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STUDIES OF THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND PORPHYRIA-INDUCING ACTIVITY—IV.

INVESTIGATIONS IN A CELL CULTURE SYSTEM*

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Abstract—A series of compounds has been synthesized and tested for porphyria-inducing activity in chick embryo liver cells. The results obtained support the idea that the critical feature for activity in a series of aliphatic and aromatic compounds studied is an ester or amide group which is sterically hindered from hydrolysis. Important features required for porphyria-inducing activity in the griseofulvin molecule were revealed by testing a series of griseofulvin analogues. The (+)— and (−)— isomers of glutethimide were shown to have equal porphyria-inducing activity. The activity of a series of analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine was investigated in which the 4-methyl substituent was replaced with other substituents. In the analogue containing a 4-isopropyl group activity was retained, whereas in analogues containing a benzyl, cyclohexyl or cyclohex-3-enyl substituent only very weak activity was retained.

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-aminolaevulic acid synthetase.[1–4] Studies on the relationship between chemical structure and the porphyria-inducing activity of a variety of chemicals[5–7] led to the suggestion[7] that the underlying critical feature for activity in the allylisopropylacetamide (AIA; Fig. 1a) and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC; Fig. 1b) series of compounds is an ester or amide group which is sterically hindered from hydrolysis. In this paper the validity of this idea has been investigated by synthesizing a series of compounds with varying degrees of steric hindrance to the hydrolysis of ester and amide groups and testing their porphyria-inducing activity.

The antifungal drug, griseofulvin (Fig. 1c) which bears no obvious structural relationship to DDC or AIA, is a highly active porphyria-inducing drug. Granick[4] compared Courtald models of DDC and griseofulvin and pointed out that it was possible to twist a Courtald model of DDC so that the two oxygen atoms (starred in Fig. 1b) were separated by the same distance as the two oxygen atoms (starred in Fig. 1c) in griseofulvin. In this paper the importance of the inter-oxygen distance in

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griseofulvin has been investigated by determing the porphyria-inducing activity of a series of griseofulvin analogues. Glutethimide (Fig. 1d), a hypnotic and sedative drug, has recently been shown to have strong porphyria-inducing activity.[4] This compound has an asymmetric carbon atom (indicated by an asterisk) and it has been resolved into its two optical isomers.[8] We have compared the porphyria-inducing activity of the two optical isomers to see if this activity resided in one or both isomers. Finally, the

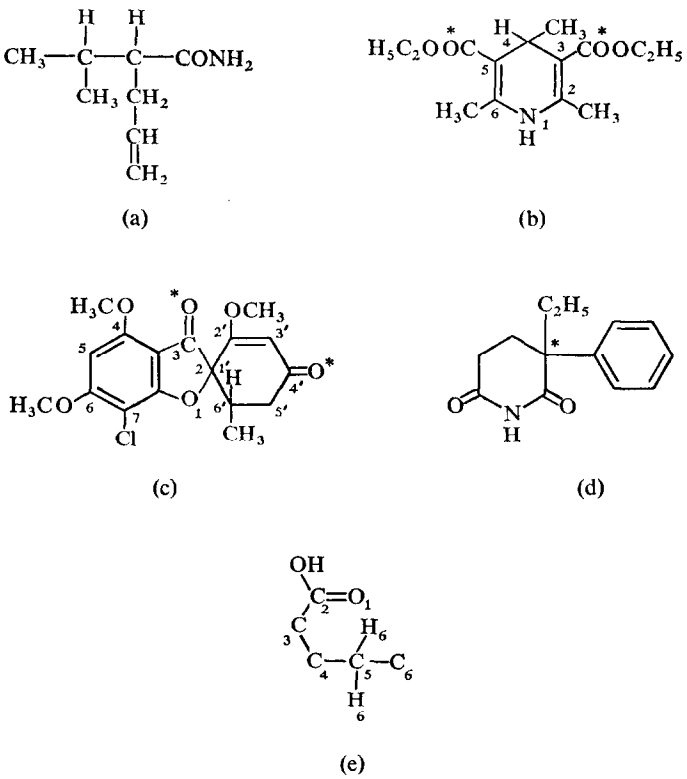


FIG. 1. Chemical structure of: (a) allylisopropylacetamide (AIA); (b) 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC); (c) griseofulvin; (d) glutethimide. In (d) the asterisk indicates an asymmetric carbon atom; (e) numbering system of Newman.

activity of several dihydropyridines was determined to obtain further information on the structural features required for activity in this series of 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 or as solutions in carbon tetrachloride. Nuclear magnetic resonance spectra were determined in carbon tetrachloride; external reference, tetramethylsilane; oscillator frequency, 60 Mc/s. Melting and boiling points are uncorrected.

