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Synthesis of *N*-oxide derivatives of metyrapone and their detection as in vitro metabolites†

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A variety of possible *N*-oxidation products of 2-methyl-1, 2-bis(3-pyridyl)propan-1-one (metyrapone) have been synthesized by peracid oxidation, and characterized using various spectroscopic techniques. Specific and sensitive chromatographic techniques have been developed for the separation and identification of its in vitro metabolites. Incubation of metyrapone with rat or mouse hepatic microsomes, in the presence of a NADPH-regenerating system, leads to the formation of metyrapol (keto-reduction), and two mono-*N*-oxides.

Metyrapone (Metopirone) (Ia) is used as a diagnostic tool for the determination of residual pituitary function in patients with hypopituitarism (Sprunt et al 1968; Meikle et al 1975). It has also been shown to inhibit a number of hepatic cytochrome P-450 dependent mono-oxygenations such as aromatic ring hydroxylation, and *O*- and *N*-dealkylation (Netter et al 1967; Kahl et al 1969; Hildebrandt 1971). The compound is bound to oxidized cytochrome P-450 to give a type II spectrum, in common with many other nitrogenous substances e.g. aniline and pyridine (Kahl et al 1969). However, in contrast with other nitrogenous compounds, metyrapone is also bound to reduced cytochrome P-450 (Hildebrandt et al 1969) to produce a difference spectrum which is similar to that produced on binding of carbon monoxide to reduced cytochrome P-450. These observations have led to the suggestion (Hildebrandt 1971) that one of the interactions of metyrapone is with the oxygen binding site of the cytochrome.

Until recently, the only reported metabolite of metyrapone was the alcohol, metyrapol (IIa) (Kahl 1970). In accord with our earlier work on metabolic *N*-oxidation of 3-substituted pyridines (Damani 1977; Cowan et al 1978), two recent studies have revealed the formation of metyrapone-*N*-oxides (Ib and Ic) as microsomal metabolites (De Graeve et al 1979; Damani et al 1979). We have now carried out a detailed investigation designed to detect a variety of possible in vitro *N*-oxidation products of metyrapone (Ib-Id, IIb-IId), the formation of which may be of relevance in understanding its cytochrome P-450 inhibitory properties (De Graeve et al 1979).

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MATERIALS AND METHODS

Compounds

Metyrapone was from Ciba Laboratories, Horsham, Sussex, *m*-chloroperoxybenzoic acid from Aldrich Chemical Company Ltd, Gillingham (Dorset) nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD) were gifts from Boehringer Corp. (London) Ltd, Lewes. Diagnostic t.l.c. analyses were on 0·25 mm Polygram sil G/uv$_{254}$ sheets and preparative t.l.c. separations were on 0·25 mm glass silica gel GF$_{254}$ plates, Type 60, Merck. Hepatic microsomal suspensions were prepared from male Sprague-Dawley rats (200–300 g) and male Swiss Albino mice (30–40 g) (see Gorrod et al 1975).

Preparation of N-*oxides of metyrapone*

Metyrapone (Ia, 1·12 g, 0·005 mol) and *m*-chloroperoxybenzoic acid (2·0 g, 0·0128 mol) were added to chloroform (50 ml) previously cooled to −10 °C (ice-salt bath) and the mixture allowed to warm to room temperature with stirring. The mixture was stirred for a further 5 min. Thin layer chromatographic analysis of the reaction mixture using chloroform–methanol–0·880 ammonia solution (90:10:1 v/v) as solvent, showed the presence of four oxidation products at R_F 0·71, 0·63, 0·44 and 0·30. Evaporation of the solvent under reduced pressure and at ambient temperature, afforded a crystalline residue which was immediately dissolved in a minimum volume of warm chloroform and the solution applied to a column of Grade H alumina, mesh 80–200 (100 g, 37 × 2·25 cm). Initial elution with chloroform afforded mainly unreacted metyrapone. Further elution with chloroform–methanol (95:5 v/v) gave fractions containing metyrapone and two unknown oxidation products. These fractions were bulked,

