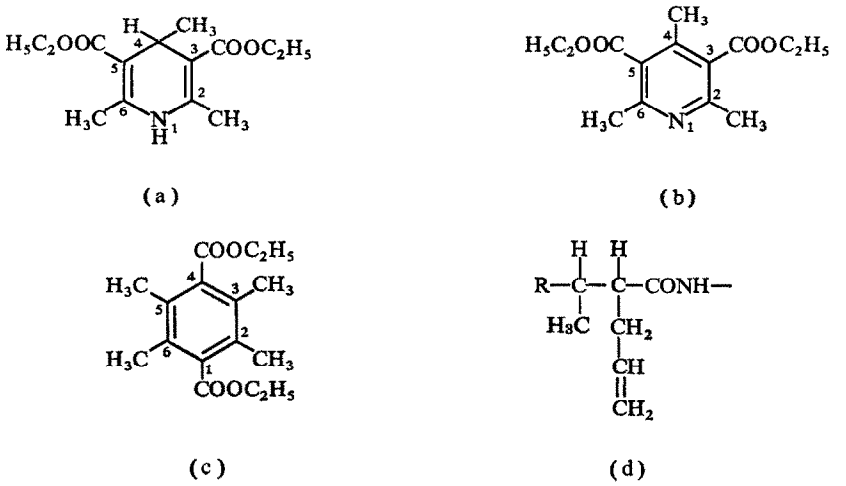

FIG. 1. Structure of porphyria-inducing compounds.

absorption spectra of analogues of DDC and the corresponding pyridine (Fig. 1b) indicated that the 2-, 4-, and 6-methyl substituents cause a twisting of the 3- and 5-ethoxycarbonyl substituents out of the plane of the ring. It was suggested[5] that this

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nonplanar relationship between the ethoxycarbonyl substituents and the pyridine ring was necessary for optimal porphyria-inducing activity. In this paper the validity of this idea has been investigated by synthesizing a series of pyridine and dihydropyridine compounds and determining their porphyria-inducing activity. The activity of a series of analogues of DDC has been investigated in which the dihydropyridine ring is replaced by a benzene ring. These studies suggested a possible relationship between the DDC and allylispropylacetamide (AIA) group of porphyria-inducing compounds.

EXPERIMENTAL

Ultraviolet absorption spectra were determined in absolute ethanol, in a Bausch & Lomb Spectronic 505 spectrophotometer. The infrared spectra were obtained with a Perkin–Elmer 137 sodium chloride spectrophotometer; solid compounds were mulled with Nujol, and liquid samples were used as liquid films. Nuclear magnetic resonance spectra (NMR) were determined in carbon tetrachloride; external reference, tetramethylsilane; oscillator frequency 60 Mc/s. Melting points are uncorrected.

Determination of porphyria-inducing activity of chemicals

Chick embryo liver cells were cultured on cover slips according to the procedure of Granick:[4] a mixture of crystallized and lyophilized trypsin (100 mg) and Pangestin (30 mg; Difco) in calcium- and magnesium-free Earle's medium (6 ml) was used to dissociate the liver cells of two chick embryos, 16–17 days old. About 3×10^5 cells of the resulting suspension were added to vials (18 × 60 mm) containing a 16-mm cover slip. Each vial contained Eagle's basal medium (1 ml) supplemented with 10 per cent fetal bovine serum, 1 per cent glutamine, and the antibiotics penicillin, streptomycin, and mycostatin. After the cells were incubated for 24 hr in an atmosphere of 5% CO_2 in air, forming a monolayer on the cover slip, the medium was renewed, drugs added in ethanol (1–5 μl), and the vials reincubated for 24 hr. The cover slip was then removed and examined in the fluorescence microscope (Table 2). The cultures of chick embryo cells to which ethanol (1–5 μl) was added exhibited no fluorescence. Fluorescence intensity was scored as follows: 4, all colonies fluoresce intensely; 3, most colonies fluoresce intensely; 2, most colonies fluoresce partially; 1, some colonies fluoresce partially.

Synthesis of pyridine and dihydropyridine compounds

For the synthesis of pyridine and dihydropyridine compounds, standard methods or modifications of standard methods were used. Details of the melting points, boiling points, and u.v. and i.r. spectra are recorded in Table 1.

Synthesis of ethyl 2,4,6-trimethylbenzoate

2,4,6-Trimethylbenzoic acid (Aldrich Chemical) was converted to the ethyl ester, b.p. 70°/0·01 mm by the method of Newman.[6] Newman records b.p. 115°/6·0–6·5 mm. The NMR spectrum contains a triplet at $\tau = 8{\cdot}66$ (CH_3 in ester), a singlet at $\tau = 7{\cdot}77$ (CH_3 on ring), a quartet at $\tau = 5{\cdot}74$ (CH_2 in ester) and a singlet at $\tau = 3{\cdot}31$ (hydrogens on ring). The relative peak areas were 3:8·6:2:2.

