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Hydroxylation of Dodecanoic Acid and (2*R*,4*R*,6*R*,8*R*)-Tetramethyldecanol on a Preparative Scale using an NADH-Dependent CYP102A1 Mutant

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Abstract: Cytochrome P450 CYP102A1 from *Bacillus megaterium* is a fatty acid hydroxylase which catalyses the highly regioselective hydroxylation of branched fatty alcohols like (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**). The product of this reaction (2*R*,4*R*,6*S*,8*S*)-tetramethyldecane-1,9-diol (**3**) can be used in synthesis of macrolide antibiotics. For setting up the biooxidation process on a preparative scale a monophasic aqueous reaction system has been established. The system was optimised using an NADH-dependent CYP102A1 mutant, dodecanoic acid as a model substrate and takes advantage of randomly methylated beta-cyclodextrins for the solubilisation of hydrophobic substrates. In the reaction with 50 mM of dodecanoic acid a total turnover number of 66,700 and substrate conversion of 66.7% could be reached. The total turnover number of a CYP102A1 mutant in the reaction with 23.4 mM (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol **4** was 17,290 and substrate conversion reached 74%. The reaction on a preparative scale yielded 420 mg of (2*R*,4*R*,6*S*,8*S*)-tetramethyldecane-1,9-diol (**3**) in 60% *de*. The major diastereomer **3a** has the (2*R*,4*R*,6*S*,8*S*,9*R*)-configuration. The configuration of **3a** was determined by an X-ray single crystal structure analysis of the corresponding bis-dinitrobenzoate **5a**.

Keywords: cofactor recycling; fatty acids; green chemistry; hydroxylation; macrolide antibiotics; P450 monooxygenase

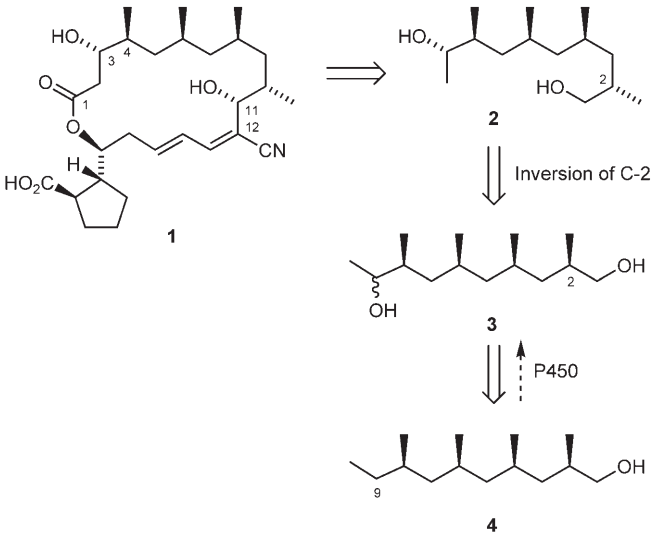
Introduction

Cytochrome P450 monooxygenases (P450s, CYPs) are versatile biocatalysts that insert an oxygen atom derived from molecular oxygen into activated and non-activated C–H bonds in a vast range of molecules, often in a regio- and stereoselective manner. A few chemical catalysts are known that directly oxidise non-activated C–H bonds, however, they work under harsh conditions and most are not selective.[1,2] Therefore, P450s have long been in the focus of chemists and biochemists as an alternative to non-selective chemical oxidation.[3]

Highly branched fatty acids, which can be found in the preen gland of the domestic goose are of particular interest as substrates for oxidation, because they have polyketide-like structures with several stereogenic centres.[4,5] The regioselective oxidation of such substances would offer a convenient route to new pharmaceutically important compounds like macrolide antibiotics. One of those is borrelidin (**1**) which displays antiangiogenetic and antimitotic properties (Scheme 1).[6] Different chemical synthetic routes, comprising 10 to 17 steps have been described in the literature for the total synthesis of borrelidin (**1**) so far.[7]

Regioselective hydroxylation of highly branched fatty acids and fatty alcohols, however, cannot be done in a classical chemical approach. We recently reported that CYP102A1 and its mutant show activity towards a broad range of highly branched fatty acids and fatty alcohols.[8] Selective biooxidation of (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) would save several steps in the total synthesis of borrelidin (**1**) as the product of this reaction, (2*R*,4*R*,6S,8S,9*R*)-tetramethyldecane-1,9-diol (**3**), can be used as C-2–C-11 fragment after inversion of C-2 (Scheme 1).[9]

Among the bacterial P450 monooxygenases, CYP102A1 from *Bacillus megaterium* (also referred to as P450 BM-3) is a well studied and characterised enzyme with exceptional properties. It is a catalytically self-sufficient P450 monooxygenase, consisting of a



Scheme 1. Retrosynthetic analysis of borrelidin (**1**) starting from (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) as C-2–C-11 fragment.

heme domain fused to an FAD- and FMN-containing P450 reductase domain.[10,11] In contrast to the majority of P450s, CYP102A1 does not require any additional redox partners except NADPH and therefore is particularly appropriate for cell-free processes. CYP102A1 converts saturated, unsaturated and branched fatty acids of chain length C_{12} to C_{22} with high activity (up to $>5000\ s^{-1}$) and partially also high stereoselectivity.[11,12]

Several biocatalytic processes using monooxygenases have been published. The high hydrophobicity of substrates hinders their accessibility for water-soluble enzymes. In order to overcome this problem and to enhance substrate accessibility either a very large aqueous reaction volume[13,14] or biphasic systems have been applied.[15]

In our earlier publication we described processes for oxidation of alkanes in a biphasic system, where the electron supply from NADPH was supported by cofactor recycling with formate dehydrogenase.[16] Based on the knowledge gained, we have developed a process for oxidation of the highly branched fatty alcohol (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) on a preparative scale. The presented reaction system is homogeneous, does not need any organic solvent and is therefore particularly benign to the environment.

Results and Discussion

Activity of CYP102A Mutants Hydroxylating (2*R*,4*R*,6*R*,8*R*)-Tetramethyldecanol (4)

Crude preparations of several NADPH-dependent CYP102A mutants (mutants based on CYP102A1 as well as on CYP102A2 and -A3 from *Bacillus subtilis*) constructed previously by site-directed mutagenesis, were screened using a spectrophotometric NADPH oxidation assay at 340 nm. The assay allows a fast identification of active mutants with high NADPH turnovers, however, it does not provide any information about products formed or coupling efficiency between NADPH oxidation and substrate hydroxylation. Therefore, product formation was additionally analysed by GC/MS. The results are shown in Figure 1.

