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Metabolism of 20-hydroxyvitamin D3 and 20,23-dihydroxyvitamin D3 by rat and human CYP24A1

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ABSTRACT

CYP11A1 hydroxylates vitamin D3 producing 20*S*-hydroxyvitamin D3 [20(OH)D3] and 20*S*,23-dihydroxyvitamin D3 [20,23$(OH)_2$D3] as the major and most characterized metabolites. Both display immuno-regulatory and anti-cancer properties while being non-calcemic. A previous study indicated 20(OH)D3 can be metabolized by rat CYP24A1 to products including 20*S*,24-dihydroxyvitamin D3 [20,24$(OH)_2$D3] and 20*S*,25-dihydroxyvitamin D3, with both producing greater inhibition of melanoma colony formation than 20(OH)D3. The aim of this study was to characterize the ability of rat and human CYP24A1 to metabolize 20(OH)D3 and 20,23$(OH)_2$D3. Both isoforms metabolized 20(OH)D3 to the same dihydroxyvitamin D species with no secondary metabolites being observed. Hydroxylation at C24 produced both enantiomers of 20,24$(OH)_2$D3. For rat CYP24A1 the preferred initial site of hydroxylation was at C24 whereas the human enzyme preferred C25. 20,23$(OH)_2$D3 was initially metabolized to 20*S*,23,24-trihydroxyvitamin D3 and 20*S*,23,25-trihydroxyvitamin D3 by rat and human CYP24A1 as determined by NMR, with both isoforms showing a preference for initial hydroxylation at C25. CYP24A1 was able to further oxidize these metabolites in a series of reactions which included the cleavage of C23-C24 bond, as indicated by high resolution mass spectrometry of the products, analogous to the catabolism of 1,25$(OH)_2$D3 *via* the C24-oxidation pathway. Similar catalytic efficiencies were observed for the metabolism of 20(OH)D3 and 20,23$(OH)_2$D3 by human CYP24A1 and were lower than for the metabolism of 1,25$(OH)_2$D3. We conclude that rat and human CYP24A1 metabolizes 20(OH)D3 producing only dihydroxyvitamin D3 species as products which retain biological activity, whereas 20,23$(OH)_2$D3 undergoes multiple oxidations which include cleavage of the side chain.

1. Introduction

CYP24A1 is the mitochondrial cytochrome P450 responsible for the catabolism of 1α,25-dihydroxyvitamin D3 [1,25$(OH)_2$D3]. Inactivation of vitamin D by CYP24A1 can take place *via* two catabolic pathways where initial hydroxylation occurs at either C24 or C23, termed the C24-oxidation and C23-oxidation pathways, respectively [1,2]. The sequential oxidation of 1,25$(OH)_2$D3 in the C24-oxidation pathway results in the formation of 24-oxo-1,23,25-trihydroxyvitamin D3 which undergoes side chain cleavage between C23 and C24 with the final product, calcitroic acid, being excreted. The C23-oxidation pathway produces 1,25-dihydroxyvitamin D3-26,23-lactone. There are species differences in the preference for these pathways, with rat CYP24A1 favoring the C24-oxidation pathway [3–5] and human CYP24A1 exhibiting both pathways [1,3,6,7].

It has been established in the last decade that CYP11A1 (also known as cytochrome P450scc) can metabolize vitamin D3 to produce many novel mono- and poly-hydroxyvitamin D metabolites, the major ones being 20*S*-hydroxyvitamin D3 [20(OH)D3]

Abbreviations: 1,25$(OH)_2$D3, 1α ,25-dihydroxyvitamin D3; 25(OH)D3, 25-hydroxyvitamin D3; 20(OH)D3, 20-hydroxyvitamin D3; 20,23$(OH)_2$D3, 20,23-dihydroxyvitamin D3; 20,23,24$(OH)_3$D3, 20,23,24-trihydroxyvitamin D3; 20,23,25$(OH)_3$D3, 20,23,25-trihydroxyvitamin D3; cyclodextrin, 2-hydroxylpropyl-β-cyclodextrin; TOCSY, ^{1}H-^{1}H total correlation spectroscopy; COSY, ^{1}H-^{1}H correlation spectroscopy; HSQC, ^{1}H-^{13}C heteronuclear single quantum correlation spectroscopy; HMBC, ^{1}H-^{13}C heteronuclear multiple bond correlation spectroscopy; PL, phospholipid.

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and 20*S*,23-dihydroxyvitamin D3 [20,23(OH)$_2$D3] [8–18]. This pathway was initially elucidated from *in vitro* studies with purified bovine CYP11A1, and more recently has been demonstrated to occur in keratinocyte cell cultures and in fragments of adrenal glands and human placenta incubated with vitamin D3 [16,18,19]. Most recently, 20(OH)D3 and 20,23(OH)$_2$D3 have been detected at relative levels similar to the classical 25(OH)D3 and 1,25(OH)$_2$D3 in human epidermal tissue [20], confirming their production *in vivo*. Possible physiological roles for these metabolites remain to be established.

20(OH)D3 and 20,23(OH)$_2$D3 are the most extensively studied of the CYP11A1-derived secosteroids in terms of their *in vitro* biological actions. Both act as biased agonists on the vitamin D receptor and thus display many, but not all, of the biological effects of 1,25(OH)$_2$D3 [16,21–23]. They can both also act as inverse agonists on RORα and RORγ [24]. They have the ability to promote differentiation and suppress proliferation of a number of normal and cancerous cells *in vitro*, such as keratinocytes, melanocytes, fibroblasts, melanoma and leukemia cells [16,21–23,25–28]. 20(OH)D3 and 20,23(OH)$_2$D3 promote anti-inflammatory activity in normal and immortalized keratinocytes by increasing the expression of IκB, thus attenuating the transcriptional activity of NF-κB [23,27,29]. In addition, both 20(OH)D3 and 20,23(OH)$_2$D3 possess anti-fibrotic properties on human dermal fibroblasts isolated from scleroderma and normal subjects [26]. In rodent models, administration of 20(OH)D3 has been found to be effective in reducing the symptoms of scleroderma [26] and rheumatoid arthritis [16] as well as protecting DNA in skin from damage caused by UV irradiation [30]. Importantly, unlike 1,25(OH)$_2$D3, both 20(OH)D3 and 20,23(OH)$_2$D3 are non-calcemic in rodents at high concentrations [25,26,31]. Thus both 20(OH)D3 and 20,23(OH)$_2$D3 have therapeutic potential for the treatment of hyperproliferative and inflammatory disorders.

Recently it has been reported that 20(OH)D3 can be further metabolized by cytochromes P450 involved in the metabolism of vitamin D3. Human CYP27A1 converts 20(OH)D3 to 20*S*,25-dihydroxyvitamin D3 [20,25(OH)$_2$D3] and 20*S*,26-dihydroxyvitamin D3 [20,26(OH)$_2$D3], whereas rat CYP24A1 produces 20*S*,24-dihydroxyvitamin D3 [20,24(OH)$_2$D3] and 20,25(OH)$_2$D3 [32,33]. Other P450 isoforms found in mouse liver microsomes also produce 20,25(OH)$_2$D3 and 20,26(OH)$_2$D3 [34]. These resulting secosteroids are more potent than the parent 20(OH)D3 in the inhibition of melanoma colony formation [33]. However, addition of the 1α-hydroxyl group to 20(OH)D3 by CYP27B1 producing 1α,20*S*-dihydroxyvitamin D3, confers some calcemic activity although less than that observed with 1,25(OH)$_2$D3 [25]. Recently we have successfully extracted and partially purified human CYP24A1, and characterized its activity toward 1,25(OH)$_2$D3 and the intermediates of the C24-oxidation pathway [35]. In the present study we used human CYP24A1, along with rat CYP24A1, to characterize the metabolism of both 20(OH)D3 and 20,23(OH)$_2$D3.

2. Materials and methods

2.1. Materials

20(OH)D3 and 20,23(OH)$_2$D3 were synthesized from vitamin D3 enzymatically using bovine CYP11A1 and were purified by TLC and HPLC, as previously described [10,17]. Vitamin D3, dioleoyl phosphaditylcholine, bovine heart cardiolipin, 2-hydroxylpropyl-β-cyclodextrin (cyclodextrin) and glucose-6-phosphate were purchased from Sigma (Sydney, Australia). Glucose-6-phosphate dehydrogenase was from Roche (Mannheim, Germany). All solvents were of HPLC grade and were purchased from Merck (Darmstadt, Germany).

2.2. Preparation of enzymes

Rat and human CYP24A1 were expressed and purified as previously described [33,35]. Human and mouse adrenodoxin, and human adrenodoxin reductase were expressed in *Escherichia coli* and purified as before [17,36,37].