evaporated to dryness, the residue dissolved in a small volume of chloroform and ether added dropwise until a white crystalline deposit was obtained. The crystals were filtered off at the pump, recrystallized twice from chloroform–ether and dried in vacuum to afford 0·21 g of 2-methyl-2-[3″-(1″-oxopyridyl)]-1-(3′-pyridyl)-propan-1-one (Ic), m.p. 144–150 °C; n.m.r. ($CDCl_3$) δ 1·63 (s, *6*, 2 × CH_3), 6·99–7·43 (m, *3*, 4″-, 5″-, and 5′-protons), 7·73–7·97 (m, *1*, 4′-proton), 8·04–8·37 (m, *2*, 6′- and 6″-protons) and 8·50–8·87 (m, *2*, 2′- and 2″-protons); mass spectrum *m/z* 242 (13), 226 (23), 123 (14), 121 (10), 120 (100), 119 (5), 106 (76), 92 (19). Found: C, 69·4; H, 5·6; N, 11·2. Calculated for $C_{14}H_{14}N_2O_2$: C, 69·4; H, 5·8; N, 11·6%; R_F = 0·71 (chloroform: methanol: 0·880 ammonia, 90 : 10 : 1 v/v). The bulked mother liquors from the recrystallization of (Ic) were submitted to preparative thin layer chromatography using chloroform–methanol–0·880 ammonia (90 : 10 : 1 v/v) as solvent and ultraviolet irradiation for band detection. This revealed the presence of three bands at R_F = 0·87, 0·79 and 0·72. The lower fluorescing band (R_F 0·72) was separated, extracted with methanol and the extracts evaporated to give 2-methyl-1-[3′-(1′-oxopyridyl)]-2-(3″-pyridyl)-propan-1-one (Ib) as a homogeneous solid (1–2 mg of material were obtained from one plate, total yield 0·021 g), m.p. 128–130°C; n.m.r. ($CDCl_3$) δ 1·60 (s, *6*, 2 × CH_3), 7·03–7·38 (m, *3*, 4″-, 5″- and 5′-protons), 7·44–7·65 (m, *1*, 4′-proton), 7·99–8·25 (m, *2*, 6′- and 6″-protons), 8·39–8·75 (m, *2*, 2′- and 2″-protons); mass spectrum *m/z* 242 (11), 226 (16), 122 (2), 121 (9), 120 (100), 119 (2), 106 (28), 95 (15), 92 (14). Found: C, 69·1; H, 5·5; N, 11·2. Calculated for $C_{14}H_{14}N_2O_2$: C, 69·4; H, 5·8; N, 11·6%; R_F = 0·63 (chloroform–methanol–0·880 ammonia, 90 : 10 : 1 v/v). Further elution of the alumina column with chloroform–methanol (95 : 5 v/v) afforded fractions containing a third oxidation product contaminated with small amounts of (Ib) and (Ic), which after bulking, evaporation to dryness and crystallization from chloroform-ether gave 0·12 g of the di-*N*-oxide of 2-[2-(3-pyridyl)propyl] nicotinate (III) as white plates, m.p. 201–205 °C; n.m.r. ($CDCl_3$) δ 1·88 (s, *6*, 2 × CH_3), 7·17–7·50 (m, *2*, 5′- and 5″-protons), 7·67–7·93 (m, *2*, 4′- and 4″-protons), 8·04–8·43 (m, *3*, 2″-, 6″- and 6′-protons) and 8·66–8·80 (m, *1*, 2′-proton); mass spectrum *m/z* 274 (6), 258 (5), 242 (4), 153 (10), 139 (24), 136 (27), 135 (100), 123 (54), 122 (12), 120 (55), 117 (18), 106 (64), 105 (25), 104 (20), 93 (15), 92 (27). Found: C, 60·9; H, 5·3; N, 9·8. Calculated for $C_{14}H_{14}N_2O_4$: C, 61·3; H, 5·1; N, 10·2%; R_F = 0·44 (chloroform–methanol–0·880 ammonia, 90 : 10 : 1 v/v). Exhaustive elution of the alumina column with chloroform–methanol (90 : 10 v/v) gave fractions containing only 2-methyl-1,2-bis-3(1-oxopyridyl)-propan-1-one (Id) which, after evaporation of solvent, was obtained as white needles, m.p. 203–206 °C, 0·19 g; n.m.r. (DMSO-d_6) δ 1·62 (s, *6*, 2 × CH_3), 7·20–7·51 (m, *3*, 4″-, 5″- and 5′-protons), 7·63–8·38 (m, *4*, 2″-, 6″-, 4′- and 6′-protons), 8·68–8·82 (m, *1*, 2′-proton); mass spectrum *m/z* 258 (6), 242 (8), 226 (11), 136 (10), 135 (18), 122 (7), 121 (8), 120 (100), 119 (11), 106 (32), 95 (12), 92 (12). Found: C, 64·8; H, 5·6; N, 10·7. Calculated for $C_{14}H_{14}N_2O_3$: C, 65·1, H, 5·4; N, 10·85%; R_F = 0·30 (chloroform: methanol : 0·880 ammonia, 90 : 10 : 1 v/v).

