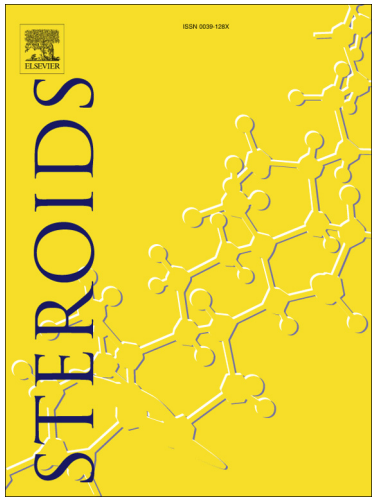

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Abstract:

Steroids and their oxyfunctionalized counterparts are valuable compounds for the pharmaceutical industry; however, the regio- and stereoselective introduction of oxygen is a challenging task for the synthetic chemistry. Thus, cytochromes P450 play an important role for the functionalization of steroidal compounds. In this study, we elucidated the main product of 11-deoxycorticosterone conversion formed by CYP260B1 from *Sorangium cellulosum* So ce56 as 9α-OH 11-deoxycorticosterone by NMR spectroscopy. This is, to the best of our knowledge, the first identification of a 9α-hydroxylase for this substrate. In addition, the major side product was identified as 21-OH pregna-1,4-diene-3,20-dione. Studies using 1α-OH 11-deoxycorticosterone as substrate suggested that the major side product is formed via dehydrogenation reaction. This side reaction was considerably decreased by employing the CYP260B1-T224A mutant, which showed an increased selectivity of about 75% compared to the 60% of the wild type for the 9α-hydroxylation. To scale up the production, an *E. coli* based whole-cell system harboring the CYP260B1-T224A variant as well as two heterologous redox partners was used. Employing growing cells in minimal medium led to a productivity of about 0.25 g/l/d at a 50 ml scale showing the biotechnological potential of this system.

Keywords:

Steroids, 11-deoxycorticosterone, CYP260B1, *Sorangium cellulosum* So ce56, cytochromes P450, 9α-hydroxylase.

Abbreviations:

AdR, adrenodoxin reductase; $Adx_{4\text{-}108}$, adrenodoxin (truncated form); P450, cytochrome P450; DOC, 11-deoxycorticosterone; HP-β-CD, 2-hydroxypropyl-β-cyclodextrin; T224A mutant, CYP260B1-T224A.

Introduction:

Steroids are sterol-derived compounds of specific structure that contain 3 cyclohexane rings and a cyclopentane ring. They have been identified in many living systems such as mammalians, insects, plants, yeasts or fungi, in which they play important roles like acting as hormones [1]. In mammalian organisms, steroid hormones are capable of controlling the salt and water balance (mineralocorticoids), the glucose metabolism (glucocorticoids) or the sexual differentiation and reproduction (sexual hormones). Their specific roles are thereby dependent on functional groups attached to the steroidal scaffold like the hydroxyl group at position C11β that is crucial for the anti-inflammatory activity of steroids [2]. Such oxyfunctionalizations are mainly performed by cytochromes P450, a superfamily of heme-thiolate proteins named due to the spectral absorbance peak of their carbon-monoxide-bound species at 450 nm. As steroids and their functionalized counterparts are of high pharmaceutical interest representing the second largest group of drugs next to antibiotics [3], the investigation of new P450s with high activity and selectivity as well as the discovery of enzymes with novel functionalities are important issues for the white biotechnology [4, 5]. Besides mammalian P450s involved in steroid biosynthesis, several bacterial P450s were already identified to act as steroid hydroxylases [6]. For instance, the selective hydroxylation of testosterone at diverse positions by bacterial P450s using an *E. coli* expression system was previously demonstrated [7]. In addition, CYP106A1 and CYP106A2 from *Bacillus megaterium* strain ATCC 13368 were identified as versatile steroid hydroxylases with a high biotechnological potential [8-11]. Improvements of CYP106A2 by different mutagenesis approaches to alter the selectivity and enhance the activity were performed, too [12, 13]. Aside from these examples, P450s from *Sorangium cellulosum* So ce56 are also able to functionalize steroids as shown for CYP260A1 and its novel 1α-hydroxylase activity towards androgens and derivatives of them as well as CYP260B1 and its 6β-hydroxylase activity towards 11-deoxycortisol [14, 15]. The selectivity of steroid conversions using bacterial P450s is often low; however, mutagenesis approaches as demonstrated for CYP260A1 as well as CYP106A2 led to an improved or even altered selectivity of hydroxylation [13, 16].

CYP260B1 was previously identified as steroid hydroxylase with a broad substrate range, in which DOC was converted with a high activity. However, the main product was not identified due to the low selectivity of hydroxylation [15]. In this study, we elucidated the structure of this main product as 9α-OH DOC by NMR spectroscopy. To reach a higher selectivity, the previously created T224A mutant of CYP260B1 that already caused an altered selectivity for progesterone hydroxylation was tested [17]. With that variant, the major side product formation was considerably decreased leading to an increased selectivity of 75% towards 9α-OH DOC during *in vitro* and *in vivo* experiments. A whole-cell system harboring the T224A variant as well as two heterologous redox partners was used to scale up the production. Utilizing M9CA medium and EtOH as solvent for DOC led to a production of about 0.25 g/l/d in shaking flasks.

Experimental:

Chemicals and strains:

Isopropyl β-D-1-thiogalactopyranoside and 5-aminolevulinic acid were purchased from Carbolution Chemicals (Saarbruecken, Germany). Bacterial media were obtained from Becton Dickinson (Heidelberg, Germany). The *E. coli* strain C43(DE3) was obtained from Novagen (Darmstadt, Germany). Standards of 9α-OH DOC and 11α-OH DOC were a kind gift from Schering AG (Berlin, Germany). 1α-OH DOC was produced and purified as described previously [18]. All other chemicals were purchased from standard sources in the highest purity available.

Expression and purification of the enzymes:

The expression and purification of CYP260B1 and its T224A variant was performed as described previously [15, 17]. The truncated bovine adrenodoxin ($Adx_{4\text{-}108}$) and adrenodoxin reductase were expressed and purified as described elsewhere [19, 20].

Determination of the binding constants:

Dissociation constants were determined by difference spectroscopy using tandem cuvettes as described elsewhere [21]. CYP260B1 and the T224A variant (5 µM) were diluted in 50 mM potassium phosphate buffer (pH 7.4) and titrated with increasing concentrations of DOC from a 0.5 mM stock solution in dimethyl sulfoxide. Difference spectra were recorded from 350 to 500 nm. To determine the binding dissociation constant (K_d), the averaged peak-to-trough absorbance differences (ΔA_{max}) were plotted against the substrate concentration (see Supplemental Figures 1a and b). Plots were fitted with Origin 8.6 software (OriginLab Corporation, Northampton, MA) by tight binding quadratic equation [$\Delta A = (Amax/2[E])\{(K_d+[E]+[S])-\{(K_d+[E]+[S])^2 - 4[E][S]\}^{1/2}\}$], whereby ΔA represents the peak-to-trough absorbance difference at every substrate concentration, A is the maximum absorbance difference at saturation, [E] is the enzyme concentration, and [S] is the substrate concentration. All titrations were performed in triplicate.

