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SHORT COMMUNICATIONS

In vitro metabolism of Calcipotriol (MC 903), a vitamin D analogue

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Calcipotriol (MC 903, Fig. 1) is a novel vitamin D analogue which combines potent effects on cell proliferation and differentiation with a decreased activity on calcium metabolism [1]. Calcipotriol is presently undergoing clinical evaluation in patients with psoriasis vulgaris.

During preliminary pharmacokinetic studies in rats and pigs, a rapid metabolism of MC 903 was observed,* and the presence of a metabolite (X) in plasma was disclosed by the HPLC method used for determination of MC 903.

The present report describes the production of this metabolite by incubation of MC 903 with post-mitochondrial supernatant from rat liver, its isolation, and its identification by comparison of UV, NMR and MS properties with synthetic material.

The same metabolite was formed by incubation with post-mitochondrial supernatants from liver of pig and man. An additional metabolite (Y) was formed by incubation with the human liver preparation, and this metabolite was identified by comparison of UV and chromatographic data with synthetic material.

Materials and methods

Chemicals. MC 903 was synthesized by the reported procedure [2]. All chemical reagents and biochemicals used were commercially available. Solvents were HPLC or spectroscopy grade.

High pressure liquid chromatography. HPLC instrumentation consisted of a Merck–Hitachi Liquid Chromatograph equipped with a Merck–Hitachi L 5000 Controller, a Merck–Hitachi 655A Variable Wavelength UV Monitor as detector and a Merck–Hitachi 833A Data Processor as recording integrator or, alternatively, a Waters 990 Photodiode Array Detector for detection and data collection. The column was stainless steel, 125 × 4 mm, packed with LiChrospher RP 18, 5 μ (Merck LiChrocart cartridge). The eluents were water–methanol gradients from about 70% methanol to 95% methanol over 15 min. Flow rate was 2 mL/min. Column effluents were monitored at 264 nm or from 210 to 360 nm using the photodiode array detector. A Varian AASP Advanced Automated Sample Processor, with C8 AASP cassettes (Analytichem International) as sorbent cassettes, was used for injection of samples. AASP purge solvent was 250 μL of methanol–water 10:90. The AASP valve reset time was 15 min.

NMR and mass spectrometry. ^{1}H-NMR spectra ($CDCl_3$) were obtained on a Bruker AC300 spectrometer. Positions of signals are given in ppm relative to Me_4Si as internal reference (δ = 0.00 ppm) and coupling constants are in Hz. EI–MS spectra (70 eV) were obtained on a Varian MAT 311A equipped with a SS200 data system.†

In vitro *metabolism.* Post-mitochondrial supernatants were prepared from livers, which were removed from male Sprague–Dawley rats (6 weeks old), washed in ice-cold 0.15 M KCl and weighed. Each liver was homogenized in 3 mL ice-cold 0.15 M KCl, using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 15,000 *g* for 20 min at 0°, the supernatant was collected and stored in liquid nitrogen. Similarly, supernatants were prepared from liver slices obtained from Göttingen minipigs or from human liver samples.‡ Incubations were carried out at 37° using an incubation mixture of 250 μL of liver supernatant and 1 mL of a 0.05 M phosphate buffer (pH 7.4) containing 7 mmol $MgSO_4$, 33 mmol KCl, 4 mmol glucose-6-phosphate and 4 mmol NADP. MC 903 was added in 2-propanol solution (5–20 μg in 5 μL). Reactions were stopped after 15, 30, 45 or 60 min by addition of 1.25 mL of acetonitrile. After removal of protein precipitate by centrifugation the supernatant was diluted with 2.5 mL of water and collected on a C8 cartridge and injected into the HPLC system.

Isolation of metabolite X. For isolation of metabolite X *in vitro* incubation with post-mitochondrial supernatant from rat liver and 5 μg of MC 903 was repeated 20 times. For each chromatogram the fraction containing metabolite X was collected in one flask and evaporated to dryness with a stream of nitrogen. Based on the areas of the collected peaks, and assuming the same detector response for metabolite X as for MC 903, the amount collected was about 10 μg.