2.3. Measurement of secosteroid metabolism by CYP24A1 in a phospholipid vesicle reconstituted system

Dioleoyl phosphaditycholine (1.08 μmol), bovine heart cardiolipin (0.19 μmol) and the secosteroid substrate (as required) were aliquotted into glass tubes and the ethanol solvent removed under nitrogen gas. Assay buffer, pH 7.4 (20 mM HEPES, 100 mM NaCl, 0.1 mM EDTA and 0.1 mM DTT) (0.5 mL) was added to the dried lipid mixture. This was purged for 30 s with nitrogen gas and then tubes sonicated for approximately 10 min in a bath-type sonicator, until the solution was clear [38]. The incubation mixture was composed of vesicles (510 μM phospholipid), P450 (0.01–0.05 μM for human CYP24A1, and 0.05–1 μM for rat CYP24A1), human or mouse adrenodoxin (15 μM), human adrenodoxin reductase (0.4 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (2 U/mL) and NADPH (50 μM), in assay buffer. Following preincubation for 3 min, reactions were started by the addition of adrenodoxin and samples (0.25–2.5 mL) incubated at 37 °C, with shaking (see for reaction times). Reactions were terminated by the addition of 2.5-volumes of ice-cold dichloromethane and samples were extracted four times with vortexing and centrifugation. The samples were dried under nitrogen gas and redissolved in ethanol for HPLC analysis. The samples were analysed on a PerkinElmer modular HPLC system which comprised a Biocompatible Binary LC pump (model 250; PerkinElmer Corporation, MA, USA) and a UV detector (LC-135C; PerkinElmer Corporation, MA, USA) set at 265 nm, equipped with a C18 analytical column (Grace Alltima, 250 × 4.6 mm, particle size 5 μm; Grace Davison Discovery Sciences, VIC, Australia). Different HPLC programs were used depending on the substrate. For the separation of monohydroxyvitamin D substrates and their products, a 20 min gradient from 45% (v/v) acetonitrile in water to 100% acetonitrile, followed by 30 min at 100% acetonitrile, all at a flow rate of 0.5 mL/min (HPLC Program A), was used. A 40 min gradient from 30% (v/v) acetonitrile in water to 100% acetonitrile, followed by 15 min at 100% acetonitrile, all at a flow rate of 0.5 mL/min, was used to separate polyhydroxyvitamin D substrates and products (HPLC Program B). The peak areas were integrated using Clarity software (DataApex, Prague, Czech Republic). Kinetic parameters were determined by fitting the Michaelis–Menten equation to the experimental data using Kaleidagraph, version 4.1 (Synergy Software, Reading, PA, U.S.A.).

2.4. Enzymatic synthesis and HPLC purification of 20,24(OH)$_2$D3

To produce 20,24(OH)$_2$D3 for NMR analysis, a large scale incubation (30 mL) of rat CYP24A1 with 20(OH)D3 was carried out, as described previously [33]. The 20,24(OH)$_2$D3 and other products were purified by HPLC, as outlined before [33], using a Grace Alltima column (as above) and a 45–58% (v/v) acetonitrile in water gradient over 25 min followed by a 10 min gradient from 58% (v/v) acetonitrile in water to 100% acetonitrile, ending with 20 min at 100% acetonitrile, all at a flow rate of 0.5 mL/min (HPLC Program C). A further HPLC purification step was carried out using the same column employing a 45 min gradient from 64% (v/v) methanol in water to 100% methanol, followed with 15 min at 100% methanol, all at a flow rate of 0.5 mL/min. Collected products were pooled and dried under nitrogen, dissolved in ethanol and the amount of

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purified secosteroid was measured spectrophotometrically using an extinction coefficient of 18,000 M^{-1} cm^{-1} [39].

2.5. Large scale enzymatic synthesis of metabolites of 20,23(OH)$_2$D3

A stock solution of 20,23(OH)$_2$D3 (0.45 mM) in cyclodextrin was prepared by drying an aliquot of 20,23(OH)$_2$D3 and redissolving it in 4.5% (w/v) cyclodextrin by stirring in the dark overnight. Expressed rat CYP24A1 (1 μM) was incubated with the 20,23(OH)$_2$D3 (56 μM) at a final cyclodextrin concentration of 0.56%, for 90 min at 37 °C, in a 20 mL incubation. Other reaction components, except phospholipids, were as described above for the phospholipid vesicle system. The extraction of the products was also as described above. Initial HPLC purification of the products was carried out using HPLC Program B. One of the products, 20,23,25(OH)$_3$D3, required further purification which was done using a 40 min gradient of 64% (v/v) methanol in water to 100% methanol, followed by 20 min at 100% methanol on the same C18 column. The yield of products formed was determined spectrophotometrically, as described before.

2.6. Mass spectrometry

The molecular masses of the intermediates of the CYP24A1 action on 20,23(OH)$_2$D3 (low resolution mass spectra) were determined by 2 dimensional (2D) UPLC tandem mass spectrometry in a similar manner to that described in detail by Clarke et al. [40]. The system consisted of two Agilent 1290 UPLC binary pumps coupled to an Agilent 6460 triple quadrupole tandem mass spectrometer with a Jetstream source. Separation of the intermediates was carried out by two pentafluorophenyl (PFP) columns (100 Å), both run isocratically with 80% (v/v) methanol in water containing 0.1% (v/v) formic acid. The mass spectrometer was operated in positive ESI (electrospray ionization) mode. Three μg of each was diluted 1/1000 with 70% (v/v) methanol in water and 20 μL was injected to the system.

High resolution mass spectra were acquired in a Waters Xevo G2-S system (Waters, Milford, MA) utilizing an ESI source with a Waters Acquity I-Class UPLC and BEH C18 column (2.1 mm × 50 mm, 1.7 μm, Waters, Milford, USA). Data were collected and processed by Masslynx 4.1 software.

2.7. NMR

NMR measurements of 20,24(OH)$_2$D3 were performed using an inverse triple-resonance 3 mm probe on a Varian Unity Inova 500 MHz spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA). Sample was dissolved in CD_3OD and transferred to a 3 mm Shigemi NMR tube (Shigemi Inc., Allison Park, PA, USA). Temperature was regulated at 22 °C and was controlled with an accuracy of ±0.1 °C. Chemical shifts were referenced to residual solvent peaks for CD_3OD (3.31 ppm for proton and 49.15 ppm for carbon). Standard two-dimensional NMR experiments [^{1}H-^{1}H total correlation spectroscopy (TOCSY, mixing time = 80 ms), ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{13}C heteronuclear single quantum correlation spectroscopy (HSQC), and ^{1}H-^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC)] were acquired in order to fully elucidate the structures of the metabolites. All data were processed using ACD software (Advanced Chemistry Development, Toronto, ON, Canada), with zero-filling in the direct dimension and linear prediction in the indirect dimension. NMR data of 20,23,25(OH)$_3$D3 and 20,23,24(OH)$_3$D3 were acquired in $CDCl_3$, using Bruker Avance III 400 MHz, with a BBO 5 mm probe with *z*-gradient (Bruker BioSpin, Billerica, MA). The spectrometer was equipped with an autosampler and IconNMR Automation was used within TopSpin 3.0 for data acquisition.

3. Results

3.1. Rat CYP24A1 metabolizes 20(OH)D3 to produce the two C24 enantiomers of 20,24-dihydroxyvitamin D3

We have previously reported that rat CYP24A1 can metabolize 20(OH)D3 to at least five products, with the major two products being identified by NMR as 20,24(OH)$_2$D3 and 20,25(OH)$_2$D3 [33]. The three other products were characterized as species of dihydroxyvitamin D3 by mass spectrometry, with the site of the CYP24A1-catalysed hydroxylation unknown [33]. In this study, we used enzymatic synthesis to produce enough of the third major product, Product C, (Fig. 1) for NMR analysis. Product C (53 μg) was produced by incubating 20(OH)D3 (53 μM in 0.29% (w/v) cyclodextrin) in a 30 mL incubation with 1 μM rat CYP24A1 (see Section 2.4), and purified by reverse phase HPLC using both acetonitrile–water (Fig. 1) and methanol–water solvent systems. The mass spectrum confirmed it was a dihydroxyvitamin D3 with the observed molecular ion m/z = 439.3188 $[M+Na]^+$, as reported before [33] (Fig. S1).

The site of hydroxylation on 20(OH)D3 in Product C was unambiguously assigned to be at the 24-position based on the NMR spectra for this metabolite. First, none of the four methyl carbons (C18, C21, C26, C27) are hydroxylated based on ^{1}H NMR (Fig. 2A). ^{1}H-^{13}C HSQC revealed the presence of a new methine group at 3.22 ppm (^{13}C at 78.3 ppm, Fig. 2B). ^{1}H-^{1}H TOCSY (Fig. 2C) clearly showed that this methine is in the same spin system as 26/27-CH_3 (^{1}H at 0.91 ppm), indicating the hydroxylation occurred in the side chain. From the ^{1}H-^{1}H COSY (Fig. 2D) spectrum, this methine (^{1}H at 3.22 ppm) showed a strong correlation to 25-CH (^{1}H at 1.62 ppm) and 23-CH_2 (^{1}H at 1.62 and 1.45 ppm). From ^{1}H-^{13}C HMBC (Fig. 2E), 26/27-CH_3 (^{1}H at 0.91 ppm) showed a strong correlation to the new methine (^{13}C at 78.3 ppm), in addition to the

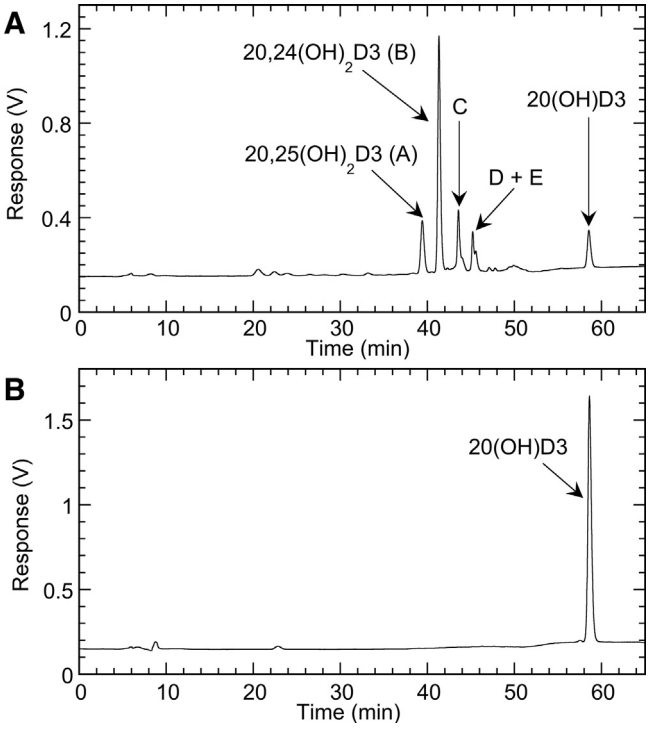

Fig. 1. 20-Hydroxyvitamin D3 is metabolized by rat CYP24A1. Rat CYP24A1 (1 μM) was incubated with 20(OH)D3 dissolved in 0.45% (w/v) cyclodextrin, for 90 min at 37 °C in a reconstituted system containing adrenodoxin and adrenodoxin reductase. The reaction mixture was analysed using HPLC Program C (see Section 2.4). (A) Chromatogram showing test reaction. (B) Chromatogram showing control reaction with NADPH omitted.