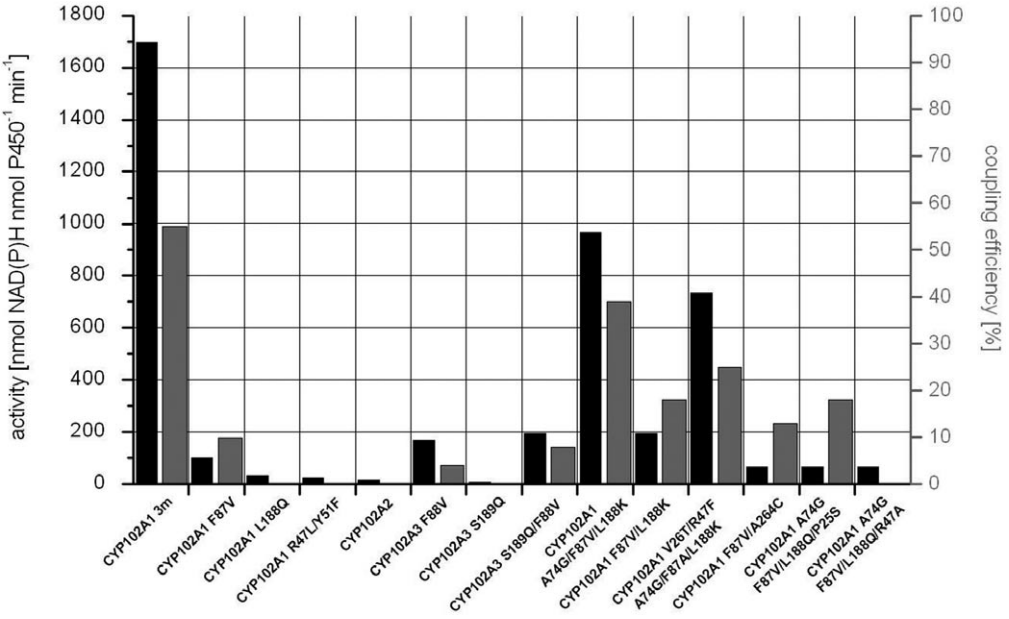


Figure 1. Results of screening several NADPH-dependent CYP102A mutants for hydroxylation activity and coupling efficiency towards (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**).

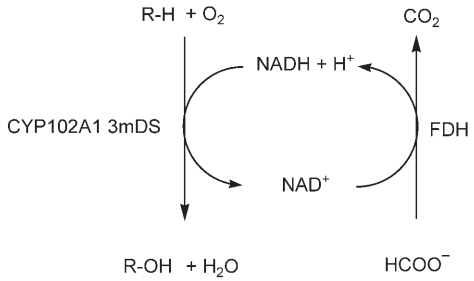
CYP102A1 3m (A74G/F87V/L188Q) showed the highest NADPH consumption in the reaction with (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) (1695 nmol NADPH per nmol P450 per min further defined as min^{-1}) compared to other CYP102A mutants tested. CYP102A1 3m displayed also the highest coupling efficiency (55%) between NADPH oxidation and product formation compared with all other mutants. Generally we found a clear correlation between NADPH oxidation rate and coupling efficiency. The faster the NADPH oxidation the higher is the coupling efficiency. All mutants tested demonstrated high regioselectivity and yielded only one oxidation product which was identified by GC/MS as (2*R*,4*R*,6*S*,8*S*)-tetramethyldecane-1,9-diol (**3**).

Since a future biocatalytic process should be economical, CYP102A1 3m was further engineered from an NADPH- to an NADH-dependent monooxygenase (CYP102A1 3mDS) by introducing the mutations R966D and W1046S in the reductase domain by site-directed mutagenesis as previously reported.[16] Although CYP102A1 3mDS showed a 4-fold lower NADH oxidation activity during the hydroxylation of (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) (462 min^{-1}) compared with the NADPH-dependent mutant, the coupling efficiency and enzyme stability were not affected. Switching the cofactor specificity of CYP102A1 3m from NADPH to NADH reduced the cofactor costs to about 20%. Thus, this mutant was chosen for optimisation of a reaction system suitable for oxidation of hydrophobic substances on a preparative scale.

Biphasic Reaction System

(2*R*,4*R*,6*R*,8*R*)-Tetramethyldecanol (**4**) represents a modified natural product derived from the preen gland of the domestic goose.[5] In order to save this valuable compound, dodecanoic acid was used as a model substrate in all experiments on reaction optimisation. CYP102A1 3mDS showed an NADH oxidation rate of 663 min^{-1} and coupling efficiency of 62% with dodecanoic acid. GC/MS measurements revealed a mixture of ω-1 to ω-5 hydroxydodecanoic acids as hydroxylation products.

Based on our previous study we started the investigation using a biphasic reaction system. Dodecanoic acid was completely dissolved in either isooctane or dodecane. Both alkanes were tested as possible substrates for CYP102A1 3mDS, but have not been converted by the enzyme. In this case the organic phase served as a substrate reservoir facilitating a constant phase transfer between organic and aqueous phase. Bovine serum albumin BSA (5 $mg\,mL^{-1}$) and catalase (600 $U\,mL^{-1}$) were also added to the aqueous phase. It has been proven previously that addition of both



Scheme 2. Scheme for CYP102A1 3mDS-mediated hydroxylation of a substrate and recycling of the cofactor NADH by NAD^+-dependent formate dehydrogenase from *Pseudomonas* sp. 101.

resulted in higher hydroxylation activity of a CYP102A1 mutant for an extended period of time.[16] For cofactor recycling the NAD^+-dependent formate dehydrogenase (FDH) from *Pseudomonas* sp. 101 was used (Scheme 2).[17] DMSO at different concentrations was added for increasing the amount of dodecanoic acid in the aqueous phase.

After 48 h the emulsions were extracted and analysed by GC/MS. The total turnover numbers (TTNs) for CYP102A1 3mDS under different reaction conditions were calculated and summarised in Table 1.

Table 1. Comparison of TTNs for CYP102A1 3mDS for the hydroxylation of dodecanoic acid in different biphasic reaction systems. TTNs were calculated after 48 h of reaction.

Organic phase	TTN
isooctane	23,600
isooctane and 1% DMSO	30,500
isooctane and 2% DMSO	33,500
dodecane and 1% DMSO	26,000

The best results (TTN of 33,500 and substrate conversion of 33.5%) could be achieved with isooctane as organic phase and 2% DMSO. The TTN was much lower without DMSO indicating a remarkable influence of DMSO on the amount of dodecanoic acid in the aqueous phase. Dodecane with 1% DMSO resulted in a lower TTN. This might be due to a higher inactivation rate of the enzyme in the presence of dodecane compared to isooctane.