In vitro conversions:

A reconstituted *in vitro* system containing CYP260B1 or its T224A variant (0.5 µM), AdR (1.5 µM), $Adx_{4\text{-}108}$ (10 µM), $MgCl_2$ (1 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (1U) in a final volume of 250 µl of potassium phosphate buffer (20 mM, pH 7.4) was used. The steroids (10 mM stock solution in EtOH) were added to a final concentration of 200 µM. The reactions were initiated by adding 500 µM NADPH. After 30 min at 30°C the reactions were quenched with 500 µl EtOAc and extracted twice. The combined organic phases were evaporated in vacuum and prepared for HPLC analyses.

Steady state kinetic measurements were performed by using *in vitro* conversions under the same conditions as described above except that substrate concentrations were varied from 10 to 200 µM and that reactions were stopped after 1 to 10 minutes by flash freezing the samples in liquid nitrogen followed by extraction with EtOAc. Data were fitted to the Michaelis–Menten equation by non-linear regression using the software Origin 8.6 to obtain K_m- and k_{cat}-values. To determine the total turnover number, reactions were performed with a substrate concentration of 1 mM and carried out for 20 h. All other parameters were equal to those described above. Mean values and standard deviations were calculated from three experiments.

Whole cell conversions:

C43(DE3) cells were transformed with two plasmids, one encoding CYP260B1 or its T224A variant (pET17b) and the other one encoding $Adx_{4\text{-}108}$ and FpR [22]. An overnight culture was prepared in NBI medium containing 100 µg/ml ampicillin and 50 µg/ml streptomycin. The main culture was inoculated with the overnight culture (1:100) and grown in M9CA medium also containing ampicillin (100 µg/ml) and streptomycin (50 µg/ml). 300 ml baffled flasks each filled with 50 ml of the main culture were used for the experiments. After 3.5 h at 37°C the cultures were induced with 1 mM isopropyl β-D-1thiogalactopyranoside and 0.5 mM 5-aminolevulinic acid and further grown for 20.5 h at 28°C followed by substrate addition (from 100 mM stock) to a final concentration of 1.5 mM DOC unless stated otherwise. Reactions were performed for 24 h and 0.5 ml samples were taken and extracted twice with ethyl acetate. The organic layers were pooled, evaporated to dryness and prepared for HPLC analyses.

Likewise, the conversion in TB medium was performed, except that the induction was done after 2.5 h and that proteins were expressed for 24 h before adding the substrate.

HPLC analysis:

HPLC analyses were performed on a system consisting of a PU-2080 HPLC pump, an AS-2059-SF autosampler, and a MD-2010 multi wavelength detector (Jasco, Gross-Umstadt, Germany). A Nucleodur 100–5 C18 column (125 x 4 mm, Macherey–Nagel, Düren, Germany) was used at 40°C. 10% (*v/v*) acetonitrile in water (A) and pure acetonitrile (B) were used as mobile phases. A gradient from 10% to 60% B over 25 min followed by 3 min of 60% B at a flow rate of 1 ml/min was used for separating the analytes. The relative amounts of the products were estimated using the relative peak area of the product compared to the combined peak areas of substrate and products at 240 nm.

Purification of the products:

For product elucidation, the reactions were performed with wild type CYP260B1 in 4x 2 l flasks each filled with 250 ml M9CA medium for 6 h with a substrate concentration of 0.5 mM. All other conditions were the same as described above. The extract of this whole cell conversion was dissolved

in ethyl acetate and loaded on a silica column. An ethyl acetate – hexane mixture of 8:2 was used as mobile phase for product separation. Fractions were collected and monitored with *p*-anisaldehyde staining. Product containing fractions were pooled, evaporated to dryness and prepared for NMR analysis.

NMR analysis:

NMR spectra were recorded on a Bruker (Rheinstetten, Germany) DRX 500 NMR spectrometer. A combination of ^{1}H, ^{13}C, $^{1}H,^{1}H$-COSY, HSQC, HMBC and NOESY was used to elucidate the structures. All chemical shifts are relative to $CHCl_3$ (δ=7.24 for ^{1}H NMR) or $CDCl_3$ (δ=77.00 for ^{13}C NMR) using the standard δ notion in parts per million (ppm).

NMR data of 21-OH pregna-1,4-diene-3,20-dione (1):

^{1}H NMR (500 MHz, $CDCl_3$): 7.01 (d, 1H, J=10.1 Hz, H1), 6.21 (dd, 1H, J= 1.9, 10.1 Hz, H2), 6.04 (s, 1H, H4), 4.15 (dd, 2H, J= 4.6, 5.9 Hz, H21), 3.23 (t, 1H, J= 4.5 Hz, OH), 2.49-2.40 (m, 2H, H17, H6a), 2.37-2.31 (m, 1H, H6b), 2.23-2.15 (m, 1H, H11a), 1.97-1.90 (m, 2H, H7a, H12a), 1.80-1.67 (m, 4H, H11b, H15a, H16), 1.67-1.56 (m, 1H, H8), 1.38-1.29 (m, 2H, H15b, H12b), 1.20 (s, 3H, H19), 1.16-1.08 (m, 2H, H14), 1.07-1.00 (m, 2H, H11b), 0.69 (s, 3H, H18); ^{13}C NMR (125 MHz, $CDCl_3$): 209.97 (C20), 186.22 (C3), 168.59 (C5), 155.43 (C1), 127.61 (C2) 123.97 (C4), 69.32 (C21), 58.87 (C17), 55.60 (C14), 52.05 (C9), 44.76 (C13), 43.37 (C10), 38.17 (C12), 35.42 (C8), 33.43 (C7), 32.66 (C6), 24.60 (C15), 22.86 (C16), 22.64 (C11), 18.64 (C19), 13.51 (C18).

