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The steroid monooxygenase from *Rhodococcus rhodochrous*; a versatile biocatalyst

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ABSTRACT

The substrate scope of a steroid monooxygenase (STMO) from *Rhodococcus rhodochrous* DSM 43269 was investigated for a large range of different ketone substrates. These studies revealed that this enzyme not only oxygenates steroids, but also ketone moieties of a series of other open-chain ketones, such as cyclohexyl methyl ketone, cyclopentyl methyl ketone, and 3-acetylindole. Furthermore, the STMO catalyzed the oxygenation of cyclobutanone derivatives. Comparative biotransformations with recombinant *Escherichia coli* resting cells harboring the STMO, the cycloalkanone monooxygenase (CAMO) from *Cylindrocarpon radicicola* or the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* revealed that the STMO is enantiodivergent compared to the CHMO-type. Moreover, the STMO resulted in a higher enantiomeric excess of the product lactones compared to the known BVMOs of the same enantiopreference, such as cyclopentanone monooxygenases.

1. Introduction

Baeyer–Villiger monooxygenases (BVMOs, EC 1.14.13.x) are flavin-dependent oxidoreductases, which convert ketones into esters or lactones utilizing molecular oxygen and NADPH. Thus, they are the enzymatic equivalents to peroxyacids or concentrated hydrogen peroxide capable of conducting the chemical Baeyer–Villiger oxidation first described by Adolf von Baeyer and Victor Villiger in 1899.[1] Due to their potential for carrying out Baeyer–Villiger oxidations in a more enantioselective and environmentally friendly manner, BVMOs have been of particular interest for biocatalysis and today more than 60 BVMOs have been expressed recombinantly.[2] Detailed investigations of the substrate scopes of these enzymes revealed that BVMOs convert a wide range of cyclic (mono-, bi-, and polycyclic) as well as aromatic[3] and open-chain[4] ketones. Furthermore, some steroid-oxygenating BVMOs have been described in bacteria[5] as well as in fungi.[6] A steroid monooxygenase (STMO, EC 1.14.13.54) from *Rhodococcus rhodochrous* IFO 3338 (identical to *R. rhodochrous* DSM 43269) was expressed recombinantly in *Escherichia coli*[7] and the structure of this enzyme was recently determined. The active site was shown to closely resemble that of phenylacetone monooxygenase and the enzyme was also shown to convert phenylacetone.[8] This observation indicates that the substrate scope of the STMO from *R. rhodochrous* might possibly be more diverse than previously described. However, the substrate scope of this enzyme as well as of other steroid monooxygenases has only been investigated rudimentally and, except for phenylacetone, virtually exclusively for steroids as substrates. The investigation of the substrate scope of the STMO for further substrates would allow for a more precise classification into specificity sub-types compared to other BVMOs. Due to the STMO's position in a phylogenetic tree separated from well-investigated BVMOs such as cyclohexanone monooxygenases, substrates could potentially be converted with different enantio- and regioselectivities. A more comprehensive substrate profile also enables a better assessment of BVMOs with regards to prototype biocatalysts for various substrate classes, as recently outlined.[9]

Herein we investigated the substrate spectrum of the STMO from *R. rhodochrous* against a large range of ketones including steroids as well as (bi)cyclic and open-chain ketones. A whole-cell recombinant biocatalyst containing the STMO was established and the enantioselectivities achieved for the oxygenation of 3-phenylcyclobutanone were compared to known BVMOs.

2. Results and discussion

In order to fully assess the potential of the STMO for biocatalytic Baeyer–Villiger oxidation reactions, the enzyme was produced recombinantly in *E. coli* BL21 (DE3). This was implemented using a pET-28b(+)-based construct allowing IPTG induction with the STMO gene fused to an N-terminal His_6-Tag for simplifying the subsequent purification procedure. The gene was expressed in high concentration and a high amount of soluble protein was formed.

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The crude extract obtained from the recombinant *E. coli* cells, which were disrupted using a FastPrep24 homogeniser, was lyophilized without any additives; the activity loss caused by lyophilisation was determined as relatively low (10%). The pH activity profile of the STMO measured for the conversion of progesterone was bell-shaped with an optimal pH value of 10 and a faster activity decrease at more alkaline pH values. Residual activities were above 60% compared to the optimal pH value at a pH between 8.0 and 10.9 (data not shown).