Determination of porphyria-inducing activity of chemicals

Chick embryo liver cells were cultured on coverslips 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 coverslip. Each vial contained Eagle's basal medium (1 ml) supplemented with 10% fetal bovine serum, 1% 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 coverslip, the medium was renewed, drugs were added in ethanol (1–5 μl) and the vials were reincubated for 24 hr. The coverslip was then removed and examined in the fluorescence microscope (Tables 1–4). The cultures of chick embryo cells to which ethanol (1–5 μl) was added did not exhibit any 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. In each experiment a drug was tested in three separate vials at a particular concentration. Each drug was tested in the above manner in three separate experiments and the value of the fluorescence intensity recorded in Tables 1–4 represents the average values obtained in three experiments. To eliminate bias in scoring of the fluorescence intensity, the experiment was arranged so that the observer did not know which drug was added to a particular vial. Moreover, in each experiment a large number of vials were included to which no drug had been added.

The griseofulvin analogues were obtained from Dr. T. P. C. Mulholland, I.C.I. Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire and the dihydropyridines from Dr. B. Loev, Smith, Kline & French, Philadelphia, Pa. Racemic glutethimide and its optical isomers were obtained from Dr. H. Keberle, Forschungslaboratorien, der Ciba Aktiengesellschaft, Pharmazeutische Abteilung, Basel. Methyl di-*t*-butylacetate and methyl isopropylisobutylacetate were obtained from Dr. C. K. Hancock, Department of Chemistry, Texas A & M University, College Station, Tex. Diethyl glutarate, diethyl α,α'-dimethylglutarate, and α,α-dimethylpropionamide were purchased from Aldrich Chemical Co. Allylisopropylacetamide was obtained from Hoffman La Roche, Montreal, and β-methylbutyramide from Professor C. Rimington, University College, London. Benzamide and ethyl benzoate were purchased from Eastman-Kodak. The preparation of α-propyl-valeramide, diethyl-β-methylglutarate, diethyl 2,3,5,6-tetramethyl-terephthalate, ethyl 2,4,6-trimethylbenzoate and 2,4,6-trimethylbenzamide has been described previously.[5, 7]

3,5-*Dicyano*-1,4-*dihydro*-2,4,6-*trimethylpyridine*. This compound was prepared by a general method described for this class of compounds by Loev and Snader.[9] After three crystallizations from methanol, the dihydropyridine had an m.p. of 184·5 to 186·5°, λ max. (ethanol) 218 and 340 mμ (ϵ 25,900 and 6,800). Hofmann *et al.*[10] record an m.p. of 184–185°, λ max. (methanol) 218 and 339 mμ (ϵ, 25,800 and 6,500).

Synthesis of aliphatic acids

3-(2,4,6-*Trimethylphenyl*)*propanoic acid*. Ethyl 2-(2,4,6-trimethylphenyl)ethanoate was reduced with lithium aluminum hydride and the product crystallized from a mixture of acetone and water. The alcohol had an m.p. of 81·5 to 82·5°; infrared

(carbon tetrachloride): max. 3330 cm^{-1} (O–H stretching). *Anal.* Calcd. for $C_{11}H_{16}O$: C, 80·5; H, 9·8. Found: C, 80·4; H, 9·7.

The alcohol was converted to the iodide with red phosphorus and iodine. The corresponding cyanide was prepared by treating the iodide with potassium cyanide. Finally, the cyanide was converted by means of alkaline hydrolysis to a product which after crystallization from petroleum ether (35–60°) yielded 3-(2,4,6-trimethylphenyl)propanoic acid as white crystals (m.p. 111–112°). *Anal.* Calcd. for $C_{12}H_{16}O_2$: C, 75·0; H, 8·4. Found: C, 74·8; H, 8·4.

Allylisopropylacetic acid. A mixture of isopropyl bromide and diethyl sodioallylmalonate in ethanol was refluxed for 6 hr and the product was isolated in the usual manner. The product was distilled in an 18 inch electrically heated fractionating column and diethyl allylisopropylmalonate was collected as a colorless liquid (b.p. 88–89°/0·7 mm). Infrared (liquid film): max. 1725 cm^{-1} (ester C=O stretching), 1645 cm^{-1} (C=C stretching), 1368 and 1388 cm^{-1} (gem-dimethyl group). Hjelt[11] records a b.p. of 232–238°.

The di-ester was hydrolyzed with potassium hydroxide dissolved in a mixture of ethanol–water (10:3), and the potassium salt was treated with hydrochloric acid. The product was crystallized from benzene–petroleum ether (35–60°) and white crystals of allylisopropylmalonic acid separated (m.p. 122–123°). Krishna Rao and Sukh Dev[12] record an m.p. of 122–123°.

The di-acid was decarboxylated at 180° (bath) under slightly reduced pressure. Distillation afforded allylisopropylacetic acid, b.p. 116–118°/13 mm, as a colorless oil. Krishna Rao and Sukh Dev[12] record a b.p. of 121–122°/20 mm. Infrared (carbon tetrachloride): Max. 1705 cm^{-1} (C=O stretching), 1645 cm^{-1} (C=C stretching), 1365 and 1388 cm^{-1} (gem-dimethyl group). *Anal.* Calcd. for $C_8H_{14}O_2$: C, 67·57; H, 9·92. Found: C, 67·63; H, 9·96.