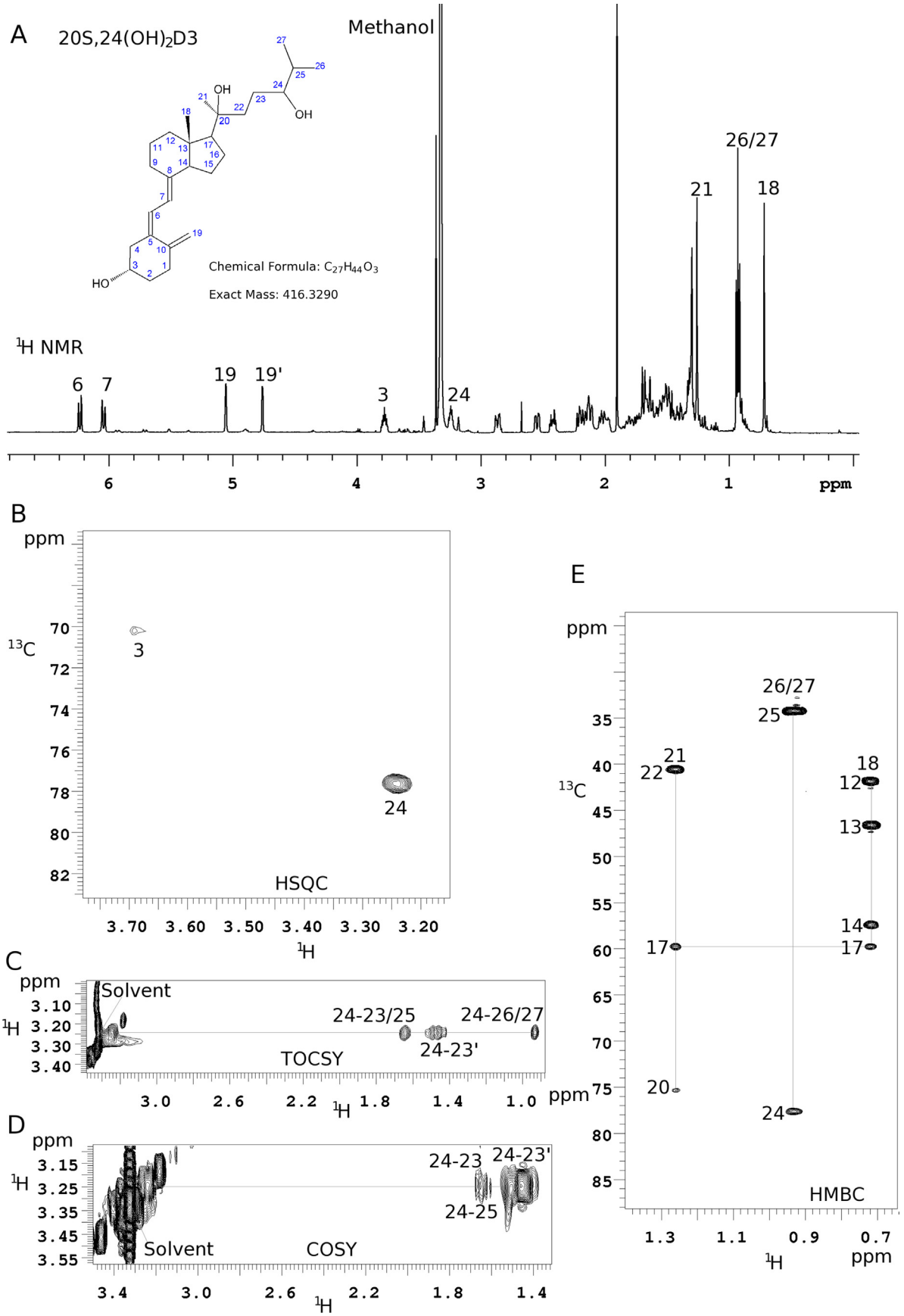

Fig. 2. NMR reveals that Product C is 20,24-dihydroxyvitamin D3. (A) 1D Proton; (B) ^{1}H-^{13}C HSQC; (C) ^{1}H-^{1}H TOCSY; (D) ^{1}H-^{1}H COSY; (E) ^{1}H-^{13}C HMBC.

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expected correlation to 25-CH (^{13}C at 34.8 ppm). Taken together, the above analysis shows that the hydroxylation site can be unambiguously assigned to the 24-position. This product therefore represents the other C24 enantiomer of the previously characterized major reaction product, 20,24(OH)$_2$D3 (Fig. 1), originally designated as Product B [33]. The full assignments for Product C are summarized in Supplementary Table 1. For comparison, we also included the assignments for the parent compound 20(OH)D3 and the other enantiomer, Product B [33]. However, we were unable to establish the absolute configurations at the 24-position for both isomers at this stage because of the lack of high resolution NMR data, due to the limiting amount of these secosteroids.

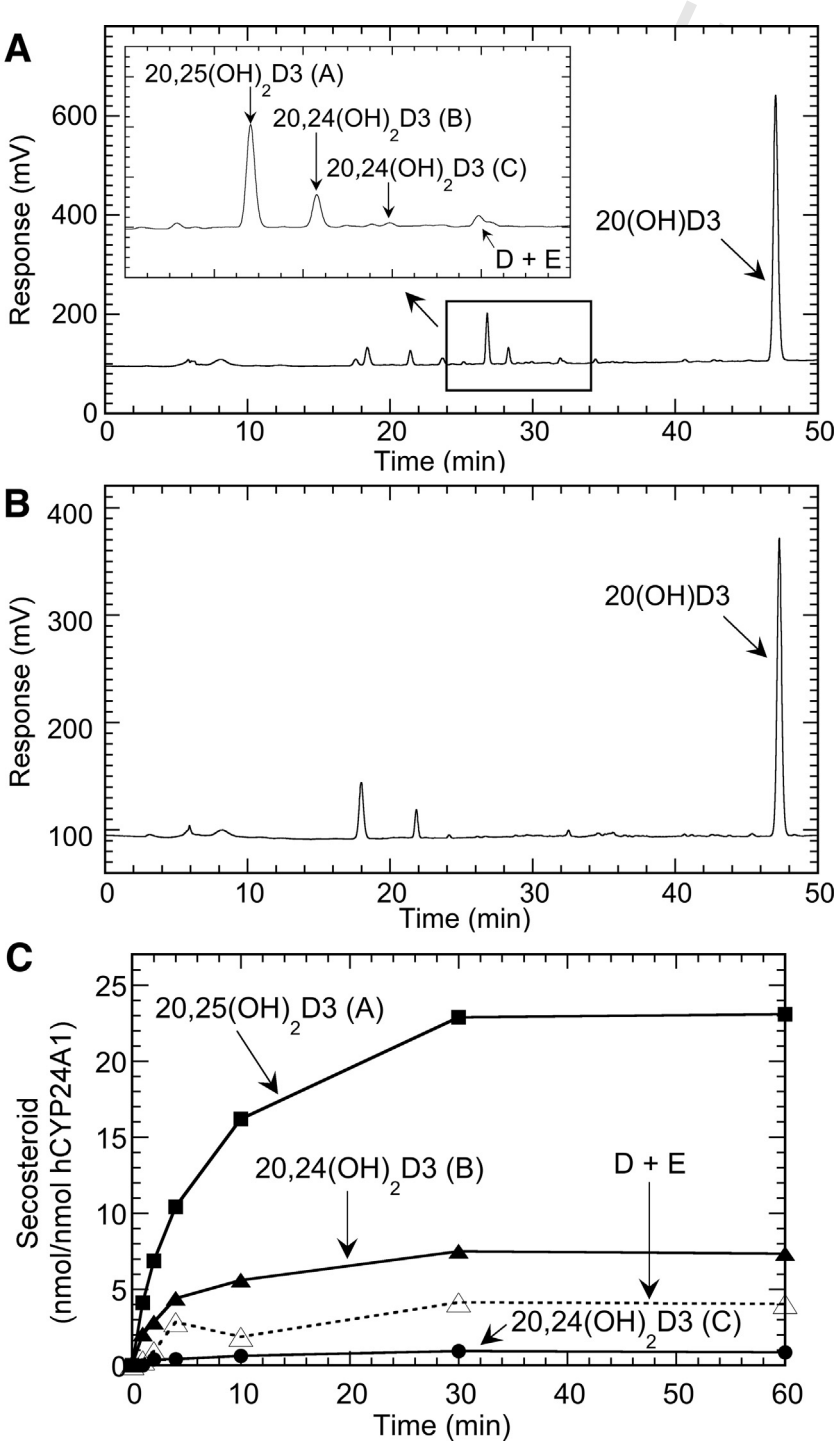

Fig. 3. Human CYP24A1 acts on 20-hydroxyvitamin D3 producing similar products to the rat enzyme. (A) 20(OH)D3 was incorporated in phospholipid vesicles at a ratio of 0.05 mol/mol phospholipid and incubated with human CYP24A1 (0.05 μM) in a reconstituted system containing adrenodoxin and adrenodoxin reductase, for 30 min at 37 °C. Samples were analysed using HPLC Program A (see Section 2.3). Inset, enlarged view of the chromatogram from 24 to 34 min. (B) Control reaction showing that lack of products when adrenodoxin was omitted. Peaks present in the control as well as the test at retention times of 18, 20 and 34 min, which appear to originate from the phospholipids, were not considered to be 20(OH)D3 products. (C) Time course for the metabolism of 20(OH)D3 in phospholipid vesicles by human CYP24A1. The same conditions were used as outlined in Fig. 3A.