General procedure for the preparation of metyrapol and its N-*oxides*

Metyrapone or the appropriate *N*-oxide derivative (0·05 m mol) was dissolved in methanol (1·0 ml) and excess sodium borohydride (0·05 g) added with stirring. The resulting solution was left to stir for 30 min, the residual sodium borohydride decomposed by addition of acetone and the mixture evaporated to dryness in a vacuum. The residue was extracted with chloroform (3 × 3 ml) and the chloroform extracts combined, dried over $MgSO_4$ and evaporated to dryness to afford the corresponding carbinol as a hygroscopic solid, which liquified rapidly in air. Reduction of (Ib) gave 2-methyl-1-[3′-(1′-oxopyridyl)]-2-(3″-pyridyl)-propan-1-ol (IIb) as a homogeneous gum. Insufficient material was available for n.m.r. spectroscopic analysis. Mass spectrum *m/z* 226 (6), 125 (14), 122 (13), 121 (100), 120 (81), 119 (18), 109 (20), 108 (34), 106 (30), 92 (33). R_F = 0·25 (chloroform–methanol–0·880 ammonia, 90 : 10 : 1 v/v). Reduction of (Ic) afforded 2-methyl-2-[3″-(1″-oxopyridyl)]-1-(3′-pyridyl)-propan-1-ol (IIc) as a thick, colourless oil; n.m.r. (DMSO-d_6/D_2O) δ 1·12 (s, *3*, one of CH_3 groups at C-2), 1·10 (s, *3*, other CH_3 group at C-2), 4·67 (s, *1*, C-1 proton), 5·66 (broad s, *1*, exchangeable with D_2O, -OH), 7·14–7·47 (m, *4*, 4′-, 5′-, 4″- and 5″-protons), 7·96–8·42 (m, *4*, 2′-, 6′-, 2″- and 6″-protons); mass spectrum *m/z* 244 (3), 226 (5), 153 (22), 138 (22), 137 (28), 135 (11), 122 (44), 121 (100), 119 (30), 118 (15), 109 (28), 108 (50), 107 (34), 106 (45), 104 (16), 95 (9), 92 (33). R_F = 0·31 (chloroform–methanol–0·880 ammonia, 90 : 10 : 1 v/v). Reduction of (Id) gave 2-methyl-1,2-bis-3-(1-oxopyridyl)-propan-1-ol (IId) as a colourless gum, n.m.r. (DMSO-d_6/D_2O) δ 1·32 (s, *3*, one of CH_3 groups at C-2), 1·38 (s, *3*, other CH_3 group at C-2), 4·66 (s, *1*, C-1 proton), 6·28 (broad s, *1*, exchangeable with D_2O, -OH), 6·88–7·43 (m, *4*, 4′-, 5′-, 4″- and 5″-

protons), 7·80–8·12 (m, *3*, 2″-, 6″- and 6′-protons), 8·22–8·40 (m, *1*, 2′-proton); mass spectrum *m/z* 260 (2), 226 (5), 178 (25), 137 (41), 135 (27), 125 (9), 124 (11), 123 (44), 122 (16), 121 (76), 120 (100), 119 (24), 118 (17), 109 (18), 108 (35), 107 (23), 106 (36), 104 (32), 95 (14), 92 (34). R_F = 0·12 (chloroform–methanol–0·880 ammonia, 90:10:1 v/v).