NMR data of 9α-OH 11-deoxycorticosterone (3):

^{1}H NMR (500 MHz, $CDCl_3$): 5.86 (d, 1H, J= 1.9 Hz, H4), 4.17 (ddd, 2H, J= 4.9, 7.8, 19.7 Hz, H21), 3.24 (t, 1H, J= 4.8 Hz, OH), 2.51 (t, 1H, J= 9.3 Hz, H17), 2.45-2.41 (m, 1H, H6a), 2.41-2.37 (m, 3H, H1a H2a, H2b), 2.31-2.16 (m, 2H, H6b, H16a), 1.92 (ddd, 1H, J= 4.1, 12.1 (x2), H8), 1.72-1.75 (m, 1H, H16b), 1.75-1.66 (m, 5H, H1b, H11a, H12a, H14, H15a), 1.66-1.54 (m, 3H, H7a, H11b, H12b), 1.43 (ddd, 1H, J= 4.9, 12.6 (x2), H7b), 1.33-1.29 (m, 1H, H15b), 1.30 (s, 3H, H19), 0.67 (s, 3H, H18); ^{13}C NMR (125 MHz, $CDCl_3$): 210.06 (C20), 198.83 (C3), 167.76 (C5), 127.23 (C4), 76.27 (C9), 69.37 (C21), 58.72 (C17), 49.46 (C14), 44.43 (C10, C13), 37.33 (C8), 33.97 (C2), 33.84 (C12), 31.59 (C6), 28.45 (C1), 26.67 (C11), 25.32 (C7), 24.26 (C15), 22.90 (C16), 19.85 (C19), 12.59 (C18).

Results:

Elucidation of the products:

Previous studies by our group showed that wild type CYP260B1 is capable of an efficient conversion of diverse steroidal compounds, in which DOC was converted with a high yield [15]. However, the

main product was not elucidated due to the low selectivity. As shown in Figure 1A, the main product (3) and a major side product (1) were formed in higher amounts compared to the other side products. Both products were purified and analyzed by NMR spectroscopy. The main product (3) showed a tertiary hydroxyl group and was identified as 9α-OH DOC. Its retention time and absorption maximum matches those of the authentic standard. The major side product showed an additional double bond between C1 and C2 and was therefore identified as 21-OH pregna-1,4-diene-3,20-dione. The minor side products were formed in insufficient amounts for structure elucidation via NMR spectroscopy. However, two of these products were compared with authentic standards and identified as 1α-OH DOC and 11α-OH DOC.

DOC conversion by the T224A mutant:

As previous docking studies suggested that threonine 224 forms a hydrogen bond with the keto group of the A ring of DOC [15], we tested the T224A mutant lacking this interaction towards its selectivity for DOC conversion. This variant showed an increased selectivity of 75% compared to the wild type showing a selectivity of 60% concerning the main product. The formation of the major side product was considerably decreased by this mutant (see Figure 1). To compare the wild type with its variant in more detail, kinetic and binding studies with DOC as substrate were performed. The results are summarized in Table 1 and showed that the wild type has a k_{cat} value of 23.5 min^{-1} compared with 12.3 min^{-1} for the mutant. Binding of DOC to the mutant was also weaker with a K_d of about 210 nM compared with 21 nM for the wild type. Moreover, the ability of the mutant to further convert the main product was investigated. The mutant T224A showed a two times lower activity than the wild type (see Supplemental Figure 2).

Investigation of the side product formation:

The formation of the major side product (1) was further investigated by *in vitro* conversions of 1α-OH DOC (2) by both enzymes (see Figure 2). A previous study demonstrated that CYP260A1 from the same strain was capable of forming the same side product out of 1α-OH DOC [18]. However, neither the wild type nor the T224A variant were able to form 21-OH pregna-1,4-diene-3,20-dione out of 1α-OH DOC (see Supplemental Figure 4). The reaction seems, therefore, to be catalyzed by a dehydrogenation reaction of the P450 itself as described for several other P450s [23].

Screening of wild type CYP260B1 and its T224A mutant towards a steroid library:

To check whether the selectivity of other steroidal compounds is also influenced by the T224A variant, a library comprising the following steroids was screened with both enzymes: androstenedione, aldosterone, corticosterone, cortisol, 11-deoxycorticosterone, 11-deoxycortisol, estradiol, estrone, 17α-OH progesterone, progesterone and testosterone. All tested steroids were converted by both enzymes, in which only progesterone and DOC were converted with an increased selectivity by the T224A

variant. The largest effect was achieved for the conversion of DOC with a selectivity of 75%, whereas the conversion of progesterone was shifted towards another main product with a selectivity of about 35%.

Optimization approaches for the whole-cell conversion:

The T224A mutant was chosen for the whole-cell experiments due to the higher selectivity for the 9α-OH DOC production and less activity to further convert this desired product. All whole-cell conversions were performed with growing cells in medium. TB medium and the minimal medium M9CA were investigated to test their effects on the bioconversion of DOC by T224A mutant. As shown in Figure 3A, the conversion in M9CA was significantly higher than that in TB medium. Therefore, all following experiments were performed in M9CA medium. In addition, the substrate concentration was varied from 0.5 to 2.0 mM showing that 1.5 mM was consumed to about 70% after 24h, whereas 0.5 mM, 1.0 mM and 2.0 mM were consumed completely, to ~90% or to ~50%, respectively (see Supplemental Figure 3). To avoid a P450 based consecutive reaction of the main product, a substrate concentration of 1.5 mM was used for the experiments. Solvents such as EtOH and DMSO to dissolve DOC or 45% HP-β-CD complexing this steroid were used to investigate their influence on the bioconversion. As shown in Figure 3B, EtOH was the most suitable solvent for the T224A dependent conversion of DOC. The biotransformation with DMSO as solvent for DOC was not as efficient as EtOH and HP-β-CD as complexing agent showed the lowest product formation. Thus, all whole-cell experiments were performed with EtOH as solvent for DOC.

As the substrate availability inside the cells is a common problem for the bioconversion of hydrophobic substrates [24, 25], the effect of diverse cell permeabilizing agents was tested. EDTA (20 mM), polymyxin B (30 µg/ml) and toluene (1%) were used for that purpose. However, none of these additives was able to increase the 9α-OH DOC production. Utilizing EDTA halved the productivity and the addition of polymyxin B and toluene even dropped the productivity to less than 10% compared with the control not containing any cell permeabilizing additive (see Figure 3C). Thus, the substrate availability inside the cells might not be limiting the bioconversion of DOC.

Time dependent bioconversion of DOC by T224A mutant:

A time dependent conversion of DOC was performed to check if the productivity is decreasing after a certain time. The reaction was performed for 48 h with a substrate concentration of 1.5 mM which was almost completely consumed after that time. The reaction process precedes a hyperbolic curve as shown in Figure 4. In the first 24 h the product formation showed no considerably decrease. Then the reaction was slowed down as only lower amounts of substrate were available. These results

demonstrate the efficiency of this enzyme towards the production of 9α-OH DOC. A product yield of about 0.25 g/l/d was obtained after 24h.