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3.2. Human CYP24A1 can metabolize 20(OH)D3 to dihydroxyvitamin D products similar to rat CYP24A1

The bacterial expression and partial purification of human CYP24A1 [35] enabled us to test this isoform of the enzyme on the metabolism of 20(OH)D3, although only at a low enzyme concentration due to its low expression. As for the rat enzyme [33], 20(OH)D3 was incorporated into phospholipid vesicles, a system that mimics the inner mitochondrial membrane where the enzyme is located *in vivo* [38,41–43]. Human CYP24A1 metabolized 20(OH)D3 to several products (Fig. 3). The two major ones were identified as 20,25$(OH)_2$D3 (Product A) and 20,24$(OH)_2$D3 (Product B) by comparison of their retention times to those of the authentic standards produced using rat CYP24A1 [33]. The other enantiomer of 20,24$(OH)_2$D3 (Product C) seen with the rat enzyme (Fig. 1) was present as a minor product of the human enzyme, and a peak with a shoulder corresponding to Products D and E was also seen (Fig. 3).

The time course for metabolism of 20(OH)D3 by human CYP24A1 is shown in Fig. 3C. After the 1 h incubation at 37 °C with 0.05 μM P450, 18% of the 20(OH)D3 substrate was consumed. None of the products measured in the time course displayed a lag,

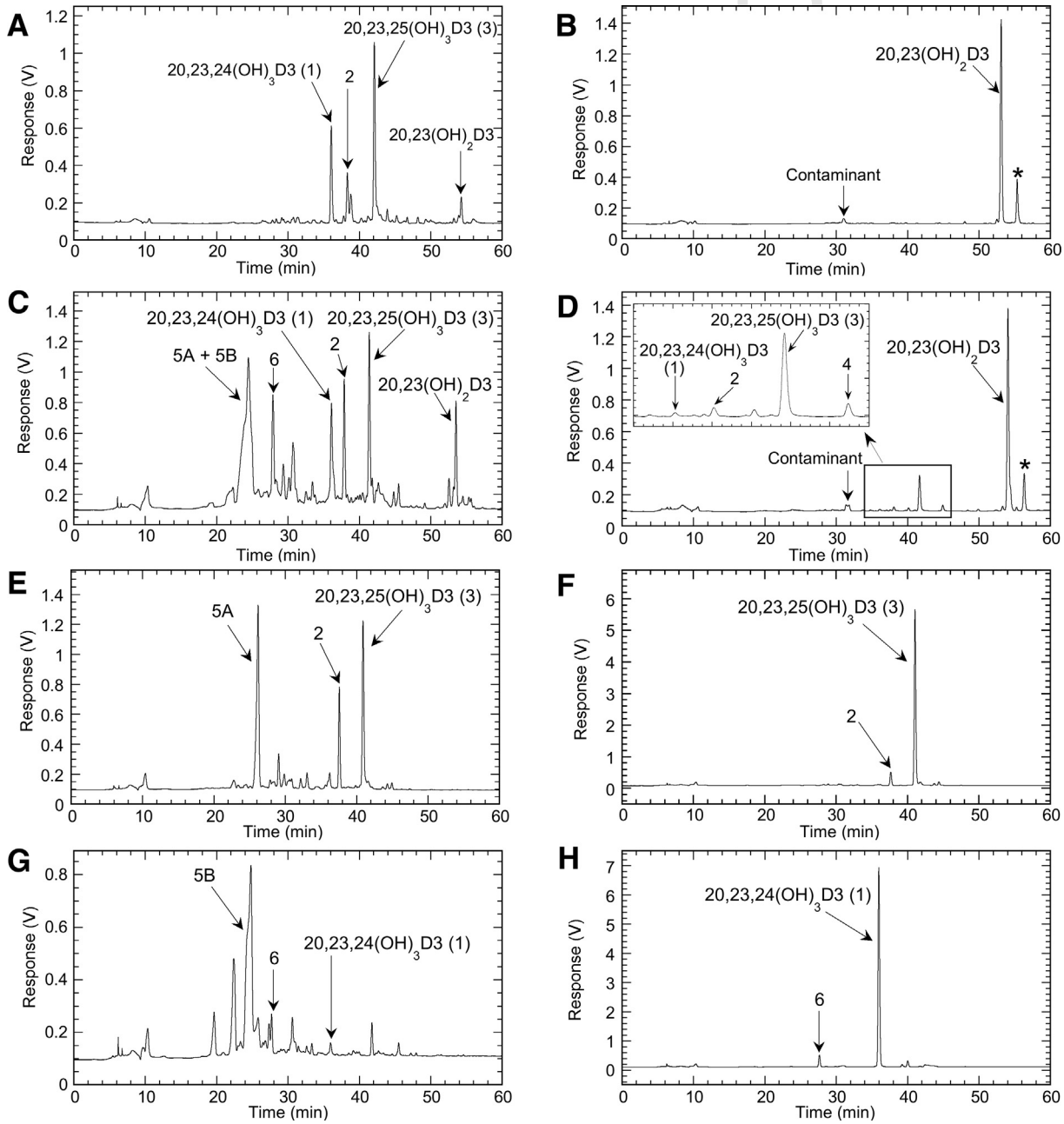

Fig. 4. Both rat and human CYP24A1 metabolize 20,23-dihydroxyvitamin D3. (A) Rat CYP24A1 (1 μM) was incubated with 20,23$(OH)_2$D3 (50 μM) dissolved in 0.45% (w/v) cyclodextrin, for 1 h at 37 °C. (B, C) Rat CYP24A1 (1 μM) was incubated with 20,23$(OH)_2$D3 incorporated in phospholipid vesicles, at a molar ratio of 0.05 mol/mol phospholipid, for 1 h at 37 °C in the absence (B) or presence (C) of adrenodoxin. (D) Human CYP24A1 (0.05 μM) was incubated with 20,23$(OH)_2$D3 incorporated in phospholipid vesicles (0.05 mol/mol), under the same conditions as described for C; inset, expanded view of the chromatogram from 34 to 46 min. 20,23,25$(OH)_3$D3 (E and F) or 20,23,24$(OH)_3$D3 (G and H) were incorporated into phospholipid vesicles (0.05 mol/mol), and incubated with 1 μM rat CYP24A1 (E and G) or 0.05 μM human CYP24A1 (F and H) at 37 °C for 30 min. Samples were analysed by HPLC using Program B (see Section 2.3). The controls for 20,23,24$(OH)_3$D3 and 20,23,25$(OH)_3$D3 (not shown) showed the same contaminant peak as seen in Fig. 4B. The asterisks denotes the putative pre-20,23$(OH)_2$D3.

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Table 1
Mass spectrometric identification of products formed from metabolism of 20,23-dihydroxyvitamin D3 by CYP24A1. Products 1, 3 and the combined product peak for 5 A and B were formed from the action of rat CYP24A1 on 20,23(OH)$_2$D3. Products 5A and 2 were made from 20,23,25(OH)$_3$D3. Products 5B and 6 were made from 20,23,24(OH)$_3$D3. Q10

Product	Exact mass	Calc. mass	Error (ppm)	Formula	Observed	Assignment
1	455.3151	455.3139	2.6	$C_{27}H_{44}O_4Na$	$M+Na^+$	20,23,24(OH)$_3$D3
	415.3222	415.3214	1.9	$C_{27}H_{43}O_3$	$M+H^+-H_2O$	
	397.312	397.3108	3	$C_{27}H_{41}O_2$	$M+H^+-2H_2O$	
	379.301	379.3002	2.1	$C_{27}H_{39}O$	$M+H^+-3H_2O$	
3	455.3138	455.3139	0.2	$C_{27}H_{44}O_4Na$	$M+Na^+$	20,23,25(OH)$_3$D3
	415.3205	415.3214	2.2	$C_{27}H_{43}O_3$	$M+H^+-H_2O$	
	397.3099	397.3108	−2.3	$C_{27}H_{41}O_2$	$M+H^+-2H_2O$	
	379.2997	379.3002	−1.3	$C_{27}H_{39}O$	$M+H^+-3H_2O$	
5A + 5B	469.2914	469.293	−3.4	$C_{27}H_{42}O_5Na$	$M+Na^+$	20(OH)D3-23COOH and Dehydro-20,23,24,Y(OH)$_4$D3
	429.3006	429.3005	0.2	$C_{27}H_{41}O_4$	$M+H^+-H_2O$	
	411.2901	411.2899	0.5	$C_{27}H_{39}O_3$	$M+H^+-2H_2O$	
5A	483.2719	483.2723	−0.8	$C_{27}H_{40}O_6Na$	?	Unknown
	357.2429	357.243	−0.3	$C_{23}H_{33}O_3$	$M+H^+-H_2O$	20(OH)D3-23COOH
	339.2329	339.2324	1.5	$C_{23}H_{31}O_2$	$M+H^+-2H_2O$	
5B	469.2921	469.293	−1.9	$C_{27}H_{42}O_5Na$	$M+Na^+$	Dehydro-20,23,24,Y(OH)$_4$D3
	429.3001	429.3005	−0.9	$C_{27}H_{41}O_4$	$M+H^+-H_2O$	
	411.2897	411.2899	−0.5	$C_{27}H_{39}O_3$	$M+H^+-2H_2O$	
	393.2803	393.2794	2.3	$C_{27}H_{37}O_2$	$M+H^+-3H_2O$	
6	469.2934	469.293	0.9	$C_{27}H_{42}O_5Na$	$M+Na^+$	Dehydro-20,23,24,*X*(OH)$_4$D3
	411.2901	411.2899	0.5	$C_{27}H_{39}O_3$	$M+H^+-2H_2O$	
	393.2804	393.2794	2.5	$C_{27}H_{37}O_2$	$M+H^+-3H_2O$	