Synthesis of esters. For the synthesis of diethyl β,β-dimethylglutarate, ethyl 3,5-dimethylbenzoate and diethyl 2,5-dimethylterephthalate, the corresponding acids were heated with ethanol containing hydrogen chloride. Ethyl di-*n*-propylacetate, ethyl 2-(2,4,6-trimethylphenyl)ethanoate and ethyl 3-(2,4,6-trimethylphenyl)propanoate were prepared by treating the corresponding acids with thionyl chloride and reacting the acid chlorides formed, with ethanol. Ethyl allylisopropylacetate was prepared by the procedure devised by Parish and Stock[13] for the esterification of sterically hindered acids. The properties of the esters are described below.

Diethyl β,β-dimethylglutarate. The di-ester had a b.p. of 236–237°; infrared (liquid film): max. 1725 cm^{-1} (ester C=O stretching). In the literature[14] the b.p. was 241–243°.

Ethyl 3,5-*dimethylbenzoate.* The ester had a b.p. of 254·5–256°; infrared (liquid film): max. 1715 cm^{-1} (ester C=O stretching). In the literature[15] the b.p. was 241°.

Diethyl 2,5-*dimethylterephthalate.* This compound was obtained as white needles from ethanol, m.p. 84–87°; infrared (carbon tetrachloride): max. 1725 cm^{-1} (ester C=O stretching). The NMR spectrum contains a triplet at $\tau = 8{\cdot}6$ (CH_3 in ester), a singlet at $\tau = 7{\cdot}48$ (CH_3 on ring), a quartet at $\tau = 5{\cdot}74$ (CH_2 in ester) and a singlet at $\tau = 2{\cdot}42$ (H on ring). The relative peak areas were 3·15: 2·95: 2·05: 1.

*Ethyl di-*n*-propylacetate.* The ester distilled at 183–185°; infrared (liquid film): max. 1725 cm^{-1} (ester C=O stretching). In the literatue,[16] the b.p. was 183°.

Ethyl 2-(2,4,6-*trimethylphenyl*)*ethanoate.* The ester distilled at 144°/12 mm; infrared

(liquid film): max. 1720 cm^{-1} (ester C=O stretching). The NMR spectrum contains a triplet at $\tau = 8{\cdot}82$ (CH_3 in ester), a doublet at $\tau = 7{\cdot}79$ (CH_3 on ring), a singlet at $\tau = 6{\cdot}52$ (CH_2 group), a quartet at $\tau = 6{\cdot}01$ (CH_2 in ester) and a singlet at $\tau = 3{\cdot}32$ (H on ring). The relative peak areas were 2:8:8·8:2·2:1·8.

Ethyl 3-(2,4,6-*trimethylphenyl*)*propanoate.* The ester distilled at 140° (bath) at 11 mm; infrared (liquid film): max. 1733 cm^{-1} (ester C=O stretching). *Anal.* Calcd. for $C_{14}H_{20}O_2$: C, 76·3; H, 9·2. Found: C, 76·2; H, 9·0.

Ethyl allylisopropylacetate. The ester distilled at 71° (bath)/12 mm; infrared (carbon tetrachloride): max. 1730 cm^{-1} (ester C=O stretching), 1647 cm^{-1} (C=C stretching), 1370 cm^{-1} and 1385 cm^{-1} (gem-dimethyl group). *Anal.* Calcd. for $C_{10}H_{18}O_2$: C, 70·6; H, 10·7. Found: C, 70·9; H, 10·5.

Preparation of amides

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

α-*Ethylbutyramide.* This amide was obtained as white crystals from ethyl alcohol, m.p. 107–108°. In the literature[17] the m.p. was 107°. Infrared (Nujol mull): max. 1667 cm^{-1} (Amide I), 1585 cm^{-1} (Amide II), 3534, 3390, 3333 and 3155 cm^{-1} (N—H stretching).

α-*Methylbutyramide.* This amide was obtained as white crystals from ether, m.p. 112–113°. In the literature[18] the m.p. was 112°. Infrared (chloroform): max. 1672 cm^{-1} (Amide I), 1590 cm^{-1} (Amide II), 3546, 3436, 3344 and 3205 cm^{-1} (N—H stretching).

2-(2,4,6-*Trimethylphenyl*)*ethanamide.* This amide was obtained as needles from ethanol–water, m.p. 211·5–213·5° (decomp.). Buchner and Schattenhammer[19] record an m.p. of 210°. Infrared (chloroform): max. 1670 cm^{-1} (Amide I), 1570 cm^{-1} (Amide II), 3550 and 3420 cm^{-1} (N—H stretching). *Anal.* Calcd. for $C_{11}H_{15}NO$: N, 7·9. Found: N, 7·53.