Chemical synthesis of MC 1046 *and MC* 1080 *(Fig.* 1*).* Reference samples of MC 1046 and MC 1080 were synthesized from the known [2] bis-*tert*-butyldimethylsilyl ethers of the corresponding (5*E*)-vitamin D analogues. Triplet-sensitized photoisomerization of these compounds (*ca.* 1 g) using the procedure described in Ref. 2 for the penultimate step in the MC 903 synthesis gave the respective (5*Z*) compounds, which were desilylated with hydrofluoric acid [2–3% solutions of substrate in acetonitrile–40% aqueous HF (25:1), 20°, 1 hr under N_2]. After basification (aqueous $NaHCO_3$) of the reaction mixtures, the products were extracted into EtOAc and purified (*ca.* 70% overall yields) by silica gel chromatography (EtOAc as eluent). MC 1046: UV (EtOH): λ_{max} 230 nm (ε_0 28000) and 264 nm (sh); i.r. ($CHCl_3$): ν_{max} 1670, 1645 and 1615 cm^{-1}; NMR: 0.59 (3H, s), 0.90 and 1.08 (each 2H, m), 1.12 (3H, d, *J* 7), 2.11 (1H, m), 2.22–2.36 (2H, m), 2.58, 2.84, 4.23 and 4.43 (each 1H, m), 4.99 and 5.33 (each 1H, bs), 6.02 (1H, d, *J* 11), 6.15 (1H, d, *J* 16), 6.37 (1H, d, *J* 11) and 6.77 (1H, dd, *J* 16 and 9); MS: *m/z* 410 ($M^{+\cdot}$), 69 (100%). MC 1080: UV (EtOH): λ_{max} 264 nm (ε_0 17500); i.r. ($CHCl_3$): ν_{max} 1685 cm^{-1}; NMR: 0.54 (3H, s), 0.86 (2H, m), 0.94 (3H, d, *J* 6), 1.00 (2H, m), 2.31 (1H, dd, *J* 7 and 13), 2.38–2.66 (3H, m), 2.82, 4.23 and 4.43 (each 1H, m), 4.99 and 5.33 (each 1H, bs), 6.02 and 6.37 (each 1H, d, *J* 11); MS: *m/z* 412 ($M^{+\cdot}$), 134 (100%), 69 (95%).

Results and discussion

Incubation of MC 903 with rat liver supernatant resulted in formation of a metabolite (X) as shown in Fig. 2a. This metabolite co-migrated on HPLC with a metabolite observed in plasma from rats dosed intravenously with MC 903 tritiated at carbon 24. The metabolite, which retained the characteristic UV spectrum of the triene system of vitamin D, was devoid of radioactivity, indicating that metabolism had taken place in the side chain of the

* H. Sørensen and E. Eilertsen, unpublished results.

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‡ The human liver samples were kindly supplied by Dr S. Henricsson, Karolinska Institutet, Huddinge University Hospital, Sweden.