consistent with all of them being primary products with the addition of just one hydroxyl group, as reported for the rat enzyme [33]. The time course also shows that human CYP24A1 favors hydroxylating at the C25 position of 20(OH)D3 over the C24 position at an approximate ratio of 3:1, the opposite of what is observed for the rat enzyme (Fig. 1 and [33]).

3.3. Metabolism of 20,23(OH)$_2$D3 by CYP24A1 results in some cleavage of the side chain

Initially, the metabolism of 20,23(OH)$_2$D3 by rat CYP24A1 was measured with substrate dissolved in cyclodextrin as this system is good for scaling reactions up to produce sufficient vitamin D metabolites for NMR. It enables high substrate concentrations to be used and has been employed extensively by us in the past for synthesizing products of other vitamin D-metabolizing enzymes [10,12,33]. Three major products were observed in a 1 h incubation of rat CYP24A1 using this system (Fig. 4A). The two major ones were identified as 20*S*,23,24-trihydroxyvitamin D3 [20,23,24(OH)$_3$D3] and 20*S*,23,25-trihydroxyvitamin D3 [20,23,25(OH)$_3$D3] by NMR, as presented in detail later. In contrast to cyclodextrin, incubation of rat CYP24A1 with 20,23(OH)$_2$D3 incorporated into phospholipid vesicles resulted in the appearance of several more products (Fig. 4C), which are likely to be downstream metabolites arising from the major products observed in Fig. 4A. This shows that the cyclodextrin reconstituted system is not conducive to the conversion of primary products to downstream secondary products. The same primary products (Products 1–3) were seen for the human enzyme based on their identical retention times to the products from the rat enzyme, although they were produced in different proportions (Fig. 4D). Due to its low expression and lability, only a low concentration of the human enzyme was used in this experiment which was carried out with 20,23(OH)$_2$D3 incorporated into phospholipid vesicles. An additional major product (Product 4) was seen for the human enzyme, but was only present in a trace amount for rat CYP24A1.

In incubations of 20,23(OH)$_2$D3 with CYP24A1, a small peak was consistently detected with a retention time slightly longer than that of the substrate, 20,23(OH)$_2$D3 (denoted by the asterisk in Fig. 4D). This peak was also present in the control incubation where adrenodoxin was omitted indicating that CYP24A1 is not involved in its formation (Fig. 4B). This peak was collected and shown to be a vitamin D3 derivative, with the same UV spectrum as 20,23(OH)$_2$D3 (not shown). Rechromatography of the collected product in the same solvent system revealed that it was partially converted back to a compound with the same retention time as 20,23(OH)$_2$D3. It would therefore appear to be pre-20,23(OH)$_2$D3, which results from the reversible thermoisomerization of 20,23(OH)$_2$D3 [44].

The products of 20,23(OH)$_2$D3 metabolism by rat CYP24A1 with the substrate dissolved in cyclodextrin (Fig. 4A) were collected and analysed by mass spectrometry. High resolution mass spectrometry of Product 1 gave the parent ion at m/z = 445.3138 $[M+Na]^+$ corresponding to a molecular weight of 432.6359, indicating that it is a trihydroxyvitamin D3 species (Table 1, Fig. S2A). Product 3 gave the parent ion at m/z = 455.3137 $[M+Na]^+$, indicating that it is also a trihydroxyvitamin D3 species (Table 1, Fig. S2B). Analysis of Product 2 by low resolution mass spectrometry gave the parent ion with m/z = 453.1 $[M+Na]^+$ and additional ions with m/z = 413.1 and 395.1, which correspond to $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$, respectively. The molecular weight of 430.1 suggests that this product is formed by oxidation of one of the three side chain hydroxyl groups in Product 1 or Product 3 to a ketone (producing a dehydro-trihydroxyvitamin D3 species), an oxidation known to occur for the 24-hydroxyl group of 1,24,25(OH)$_3$D3 and 24,25(OH)$_2$D3 in the C24-oxidation pathway of vitamin D inactivation [35,45]. The broad peak labeled as Product 5A and B (Fig. 4C), produced from 20,23(OH)$_2$D3 in phospholipid vesicles, was a mixture of two secosteroids based on its retention time and mass spectrum, and will be described later.

3.4. Enzymatic production of 20,23,24(OH)$_3$D3, and 20,23,25(OH)$_3$D3 by CYP24A1 for structure determination

As seen in Fig. 4A, rat CYP24A1 almost completely metabolized 20,23(OH)$_2$D3 solubilized in cyclodextrin and converted it to two

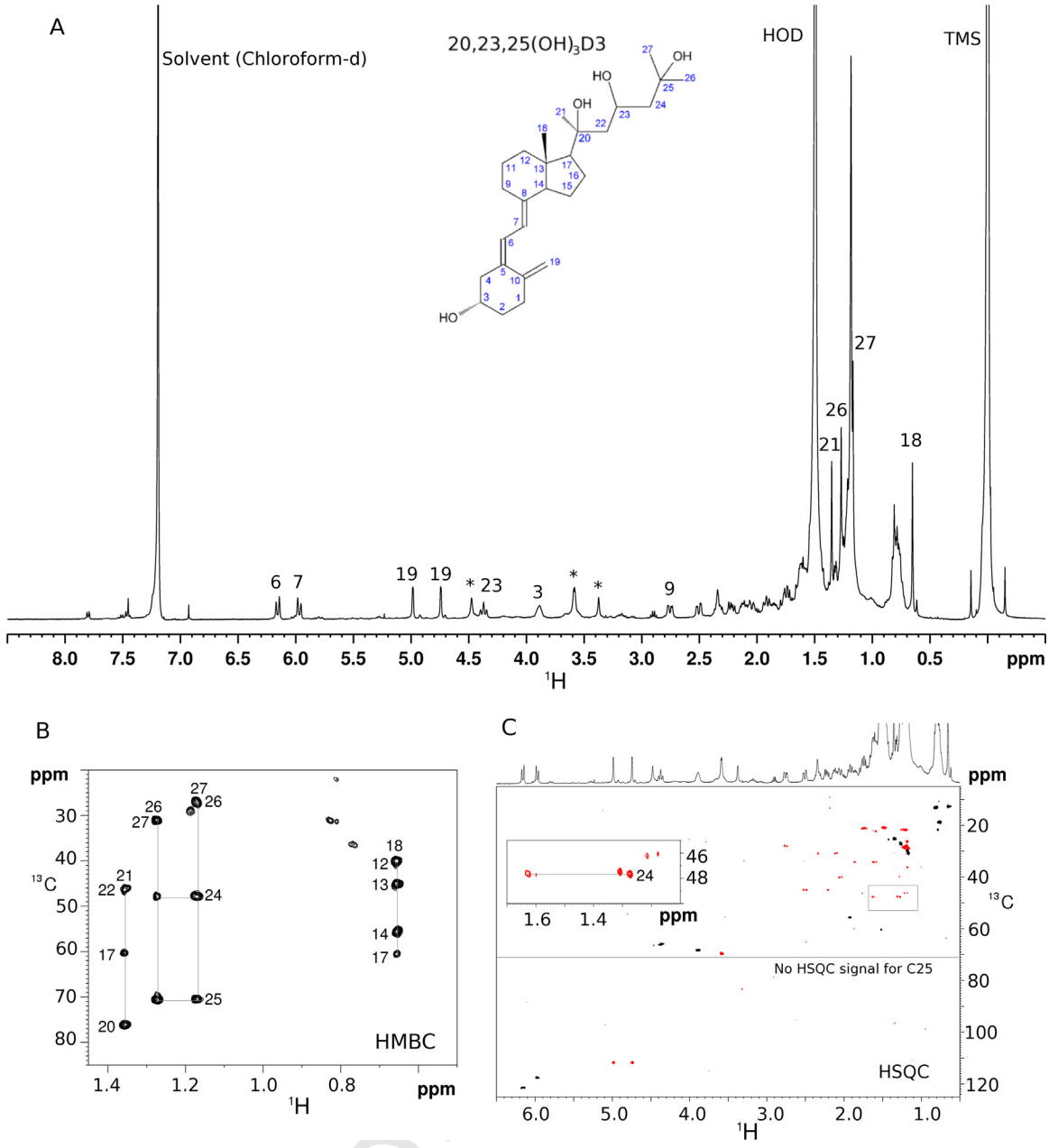

Fig. 5. NMR reveals that Product 3 is 20,23,25-trihydroxyvitamin D3. (A) 1D Proton; (B) ^{1}H–^{13}C HMBC; (C) ^{1}H–^{13}C HSQC. Peaks labeled with asterisk (*) sign were not identified.

major trihydroxyvitamin D3 species, labeled as Product 1 and Product 3. This reaction was scaled up to 20 mL to produce enough of Products 1 and 3 to enable their structure to be determination by NMR. Almost complete conversion of 20,23$(OH)_2$D3 was achieved with 1 μM rat CYP24A1 in a 90 min incubation. Overall, 83 μg of Product 1 and 108 μg of Product 3 were obtained following their purification in acetonitrile–water and methanol–water solvent systems, which was sufficient for structure determination by NMR.