Monophasic Aqueous Reaction System

Next we investigated a monophasic aqueous reaction system with different cosolvents and emulsifiers promoting the solubility of dodecanoic acid. Reactions were performed in a 20 mL scale with 200 mg dodecanoic acid (final concentration 50 mM), NAD^+-depen-

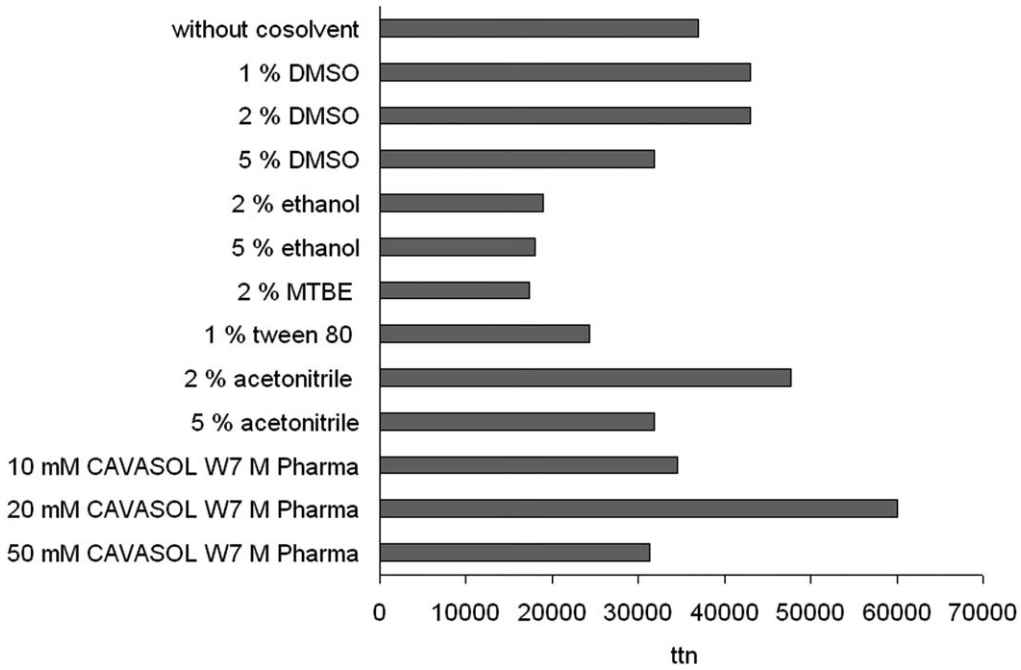


Figure 2. TTNs for CYP102A1 3mDS for hydroxylation of dodecanoic acid in monophasic aqueous reaction system with different cosolvents and emulsifiers. After 48 h reactions were extracted and TTNs were calculated.

dent FDH for cofactor recycling and 5 mg mL^{-1} BSA for enzyme stabilisation. Additionally, catalase (600 U mL^{-1}) was added to decompose hydrogen peroxide to water and oxygen. Hydrogen peroxide can be formed during the reaction as a result of uncoupling of NADH oxidation and substrate hydroxylation. After 48 h the reaction mixtures were extracted and analysed by GC/MS. Totally, on average 200 mg of dodecanoic acid and hydroxylated products were extracted. The corresponding TTNs are shown in Figure 2.

In a monophasic aqueous reaction system without cosolvent a higher TTN (37,000) was observed than in the biphasic reaction system with isooctane and 2% DMSO (33,500). Possible inactivation of the enzyme by isooctane or very poor substrate transfer in a biphasic reaction system seems to outweigh the increase of solubility of dodecanoic acid. Enhancement of accessibility of the substrate by adding DMSO could also be observed in a monophasic aqueous reaction system. With 1% and 2% DMSO TTNs were higher compared to the reaction without cosolvent.

The presence of ethanol, methyl *tert*-butyl ether (MTBE) or Tween 80 diminished enzyme activity or stability. Addition of 2% acetonitrile, in contrast, increased the TTN up to 47,700, however 5% acetonitrile reduced the TTN again to 32,000.

The best result (TTN of 60,000) was obtained when 20 mM CAVASOL W7 M Pharma, a randomly methylated beta-cyclodextrin, was used for substrate solubilisation. Cyclodextrins build a hydrophilic exterior and a hydrophobic central cavity and form host-guest complexes with hydrophobic compounds. They have already been successfully used in enzymatic synthesis and for pharmaceutical purposes.[18]

Further we tested different concentrations of CAVASOL W7 M Pharma. The highest TTN was achieved applying 20 mM CAVASOL W7 M Pharma with 50 mM dodecanoic acid (molar ratio 1:2.5) (Figure 2). To investigate the effect of substrate complexation by CAVASOL W7 M Pharma on enzyme activity, a molar ratio of 1:1 was tested. When we applied 50 mM CAVASOL W7 M Pharma in combination with 50 mM dodecanoic acid, the TTN drastically decreased. The observed decrease in TTN is probably due to lower substrate availability, because of better complexation of the substrate. Another reason for decreased enzyme efficiency could be an inhibitory effect of the solubiliser at 50 mM. Already at 25 mM CAVASOL W7 M Pharma the TTN was reduced (TTN 55,600, data not shown), in fact indicating enzyme inhibition by CAVASOL W7 M Pharma concentrations of 25 mM or higher. An additional test was performed at 10 mM CAVASOL and 50 mM dodecanoic acid. The TTN reached only 34,500. Therefore 20 mM CAVASOL W7 M Pharma can be used as optimal concentration of solubiliser. The effect of CAVASOL W7 M Pharma on substrate availability was further investigated at a molar ratio of 1:1, using lower concentrations of dodecanoic acid (20 mM). Under these conditions the proposed inhibitory effect is excluded. However, also in this case the TTN de-