Incubation of metyrapone with microsomal enzymes

Incubation conditions were essentially the same as described before for the microsomal *N*-oxidation of 3-substituted pyridines (Gorrod & Damani 1979), except that they were scaled up ten fold. A typical 20 ml incubation consisted of the following: 10 ml phosphate buffer (0·2 M, pH 7·4) containing metyrapone (5·65 mg, 25 μ mol), NADP (15·75 mg, 20 μ mol) G6P (30·42 mg, 100 μmol), G6PD (14 μl, 10 units enzyme activity or 70 μg protein) and magnesium chloride (50·83 mg, 250 μmol) *plus* 10 ml Tris/KCl buffer (pH 7·4) containing microsomal enzymes equivalent to 5·0 g of original wet liver. Incubations were performed in 100 ml conical flasks at 37 °C for 1 h, using a Grants shaking water bath. An aliquot (ca 1 ml) was taken for h.p.l.c. analysis (see below), while the rest of the incubate was treated as follows.

Extraction of metyrapone and its N*-oxide metabolites*

The incubation mixture was transferred to a 250 ml separating funnel, and enzyme activity terminated by the addition of sodium hydroxide (5 ml, 1 M), and sodium chloride (10 g). The metabolites were extracted using dichloromethane (3 × 50 ml). The dichloromethane extracts were pooled, concentrated (0·5 ml) and the individual metabolites isolated by preparative t.l.c. as described before.

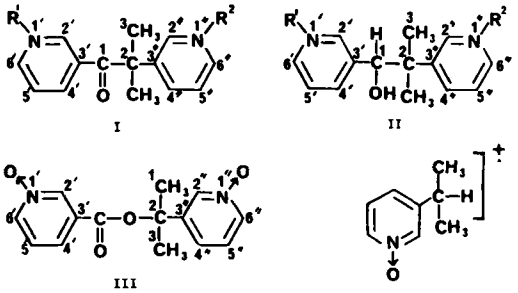
High performance liquid chromatography

A reverse phase h.p.l.c. assay has been developed for the detection and quantitation of metyrapone, metyrapol and their mono- and di-*N*-oxides (Damani et al 1979). The separation of these compounds was achieved using a Whatman Partisil PXS 10 ODS column (4·6 mm × 25 cm), and a mobile phase of acetonitrile: phosphate buffer (pH 7·4, 0·067 M) (20:80 v/v) at a flow rate of 2·5 ml min^{-1}. Detection was by u.v. absorbance at 261 nm. For qualitative work, aliquots from the incubate were injected onto the column either directly or after protein precipitation using an equal volume of acetonitrile.

RESULTS AND DISCUSSION

Structure of synthetic products

Initial attempts at preparing the *N*-oxides of metyrapone by oxidation with hydrogen peroxide-acetic acid mixtures were unsuccessful, producing multi-component mixtures which contained only very small amounts of the *N*-oxide products. Oxidation of metyrapone with *m*-chloroperoxybenzoic acid in chloroform or ether at low temperatures, although affording only low yields of oxidation products, was found to be a superior method, since products could be separated by chromatographic methods. In all reactions carried out with *m*-chloroperoxybenzoic



I II III IV

(a) R^1 = R^2 = nothing
(b) R^1 = O, R^2 = nothing
(c) R^1 = nothing, R^2 = O
(d) R^1 = R^2 = O

acid, four oxidation products were consistently obtained and could be separated by a combination of column and preparative thin layer chromatography. The oxidation products were characterized as the three *N*-oxides (Ib), (Ic) and (Id) of metyrapone, and the ester dioxide (III). The formation of the latter product involves oxidative conversion of a ketone to an ester, preceded by or with subsequent oxidation of the two pyridyl nitrogens. Therefore, the product (III) is presumably formed via a Baeyer-Villiger type of reaction, mechanistic features of which are reasonably well understood (Palmer & Fry 1970).