Discussion:

Steroids are an important group of pharmaceuticals primarily used in birth control, hormone-replacement therapy, inflammatory conditions, and cancer treatment [26]. Their functionalization is of high interest, whereby hydroxylation at positions C11 and C16 is so far mainly produced by microbial transformations in steroid industry revealing the lack of efficient chemical techniques for the selective introduction of oxygen into steroids. During the past years, however, a novel and challenging procedure using a recombinant yeast strain has been developed for the production of hydrocortisone [27]. As other positions like C9 and C14 are also of industrial interest, the discovery of alternative and more effective methods is an important issue [28]. For that reason, bacterial P450s might be used to perform such reactions due to their high product spectrum, which can be further improved towards activity and selectivity with molecular biological methods as shown for P450BM3(F87A). The directed evolution of this enzyme led to either 2β-OH testosterone, 15β-OH testosterone, 2β-OH progesterone or 16β-OH progesterone with almost complete regio- and stereoselectivity [29].

Here we elucidated the main product of DOC conversion by CYP260B1 from So ce56 as 9α-OH DOC by NMR spectroscopy. To best of our knowledge, this product was so far only achieved by biotransformation with whole organisms and not by a specified P450. Fungi like C*ircinella strains* or *Ascochyta linicola* and bacteria such as *Nocardia* strains were previously reported to metabolize DOC to its 9α-hydroxy derivative [30-32]. Another approach to obtain 9α-OH DOC was performed by hydroxylating androstenedione at position C9 by *Mycobacterium* species followed by synthetic routes of C17 side chain modification [33]. However, the utilization of a whole-cell organism lacks the information on which specific enzyme is capable of performing the desired reaction. As a result, strategies for enzyme engineering via molecular biological methods are not feasible hampering the optimization of such a biotechnological process. In contrast, we identified here a specific P450, whose crystal structure has been recently solved. Previous docking studies of steroidal compounds into the substrate-free crystal suggested that threonine 224 forms a hydrogen bond with the C3 keto-group of C21-steroids [15]. For that reason, we tested the previously created T224A mutant of CYP260B1 that already showed an altered selectivity for progesterone conversion [17] towards the selectivity of DOC conversion. This variant showed an increased selectivity of 75% compared with the 60 % of the wild type concerning the 9α-hydroxylation of DOC. Albeit the activity and binding strength of the mutant were lower than those of the wild type, we have chosen the T224A mutant for whole-cell conversions due to its higher selectivity and less activity to further convert the desired 9α-OH DOC. The increased selectivity for 9α-OH DOC was thereby mainly caused by the lower 21-OH pregna-1,4-diene-3,20-

dione formation. This side product formation was further investigated as the family member CYP260A1 from the same strain was able to form this product out of 1α-OH DOC [18]. However, neither the wild type nor the T224A variant catalyzed this reaction leading to the assumption that the side product was formed via dehydrogenation catalyzed by the P450 itself.

With the biocatalytic system harboring the T224A mutant and employing growing cells in M9CA medium, a production of about 0.25 g/l/d was achieved. Cell permeabilizing agents or expensive substrate complexing compounds like 2-HP-β-CD were not necessary to reach a high productivity. The time dependent biotransformation showed a continuous conversion for 48h until nearly all substrate was consumed demonstrating the durability of the system. Nevertheless, this process might be further improved by site directed mutagenesis of additional amino acids that are important for the binding and orientation of DOC to reach a higher selectivity and activity. Such an approach has been successfully used to change the regioselectivity of CYP106A2 and improve the obtained mutants concerning their activities [13]. Moreover, performing the reactions in a bioreactor instead of shaking flasks could increase the space-time yield of this bioconversion [25]. To improve the selectivity of this enzyme towards the other screened steroidal compounds, saturation mutagenesis [34] of supposed amino acids binding and interacting with functional groups of the C- or D-ring such as tyrosine 63, serine 274 or alanine 275 might be performed [15].

The resulting 9α-OH DOC can be used as intermediate in the synthesis of 9α-halo-11β-hydroxysteroids that show a high physiological activity [35]. In the 1950s, Fried and Sabo discovered that the anti-inflammatory bioactivity of steroids correlates with the size of the halogen substituent attached at position C9, in which fluorine showed a stronger effect as chlorine, bromine or iodine. The bioactivity of the 9α-fluoro steroids was 10 times higher than that of the parent hormones [36, 37]. Since that time, many steroidal drugs were marketed and the most potent corticosteroid hormones are 9α-fluoro derivatives [35, 38]. These 9α-halo-11β-hydroxysteroids diminish the oxidation of the 11-hydroxyl group providing an increased duration of activity on the receptor which leads to their high anti-inflammatory effect [39]. Synthetic routes for the production of 9α-fluoro derivatives often begin with 9(11)-dehydro steroids followed by an epoxidation step and subsequent ring opening [36, 37, 40]. Therefore, the 9α-OH DOC product can serve as precursor for the 9(11)-dehydro compound that can be further derivatized to the corresponding 9α-fluoro-11β-hydroxysteroid [41]. Alternatively, the hydroxyl group might be replaced by fluorine via nucleophilic fluorination [42].

In conclusion, we identified 9α-OH DOC as major product for DOC conversion by CYP260B1. The previously created T224A variant showed an increased selectivity of 75% compared to 60% of the wild type. With our whole-cell system harboring this mutant, a productivity of about 0.25 g/l/d was achieved at a 50 ml scale demonstrating the biotechnological potential of this system.

Declaration of interest:

Conflict of interest: none

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Table 1: Dissociation constants, selectivity and kinetic parameters of DOC oxidation catalyzed by wild-type CYP260B1 and its T224A variant.

Enzyme	K_m [µM]*	k_{cat} [min^{-1}]*	TTN*	K_d [nM]	Selectivity
Wild type	7.94 ± 2.99	23.52 ± 0.96	960	21 ± 10	60%
T224A	3.67 ± 2.01	12.33 ± 0.39	610	213 ± 70	75%

* Values were determined by measuring the total amount of products.
Results are obtained from triplicate experiments, ± standard deviation.

Figure captions:

Figure 1: Chromatograms of *in vitro* reactions of DOC by purified wild type (A) and its T224A variant (B) after almost complete conversion. Marked peaks 1-3 were elucidated by NMR spectroscopy (1 and 3) or by comparison with an authentic standard of 1α-OH DOC (2).

Figure 2: Reactions catalyzed by CYP260B1 and its T224A variant.

Figure 3: Influences of the media, solvents and cell permeabilizing agents on the whole-cell conversion of DOC by T224A mutant. The comparison of M9CA and TB medium (A), different solvents and complexing agents to dissolve DOC (B) and diverse cell permeabilizing agents (C) are shown. Results are obtained from triplicate experiments, ± standard deviation.

Figure 4: Time dependent production of 9α-OH DOC by the T224A mutant. Results are obtained from triplicate experiments, ± standard deviation.