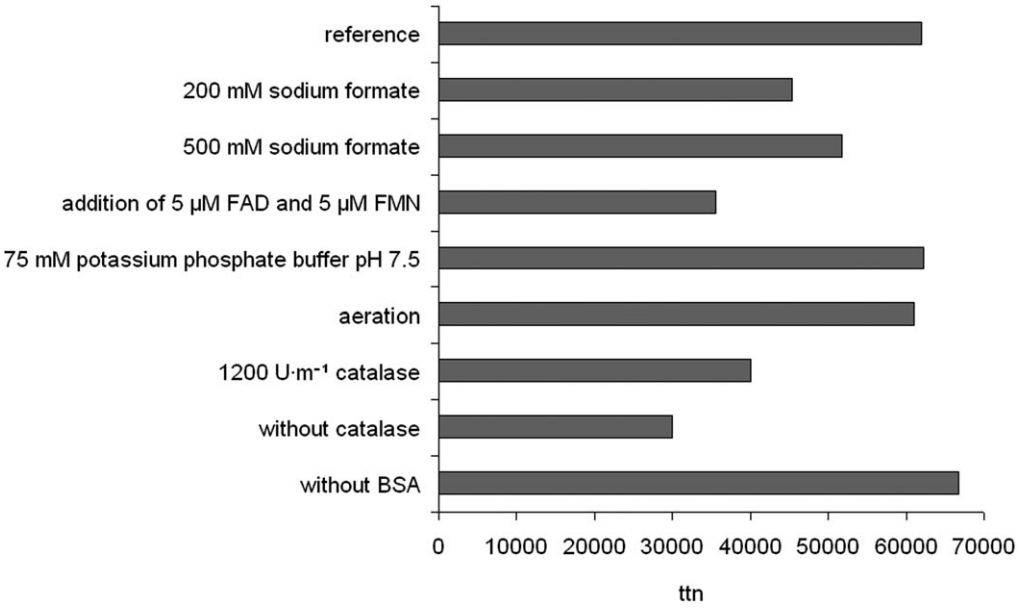


Figure 3. Altering of reaction conditions for hydroxylation of dodecanoic acid by CYP102A1 3mDS in a monophasic aqueous reaction system. The control reaction contained 300 mM sodium formate, 600 U mL^{-1} catalase, 5 mg mL^{-1} BSA and 50 mM KPi, pH 7.5.

creased to 40,000 compared to the data obtained at a higher concentration of substrate. These results suggest that substrate availability is strongly dependent on the ratio between substrate and solubiliser.

The monophasic aqueous reaction system including 20 mM CAVASOL W7 M Pharma for the solubilisation of 50 mM dodecanoic acid was used for further investigations.

Optimisation of Reaction Conditions

Enzymatic reactions are often strongly affected by changes in pH. Therefore, conversion of dodecanoic acid was tested in 50 mM potassium phosphate buffer (KPi) at different pH values.

The highest TTN of 62,000 could be achieved at pH 7.5 (data not shown). Lowering as well as increasing pH by about 0.5 pH units led to a remarkable decrease in TTN. This observation indicates a strong impact of even slight changes in pH on the activity or stability of CYP102A1 3mDS.

During hydroxylation of dodecanoic acid the pH increased from 7.5 to 8.4, because one proton required for hydroxylation is consumed in each reaction cycle. An increase of potassium phosphate buffer concentration from 50 to 75 mM did not significantly increase the TTN. Use of KPi buffers of higher molarity decreased the activity of CYP102A1 3mDS towards dodecanoic acid (data not shown).

For further reaction optimisation other reaction conditions, shown in Figure 3, were altered. The results were compared with a control reaction containing 200 mg dodecanoic acid, 20 mM CAVASOL W7 M Pharma, 300 mM sodium formate, 600 U mL^{-1} catalase, 5 mg mL^{-1} BSA, 0.1 mM NAD^+, 7 U FDH and 0.5 µM CYP102A1 3mDS in 20 mL 50 mM KPi pH 7.5 (TTN of 62,000).

First we investigated the influence of different concentrations of sodium formate, the substrate for formate dehydrogenase, as sodium ions as well as formate can affect the activity of enzymes.[19] Lower TTNs were reached with 200 mM and 500 mM sodium formate compared to the reaction with 300 mM sodium formate.

As loss of flavins from the reductase domain of CYP102 A1 3mDS could be a reason for the insufficient operational stability of CYP102A1,[20] we decided to add external FAD and FMN for enzyme stabilisation. This caused, however, a decrease of TTN to 35,600. Probably this effect is due to enzyme inhibition by the 10-fold excess of cofactors or the loss of reducing equivalents channelled away from product formation.

Additional oxygen supply by aeration of the reaction mixture did not influence the TTN. Increased catalase concentration (1200 U mL^{-1} instead of 600 U mL^{-1}) decreased the TTN, probably due to an inhibitory effect of high concentrations of catalase. Without catalase the TTN was halved, indicating that hydrogen peroxide was formed during the reaction as the product of uncoupling. H_2O_2 is known to reduce enzyme stability. In the beginning BSA was also added to the reaction mixture because it showed a

stabilising effect in biphasic reaction systems.[16] Surprisingly the TTN in the reaction without BSA was raised from 62,000 to 66,700 and substrate conversion reached 66.7%. We assume that BSA interacts with CYP102A1 3mDS which may hinder the access of dodecanoic acid to the active centre.

All reactions have been performed with the crude cell lysate. When purified CYP102A1 3mDS was used a TTN of only 52,000 was reached. This indicates the presence of some stabilising agents in the crude cell lysate of *Escherichia coli*.

In summary, the best results for the hydroxylation of dodecanoic acid by NADH-dependent CYP102A1 3mDS have been achieved in a monophasic aqueous reaction system with 20 mM CAVASOL W7M Pharma, 300 mM sodium formate, 600 U mL^{-1} catalase, 0.1 mM NAD^+, 7 U FDH and 0.5 μM CYP102A1 3mDS (crude cell lysate) in 20 mL 50 mM KPi at pH 7.5.

Hydroxylation of (2*R*,4*R*,6*R*,8*R*)-Tetramethyldecanol (4)

The hydroxylation of (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) was performed under the optimal reaction conditions described above. Alternatively, 100 mg tetramethyldecanol (**4**) (final concentration 23.4 mM), 1 μM CYP102A1 3mDS and 18.4 U FDH were used. Samples were taken during 8 h, extracted and subsequently analysed by GC/MS. The results are shown in Figure 4.

As shown in Figure 4 the concentration of (2*R*,4*R*,6*S*,8*S*,)-tetramethyldecane-1,9-diol (**3**) constantly increased during the first five hours. After that, the reaction stopped and the product yield could not be enhanced either by a new portion of CYP102A1 3mDS or FDH. In this reaction we obtained a TTN of 17,290 and substrate conversion of 74%. The space-time yield of this reaction was 11.1 g L^{-1} d^{-1} and the TTN for the cofactor NAD^+ was 179. At a molar ratio of tetramethyldecanol to CAVASOL W7M Pharma of 1:1 (86 mg substrate) the TTN was lower (15,000) than that in the reaction with 100 mg tetramethyldecanol (molar ratio 1:1.16) (17,290). Further increases of substrate concentration up to 50 mM (214 mg) did not lead to better total turnover numbers; at a ratio 1:2.5 the TTN reached 16,900. Also here the best results were observed at a concentration of CAVASOL W7M Pharma of 20 mM.