TABLE 1. PYRIDINE AND DIHYDROPYRIDINE DERIVATIVES

Compound	Reference	m.p. (°)	b.p. (°)	Ultraviolet spectrum λmax.	Ultraviolet spectrum $\epsilon \times 10^{-3}$	Ultraviolet spectrum λmax.	Ultraviolet spectrum $\epsilon \times 10^{-3}$	Infrared spectrum (cm^{-1}) Secondary amine	Infrared spectrum (cm^{-1}) Carbonyl group	Infrared spectrum (cm^{-1}) Pyridine ring
3,5-Diethoxycarbonyl-4-methylpyridine*			116/0·05 mm	264	1·86				1720	1565
3-Ethoxycarbonyl-2,4,6-trimethylpyridine†	17, 18		83/0·3 mm; 250–260/760 mm	267	3·65				1720	1500 and 1595
3,5-Diethoxycarbonylpyridine	19	50·5–51		269	2·08				1720	1597
3-Ethoxycarbonyl-2,4,6-trimethyl-5-pyridinecarboxylic acid	17	155–156		272	3·34				1715	1570
3,5-Diethoxycarbonyl-1,4-dihydro-4-methyl-2,6-diethylpyridine‡		112–114·5		235	17·8	353	7·8	3350	1700	
3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-triethylpyridine§		78–80		236	17·5	350	7·55	3350	1700	

* Potassium 2,4,6-trimethyl-3,5-pyridinedicarboxylate[20] was oxidised to the potassium salt of the tetracarboxylic acid.[21] The procedure[21] for the isolation of the tetracarboxylic acid from the potassium salt was modified as follows. The aqueous solution of the potassium salt was passed through a column of Dowex 50-X8 (H^+ form) and the eluate evaporated to dryness on a rotatory evaporator. The residue was extracted into ether (Soxhlet) and the ether evaporated. The residue had m.p. 188° (Hantzsch[21] records m.p. 199°); 51·0 mg of the tetracid, dried for 2 hr at 111°, required 38·3 ml of 0·02 N NaOH; calculated for tetraacid, 37·9 ml. The tetracarboxylic acid was decarboxylated[22] to 4-methyl-3,5-pyridinedicarboxylic acid, m.p. 280–1° (decomp., fast heating). Wolff[22] records m.p. 282–4° (decomp., fast heating); 45·35 mg of the diacid, dried for 1·5 hr at 111° required 5·0 ml of 0·1 N NaOH. Calculated for diacid, 5·0 ml. The diester was prepared by passing dry hydrogen chloride into an ethanolic solution of the diacid and refluxing the mixture for 4 hr. The NMR spectrum of the purified diester contains a triplet at $\tau = 8{\cdot}57$ (CH_3 in ester group), a singlet at $\tau = 7{\cdot}26$ (CH_3 on ring), a quartet at $\tau = 6{\cdot}60$ (CH_2 in ester group), and a singlet at $\tau = 1{\cdot}03$ (hydrogens on ring). The relative peak areas were 6·1:3:4:1·9 respectively. The picrate was prepared by refluxing 3,5-diethoxycarbonyl-4-methylpyridine (100 mg) with a saturated solution of picric acid in ethanol (1 ml). On cooling, an oil separated, which crystallised upon trituration with ether. On recrystallisation, the picrate was obtained as needles, m.p. 75·5–76·5°. Calculated for $C_{18}H_{18}N_4O_4$: C, 46·35; H, 3·9; N, 12·0. Found C, 46·0; H, 4·45; N, 12·1.

† The picrate was prepared by refluxing the pyridine (200 mg) with a saturated solution of picric acid in ethanol (1 ml). On cooling, the product separated and was recrystallised from ethanol, yielding needles, m.p. 151–2°. Calculated for $C_{17}H_{18}O_9N_4$: C, 48·3; H, 4·3. Found: C, 48·2; H, 4·1.

‡ The dihydropyridine was prepared by passing dry ammonia gas into a solution of acetaldehyde (0·021 mole) and ethyl propionylacetate[23] (0·042 mole) in alcohol (15 ml) for 0·5 hr and keeping the solution at room temperature for an additional 12 hr. After heating on a boiling water-bath for 2 hr and then removing the alcohol, the residue was dissolved in ether and washed with dilute hydrochloric acid and water. The ether was removed and the dihydropyridine crystallised from *n*-heptane as white needles (yield 10·5%). Calculated for $C_{16}H_{25}O_4N$: C, 65·1; H, 8·5. Found: C, 65·1; H, 8·0.