3-(2,4,6-*Trimethylphenyl*)*propanamide.* This amide was obtained as white needles after four crystallizations from benzene, m.p. 164–165°. Infrared (Nujol mull): max. 1664 cm^{-1} (Amide I), 1642 cm^{-1} (Amide II), 3390 and 3226 cm^{-1} (N—H stretching). *Anal.* Calcd. for $C_{12}H_{17}NO$: C, 75·35; H, 8·95; N, 7·3. Found: C, 74·95; H, 8·9; N, 7·75.

3,5-*Dimethylbenzamide.* This amide was obtained as white needles from methanol–water, m.p. 133–134°. In the literature[20] the m.p. was 133°. Infrared (Nujol mull): max. 1645 cm^{-1} (Amide I), 1620 cm^{-1} (Amide II), 3400 and 3200 cm^{-1} (N—H stretching).

RESULTS AND DISCUSSION

It has been suggested that the critical feature for porphyria-inducing activity in a series of aliphatic and aromatic compounds studied is an ester or amide group which is sterically hindered from hydrolysis by acid or base.[7] To test this idea, a series of compounds has been synthesized with varying degrees of steric hindrance to the hydrolysis of ester and amide groups and their porphyria-inducing activity measured. Our attention was first directed to a series of aliphatic esters and amides. In order to estimate the degree of steric hindrance to hydrolysis in these compounds, we have

utilized several concepts formulated by Newman.[21] Newman has pointed out that steric factors in a given compound are believed to be the same for esterification and hydrolysis under acidic and basic conditions. It follows that an assessment of the degree of steric hindrance to hydrolysis in a molecule is possible by comparing the rate of esterification of the parent acid with the rate of esterification of acetic acid (taken as a standard). This assessment of the degree of steric hindrance in a particular molecule (RCOOH) is recorded in Table 2 as $K^{40°}$ $CH_3COOH/K^{40°}$ RCOOH. It follows that the greater the magnitude of this number, the greater will be the steric hindrance to esterification in the acid RCOOH and the greater the steric hindrance to hydrolysis in the corresponding ester or amide. In cases where data were not available on the rates of esterification of the acids, we have utilized the empirical "rule of six" which was derived by Newman[21] from a study of the rates of esterification of aliphatic acids. Newman[21] numbered the atoms in an acid consecutively, starting with the carbonyl oxygen as one (Fig. 1e) and called the number of atoms in the six position the six-number. "The rule of six states that in reactions involving addition to an unsaturated function containing a double bond, the greater the number of atoms in the six position the greater will be the steric effect." Accordingly we have estimated the degree of steric hindrance to hydrolysis by the magnitude of the six-number.

Granick[4] measured the activity of a series of aliphatic di-esters and found that diethyl β-methylglutarate (six-number = 6) was active while diethyl glutarate (six-number = 3) was inactive. In the present study the activity of diethyl β,β-dimethylglutarate (six-number = 9) and diethyl α,α′-dimethylglutarate (six-number = 3) was measured and compared to the activity of diethyl β-methylglutarate and diethyl glutarate. The results (Table 1), which are in accordance with expectation,

TABLE 1. PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO LIVER CELLS INDUCED BY A VARIETY OF CHEMICALS AND MEASURED BY FLUORESCENCE MICROSCOPY*

Porphyria-inducing compound	Six-No.	Concentration (μg)	Concentration ($M \times 10^{-5}$)	Intensity of fluorescence
Diethyl β,β-dimethylglutarate	9	200	93	3
		40	19	1
Diethyl β-methylglutarate	6	200	99	1
		40	19	0
Diethyl α,α′-dimethylglutarate	3	200	93	trace
		40	19	0
Diethyl glutarate	3	200	106	0
		40	21	0

* Tests were made with the coverslip technique (see Experimental).

show that the porphyria-inducing activity is related to the magnitude of the six-number.

The aliphatic mono-ester, ethyl di-*n*-propylacetate (six-number = 6), was found to be inactive while ethyl allylisopropylacetate (six-number = 8) showed only slight

activity (Table 2). These results were unexpected since the corresponding amides, α-propylvaleramide (six-number = 6) and allylisopropylacetamide (six-number = 8), were active (Table 2). Several aliphatic mono-esters with a higher six-number were then tested. However, both methyl isopropylisobutylacetate (six-number = 12) and methyl di-*t*-butylacetate (six-number = 18) showed only slight activity (Table 2). In comparing the results obtained with the aliphatic mono- and di-esters, it is apparent that one sterically hindered ester group confers slight activity on a molecule, while a second sterically hindered ester group in a molecule reinforces this activity. This result is similar to the results obtained with aromatic mono- and di-esters.[7] However, the activity obtained with aromatic esters was considerably greater than with the aliphatic esters.