For the determination of the substrates converted and the kinetic parameters, the enzyme was purified to homogeneity by Ni^{2+}-based metal ion affinity chromatography, yielding a pure protein of 61 kDa on a SDS gel. Since only a small series of steroid compounds have been identified as substrates of this enzyme so far, and no extensive investigation of the enzymatic activity toward substrates other than steroids has been reported in the literature, a wide range of potential substrates was investigated (Table 1, Section 4). Since a steroid monooxygenase from *Cylindrocarpon radicicola* converts open-chain ketones, such as progesterone, preferably at higher pH values than cycloaliphatic steroids, such as 4-androstene-3,17-dione,[5] this was assumed to be also applicable to the STMO from *R. rhodochrous*, in case it would convert any cycloaliphatic ketones. Therefore, in order not to impede the discovery of cycloaliphatic ketone substrates, all activity measurements were performed in a sodium phosphate buffer (50 mM, pH 8.0). Results show that the enzyme exhibits its highest activities against a small panel of steroids as previously described, particularly progesterone and 11-α-hydroxyprogesterone.[5] Furthermore, 11-ketoprogesterone and, with a rather low activity, corticosterone were also converted, which had not been shown so far for this enzyme. More interestingly, the enzyme also converted other open-chain ketones such as 2-decanone, 3-acetylindole as well as cyclohexyl- and cyclopentyl methyl ketone. These substrates are all open-chain subterminal ketones such as progesterone and particularly the two latter substrates might be regarded as a type of minimal substrates mimicking the steroidal D-ring. This is also supported by the fact that cyclohexyl- and cyclopentyl methyl ketones are the non-steroids, which were converted with the highest activity. Furthermore, the conversion of 3-acetylindole might open up a possibility for screening mutant libraries of the STMO for their activity toward open-chain ketones. Besides the converted non-steroid open-chain ketones, an oxygenation of cycloaliphatic ketones was of particular interest. Cyclobutanone and several of its substituted derivatives were found to be substrates of this enzyme in contrast to other cycloaliphatic ketones such as cyclopentanone and -hexanone, which were not converted. In contrast, cyclopentanone and -hexanone monooxygenases, which have typically been reported to catalyze the oxygenation of cyclobutanone[10] also convert other cycloaliphatic ketones.

Whole cell biotransformations established for selected substrates with glucose added for cofactor regeneration using resting *E. coli* BL 21 (DE3) producing the STMO allowed us to prove the conversion of the substrates shown to be active in previous activity studies. Substrate and product concentrations were monitored using GC–MS and it was found that the open-chain ketone cyclohexyl methyl ketone was fully converted after 17 h as well as the steroids progesterone and 11-ketoprogesterone. Additionally, further non-steroid open-chain ketones such as cyclopentyl methyl ketone as well as 3-acetylindole and 2-decanone were converted in agreement with the spectrophotometric activity data given in Table 1. Interestingly, also conversion of the cyclobutanone derivative 3-phenylcyclobutanone was observed and confirmed by GC–MS analysis.

The kinetic constants of the STMO were determined against a small panel of steroids, as well as the two non-steroids cyclobutanone and cyclohexyl methyl ketone, for which the highest activities were found in initial activity studies (Table 2). The results confirmed the previous findings that progesterone is the main substrate of this enzyme,[5] being converted with the highest catalytic efficiency (k_{cat}/K_m). However, turnover numbers are in a comparable range of values for all of the substrates tested and mainly the Michaelis constants varied, resulting in different catalytic efficiencies. These data show that steroids are indeed the preferred substrates of this enzyme. Still, the activities against non-steroids are sufficient enough for biocatalytic applications.