During 8 h of reaction the pH increased from 7.5 to 9.4, as one proton is required for one catalytic cycle. At pH 9.4 the activity of CYP102A1 3mDS is decreased to 15% of the initial activity at pH 7.5 (data not shown). We assume that this caused the stop of the reaction after approximately 6 h. The activity of FDH is also reduced at pH 9.4, but still reaches 75%.

(2*R*,4*R*,6*S*,8*S*)-Tetramethyldecane-1,9-diol (**3**) was the only product obtained from this reaction. It was identified as an (80:20) mixture of diastereomers according to GC/MS (for assignment of the configuration at C-9 see below). For preparative production of (2*R*,4*R*,6*S*,8*S*)-tetramethyldecane-1,9-diol (**3**) 800 mg (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) were used. After purification of the hydroxylation product on a silica gel column 420 mg product with a purity of 97.4% (GC/MS) could be obtained.

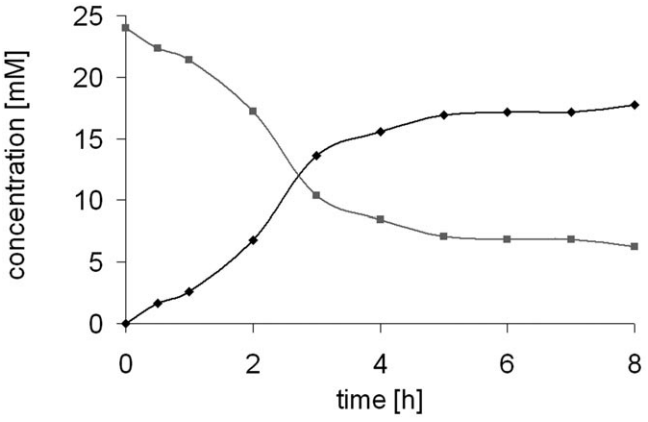
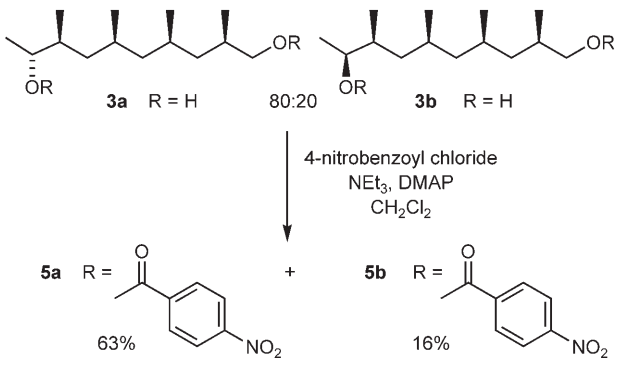


Figure 4. Hydroxylation of (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) by CYP102A1 3mDS in the monophasic aqueous reaction system for 8 h. Square/grey line: educt concentration. Diamond/black line: product concentration.

Determination of the Absolute Configuration of (2*R*,4*R*,6*S*,8*S*)-Tetramethyldecane-1,9-diol (3)

In order to determine the configuration of the newly formed stereocentre at C-9 of (2*R*,4*R*,6*S*,8*S*)-tetramethyldecane-1,9-diol (**3**), the (80:20) diastereomeric mixture of compound **3a**,**b** was treated with 4-nitrobenzoyl chloride in the presence of triethylamine and DMAP in dichloromethane at room temperature for



Scheme 3. Esterification of the diols **3a**,**b** to the corresponding nitrobenzoates **5a**,**b**.