In a previous study several aliphatic compounds with varying degrees of steric hindrance to hydrolysis of the amide group were tested for activity.[7] This study has now been extended and the results (Table 2) show that the porphyria-inducing activity

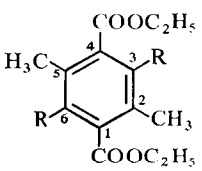
TABLE 2. PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO LIVER CELLS INDUCED BY A VARIETY OF CHEMICALS AND MEASURED BY FLUORESCENCE MICROSCOPY*

Porphyria-inducing compound	Six-No.	$K^{40°}$ CH_3COOH/ $K^{40°}$ RCOOH	Concentration (μg)	Concentration ($M \times 10^{-5}$)	Intensity of fluorescence
Allylisopropyl-acetamide	8	†	100	71	3
			20	14·2	2
			5	3·5	1
			1	0·7	trace
α-Propylvaleramide	6	108	100	69·9	2·5
			50	34·9	2
			25	17·5	2
α-Ethylbutyramide	6	100	100	86·9	2
			10	8·7	0
α-Methylbutyramide	3	10·1	100	99	0
			10	9·9	0
β-Methylbutyramide	6	8.57	100	99	0
			50	49·5	0
			25	24·7	0
α,α-Dimethyl-propionamide (trimethylacetamide)	0	26·8	100	98	0
Methyl di-*t*-butyl-acetate	18	reaction too slow to measure	100	53·7	0·5
			20	10·7	0
Methyl isopropyl-isobutylacetate	12	too slow to measure	100	58·5	trace
			20	11·7	0
Ethyl allyl-isopropylacetate	8	†	100	58·8	trace
			20	11·8	0
Ethyl di-*n*-propylacetate	6	108	100	58·1	0
			25	14·5	0

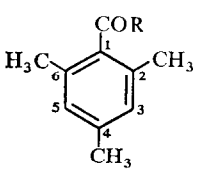
* Tests were made with the coverslip technique (see Experimental).
† Data not available.

is related to the degree of steric hindrance in the molecule calculated from the ratio $K^{40°}$ $CH_3COOH/K^{40°}$ RCOOH. The inactivity of β-methylbutyramide (six-number = 6) appears to be anomalous. However the "six-number" is only an approximation of the degree of steric hindrance in the molecule, whereas the ratio $K^{40°}$ $CH_3COOH/K^{40°}$ RCOOH provides a more accurate assessment of this parameter.

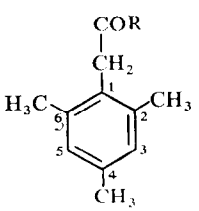
In a previous study[7] the high activity of diethyl 2,3,5,6-tetramethylterephthalate (Fig. 2a) was attributed to the fact that the molecule contained two ethoxycarbonyl substituents, each of which was sterically hindered from hydrolysis by two ortho-methyl substituents. In the present study this idea was supported by the inactivity of diethyl 2,5-dimethylterephthalate (Fig. 2b) in which the steric hindrance to hydrolysis of the ethoxycarbonyl substituents is largely removed (Table 3). The activity of ethyl 2,4,6-trimethylbenzoate (Fig. 2c) has similarly been attributed to the ethoxycarbonyl group, which is sterically hindered from hydrolysis by two ortho-methyl substituents.[7] In the present study this idea has been supported (Table 3) by the inactivity of ethyl benzoate, ethyl 3,5-dimethylbenzoate, ethyl 2-(2,4,6-trimethylphenyl)ethanoate (Fig. 2d) and ethyl 3-(2,4,6-trimethylphenyl)propanoate (Fig. 2e) in which the steric hindrance to hydrolysis of the ethoxycarbonyl substituent is removed. The idea that the activity of 2,4,6-trimethylbenzamide (Fig. 2f) was due to a sterically hindered amide group[7] received support from the inactivity of benzamide and the low activity of 3,5-dimethylbenzamide (Table 3). However, the activity of 2-(2,4,6-trimethylphenyl)-ethanamide (Fig. 2g) and 3-(2,4,6-trimethylphenyl)propanamide (Fig. 2h) was not expected (Table 3), since the steric hindrance to hydrolysis of the amide group is removed in these two compounds.



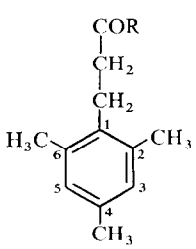
(a); R = CH_3
(b); R = H



(c); R = OEt
(f); R = NH_2



(d); R = OEt
(g); R = NH_2



(e); R = OEt
(h); R = NH_2

FIG. 2. Chemical structure of: (a) diethyl 2,3,5,6-tetramethylterephthalate; (b) diethyl 2,5-dimethylterephthalate; (c) ethyl 2,4,6-trimethylbenzoate; (d) ethyl 2-(2,4,6-trimethylphenyl)ethanoate; (e) ethyl 3-(2,4,6-trimethylphenyl)propanoate; (f) 2,4,6-trimethylbenzamide; (g) 2-(2,4,6-trimethylphenyl)ethanamide; and (h) 3-(2,4,6-trimethylphenyl)propanamide.