Table 1
Substrate scope of recombinant *R. rhodochrous* STMO

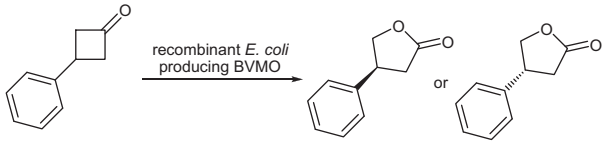
Substrate[a]	Activity (mU/mg)	Activity[b] (%)
Steroids		
Progesterone	460 ± 2	100
11-α-Hydroxyprogesterone	253 ± 23	54.9
11-Ketoprogesterone	177 ± 2	38.5
Corticosterone	8 ± 0	1.7
Cyclic and (bi)cyclic ketones		
Cyclobutanone	38 ± 6	8.3
2-Hydroxy cyclobutanone	53 ± 4	11.5
Bicyclo[3.2.0]-hept-2-ene-6-one	8 ± 1	1.7
Open-chain ketones		
Cyclohexyl methyl ketone	189 ± 5	41.1
Cyclopentyl methyl ketone	23 ± 1	5.1
2-Acetyl cyclopentanone	43 ± 5	9.4
3-Acetylindole	35 ± 2	7.5
2-Decanone	41 ± 1	8.9
3-Decanone	11 ± 2	2.3
4-Decanone	11 ± 1	2.3

[a] All measurements were made at 1 mM concentration, except for the steroids (200 μM) with purified enzyme and in triplicate.
[b] Activity relative to progesterone (100% value).

Table 2
Kinetic constants determined for STMO substrates using purified enzyme

Substrate	V_{max} (U/mg)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Progesterone	0.676	0.085	0.702	8.25
11-α-Hydroxyprogesterone	0.665	0.299	0.690	2.31
11-Ketoprogesterone	0.485	0.323	0.504	1.56
Cyclohexyl methyl ketone	0.457	1.461	0.474	0.33
Cyclobutanone	0.540	18.79	0.561	0.03

Since the STMO exhibits an interesting activity in the formation of butyrolactones, and considering that the substrate scope of this enzyme differs significantly from BVMOs typically reported to convert cycloaliphatic ketones, it was anticipated that the STMO might possibly exhibit different enantiopreferences and -selectivities. Since a cycloalkanone monooxygenase (CAMO) from *C. radicicola* ATCC 11011 has been expressed and characterized recently exhibiting an interestingly high catalytic efficiency against cyclobutanone[11] the selectivity of the STMO was compared to this enzyme as well as to the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus*. In order to establish these properties, the enantioselectivity of these three enzymes was investigated through biocatalytic reactions using the substrate 3-phenylcyclobutanone and compared to the values measured for the CHMO from *A. calcoaceticus* (see Scheme 1).



Scheme 1. Formation of 3-phenylbutyrolactone using BVMOs.

These experiments revealed that the CAMO exhibits a higher enantioselectivity compared to the CHMO (Table 3). In contrast, the STMO turned out to be enantiodivergent compared to the CHMO, and STMO also shows higher enantioselectivity than previously known (*S*)-selective BVMOs. Thus, the BVMOs investigated herein might possibly enable the synthesis of chiral butyrolactone derivatives, which represent valuable building blocks for the synthesis of natural products.

Table 3
Enantioselectivities and conversions of the BVMOs[a]

Enzyme	Organism	Selectivity	ee (%)	Conversion (%)
CHMO	*A. calcoaceticus*	(*R*)	58.6	45.9
CAMO	*C. radicicola*	(*R*)	90.9	70.8
STMO	*R. rhodochrous*	(*S*)	76.3	72.4

[a] After 20 h of biotransformation with whole cells.

3. Conclusion

An extensive study of the substrate scope of the steroid monooxygenase from *R. rhodochrous* revealed that this enzyme oxygenates a range of non-steroid ketones in addition to its well-established steroidal substrates. In particular the conversion of cyclobutanone derivatives and a series of open-chain ketones such as cyclohexyl methyl ketone by STMO indicates a significant overlap with the substrate scope of a variety of other BVMOs. Hence, the STMO from *R. rhodochrous* is a far more versatile biocatalyst than it was previously assumed. The enzyme converts (aryl-)aliphatic ketones such as 3-acetylindole, phenylacetone, and 2-decanone, which have previously been described as typical substrates of 4-hydroxyacetophenone monooxygenase (HAPMO), PAMO, and aliphatic ketone monooxygenase (AKMO), respectively. The STMO-catalyzed oxygenation of cyclobutanone derivatives, which are typically converted by CHMOs and CAMO, was of particular interest since this enzyme only converts open-chain ketones except for this substrate group. The STMO produces the enantiocomplementary (*S*)-3-phenyl-4-butyrolactone product by the oxygenation of 3-phenylcyclobutanone compared to CHMO and CAMO. Furthermore, the STMO shows similar or higher enantioselectivity than the previously described (*S*)-selective BVMOs indicating the potential of this enzyme for the biocatalytic preparation of chiral butyrolactones. A whole-cell recombinant biocatalyst based on *E. coli* producing the STMO, with glucose added for cofactor regeneration, has been established with good conversion and enantioselectivity, pointing the way toward future applications in non-steroid biotransformations using this STMO.