The *N*-oxides (IIb), (IIc) and (IId) of metyrapol were prepared by sodium borohydride reduction of the corresponding metyrapone *N*-oxide. The above hydride reagent selectively reduces the carbonyl function to the carbinol with insignificant *N*-oxide reduction (Damani et al 1980). Thin layer chromatographic or h.p.l.c. monitoring of these reductions showed that the appropriate carbinol was formed quantitatively within 15 min. Structures for the two metyrapone mono-*N*-oxides could not be assigned from their n.m.r. spectra and mass spectral fragmentation patterns. Structural assignment was achieved by examination of the mass spectra of the sodium borohydride reduction products obtained from each of the synthetic mono-*N*-oxides. The *N*-oxide assigned as (Ic) afforded a reduction product which exhibited a diagnostic ion at *m/z* 137 attribut-

able to fragment (IV). This is consistent with the expected characteristic mass fragmentation observed with metyrapol (IIa) (Damani et al 1981) and unequivocally assigns this product as (IIc). The alternative isomeric *N*-oxide (Ib) gave a reduction product (IIb) which did not give an ion at *m/z* 137 in its mass spectrum. (For fragmentation processes see Damani et al 1981).

Microsomal metabolism of metyrapone

The extraction procedure used recovered sufficient (50–60%) amounts of the mono-*N*-oxides (Ib, Ic) of metyrapone from simulated incubates, but failed to extract significant amounts of the very water-soluble metyrapone-*NN*-dioxide (Id). This necessitated the examination of the intact incubate by h.p.l.c. for detecting the *NN*-dioxide (if any).

Initial examination of the dichloromethane extracts by t.l.c. revealed the presence of five compounds. The compound migrating at $R_F = 0{\cdot}84$ was identified as unchanged metyrapone (Ia) by comparison of its chromatographic (h.p.l.c., Rt = 14·3 min) and spectroscopic properties with that of authentic metyrapone. The material at $R_F = 0{\cdot}50$ (h.p.l.c. Rt = 12·1 min) was similarly identified as the keto-reduction product metyrapol (IIa), whereas the compound at $R_F = 0{\cdot}38$ (h.p.l.c., Rt = 1·8 min) was nicotinamide, resulting presumably from breakdown of NADP. The compounds with R_F values 0·71 and 0·63 (h.p.l.c., Rt = 5·6 and 7·2 min respectively) were tentatively identified as metyrapone-*N*-oxides (Ic and Ib). This was confirmed by measurement of u.v. and mass spectra (see above), and comparison with data from the synthetic compounds. An h.p.l.c. examination of the complete incubate confirmed the presence of metyrapone, metyrapol, and the two mono-*N*-oxides, and the *absence* of any of the other possible *N*-oxidized derivatives of metyrapone (Id, IIb–IId).

Kahl and coworkers have reported very fully on the enzymology of metyrapone reduction. There are at least two metyrapone reductases, one in the soluble hepatic fraction, and one in the microsomal fraction. The cofactor for both these appears to be reduced NADP (Kahl 1970; Dünges & Kahl 1970). The in vivo and in vitro *N*-oxidation of the pyridyl nitrogen in the plethora of drugs and foreign compounds having this type of amine function has not been extensively studied (Gorrod 1971). Recent work on heteroaromatic *N*-oxidation (Damani 1977; Cowan et al 1978; Gorrod & Damani 1980) suggests that this may be an important route of in vitro and in vivo metabolism. Preliminary reports from our laboratory (Damani et al 1979) and from those of others (De Graeve et al 1979) have suggested that metyrapone, like other 3-substituted pyridines, is also a substrate for a cytochrome P-450 dependent pyridine-*N*-oxidase. The results from the present study, using synthetic standards, confirm and extend these observations. We have unequivocally shown that metyrapone only yields the mono-*N*-oxides as in vitro metabolites, and that the metabolites so formed are not further reduced to any significant extent to yield metyrapol *N*-oxides. Further work is being carried out to determine the binding of these *N*-oxides to cytochrome P-450 and their inhibitory potency. Our results may help in understanding the role of *N*-oxidation in the inhibition of cytochrome P-450 mediated reactions by metyrapone.

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