Figure 1A

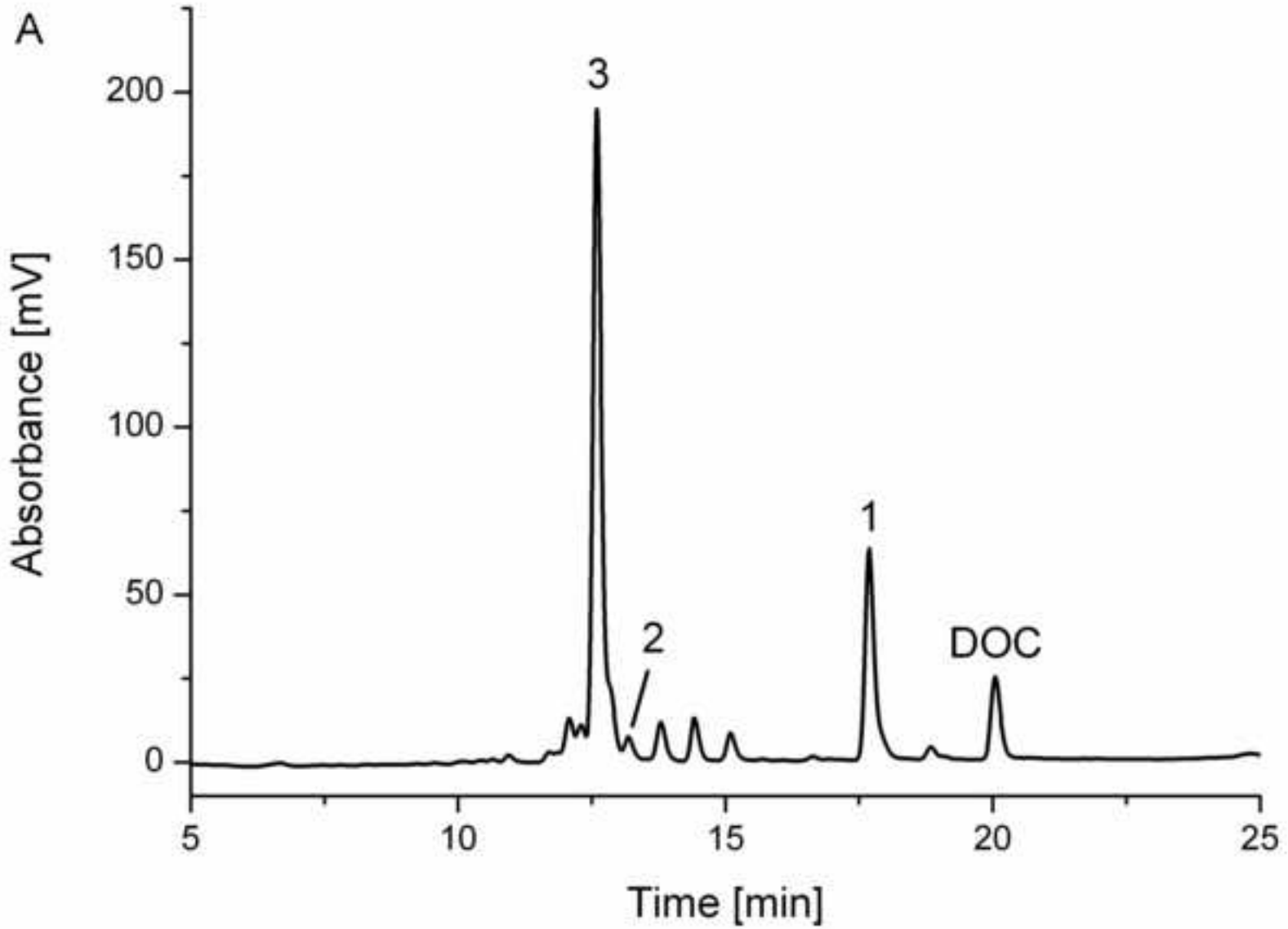


Figure 1B

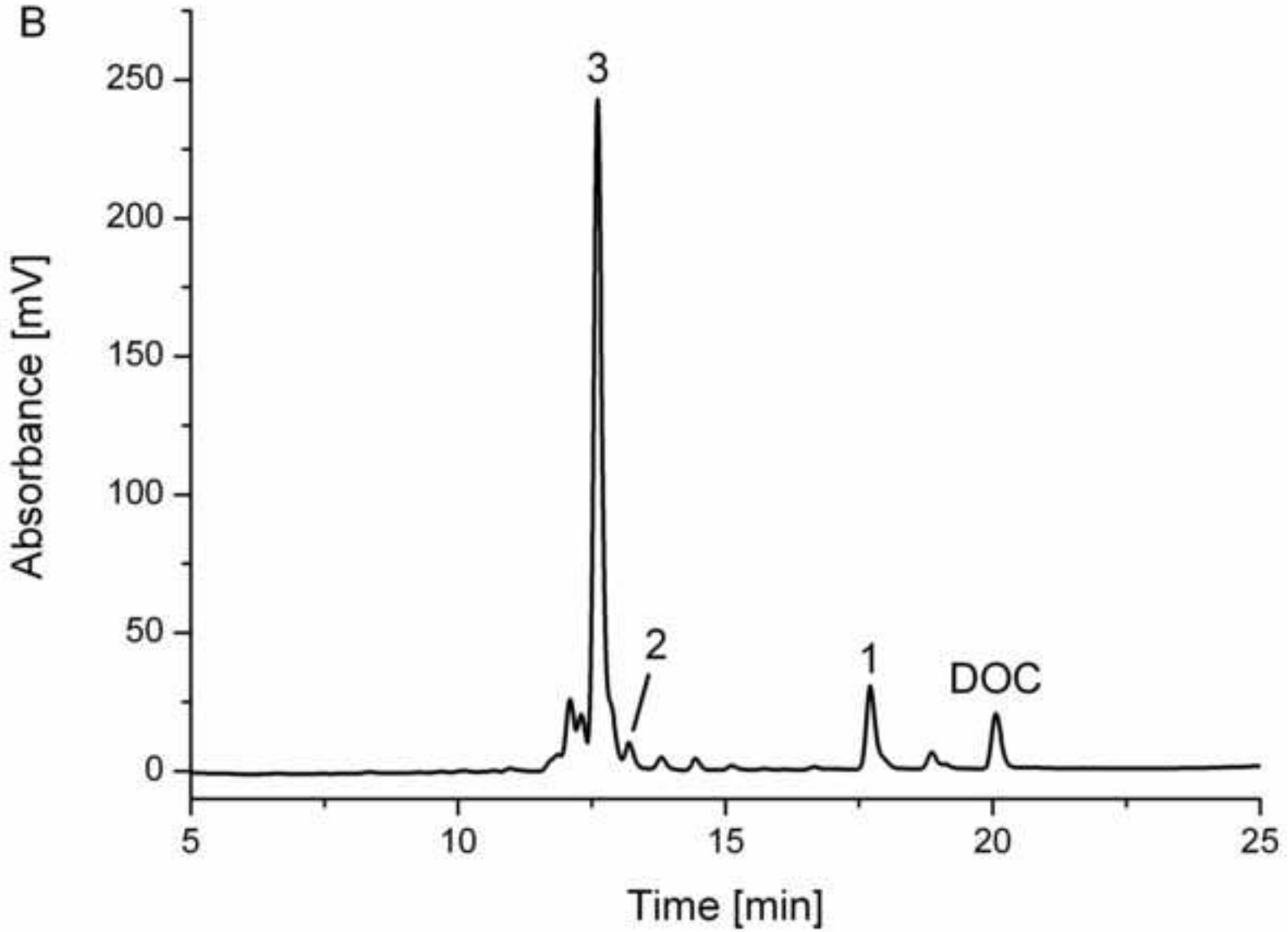


Figure 2

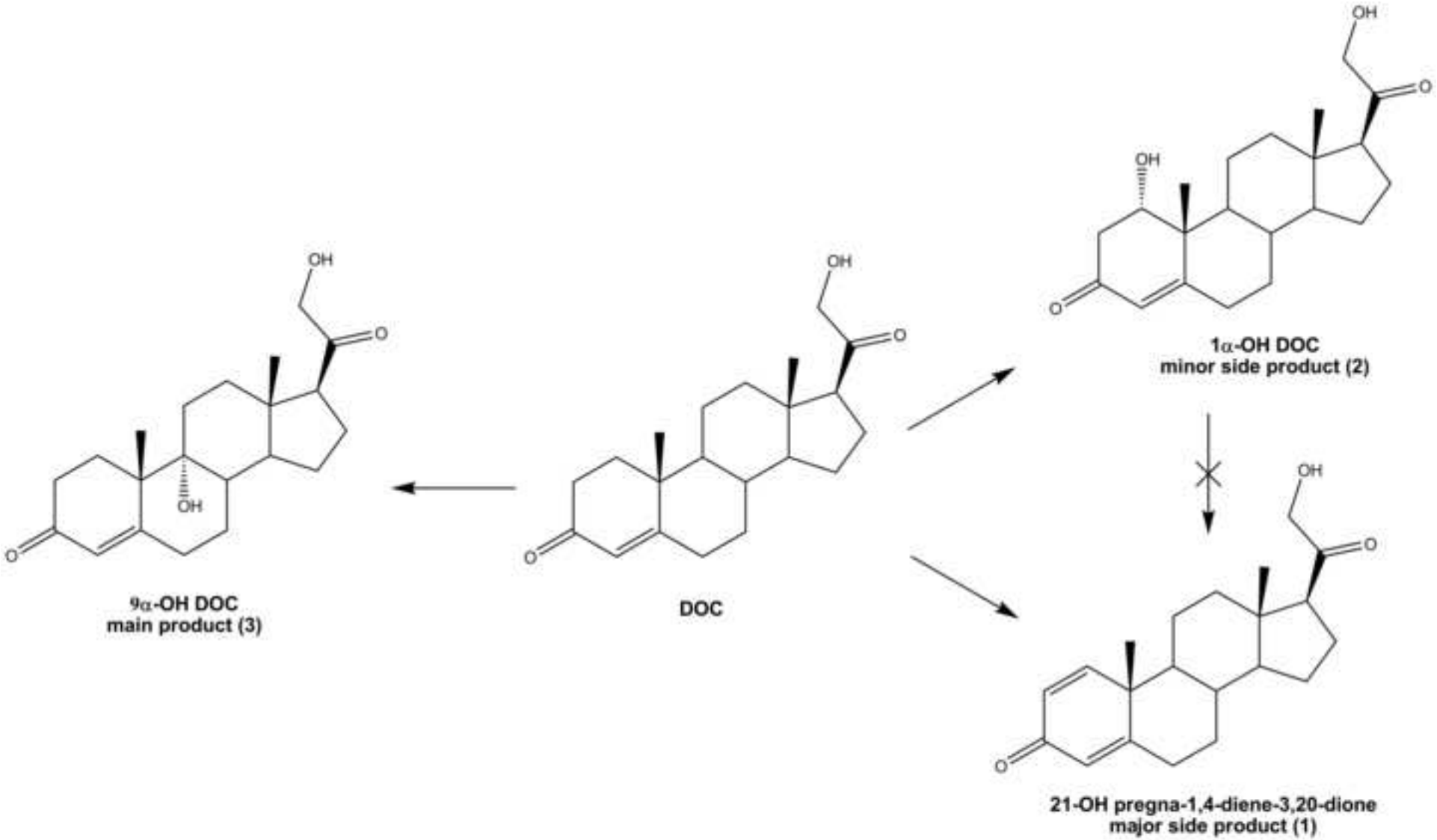