TABLE 3. PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO LIVER CELLS INDUCED BY A VARIETY OF CHEMICALS AND MEASURED BY FLUORESCENCE MICROSCOPY*

Porphyria-inducing compound	Concentration (μg)	Concentration ($M \times 10^{-5}$)	Intensity of fluorescence
Diethyl 2,3,5,6-tetra-methylterephthalate	10	3·6	3·5
	3	1·1	2
	1	0·4	1
Ethyl 2,4,6-trimethylbenzoate	50	26·1	2
	20	10·4	2
	10	5·2	0·5
Ethyl 2-(2,4,6-trimethyl-phenyl)ethanoate	50	24.3	0·5
	20	9·7	trace
	10	4·9	0
Ethyl 3-(2,4,6-trimethyl-phenyl)propanoate	100	45·4	0
	20	9·1	0
Diethyl 2,5-dimethyl-terephthalate	100	40·0	0
	10	4·0	0
Ethyl 3,5-dimethylbenzoate	100	56	0
	10	5·6	0
Ethyl benzoate	100	67	0
	10	6·7	0
2,4,6-Trimethylbenzamide	100	61·4	2
	50	30·7	2
	25	15·3	0·5
	5	3·1	0
2-(2,4,6-Trimethylphenyl) ethanamide	100	56·5	2
	50	28·3	2
	25	14·1	2·5
	5	2·8	2
3-(2,4,6-Trimethylphenyl) propanamide	50	26·2	2·5
	25	13·1	2
	5	2·6	1
3,5-Dimethylbenzamide	100	67	1
	10	6·7	0
Benzamide	50	41·3	0
	5	4·1	0
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-phenylpyridine	5	1·5	1·5
	0·5	0·2	0·5
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-benzylpyridine	50	14·6	0·5
	5	1·5	trace
	0·5	0·2	0
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-cyclohexylpyridine	50	14·3	trace
	5	1·4	trace
	0·5	0·14	0
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-cyclohexenylpyridine	50	14·4	1
	5	1·4	0·5
	0·5	0·14	0

TABLE 3.—*continued.*

Porphyria-inducing compound	Concentration (μg)	Concentration (M $\times 10^{-5}$)	Intensity of fluorescence
3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-isopropylpyridine	50	17·0	3
	5	1·7	2
3,5-Dicyano-1,4-dihydro-2,4,6-trimethylpyridine	50	29·2	trace
	5	2·9	0
3,5-Dicyano-1,4-dihydro-2,6-dimethyl-4-t-butylpyridine	50	23·3	0·5
	5	2·3	trace
(±)—Glutethimide	10	4·6	2
	2	0·9	0·5
(—)—Glutethimide	10	4·6	2
	2	0·9	0·5
(+)—Glutethimide	10	4·6	2
	2	0·9	0·5

* Tests were made with the coverslip technique (see Experimental).

In summary, it appears that in the series of aliphatic and aromatic esters a sterically hindered ester group is a requirement for porphyria-inducing activity. Similarly, in the series of aliphatic amides a sterically hindered amide group is required for activity. On the other hand, in the series of aromatic amides studied, a sterically hindered amide group does not appear to be necessary for activity. In attempting to interpret the above results, the recent work of Granick[4] is pertinent. This author has suggested that porphyria-inducing drugs are oxidatively metabolized and that excess porphyrin is produced in response to an increased requirement for heme. This suggests a possible explanation of our results: drugs that cannot be metabolized by a hydrolytic mechanism, because of steric factors, are oxidatively metabolized and increased heme and porphyrin formation is required. In order to ascertain whether this explanation is correct, it remains to determine to what extent the "six-number" and the magnitude of the ratio $K^{40}CH_3COOH/K^{40}RCOOH$ are indices of the difficulty of enzymic hydrolysis of the compounds studied. For this reason a study of the rates of hydrolysis of the above compounds by chick liver esterases and amidases is in progress. It is of interest that the hydrolysis of a series of esters has been found to be more susceptible to steric factors when catalyzed by cholinesterase than when catalyzed by hydroxide ion.[22]

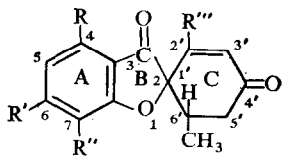
Granick[4] found that it was possible to twist a Courtald model of DDC so that the two oxygen atoms (starred in Fig. 1b) were separated by the same distance as the two oxygen atoms (starred in Fig. 1c) in griseofulvin. This finding indicated that the two keto-oxygen atoms in griseofulvin were responsible for its porphyria-inducing activity, and this possibility has been investigated by determining the activity of a series of griseofulvin analogues (Table 4). The numbering system[23, 24] used for griseofulvin and its analogues is shown in Fig.3. The systematic nomenclature is based on the trivial name grisan for the tricyclic system and according to this nomenclature griseofulvin is designated as 7-chloro-4,6,2′-trimethoxy-6′-methylgris-2′-en-3,4′-dione.