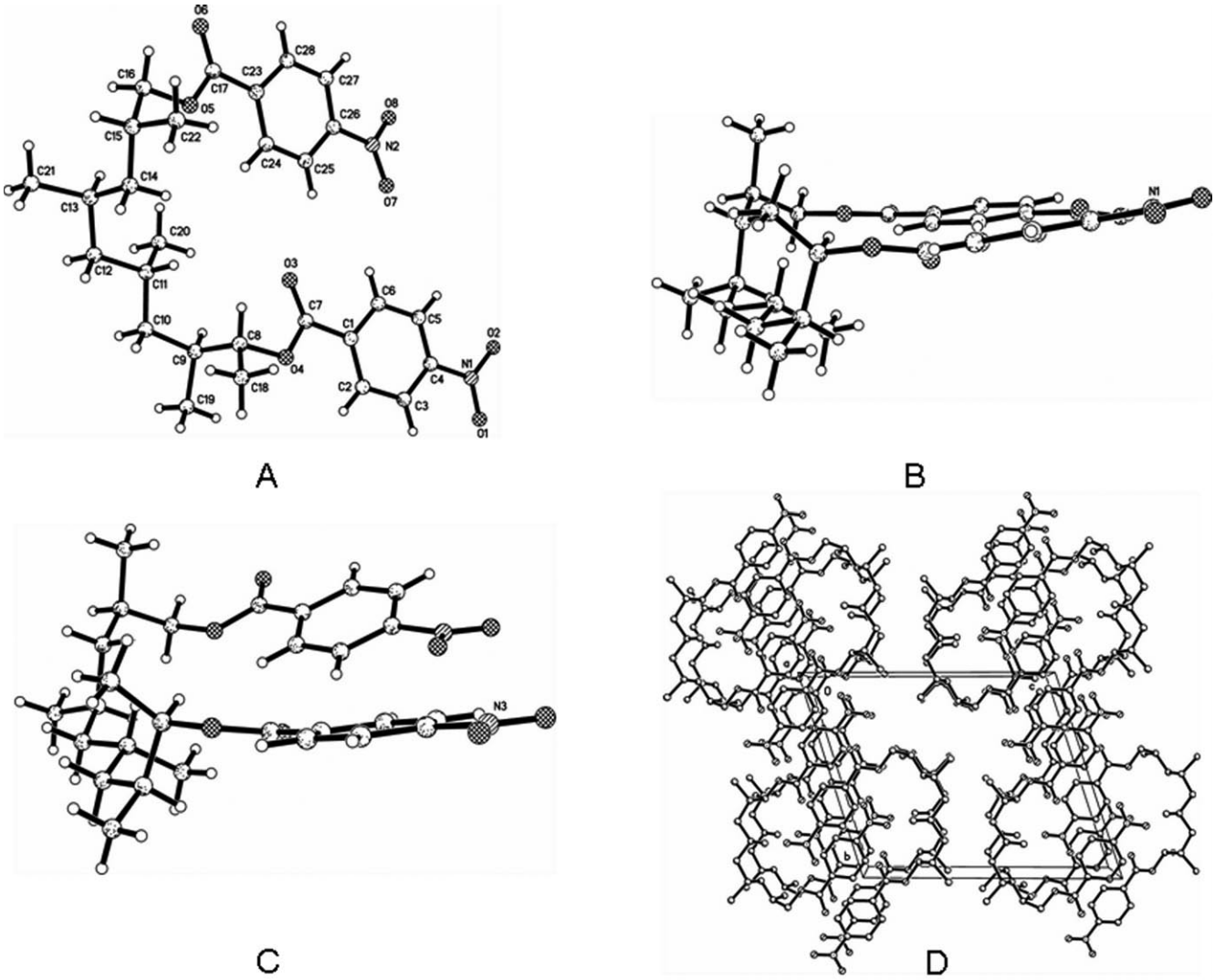


Figure 5. Structures of two conformers of the major diastereomer **5a**, elucidated by X-ray analysis. **A:** Structure of dinitrobenzoate **5a** in the solid state. **B:** Side view of the molecular structure of the first conformer of **5a. C:** Side view of the molecular structure of the second conformer. **D:** b,c-axis view along the a-axis of the packing diagram of both conformers of **5a**.

1 h (Scheme 3). After aqueous work-up, the diastereomeric nitrobenzoates **5a**,**b** could be separated by flash chromatography. The major diastereomer **5a** was isolated as a colourless crystalline solid in 63% yield and the minor diastereomer **5b** as an amorphous solid in 16% yield. Fortunately, the crystals of compound **5a** were suitable for single crystal X-ray structure analysis (Figure 5). As shown in Figure 5A, the relative configuration at C-8,C-9 is *anti*.[21] Thus the major diastereomer **5a** and its precursor diol **3a** both have the (9*R*)-configuration, whereas the minor diastereomer **5b** and its precursor diol **3b** have the (9*S*)-configuration. Besides this stereochemical assignment, the X-ray data offer other interesting features. In the solid state compound **5a** adopts two different conformations, avoiding any unfavourable *syn*-pentane interactions.[7a,d] Furthermore, the benzoate moieties are almost coplanar oriented with a slight twist of 11.2° (Figure 5B) and 32.8° (Figure 5C) to each other. The nitro groups are also twisted out of plane by 4.7° and 15.7° for the first conformer (Figure 5B) and 2.9° and 24.4° for the second conformer (Figure 5C). This leads to a sheet-like packing with aryl stacks and branched alkyl bends separated from each other (Figure 5D).

Determination of the absolute configuration of (2*R*,4*R*,6*S*,8*S*)-tetramethyldecane-1,9-diol (**3**) allowed us to get information about the diastereoselectivity of our mutant in this reaction. CYP102A1 3mDS demonstrated a preference for the (*R*)-configuration, although in a quite moderate rate (60% *de* for C-9 stereocentre). Nevertheless, the (2*R*,4*R*,6*S*,8*S*,9*R*)-tetramethyldecane-1,9-diol (**3a**) corresponds to the natural configuration at the C-9 stereocentre in borrelidin (**1**).

Conclusions

The present work describes the oxidation of the highly branched fatty alcohol (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) by P450 monooxygenase yielding (2*R*,4*R*,6*S*,8*S*,9*R*)-tetramethyldecane-1,9-diol (**3a**) in 60% *de* as a single regioisomer on a preparative scale. Attempts to use compound **3a** and its minor diastereomer **3b** for the total synthesis of borrelidin (**1**) are currently in progress.

For setting up and optimisation of the biocatalytic process different reaction systems have been investigated and compared. As a model substrate dodecanoic acid was used. Although a biphasic reaction system, buffer/isooctane, where the substrate was dissolved in the organic phase is appropriate for the hydroxylation of highly hydrophobic compounds like fatty acids, it led to worse results than a monophasic aqueous reaction system with water-miscible cosolvents or emulsifier. Not all cosolvents or emulsifiers are applicable for the biocatalytic process. DMSO, for example, although showing a positive effect, is not a desired cosolvent if one thinks on further process application and product separation. Ethanol, MTBE and the detergent Tween 80 had a negative effect on the enzyme. Acetonitrile showed a positive enhancing effect on system productivity. Nevertheless, a broader use of acetonitrile is restricted by its toxicity. The best results were observed with CAVASOL W7 M Pharma. This reaction system was applied for the hydroxylation of (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) by P450 monooxygenase and resulted in a total turnover number of 17,290 and a volumetric productivity of 11 $g L^{-1} d^{-1}$. CAVASOL W7 M Pharma is a randomly methylated beta-cyclodextrin, which is permitted for use in food and pharmaceutical industries. It is nontoxic, highly water soluble, easily hydrolysed at low pH values and therefore perfectly suitable also for other biocatalytic applications.

Experimental Section

Chemicals, Enzymes and Strains

All chemical reagents were of analytical grade or higher and purchased from Fluka (Buchs, Switzerland), Sigma (Deisenhofen, Germany) or Riedel-de-Haën (Seelze, Germany). *(2R,4R,6R,8R)*-Tetramethyldecanol (**4**) was kindly provided by Dr. Michael Morr (GBF Braunschweig).[5] NAD^+ and FDH from *Pseudomonas* sp. 101 were from Jülich Fine Chemicals (Jülich, Germany). CAVASOL W7 M Pharma was purchased from Wacker Fine Chemicals (Germany). *E. coli* strain BL21(DE3) and vector pET28a+ were obtained from Novagen (Madison, Wisconsin, USA).

Expression and Purification of CYP102A Mutants

Construction of an NADH-dependent mutant of CYP102A1 3m has been described previously.[16] Heterologous expression of the enzymes in *Escherichia coli* BL21 (DE3) using the pET28a(+) expression vector was performed as described elsewhere.[22]

Purification was performed using the N-terminal His_6-tag of CYP102A1 3mDS and a Ni-sepharose HP column (Amersham Biosiences, Sweden). After a washing step with 30 mM imidazole, protein elution was performed by 200 mM imidazole. As in further experiments a partial aggregation and inactivation of CYP102A1 by imidazole was observed,[22] the enzyme solution was dialysed against 50 mM KPi, pH 7.5 at 8 °C.

Activity Measurements

The concentration of correctly folded P450 enzymes was determined from the CO-binding difference spectra of the reduced heme iron using an extinction coefficient of 91 $mM^{-1} cm^{-1}$ as reported elsewhere.[23]

All activity measurements were done in triplicate. One mL reaction mixture contained 20 μL of 10 mM dodecanoic acid or *(2R,4R,6R,8R)*-tetramethyldecanol (**4**) dissolved in DMSO to 880 μL of 50 mM KPi, pH 7.5 with 0.2 μM P450 enzyme. Reactions were started by addition of 100 μL of 1 mM NAD(P)H in 50 mM KPi, pH 7.5. CYP102A activity was monitored by measuring decrease in the absorption of NAD(P)H at 340 nm for 1 min. For calculations of turnover numbers an extinction coefficient for NAD(P)H of 6.22 $mM^{-1} cm^{-1}$ at 340 nm was used.