§ This compound was prepared in a similar manner to the above but propionaldehyde replaced acetaldehyde. The dihydropyridine crystallised from *n*-heptane (yield 6%). Calculated for $C_{17}H_{27}O_4N$: C, 66·0; H, 8·8; N, 4·5. Found: C, 66·5; H, 8·5; N, 4·55.

Synthesis of di-esters of 2,3,5,6-*tetramethylterephthalic acid*

The method of Newman[6] for the esterification of sterically hindered acids was used. 2,3,5,6-Tetramethylterephthalic acid (1 g; Aldrich Chemical) was dissolved in 16 ml of 100% sulphuric acid; after standing at room temperature for a few minutes, the solution was poured into an excess (40 ml) of the appropriate cold alcohol. The product was isolated as previously described,[6] crystallized and identified by C, H analysis and by NMR spectroscopy. The yield, properties, and analysis of each ester are reported below. The yields refer to the pure crystalline esters.

Diethyl 2,3,5,6-*tetramethylterephthalate*

Obtained in 16 per cent yield, as white crystals from a mixture of carbon tetrachloride and *n*-heptane; m.p. 157–9°. *Anal.* Calculated for $C_{16}H_{22}O_4$: C, 69·0; H, 8·0. Found: C, 69·1; H, 7·6%. The NMR spectrum contains a triplet at $\tau = 8{\cdot}65$ (CH_3 in ester), a singlet at $\tau = 7{\cdot}88$ (CH_3 in ring), and a quartet at $\tau = 5{\cdot}72$ (CH_2 in ester). The relative peak areas were 3:5·8:2.

Dimethyl 2,3,5,6-*tetramethylterephthalate*

Obtained in 12 per cent yield, as white crystals from a mixture of carbon tetrachloride and *n*-heptane, m.p. 161–3°. *Anal.* Calculated for $C_{14}H_{18}O_4$: C, 67·2; H, 7·25. Found: C, 67·0; H, 7·1. The NMR spectrum contains a singlet at $\tau = 7{\cdot}90$ (CH_3 on ring) and a singlet at $\tau = 6{\cdot}18$ (CH_3 in ester). The relative peak areas were 2·05:1.

*Di-*n*-propyl* 2,3,5,6-*tetramethylterephthalate*

Obtained in 16 per cent yield, as white crystals from *n*-heptane, m.p. 113–5°. *Anal.* Calculated for $C_{18}H_{26}O_4$: C, 70·6; H, 8·55. Found: C, 71·1; H, 8·15. The NMR spectrum contains a triplet at $\tau = 8{\cdot}99$ (CH_3 in ester), a singlet at $\tau = 7{\cdot}87$ (CH_3 on ring), and a triplet at $\tau = 5{\cdot}82$ (O—CH_2 in ester). The relative peak areas were 3:6·2:2.

Di-isopropyl 2,3,5,6-*tetramethylterephthalate*

Obtained in 7 per cent yield, as white crystals from a mixture of carbon tetrachloride and *n*-heptane, m.p. 195–6°. *Anal.* Calculated for $C_{18}H_{26}O_4$: C, 70·6; H, 8·55. Found: C, 70·1; H, 8·2. The NMR spectrum contains a doublet at $\tau = 8{\cdot}66$ (CH_3 in isopropyl group) and a singlet at $\tau = 7{\cdot}87$ (CH_3 on ring). The relative peak areas were 1:1.

Preparation of amides

The appropriate acid was refluxed with excess thionyl chloride for 2 hr. The acid-chloride was distilled *in vacuo*, dissolved in ether, and ammonia gas passed into the solution for 2 hr. The product was isolated in the usual manner. The properties of the amides are reported below.

2,4,6-*Trimethylbenzamide*

Obtained as white crystals from hot water, m.p. 189–191°. Hantzsch and Lucas[7] record m.p. 189°. Infrared (Nujol mull): max. 1645 cm^{-1} (Amide I), 1600 cm^{-1} (Amide II); 3100, 3240, 3380 cm^{-1} (N–H stretching). Calculated for $C_{10}H_{13}NO$: C, 73·6; H, 8·0; N, 8·6. Found: C, 73·2; H, 7·9; N, 8·8.

2-Propylvaleramide

Obtained as white crystals from water containing a small amount of alcohol, m.p. 123°. Infrared (Nujol mull): max. 1650 cm^{-1} (Amide I), 1625 cm^{-1} (Amide II); 3180, 3380 cm^{-1} (N–H stretching). Literature[8] m.p. 123–4°.