For convenience in the following discussion, the more common names of the analogues will be used where available; the structures of the analogues are presented in Fig. 3. Griseofulvin has two asymmetric carbon atoms which are located at positions 2 and 6′. The configuration at these asymmetric centers is designated as d or *l*. Griseofulvin is prefixed by (d,d), the optical antipode by (*l*,*l*) and the diastereoisomer epimeric at position 2 by (*l*,d). The spiran center (position 2) is the position first mentioned.[23, 24]

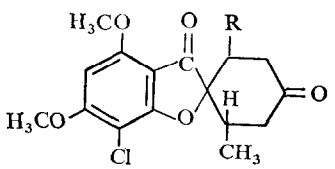
TABLE 4. PORPHYRIN ACCUMULATION IN PRIMARY CULTURE OF CHICK EMBRYO LIVER CELLS INDUCED BY GRISEOFULVIN ANALOGUES AND MEASURED BY FLUORESCENCE MICROSCOPY*

Porphyria-inducing compound	Concentration (μg)	Concentration (M × 10^{-5})	Intensity of fluorescence
3a (Griseofulvin)	10	2·8	2·5
	2	0·6	1
3b (Diastereiosomer of griseofulvin)	10	2·8	3
	2	0·6	1·5
3c (Bromogriseofulvin)	10	2·5	2
	2	0·5	1
3d (Dechlorogriseofulvin)	10	3·1	2·5
	2	0·6	0·5
3e	10	3·4	3·5
	2	0·7	2·5
3f (Homogriseofulvin)	10	2·7	1
	2	0·5	1
3g (Desmethoxygriseofulvin)	10	3·1	0
	2	0·6	0·5
3h (Griseofulvamine)	10	3·0	trace
	2	0·6	0
3i	10	3·1	1
	2	0·6	trace
3j (Dihydrogriseofulvin)	10	2·8	0·5
	2	0·6	trace
3k	10	3·2	1·5
	2	0·6	1
3l (Griseofulvic acid)	10	3·0	0
	2	0·6	0
3m (Isogriseofulvin)	10	2·8	1·5
	2	0·6	0·5
3n	10	3·2	1
	2	0·6	2·5
3o	10	3·7	1
	2	0·8	1
3p (Tetrahydroisogriseofulvin)	10	2·9	0
	2	0·6	0

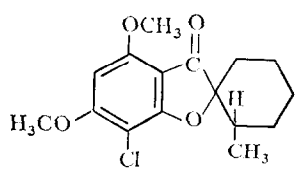
* Tests were made with the coverslip technique (see Experimental).



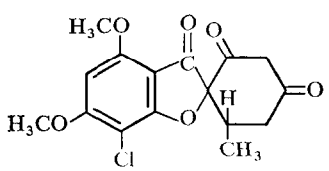
(a); R = R′ = R‴ = OCH$_3$; R″ = Cl (d,d)
(b); R = R′ = R‴ = OCH$_3$; R″ = Cl (l,d)
(c); R = R′ = R‴ = OCH$_3$; R″ = Br (d,d)
(d); R = R′ = R‴ = OCH$_3$; R″ = H (d,d)
(e); R = R′ = H; R‴ = OCH$_3$; R″ = Cl (dd,ll)
(f); R = R′ = OCH$_3$; R‴ = OC$_2$H$_5$; R″ = Cl (d,d)
(g); R = R′ = OCH$_3$; R‴ = H; R″ = Cl (d,d)
(h); R = R′ = OCH$_3$; R‴ = NH$_2$; R″ = Cl (d,d)



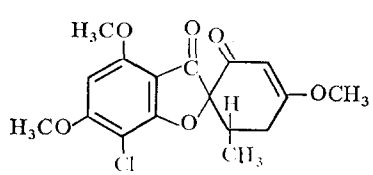
(i); R = H (d,d)
(j); R = OCH$_3$ (d,d)



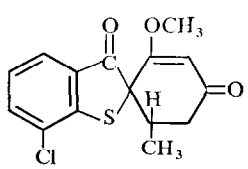
(k); (d,d)



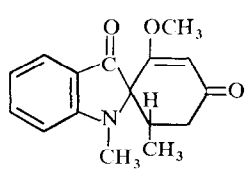
(l); (d,d)



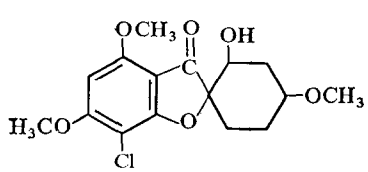
(m); (d,d)



(n); (dd, ll)



(o); (dd, ll)



(p); (d,d)

FIG. 3. Chemical structure of various griseofulvin analogues.