3.5. NMR identification of 20,23,24(OH)$_3$D3 and 20,23,25(OH)$_3$D3 as major products of rat CYP24A1 action on 20,23(OH)$_2$D3

Based on the current NMR spectra and previous data for 20,23$(OH)_2$D3 [17], the site of the third hydroxylation of Product 3 was assigned to be at the 25-position (Fig. 5A). The carbon chemical shift of C25 moved from 25.2 ppm in 20,23$(OH)_2$D3 to 70.7 ppm in Product 3 [17] (Fig. 5B). This carbon does not have any proton directly attached to it based on the ^{1}H-^{13}C HSQC (Fig. 5C). The chemical shift of C24 of Product 3 is at 47.7 ppm and it is a methylene group (—CH_2) as revealed by ^{1}H-^{13}C HSQC data (Fig. 5C). Based on the above analysis, Product 3 was assigned 20,23,25$(OH)_3$D3.

Product 1 was positively identified as 20,23,24$(OH)_3$D3 (Fig. 6). There are two sets of peaks centered at 3.48 ppm, and 3.30 ppm on the proton spectrum of Product 1 (Fig. 6A), which are not present in Product 3. Based on ^{1}H-^{1}H TOCSY (Fig. 6B), both signals at 3.48 ppm, and 3.30 ppm shared the same spin system as 26/27-CH_3 (0.83/0.79 ppm). They also showed correlations to 22-CH_2, and 21-CH_3 (Fig. 6B). By lowering the contour levels, weak correlations to 23-CH were also positively identified (Fig. 6B). In addition, they showed correlation to each other in COSY indicating that they should be next to each other in the structure (Fig. 6C). Based on the above analysis, they were assigned to 24-CH (3.30 ppm) and 25-CH (3.48 ppm), and Product 1 was assigned 20,23,24$(OH)_3$D3. The full

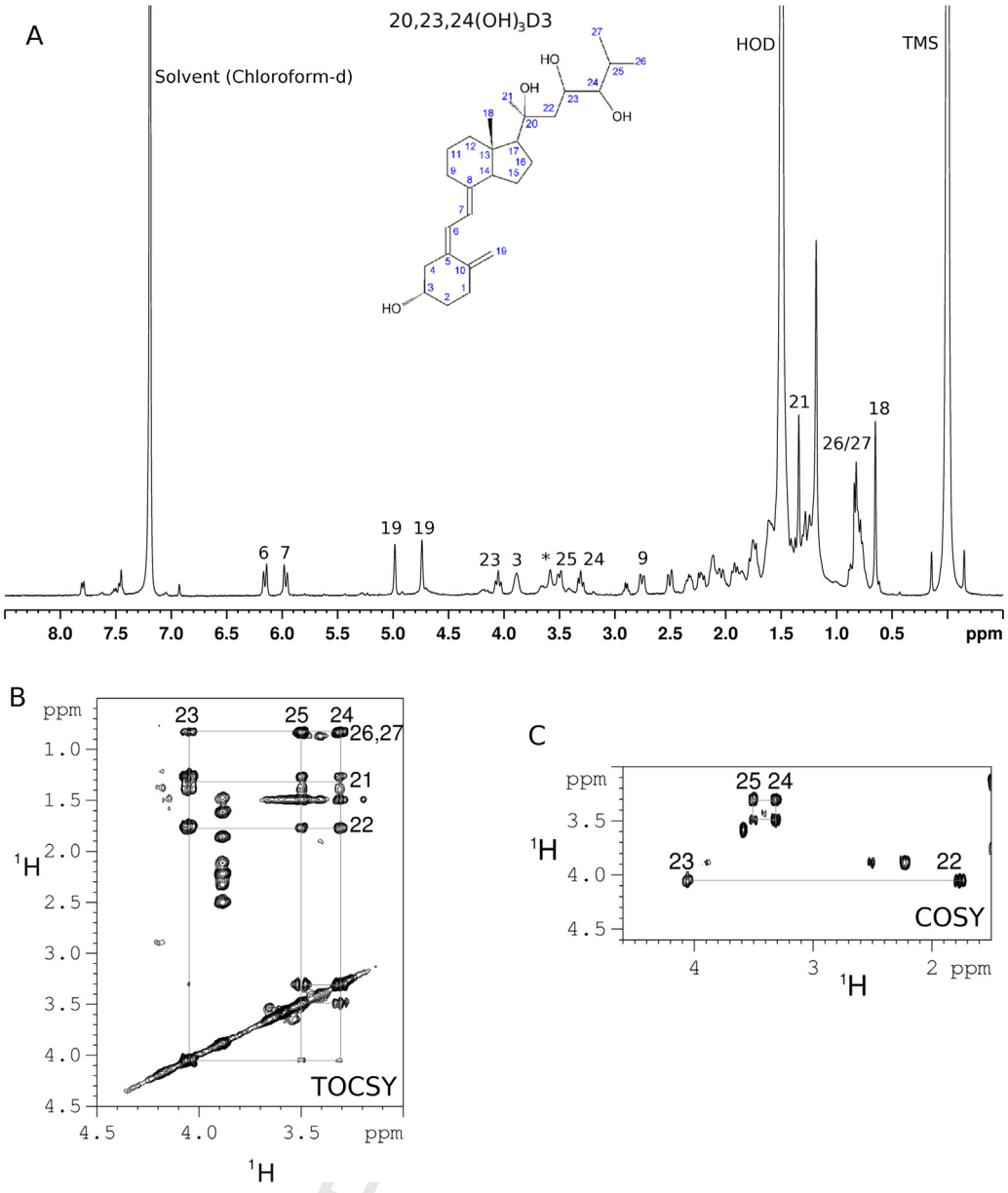

Fig. 6. Product 1 was identified as 20,23,24-trihydroxyvitamin D3 by NMR. (A) 1D Proton; (B) ^{1}H–^{1}H TOCSY; (C) ^{1}H–^{1}H COSY. Peaks labeled with asterisk (*) sign were not identified.

assignments for 20,23,24$(OH)_3$D3 (Product 1) and 20,23,25$(OH)_3$D3 (Product 3) are summarized in Supplementary Table 2.

3.6. Time course for the metabolism of 20,23$(OH)_2$D3 by CYP24A1

The time course for metabolism of 20,23$(OH)_2$D3 incorporated into phospholipid vesicles by human CYP24A1 shows that 20,23,25$(OH)_3$D3 was the major product of the reaction throughout the 1 h incubation (Fig. 7A). The two minor products, 20,23,24$(OH)_3$D3 and Product 2, were not detected until 30 min of incubation but this was likely due to the sensitivity of detection rather than there being a lag in their production, especially for the primary product, 20,23,24$(OH)_3$D3. The reaction was linear for the first 2 min and 18% of substrate was consumed in 1 h with 0.05 μM human CYP24A1.

The time course for the metabolism of 20,23$(OH)_2$D3 incorporated into phospholipid vesicles by rat CYP24A1 (Fig. 7B) shows that there is immediate formation of 20,23,24$(OH)_3$D3 (Product 1) and 20,23,25$(OH)_3$D3 (Product 3). After the 1 h incubation with 1 μM rat CYP24A1, only 15% of substrate remained. The proportions of the two major products diminished after the initial 10 min, suggesting that they serve as precursors that give rise to some of the minor products (Fig. 7B). Consistent with this, Products 2, 5A, 5B and 6 displayed lags in their time courses suggesting that they are downstream secondary metabolites, arising from further metabolism of 20,23,24$(OH)_3$D3 or 20,23,25$(OH)_3$D3. Both rat and human CYP24A1 displayed a preference for hydroxylating at C25 of 20,23$(OH)_2$D3, with the proportion of 20,23,25$(OH)_3$D3 to 20,23,24$(OH)_3$D3 being 2.3:1 and 19:1 for the rat and human CYP24A1, respectively, at the end of the incubation.