Figure 3A

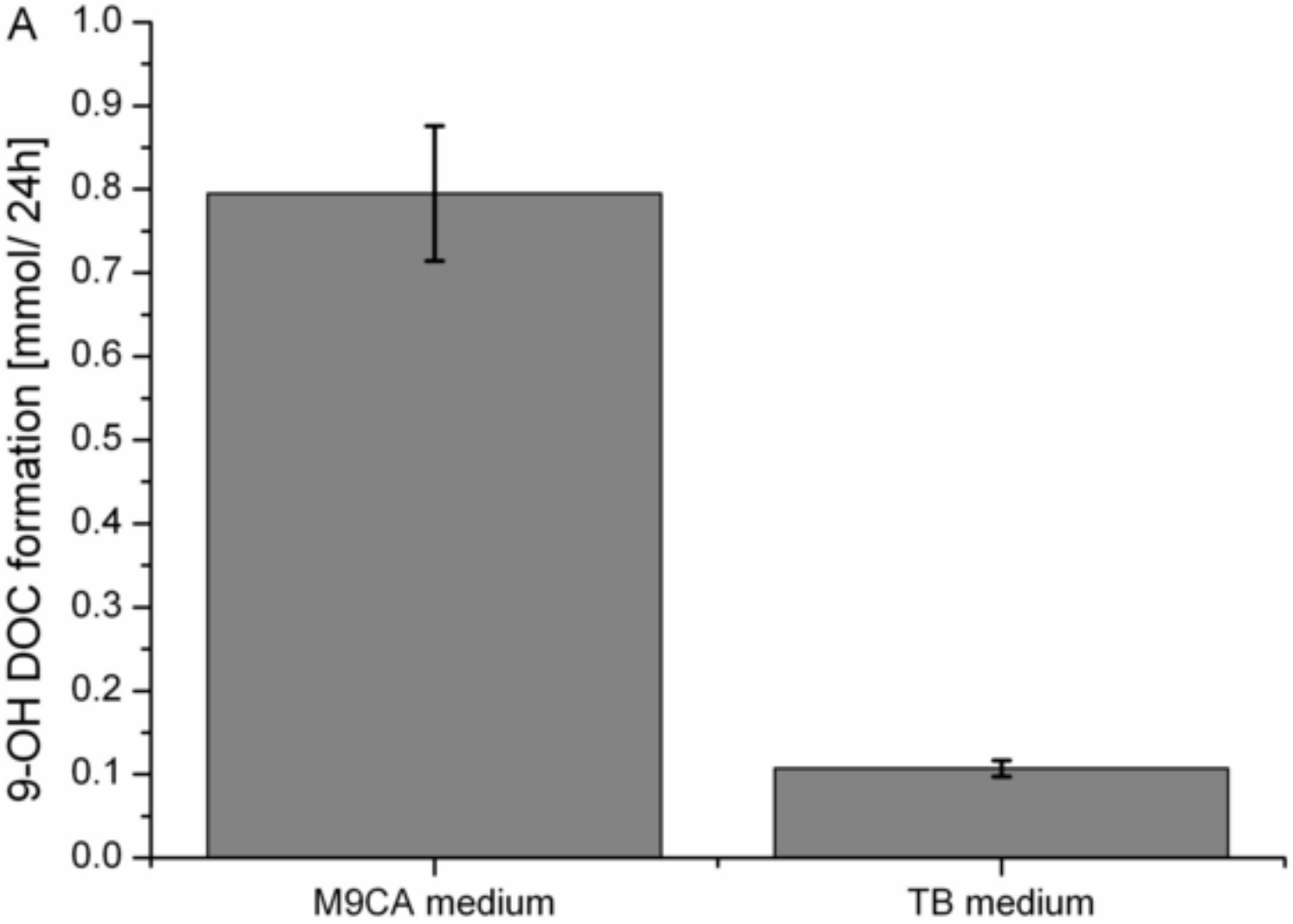


Figure 3B

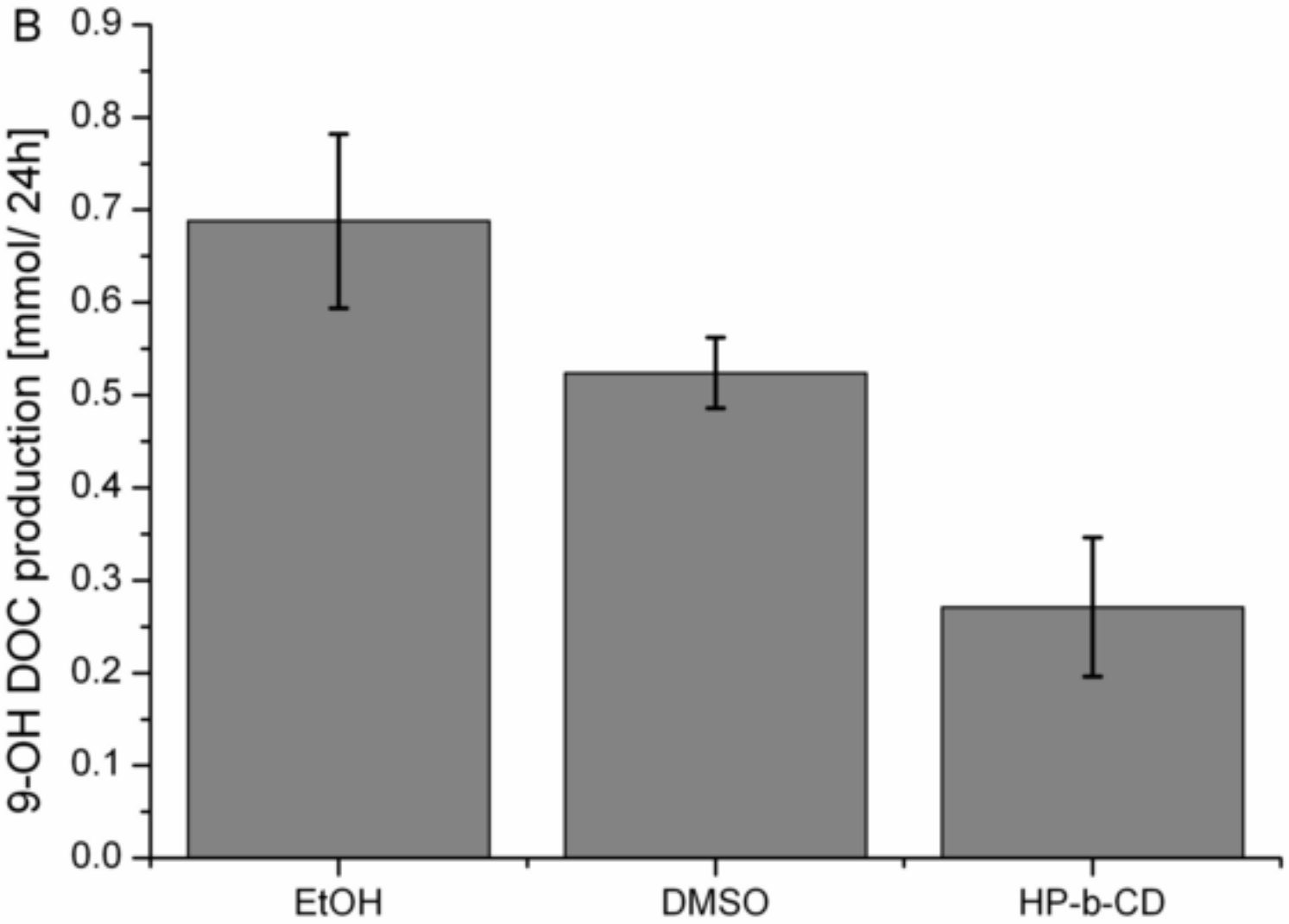


Figure 3C