The acidic griseofulvic acid (3*l*) was inactive and the basic griseofulvamine (3h) was very weakly active, while the neutral analogues of griseofulvin, with the single exception of tetrahydroisogriseofulvin (3p), all exhibited activity. This is probably due to the fact that griseofulvic acid and griseofulvamine exist as charged molecules at physiological pH and would experience difficulty in crossing the cell membrane.[25] It is of interest that antifungal activity is only found in neutral analogues of griseofulvin.[23]

In the ring A analogues of griseofulvin (3a), *viz.* bromogriseofulvin (3c), dechlorogriseofulvin (3d) and 7-chloro-2^1-methoxy-6^1-methylgris-2^1-en-3,4^1-dione (3e), activity was retained. However, activity was found to be reduced in an analogue containing only one keto-oxygen atom, *viz.* 7-chloro-4,6-dimethoxy-6^1-methylgrisan-3-one (3k) and in isogriseofulvin (3m), an analogue in which the distance between the two keto-oxygen atoms is decreased. These findings supported the interpretation of Granick[4] that the two keto-oxygen atoms and the distance between them were important structural features for activity.

However, the importance of other features in the molecule for optimal activity was revealed by further studies. Thus the importance of the double bond and methoxy group in ring C was revealed by the reduced activity of desmethoxygriseofulvin (3g), dihydrogriseofulvin (3j), tetrahydroisogriseofulvin (3p), homogriseofulvin (3f) and 7-chloro-4,6-dimethoxy-6^1-methyl-grisan-3,4^1-dione (3i). It is of considerable interest that (*l*,d)-griseofulvin (3b) exhibited greater porphyria-inducing activity than (d,d)-griseofulvin (3a) in the present study (Table 4). In previous studies on the antifungal activity of griseofulvin analogues, stereochemistry was found to have a decisive influence and of the four stereoisomers of griseofulvin, only the naturally occurring (d,d)-griseofulvin was found to be active.[23, 26] This finding strongly suggests that the mechanism by which griseofulvin induces porphyria is unrelated to the mechanism by which it exerts its antifungal effect. Recently De Matteis[27] showed that isogriseofulvin (3m) was much more active than griseofulvin in inducing porphyria in mice. In our studies griseofulvin was shown to be considerably more active than isogriseofulvin. This difference between the results observed in a system *in vitro* and *in vivo* requires further investigation.

Glutethimide, a hypnotic and sedative drug, has been shown to have strong porphyria-inducing activity[4] and it has been recommended that the drug should not be used in patients with porphyria.[28] Glutethimide has an asymmetric carbon atom and the (+) form of the drug has twice the hypnotic and sedative activity of the (—) form.[29] It appeared possible that one of the enantiomorphs might be devoid of porphyria-inducing activity and might therefore be useful for the treatment of patients with porphyria. However, the results shown in Table 3 reveal that both optical isomers have equal porphyria-inducing activity.

In a previous study[5] it was shown that replacement of the 3- and 5-ethoxycarbonyl substituents of DDC (Fig. 1b) with acetyl substituents led to a loss of activity. In this study it was shown (Table 3) that analogues of DDC, *viz.* 3,5-dicyano-1,4-dihydro-2,4,6-trimethylpyridine and 3,5-dicyano-1,4-dihydro-2,6-dimethyl-4-t-butylpyridine, in which the ethoxycarbonyl groups were replaced with cyano substituents, were also inactive, thus emphasizing the importance of the ethoxycarbonyl substituents for activity in this series.

The importance of an alkyl substituent in the 4-position of DDC has been emphasized

previously. In this study the activity of a series of analogues of DDC containing a variety of substituents in the 4-position was investigated. The first analogue tested was 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-phenylpyridine, which had been reported to be inactive when administered to guinea pigs by gastric intubation.[5] In the chick embryo liver cell system, however, this analogue exhibited moderate activity. This phenomenon of a compound displaying activity in the system *in vitro* and yet manifesting no activity *in vivo* has been encountered previously[5] with several drugs and remains to be explained. In further experiments a series of DDC analogues was investigated in which the 4-methyl group was replaced with other substituents. In the analogue containing a 4-isopropyl substituent (Table 3), activity was retained, while in those analogues containing a benzyl, cyclohexyl or cyclohex-3-enyl substituent, only very weak activity was found. In order to obtain a better understanding of the reasons for the change in activity with the change in the nature of the 4-substituent, a study of the metabolism of these dihydropyridines is in progress. It is possible that the inactivity of some of these dihydropyridines may be attributed to the loss of the 4-substituent due to the action of liver enzymes. Such a transformation appears possible in view of the work of Loev and Snader,[9] who have demonstrated that loss of the 4-substituent in addition to aromatization occurs upon oxidation of the dihydropyridines containing a 4-isopropyl, 4-cyclohexyl, 4-cyclohexenyl or 4-benzyl substituent.

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