RESULTS AND DISCUSSION

Previous studies[5] of structure–activity relationships of analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) revealed the importance of a 4-alkyl substituent for porphyria-inducing activity. This was attributed to the fact that a 4-alkyl substituent causes a twisting of the 3- and 5-ethoxycarbonyl substituents out of the plane of the ring, and this nonplanar relationship was thought to be necessary for optimal porphyria-inducing activity. To test the validity of this concept the 2- and 6-methyl substituents of 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine have been replaced by hydrogen atoms and the porphyria-inducing activity of this compound, viz. 3,5-diethoxycarbonyl-4-methylpyridine, was tested (Table 2). The inactivity of this compound in which the ethoxycarbonyl substituents are coplanar with the pyridine ring provides support for the correctness of this idea. Further support for this concept is provided by the inactivity of the planar 3,5-diethoxycarbonylpyridine and the activity of two DDC analogues in which methyl substituents on the ring are replaced by ethyl substituents, viz. 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-triethylpyridine and 3,5-diethoxycarbonyl-1,4-dihydro-4-methyl-2,6-diethylpyridine (Table 2).

The next compound studied was 3-ethoxycarbonyl-2,4,6-trimethylpyridine, which was expected to be inactive since it has only one ethoxycarbonyl substituent out of the plane of the ring. This compound, although less active than 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine which has two ethoxycarbonyl substituents out of the plane of the ring, nevertheless was shown to have considerable activity (Table 2). This observation led to a revision of our views of the critical features necessary for activity in these compounds. Since 2,4,6-trimethylpyridine was shown to have only very weak activity, in agreement with the report of Granick,[4] it appeared likely that the critical feature for activity in these compounds was an ethoxycarbonyl group with two *o*-alkyl substituents. This observation indicated that the pyridine or dihydropyridine rings might not be essential for activity and that they might be replaced by other ring systems. For this reason two benzene analogues were tested for porphyria-inducing activity, viz. ethyl 2,4,6-trimethylbenzoate and diethyl. 2,3,5,6-tetramethylterephthalate (Fig. 1c). The fact that the activity of ethyl 2,4,6-trimethylbenzoate resembled that of 3-ethoxycarbonyl-2,4,6-trimethylpyridine and that the activity of diethyl 2,3,5,6-tetramethylterephthalate was similar to that of 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine clearly showed that the pyridine or dihydropyridine rings were not essential for activity. The results obtained with the benzene analogues confirm the observations in the pyridine series that one ethoxycarbonyl group with two *o*-methyl substituents is necessary for activity, while a second ethoxycarbonyl group in the molecule with two *o*-methyl substituents reinforces this activity. The high activity of a variety of esters of 2,3,5,6-tetramethylterephthalic acid (Table 2) shows that the nature of the alcohol moiety of the ester groups can be varied within considerable limits without any major change in activity. The inactivity of 2,3,5,6-tetramethylterephthalic acid, 2,4,6-trimethylbenzoic acid, and 3-ethoxycarbonyl-

2,4,6-trimethyl-5-pyridinecarboxylic acid is probably the result of the fact that they exist predominantly as anions at physiological pH and would experience difficulty in crossing the cell membrane.[9]

The porphyria-inducing activity of the ethyl ester of 2,4,6-trimethylbenzoic acid is of particular interest since this compound has played an important historical role

TABLE 2. PORPHYRIN ACCUMULATION IN PRIMARY CULTURES OF CHICK EMBRYO LIVER CELLS INDUCED BY A VARIETY OF CHEMICALS AND MEASURED BY FLUORESCENCE MICROSCOPY

Tests were made with the cover-slip technique (see Experimental).