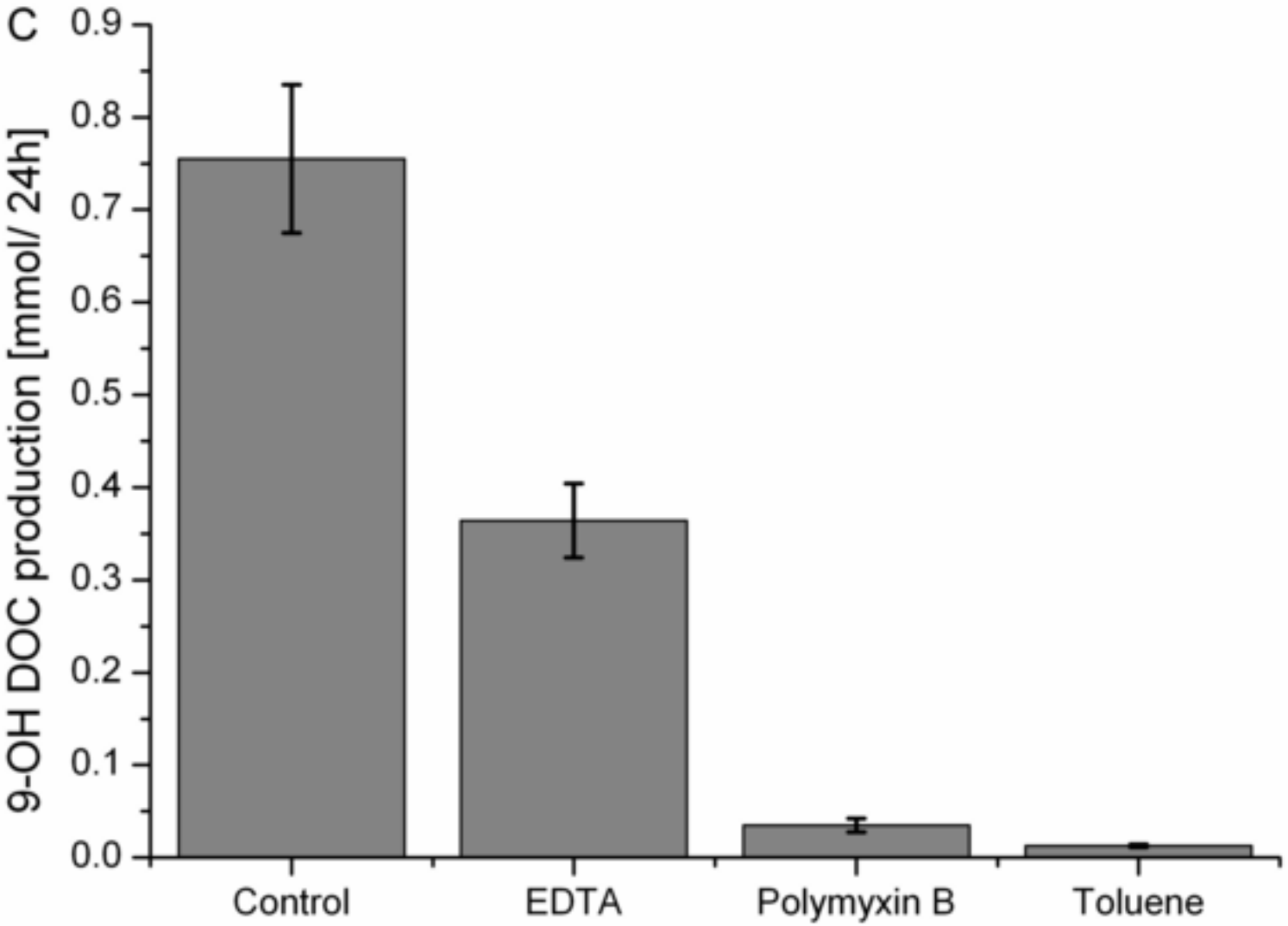
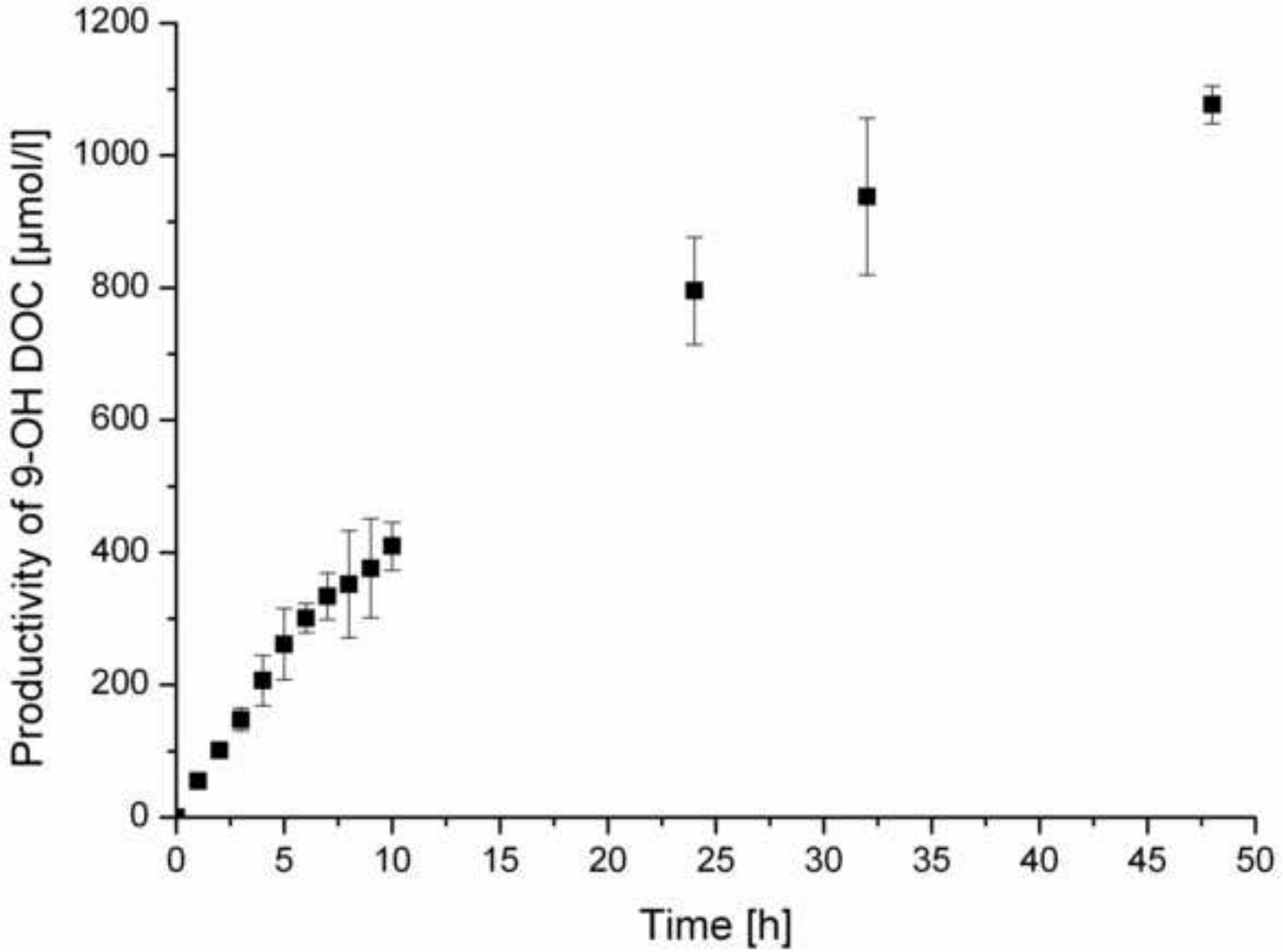


Figure 4



Highlights:

- CYP260B1 acts as 9α-hydroxylase for 11-deoxycorticosterone
- The T224A mutant of CYP260B1 increases the selectivity
- A productivity of about 0.25 g/l/d can be achieved