NAD(P)H oxidation and CO-difference spectra were recorded on an Ultrospec 3000 UV/vis spectrometer (Amersham Biosciences, Sweden).

After 30 min the reaction mixture was acidified with dilute HCl, extracted three times with 300 μL diethyl ether and evaporated to dryness. The solid was dissolved in *N,O*-bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane, incubated at 80 °C for 30 min and analysed by GC/MS as described in the section on GC/MS measurements. From the proportion of substrate converted, the coupling of NAD(P)H oxidation to product formation can be calculated.

The FDH activity assay was performed by adding 100 μL FDH (diluted in 50 mM KPi, pH 7.5) to 800 μL FDH reaction buffer (50 mM KPi pH 7.5, 300 mM sodium formate). The reaction was started by addition of 100 μL 1 mM NAD^+ in 50 mM KPi, pH 7.5. FDH activity was monitored by measuring the development of NADH at 340 nm.

Hydroxylation in Biphasic Reaction System

The biphasic reaction system consisted of 10 mL aqueous phase and 10 mL organic phase. The aqueous phase contained 300 mM sodium formate, 600 U mL^{-1} catalase to destroy traces of hydrogen peroxide, 5 mg mL^{-1} BSA for protein stabilisation, 0.1 mM NAD^+, 0.5 μM CYP102A1 3mDS and 7 U NAD^+-dependent FDH in 50 mM KPi pH 7.5. Isooctane and dodecane were added as organic phase containing 100 mM dodecanoic acid. 1% and 2% DMSO, respectively, were added to the reaction mixture to improve the solubility of dodecanoic acid in the aqueous solution.

After 48 h reaction mixtures were acidified to pH 2–3 by addition of dilute HCl, extracted three times with diethyl ether, the organic phases were combined and evaporated to dryness. The solid was weighed after each reaction. The recovery of substrates and products was quantitative. The efficacy of extraction was confirmed by GC/MS analysis of the fourth diethyl ether extraction phase, which contained neither substrates nor products. The solid rest was dissolved in *N,O*-bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane, incubated at 80 °C for 30 min and analysed by GC/MS as described in the section on GC/MS measurements.

Hydroxylation in Monophasic Aqueous Reaction System

The hydroxylation of dodecanoic acid was performed in 20 mL 50 mM KPi, pH 7.5 containing 200 mg dodecanoic acid (final concentration 50 mM), 300 mM sodium formate, 600 U mL^{-1} catalase, 5 mg mL^{-1} BSA, 0.1 mM NAD^+, 7 U FDH and 0.5 μM CYP102A1 3mDS unless otherwise noted. Reactions also contained different concentrations of DMSO, ethanol, MTBE, Tween 80, acetonitrile or CAVASOL W7 M Pharma to enhance the solubility of dodecanoic acid. Respective values are given in the "Results and Discussion" section. After 48 h reactions were stopped and analysed as described in the previous section "Hydroxylation in Biphasic Reaction System". Substrate and products (on average 200 mg) were completely extracted from the reaction mixture.

Hydroxylation of (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) was performed in 20 mL 50 mM KPi pH 7.5 containing 100 mg (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) (final concentration 23 mM), 300 mM sodium formate, 600 U mL^{-1} catalase, 0.1 mM NAD^+, 20 mM CAVASOL W7 M Pharma, 18.4 U FDH and 1 μM CYP102A1 3mDS. Monitoring of the reaction progress was achieved by taking samples from the reaction mixture, acidifying them with dilute HCl to pH 3, in order to hydrolyse CAVASOL W7 M Pharma, extracting with diethyl ether and evaporating to dryness. The solid was derivatised as described above and analysed by GC/MS.

For the preparation of (2*R*,4*R*,6*S*,8*S*)-tetramethyldecane-1,9-diol (**3**) on a mg-scale, 800 mg of (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**) were used. After 8 h the reaction mixtures were acidified with dilute HCl to pH 3, extracted with diethyl ether, evaporated and purified on silica gel column. Elution was performed by an *n*-hexane:ethyl acetate mixture (ratio 2:1).

GC/MS Measurements

Products were identified on a Shimadzu GC/MS-QP2010 equipped with a 30 m FS-Supreme column (internal diameter 0.25 mm, film thickness 0.25 μm) using helium as carrier gas at a linear velocity of 30 cm s^{-1}. The following GC programs were used.

For dodecanoic acid: 1) 160 °C for 1 min, 2) 160 °C to 240 °C at 5 °C min^{-1}, 3) 240 °C to 300 °C at 20 °C min^{-1}, 4) 300 °C for 1 min. Trimethylsilylated hydroxy derivatives of dodecanoic acid were identified by their characteristic MS fragmentation patterns.

For (2*R*,4*R*,6*R*,8*R*)-tetramethyldecanol (**4**): 1) 150 °C for 1 min, 2) 150 °C to 300 °C at 15 °C min^{-1}. Trimethylsilylated (2*R*,4*R*,6*S*,8*S*)-tetramethyldecane-1,9-diol (**3**) was identified by its characteristic MS fragmentation pattern.

Determination of the Configuration of (2*R*,4*R*,6*S*,8*S*)-Tetramethyldecane-1,9-diol (3)

General experimental: IR spectra were recorded on a Bruker Vector 22 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX 300 or a Bruker ARX 500 spectrometer with TMS as an internal standard. Mass spectra were performed on Bruker Daltonics micrOTOF Q spectrometer. MPLC was performed on a LKB-Bromma using a 370 mm × 25 mm column with LiChroprer, 10–15 μm Silica gel from Merck and was detected by 254 nm and 20 bar flow pressure. Optical rotations were measured using a Perkin–Elmer Polarimeter 241 at room temperature in 1 mL-cuvettes (l = 0.1 dm).