Porphyria-inducing compound	Concentration (μg)	Concentration ($M \times 10^{-5}$)	Intensity of fluorescence
3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine	10	3·8	3
	3	1·1	2
	1	0·38	0·5
3-Ethoxycarbonyl-2,4,6-trimethyl-5-pyridinecarboxylic acid	100	42·1	0
	10	4·2	0
3,5-Diethoxycarbonyl-4-methylpyridine	100	42·1	0
	10	4·2	0
3,5-Diethoxycarbonylpyridine	100	44·8	0
3-Ethoxycarbonyl-2,4,6-trimethylpyridine	100	51·7	3·5
	10	5·2	1
	3	1·6	trace
3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-triethylpyridine	2	0·65	2·5
	0·2	0·06	1·5
	0·02	0·006	trace
3,5-Diethoxycarbonyl-1,4-dihydro-4-methyl-2,6-diethylpyridine	2·5	0·85	4
	0·25	0·08	2
2,4,6-Trimethylpyridine	100	83	1
	10	8·3	0
Diethyl 2,3,5,6-tetramethylterephthalate	10	3·6	3·5
	3	1·1	2
	1	0·4	1
Dimethyl 2,3,5,6-tetramethylterephthalate	10	4	3·5
	1	0·4	1
Di-*n*-propyl 2,3,5,6-tetramethylterephthalate	10	3·3	4
	1	0·3	2
Diisopropyl 2,3,5,6-tetramethylterephthalate	10	3·3	3·5
	1	0·3	1·5
2,3,5,6-Tetramethylterephthalic acid	100	45	0
	10	4·5	0
2,4,6-Trimethylbenzoic acid	100	60·9	0
	10	6·1	0
2,4,6-Trimethylbenzamide	100	61·4	2
	10	6·1	1
Ethyl 2,4,6-trimethylbenzoate	100	52	2·5
	10	5·2	1
2-Propylvaleramide	100	70	2·5
	25	17·5	2
3-Methylbutyramide	100	99	0
Trimethylacetamide	100	98	0

in the development of concepts of steric hindrance.[10] It has been known since the end of the last century that the hydrolysis of ethyl benzoate is blocked by the presence of substituents in the two *ortho* positions. It therefore appeared possible that the *o*-alkyl substituents in the above porphyria-inducing compounds were required to protect the ethoxycarbonyl substituents from enzymic hydrolysis. Talman *et al.*,[11] who studied

the structure–activity relationships of a large series of compounds related to allyl-isopropylacetamide (AIA), concluded that the molecular structure necessary to produce an experimental porphyria in chick embryos was a dialkyl-substituted acetamide or acetamide derivative where one substituent is an allyl group and the other contains at least three carbon atoms, preferably in a branched chain (Fig. 1d). Similar conclusions were reached by other workers.[12, 13] The recent studies of Hirsch *et al.*[14] have shown that the allyl group is not essential for activity and can be replaced by a propyl group when this series of compounds is tested in the chick embryo liver cell system. Since there is branching at the α- and β-carbon atoms in this molecule it is clear from the work of Newman[15] that considerable steric hindrance to hydrolysis of the amide group exists.

The above considerations led us to consider the possibility that the underlying critical feature for activity in the AIA and DDC series of compounds is an ester or amide group which is sterically hindered from hydrolysis. If this view is correct, it follows that the ester group of ethyl 2,4,6-trimethylbenzoate should be capable of being replaced with an amide group with retention of activity. For this reason the porphyria-inducing activity of 2,4,6-trimethylbenzamide was determined, and it was found to have similar activity to ethyl 2,4,6-trimethylbenzoate (Table 2). As a further test of this idea several aliphatic compounds with varying degrees of steric hindrance to hydrolysis of the amide group were tested for activity. The degree of steric hindrance in these compounds as deduced from the work of Newman[15] was 2-propylvaleramide $>$ trimethylacetamide $>$ 3-methylbutyramide. Only 2-propylvaleramide was found to be active (Table 2), thus providing support for this view. Recently Prato *et al.*[16] have demonstrated porphyria-inducing activity in 2-allyloxy-3-methylbenzamide and have pointed out that the side-chain groupings of this compound are similar to those in AIA. It is possible that in this compound the activity is dependent upon steric hindrance to hydrolysis of the amide group provided by the *o*-allyloxy group.

Granick[4] has recently studied the prphyria-inducing activity of several aliphatic diesters. He noted that diethyl β-methylglutarate was active, whereas dimethyl glutarate and diethyl β-methyl succinate were inactive. It is of interest that the degree of steric hindrance as deduced from the work of Newman[15] is greatest in the active diethyl β-methylglutarate. Further support for the correctness of these ideas is being sought by attempting to replace the amide group of allylisopropylacetamide with an ester group and investigating the porphyria-inducing activity of this ester.

Granick[4] has recently shown that a variety of drugs such as glutethimide, mesantoin, celontin, meprobamate, and griseofulvin, which are unrelated chemically to DDC or AIA, have porphyria-inducing activity. This author suggested that porphyria-inducing drugs are oxidatively metabolized and that the excess porphyrin is produced in response to an increased requirement for heme. This suggests a possible explanation of our results: drugs that may be metabolized by a hydrolytic mechanism do not require increased heme production in liver cells. However, where hydrolysis is prevented by steric factors, the drugs are oxidatively metabolized, and increased heme and porphyrin formation is required.

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