3.7. 20,23,24$(OH)_3$D3, and 20,23,25$(OH)_3$D3 can be further metabolized by CYP24A1

Following NMR, 20,23,24$(OH)_3$D3 and 20,23,25$(OH)_3$D3 were repurified and used as substrates for CYP24A1. Due to the higher

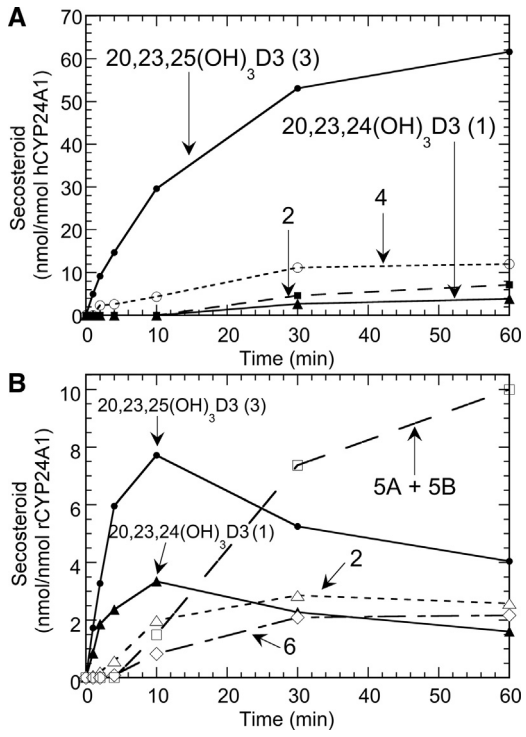

Fig. 7. Time course for the metabolism of 20,23-dihydroxyvitamin D3 in phospholipid vesicles by CYP24A1. (A) Human CYP24A1 (0.05 μM) and (B) rat CYP24A1 (1 μM) were incubated with 20,23(OH)$_2$D3 (at a molar ratio of 0.05 mol/mol phospholipid) in a reconstituted system with adrenodoxin and adrenodoxin reductase. The samples were analysed using HPLC Program B (see Section 2.3). Product numbers refer to the peaks shown in Fig. 4.

expression of rat CYP24A1 than the human enzyme, enabling us to use higher final concentration of P450, rat CYP24A1 was used to generate more downstream metabolites from 20,23,24(OH)$_3$D3 and 20,23,25(OH)$_3$D3 for mass spectral analysis. The human CYP24A1 enzyme was used at a final concentration that was 20-fold lower than for rat CYP24A1, but this still permitted some of the earlier metabolites to be observed (Fig. 4F and H).

When 20,23,25(OH)$_3$D3 incorporated into phospholipid vesicles was incubated with rat CYP24A1, two major products were observed (Fig. 4E). These were labeled as Products 2 and 5A, based on alignment of their HPLC retention times with products from the reaction of CYP24A1 on 20,23(OH)$_2$D3 (Fig. 4A and C). After the 30 min incubation with 1 μM rat CYP24A1, 32% of the substrate remained and Product 5A accounted for 75% of the total products formed. Incubation of 20,23,25(OH)$_3$D3 with human CYP24A1 yielded only Product 2 (Fig. 4F), suggesting that it is a primary product that may give rise to Product 5A. As described earlier, it was tentatively identified as a dehydro-trihydroxyvitamin D3 species based on its mass spectrum. Product 5A was tentatively identified as 20-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D3 by high resolution mass spectrometry with an exact mass of 374.2457. The mass spectrum gave ion fragments with m/z = 357.2429, 339.2329 and 321.2215, which correspond to $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$ and $[M+H-3H_2O]^+$, respectively (Table 1, Fig. S3). This identification provides strong evidence that CYP24A1 can cleave the side chain of 20,23(OH)$_2$D3 between C23 and C24 subsequent to other oxidation steps, and then oxidize the product to the C23 carboxylic acid, similar to the final steps in the C24-oxidation pathway of 1,25(OH)$_2$D3 metabolism [1,35].

20,23,24(OH)$_3$D3 was observed to be almost completely metabolized by rat CYP24A1 and gave one major and several minor products (Fig. 4G). The main product observed was Product 5B and accounted for 66% of the total products. Product 6 was labeled according to its identical retention time to Product 6 produced from the action of CYP24A1 on 20,23(OH)$_2$D3 (Fig. 4C) and was classified as a dehydro-tetrahydroxyvitamin D3 species by high resolution mass spectrometry (Table 1, Fig. S4). Since it is made from 20,23,24(OH)$_3$D3, it can be further classified as dehydro-20,23,24,X(OH)$_4$D3 where both the position (X) of the new hydroxylation and which hydroxyl group is reduced to a ketone are unknown. Two peaks with shorter retention times than Product 5B (20 and 22 min, Fig. 4G) were observed and may represent further downstream metabolites.

Like Product 6, Product 5B gave the parent with m/z = 469.2921 $[M+Na]^+$, as well as other ion fragments with m/z = 429.3001, 411.2897 and 393.2803, which correspond to $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$ and $[M+H-3H_2O]^+$, respectively (Table 1, Fig. S5). This indicates that Product 5B has an exact mass of 446.3032 and is also a dehydro-tetrahydroxyvitamin D3 species (dehydro-20,23,24,Y(OH)$_4$D3). The high resolution mass spectrum of the combined peak labelled as Product 5A and 5B in Fig. 4C showed ions with m/z values seen in the mass spectrum of Product 5A produced from 20,23,25(OH)$_3$D3 (from the peak in Fig. 4E) and the mass spectrum of Product 5B produced from 20,23,24(OH)$_3$D3 (from the peak in Fig. 4G) (Fig. S6). These two products have almost identical retention times thus both products run together in the reaction starting from 20,23(OH)$_2$D3 (Fig. 4C).

3.8. Kinetics of the metabolism of 20(OH)D3 and 20,23(OH)$_2$D3 in phospholipid vesicles by CYP24A1

Previously we reported the kinetic parameters for the metabolism of 1,25(OH)$_2$D3 and its C24-oxidation pathway intermediates by human CYP24A1 [35]. In the current study, we observed that 20(OH)D3 was metabolized with a k_{cat} of 16.8 ± 1.1 mol/min/mol CYP24A1 and a K_m of 0.031 ± 0.005 mol/mol phospholipid (Fig. 8). The 20,23(OH)$_2$D3 substrate was metabolized with similar kinetic values; k_{cat} = 19.5 ± 2.1 mol/min/mol CYP24A1 and K_m = 0.045 ± 0.014 mol/mol phospholipid (Fig. 8). On the basis of catalytic efficiency, 20(OH)D3 and 20,23(OH)$_2$D3 are both relatively poor substrates for human CYP24A1 being metabolized with k_{cat}/K_m values of 560 and 433 min^{-1}(mol substrate/mol

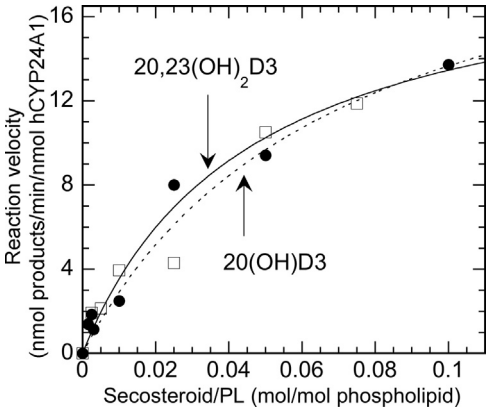

Fig. 8. 20-Hydroxyvitamin D3 and 20,23-dihydroxyvitamin D3 are metabolized with similar K_m and k_{cat} values by human CYP24A1. Secosteroids at varying ratios to phospholipid were incorporated into phospholipid vesicles and incubated with human CYP24A1 (0.02 μM and 0.05 μM for 20(OH)D3 and 20,23(OH)$_2$D3, respectively) for 2 min in a reconstituted system containing adrenodoxin and adrenodoxin reductase. The products were analysed by HPLC using Program A for 20(OH)D3 and HPLC Program B for 20,23(OH)$_2$D3 (see Section 2.3). Hyperbolic curves were fitted by non-linear least squares analysis by Kaleidagraph 4.0. The r values were 0.998 and 0.988 for the curve fit for 20(OH)D3 and 20,23(OH)$_2$D3, respectively. □ 20,23(OH)$_2$D3; ● 20(OH)D3.

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phospholipid)$^{-1}$, respectively, which are markedly lower than for the metabolism of 25(OH)D3 or 1,25$(OH)_2$D3 [35] (see Section 4).

4. Discussion

The novel vitamin D metabolic pathway catalyzed by CYP11A1 produces a number of secosteroids with hydroxyl groups along the C20-27 side chain [9,10,15]. The most extensively characterized secosteroids produced from this pathway with respect to biological activity are 20(OH)D3 and 20,23$(OH)_2$D3 [16,22,23,25–28,32–34,46,47]. In the present study, we show that these two biologically active secosteroids can be metabolized by both rat and human CYP24A1. The rat and human enzymes produce the same major products from 20(OH)D3 and both isoforms can hydroxylate at the two alternate C24 positions of this chiral center to produce the two enantiomers of 20,24$(OH)_2$D3. Interestingly, human CYP24A1 prefers to initially hydroxylate 20(OH)D3 at C25 whereas rat CYP24A1 favors initial hydroxylation at C24. This is not surprising due to the known differences between the rat and human enzymes where rat CYP24A1 catalyses the C24-oxidation pathway, while human CYP24A1 can catabolize vitamin D through the C23- and C24-oxidation pathways. Metabolism of 20(OH)D3 by CYP24A1 is clearly different from that of 25(OH)D3 and 1,25$(OH)_2$D3, as no subsequent oxidations were observed with 20(OH)D3, with all products being dihydroxyvitamins D3 species [33]. The presence of the 20-hydroxyl group prevents 23-hydroxylation as no 20,23$(OH)_2$D3 was seen among the products [33]. Rather, the presence of the 20-hydroxyl group shifts the position of the side chain in the active site such that 20,25$(OH)_2$D3 is the one of the major products of CYP24A1 action on 20(OH)D3. The sites of hydroxylation in the minor products remain to be established.

Like 20(OH)D3, 20,23$(OH)_2$D3 is also hydroxylated at C24 and C25 by CYP24A1, producing 20,23,24$(OH)_3$D3 and 20,23,25$(OH)_3$D3 as initial products for both the rat and human enzymes. The introduction of a hydroxyl group at C23 of 20(OH)D3 (as in 20,23$(OH)_2$D3), shifts the favored site of hydroxylation by the rat enzyme from C24 to C25, the site preferred by the human enzyme with both 20(OH)D3 and 20,23$(OH)_2$D3 as substrates. It is therefore evident that CYP24A1 prefers hydroxylating the secosteroid at the more terminal carbons of the vitamin D side chain when a 23-hydroxyl group is present. This is seen in the C23-oxidation pathway of 25(OH)D3 metabolism where after initial C23 hydroxylation, the next hydroxylation is at C26, with C25 already having a hydroxyl group present [1,3].