*Bis-1,9-(4-nitrobenzoyl)-(2*R*,4*R*,6*S*,8*S*,9*R*)-tetramethyldecane-1,9-diol* (**5a**) *and bis-1,9-(4-nitrobenzoyl)-(2*R*,4*R*,6*S*,8*S*,9*S*)-tetramethyldecane-1,9-diol* (**5b**)*:* To a solution of **3a**,**b** (80 mg, 347 μmol, **3a**:**3b** = 80 : 20) in absolute CH_2Cl_2 (3 mL) was added at room temperature absolute NEt_3 (195 μL, 1.39 mmol), DMAP (42 mg, 347 μmol) and dropwise a solution of 4-nitrobenzoyl chloride (258 mg, 1.39 mmol) in absolute CH_2Cl_2 (1 mL). After stirring at room temperature for 1 h the reaction mixture was quenched by 1 M HCl (1 mL). The water layer was separated and extracted with diethyl ether (3 × 5 mL). The combined organic layers were dried (Na_2SO_4) and concentrated under vacuum. Flash chromatography by MPLC hexanes/ethyl acetate (20:1) afforded the minor diastereomer **5b** (yield: 29 mg, 59 μmol, 16%) as an amorphous colourless solid and the major diastereomer **5a** (yield: 117 mg, 221 μmol, 63%) as a colorless crystalline solid.

Minor diastereomer 5b: mp 57 °C; $[\alpha]_D^{20}$: +2.6 (*c* 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.81 (d, 3H, J = 6.5 Hz, , 8-Me), 0.85 (d, 3H, J = 6.5 Hz, 6-Me), 0.88 (m, 2H, 5-H), 0.99 (d, 3H, J = 6.6 Hz, 4-Me), 1.02 (d, 3H, J = 6.7 Hz, 2-Me), 1.12–1.20 (m, 1H, 4-H), 1.33 (d, 3H, J = 6.7 Hz, 10-H), 1.35–1.45 (m, 2H, 3-H), 1.50–1.65 (m, 3H, 7-H, 8-H), 1.80 −1.90 (m, 1H, 6-H), 1.96–2.06 (m, 1H, 2-H), 4.03–4.09 (dd, 1H, J = 6.9/10.7 Hz, 1a-H), 4.20–4.25 (dd, 1H, J = 6.0/10.7 Hz, 1b-H), 5.14–5.20 (m, 1H, 9-H), 8.16–8.21 (m, 4H, Ph), 8.25–8.31 (m, 4H, Ph); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 15.5 (Me-8), 17.2 (Me-2), 18.3 (Me-4), 20.7 (Me-6), 20.9 (C-10), 27.4 (C-6), 27.5 (C-4), 30.0 (C-2), 34.9 (C-8), 40.6 (C-7), 41.0 (C-3), 45.3 (C-5), 70.3 (C-1), 74.9 (C-9), 123.5, 123.6 (C-3′), 130.5, 130.6 (C-2′), 135.8, 136.2 (C-1′), 150.4, 150.5 (C-4′), 164.2, 164.7 (C=O); IR (film): $\tilde{\nu}$ = 3110, 2958, 2928, 2843, 1711, 1607, 1525, 1456, 1346, 1277, 1100, 1009, 933, 848 cm^{-1}; ESI-MS m/z = 551.2364, calcd. for $C_{28}H_{36}N_2O_8Na$ $[M + Na]^+$: 551.2372; ESI-MS: m/z (%) = 551 (100) $[M+Na]^+$, 384 (20), 359 (20), 301 (5), 226 (5), 190 (20), 159 (3), 132 (3).

Major diastereomer 5a: mp 78 °C; $[\alpha]_D^{20}$: −21 (c 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.90 (d, 3H, J = 6.7 Hz, , 8-Me), 0.92 (d, 3H, J = 6.7 Hz, 6-Me), 0.99 (d, 3H, J = 6.9 Hz, 4-Me), 0.98–1.02 (m, 2H, 5-H), 1.05 (d, 3H, J = 6.9 Hz, 2-Me), 1.19–1.25 (m, 1H, 4-H), 1.27 (d, 3H, J = 6.3 Hz, 10-H), 1.32–1.39 (m, 2H, 3-H), 1.40–1.49 (m, 1H, 8-H), 1.57–1.68 (m, 2H, 7-H), 1.94–2.02 (m, 1H, 6-H), 2.04–2.12 (m, 1H, 2-H), 4.10–4.15 (dd, 1H, J = 6.9/10.7 Hz, 1a-H), 4.27–4.33 (dd,

1H, $J=6.0/10.7$ Hz, 1b-H), 5.11–5.17 (m, 1H, 9-H), 8.17–8.22 (m, 4H, Ph), 8.26–8.32 (m, 4H, Ph); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=14.9$ (Me-8), 15.3 (Me-2), 18.4 (Me-4), 20.8 (Me-6), 20.9 (C-10), 27.5 (C-6), 27.6 (C-4), 30.1 (C-2), 34.4 (C-8), 40.8 (C-7), 40.9 (C-3), 45.3 (C-5), 70.3 (C-1), 75.6 (C-9), 123.5, 123.6 (C-3′), 130.5, 130.6 (C-2′), 135.8, 136.3 (C-1′), 150.4, 150.5 (C-4′), 164.2, 164.7 (C=O); IR (film): $\tilde{\nu}=3112$, 2959, 2913, 2839, 1712, 1606, 1523, 1454, 1322, 1274, 1100, 1013, 924, 840 cm^{-1}; ESI-MS: $m/z=$ 551.2364, calcd. for $C_{28}H_{36}N_2O_8Na$ $[M+Na]^+$: 551.2372; ESI-MS: m/z (%) = 551 (100) $[M+Na]^+$, 384 (20), 359 (20), 301 (20), 273 (50), 242 (3), 226 (5), 190 (25).

Crystal Structure Determination of 5a

Suitable single crystals were obtained by crystallisation from hexane. Data were collected on a Siemens P4 diffractometer with graphite-monochromated Cu-K_α radiation ($\lambda=$ 1.54178 Å) at 293 K. The structures were solved by direct methods and refined against F^2 for all observed reflections.

Crystal data: $C_{28}H_{36}N_2O_8$, $M=528.59$, triclinic, space group *P1*; $a=6.8250(10)$, $b=13.594(2)$, $c=16.279(3)$ Å, $\alpha=$ 72.040(10)°; $\beta=87.920(10)$°; $\gamma=87.690(10)$; $V=$ 1435.1(4) Å^3, $Z=2$; $\rho_{calcd.}=1.223$ Mg m^{-3}.

Data collection: crystal size $0.55\times0.15\times0.15$ mm, 5153 reflections in the range $\theta=3.89$–67.48°, 5135 unique reflections.

Structure refinement: 5153 reflection data [$I>3\sigma(I)$], 686 parameters; the final R indices were $R=0.0716$, $Rw=$ 0.1769; residual electron density between 0.297 and -0.200 e Å^{-3}.

Crystallographic data for the structure have been deposited with the Cambridge Crystallographic Data Centre, CCDC 634600. Copies of the data can be obtained free of charge on application to The Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (Fax: int.code+ (1223)336–033; e-mail for inquiry: fileserv@ccdc.cam.ac.uk).

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