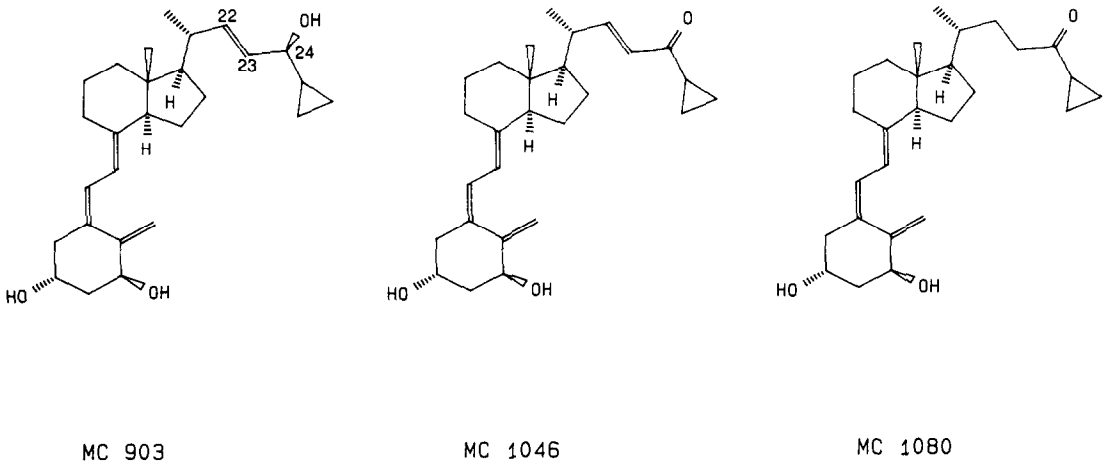

Fig. 1.

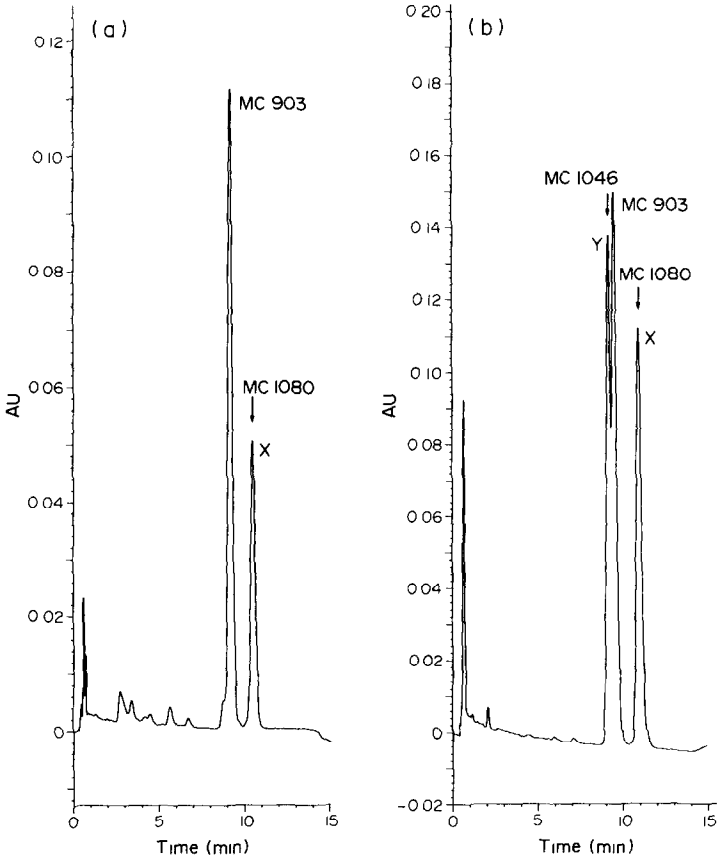

Fig. 2. Chromatograms from incubations of MC 903 with post-mitochondrial supernatants: (a) rat liver (5 μg MC 903, 15 min); (b) human liver (12.5 μg MC 903, 15 min). Migration positions of MC 1080 and MC 1046 are indicated by arrows.

molecule, and in particular involved the 24-position.

The mass spectrum of the isolated metabolite X shows the same molecular peak (m/z 412) and fragmentation pattern as found in MC 903. They both have the base peak m/z 134, but the peak at m/z 69 is more prominent in metabolite X (90–95%) than in MC 903 (50–55%).

In the proton NMR spectrum there was no indication of either the 22, 23 double bond or the proton at position 24 present in MC 903. Furthermore the signals characteristic of the cyclopropane ring system were found to be shifted downfield indicating metabolism around the 22–24 positions.

The UV, NMR and MS data obtained were found to be identical with data obtained for MC 1080, which also co-migrated with metabolite X on HPLC, identifying the metabolite as MC 1080.