The present study shows that human CYP24A1 can act on 20(OH)D3 and 20,23$(OH)_2$D3 with similar K_m and k_{cat} values, and thus comparable catalytic efficiencies (k_{cat}/K_m). In comparing the kinetics of 20(OH)D3 metabolism by rat CYP24A1 to human CYP24A1 in the phospholipid vesicle system, we found that the K_m values are comparable (0.028–0.031 mol substrate/mol phospholipid) but human CYP24A1 had a 1.6-fold higher k_{cat} than rat CYP24A1. Previously we reported the kinetic parameters for the metabolism of 25(OH)D3 and 1,25$(OH)_2$D3 by human CYP24A1 using the same phospholipid-vesicle reconstituted system employed in the current study, thus permitting a direct comparison of values. 1,25$(OH)_2$D3 is metabolized by human CYP24A1 with k_{cat} and K_m values that are 1.6-fold higher and 7-fold lower than that for 20(OH)D3, respectively, with an overall 11-fold higher catalytic efficiency. The relative efficiency for human CYP24A1 is even higher for 25(OH)D3 where k_{cat}/K_m is 34-fold higher than for 20(OH)D3. In contrast, 24,25$(OH)_2$D3, the first reaction intermediate of the C24-oxidation pathway of 25(OH)D3 metabolism, is oxidized by human CYP24A1 with a catalytic efficiency only 3-fold higher than for 20(OH)D3, and actually displays a 3-fold higher K_m value [35]. Overall, the introduction of a C23 hydroxyl group to 20(OH)D3 to form 20,23$(OH)_2$D3 does not alter the kinetic parameters for the initial rates compared to those seen with only the C20 hydroxyl group present. Having the hydroxyl group at C20 rather than at C25 decreases the K_m, suggesting it weakens binding to the active site of the enzyme, and reduces the overall catalytic efficiency dramatically.

Even though the initial rates of metabolism of 20(OH)D3 and 20,23$(OH)_2$D3 by CYP24A1 are similar, the addition of the 23-hydroxyl group to 20(OH)D3 by CYP11A1, producing 20,23$(OH)_2$D3, dramatically alters the metabolic pathway catalyzed by this enzyme. Only a single hydroxyl group is added to various positions of the side chain of 20(OH)D3, and no secondary

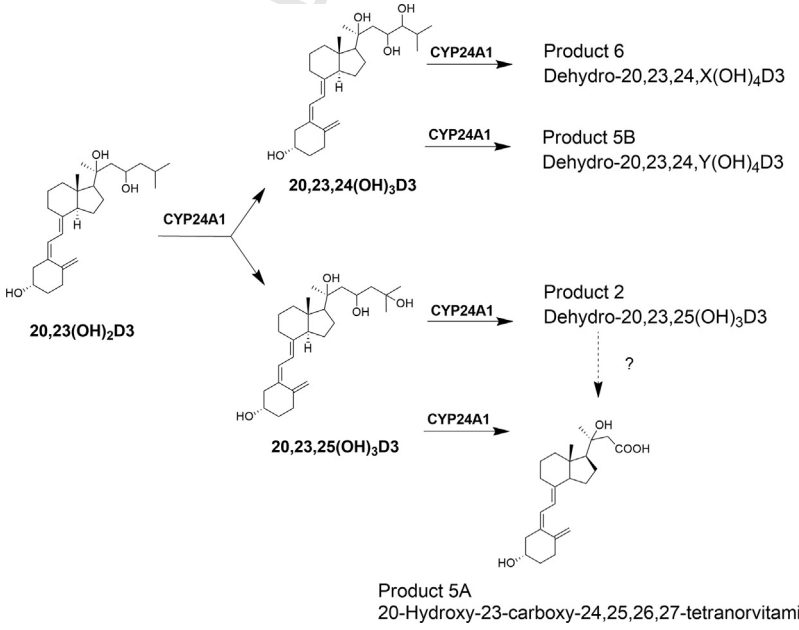

Fig. 9. Pathways illustrating the multiple reactions carried out on 20,23-dihydroxyvitamin D3 by CYP24A1. The potential for Product 2 to be an intermediate for production of Product 5A remains to be established (dashed arrow).

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metabolites are observed. In contrast, following hydroxylation of 20,23(OH)$_2$D3 to 20,23,24(OH)$_3$D3 and 20,23,25(OH)$_3$D3, CYP24A1 can further oxidize these primary metabolites in a complex series of reactions that appear to include side chain cleavage between C23 and C24. Identified steps in the likely pathway are illustrated in Fig. 9. CYP24A1 initially metabolizes 20,23(OH)$_2$D3 to the primary products, 20,23,24(OH)$_3$D3 and 20,23,25(OH)$_3$D3, where the proportion of each primary product is species dependent. Subsequent metabolism of 20,23,24(OH)$_3$D3 yields two different products which have three hydroxyl groups and one keto-group on the side chain and maybe either 23-oxo-20,24,X(OH)$_3$D3, 24-oxo-20,23,X(OH)$_3$D3 or X-oxo-20,23,24(OH)$_3$D3. Note that the tertiary hydroxyl groups at C20 or C25 cannot be oxidized to a ketone. It is also possible that the two dehydro-tetrahydroxy- products (Products 5B and 6) are stereoisomers as this study shows that CYP24A1 can catalyze the formation of the two 20,24(OH)$_2$D3 enantiomers from 20(OH)D3.

High resolution mass spectrometry of Product 5B derived from rat CYP24A1 action on 20,23,25(OH)$_3$D3, suggests that it is 20-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D3. This is analogous to the final product of the C24-oxidation pathway of 1,25(OH)$_2$D3 metabolism, calcitroic acid (1α-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D3) but with a 20-hydroxyl group rather than a 1α-hydroxyl group. This indicates that CYP24A1 can cleave the side chain of 20,23(OH)$_2$D3 and oxidize the product (likely to be 20-hydroxy-23-oxo-24,25,26,27-tetranorvitamin D3) to the C23 carboxylic acid, as occurs in the metabolism of 1,25(OH)$_2$D3 by the C24-oxidation pathway [1,35]. The likely substrate for the cleavage reaction is 24-oxo-20,23,X(OH)$_3$D3 which has the adjacent ketone and hydroxyl groups required for C23—C24 bond cleavage, but the presence of this intermediate remains to be established. It may also be possible for cleavage to occur between the C23 and C24 in 23-oxo-20,24,X(OH)$_3$D3. While the tentative identification of 20-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D3 by the action rat CYP24A1 on 20,23,25(OH)$_3$D3 provides evidence for C23-C24 bond cleavage, this intermediate was not seen for human CYP24A1. However, this is likely to be due to the low concentrations of human CYP24A1 used in these experiments compared to rat CYP24A1, due to its low expression and the difficulty in purifying substantial amounts of the active enzyme [35]. Some metabolism of 20,23,25(OH)$_3$D3 and 20,23,24(OH)$_3$D3 was seen for the human enzyme with Product 2 (dehydro-20,23,25(OH)$_3$D3) and Product 6 (dehydro-20,23,24,X(OH)$_4$D3) being observed.

Both 20(OH)D3 and 20,23(OH)$_2$D3 are biologically active *in vitro* on a range of cells including keratinocytes, fibroblasts, melanocytes, melanoma and leukemia cells [16,21–23,25–29]. They are also active *in vivo* in rats causing the suppression of inflammation associated with arthritis and collagen synthesis in scleroderma, in mouse models [16,26], without the toxic hypercalcemic effect of 1,25(OH)$_2$D3 [25,26,31] (see Section 1). They thus have therapeutic potential for treating hyperproliferative and inflammatory disorders. Therefore, their metabolism by CYP24A1 is relevant to their possible therapeutic use. They are metabolized with lower catalytic efficiency than 1,25(OH)$_2$D3 as described above. In contrast to the inactivating role CYP24A1 has towards 1,25(OH)$_2$D3 [48], biological testing of the products of CYP24A1 action on 20(OH)D3, namely 20,24(OH)$_2$D3 and 20,25(OH)$_2$D3, showed that these products cause significantly higher inhibition of colony formation by melanoma cells in soft agar than 1,25(OH)$_2$D3 or the parent 20(OH)D3 [33]. Thus, it is possible that CYP24A1 will act as a potentiator of the anti-cancer activity of 20(OH)D3, which would explain recent results showing an inverse correlation between melanoma progression and CYP24A1 expression [49]. It remains to be established whether CYP24A1 causes activation or inactivation of 20,23(OH)$_2$D3. Based on studies of other side chain hydroxylated 20(OH)D3 products such as 17,20,23(OH)$_3$D3 [21,26], it is likely that primary products of CYP24A1 action on 20,23(OH)$_2$D3 will remain active, but later products with the side chain cleaved are likely to be inactive, as is observed for calcitroic acid [1,4].

In conclusion, this study shows that both human and rat CYP24A1 hydroxylate 20(OH)D3 to produce only dihydroxyvitamin D3 derivatives, which retain biological activity. 20,23(OH)$_2$D3 undergoes multiple oxidation steps in a pathway that is analogous to the C24-oxidation pathway of 1,25(OH)$_2$D3 metabolism, with evidence for side chain cleavage between C23 and C24.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2015.02.010.

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