The formation of MC 1080 was also observed in incubations of MC 903 with post-mitochondrial supernatant from liver from mini-pig and man. In the experiment with human liver an additional metabolite (Y) was prominent (Fig. 2b). The UV spectrum was different from that of MC 1080, but identical to that of MC 1046, with which compound metabolite Y also co-migrated exactly on HPLC. These results strongly indicate that metabolite Y is identical with MC 1046. Having observed MC 1046 in the experiments with human liver preparations further experiments were performed with rat and pig liver preparations (variation of incubation time and substrate concentrations). In these experiments the presence of minor amounts of MC 1046 was also observed, indicating the same qualitative pattern of metabolism in the three species.

The metabolism of MC 903 to MC 1080 and MC 1046 involves oxidation at the 24-position, similar to the C-24 oxidation pathway of metabolism of 1,25-dihydroxyvitamin D_3, the active form of vitamin D_3 [3, 4]. However, the 24-oxidation of 1,25-dihydroxy vitamin D_3 takes place mainly in the kidney and in the intestine, whereas the presently described metabolism is hepatic. Furthermore, the metabolism to MC 1080 involves a reduction of the 22,23 double bond in addition to the 24-oxidation step.

The formation of 24-oxidized metabolites appears to constitute a deactivation pathway for MC 903, since *in vitro* investigations of the effects of MC 1080 and MC 1046 on cell proliferation and differentiation show a considerably reduced activity compared to MC 903. These results and the results from *in vivo* investigations of metabolism of MC 903 in rat and pig will be published later.

In summary, a metabolite of MC 903 observed *in vivo* has been produced by incubation *in vitro* of MC 903 with liver post-mitochondrial supernatants from rat, pig and man. This metabolite was identified as MC 1080 by UV, NMR, MS and HPLC. An additional metabolite formed by incubation of MC 903 with human liver post-mitochondrial supernatant was identified as MC 1046. This metabolite was also detectable in rat and pig liver incubations.

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Effect of phenobarbital induction, charcoal treatment and storage on the spectral binding characteristics and NADPH–cytochrome P-450 reductase activity of hepatic microsomes

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The rate-limiting step in the hydroxylation of many foreign compounds and steroids by the hepatic microsomal cytochromes P-450 is the reduction of the cytochrome heme iron to Fe^{2+} by NADPH–cytochrome P-450 reductase [1–3]. Gigon *et al.* [4, 5] and Schenkman [6] found that, for a large number of water-soluble drugs, the substrate increases the rate of this reduction. Further, Gigon *et al.* [4] found that this stimulation showed a 1:1 stoichiometry with the hydroxylase activity. A number of studies from our laboratory have confirmed this observation [1, 7–10]. We and others have observed that there is an association between this substrate-induced increase in reductase activity and the presence of a type I difference binding spectrum [10–17], suggesting that the increased activity is associated with an increased fraction of the heme in the high spin state [10–17].

We have examined this hypothesis during studies that were stimulated by an apparent difference between the observations cited above and those of Peterson *et al.* [18]. This group reported that ethylmorphine does not stimulate NADPH–cytochrome P-450 reductase activity (cf. Table 3 of Ref. 18). A major difference between their studies and ours was that they utilized microsomes from phenobarbital-induced rats. Their failure to observe a stimulation with ethylmorphine could be due to residual phenobarbital, which we have detected previously in microsomes from treated animals [19]. This bound drug could be blocking the stimulatory action of ethylmorphine.

In the current study we examined this question by removing the residual phenobarbital from microsomes with charcoal. We found that the addition of ethylmorphine to this preparation gave a reverse type I difference binding spectrum and also markedly reduced the NADPH–cytochrome P-450 reductase activity. Charcoal treatment had no effect on either the spectral binding properties or the stimulation of cytochrome P-450 reduction by benzphetamine for microsomes from either control or phenobarbital-induced animals or on the effect of ethylmorphine