4. Experimental

4.1. Chemicals

All chemicals were of highest purity and purchased from Fluka (Buchs, Switzerland), Sigma–Aldrich (Munich, Germany), Roth GmbH (Karlsruhe, Germany), Alfa Aesar (Karlsruhe, Germany), and Acros Organics (Geel, Belgium) unless otherwise specified. Restriction enzymes were obtained from New England Biolabs (Beverly, MA, USA), DNA polymerases were from Roboklon (Berlin, Germany). HisTrap™ 5 ml FF columns were purchased from GE Healthcare (Uppsala, Sweden). 3-phenylcyclobutanone was prepared as described previously.[10]

4.2. Organisms, plasmids and culture conditions

Rhodococcus rhodochrous DSM 43269 (equivalent to IFO 3338) was purchased from the German National Resource Centre for Biological Material (DSMZ). *Escherichia coli* DH5α [Δ*lacU169*(Φ80-*lacZ*ΔM15) *hsdR17 recA1 end A1 gyrA96 thi-1 relA1*] was purchased from Clontech (Mountain View, USA). *E. coli* BL21 (DE3) [F^- *ompT hsdSB* (r_B-m_B-) *gal dcm rne131* (DE3)] and the plasmid pET28b(+) were from Novagen (Darmstadt, Germany).

E. coli strains were routinely cultured in a lysogeny broth (LB) medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) and supplemented with the antibiotic kanamycin (20 μg/ml) when necessary. *R. rhodochrous* was grown at 30 °C on GYM Streptomyces medium containing 0.4% (w/v) glucose, 0.4% (w/v) yeast extract, 1% (w/v) malt extract, 0.2% (w/v) $CaCO_3$, and 1.2% (w/v) agar and adjusted to an pH of 7.2 (DSMZ medium 65). For cultivation in liquid phase, the same medium, but without $CaCO_3$ and agar was utilized.

4.3. Cloning and recombinant expression of the STMO in *E. coli*

The gene sequence of the STMO from *R. rhodochrous* was amplified from the genomic DNA of the wild type strain, which was isolated using a TRI reagent (Sigma) according to the manufacturer's instructions.[13] Amplification was carried out using *Pfu*Plus DNA Polymerase and primers incorporating restriction sites for cloning the BVMO gene into the expression vector pET28b(+) containing a N-terminal (pHisSTMO), C-terminal (pSTMOHis), and no His_6-Tag (pSTMO), respectively. The fragment for cloning pHisSTMO was amplified using the primers SMO_ClonN_FW (5′-CATATGGA CGGCCAGCATCCCCGATC-3′) and SMO_ClonC_RV (5′-GAATT CAGCCCTCGAGGATCGCAAACCCC-3′), and pSTMOHis was constructed using SMO_ClonN_FW (5′-CCATGGACGGCCA GCATCCCC GATC-3′) and SMO_ClonCH_RV (5′-AAGCTT GCCCTCGAGGATCG CAAACCCC-3′). The primers SMO_ClonN_FW and SMO_ClonC_RV were used for the construction of the STMO-bearing construct without a N-terminal His_6-Tag. The amplified products were subcloned into the pCR® II-TOPO® vector, digested with NdeI and EcoRI (pHisSTMO), NcoI and HindIII (pSTMOHis) as well as NcoI and EcoRI (pSTMO), respectively (37 °C, 4 h) and ligated into an appropriately linearized pET28b(+) vector. The level of STMO expression of the different constructs was evaluated and the results showed that the highest production of soluble protein was achieved as a fusion protein with a N-terminal His_6-Tag (data not shown). In order to establish expression of the STMO with a His_6-Tag fused to different termini of the protein, NcoI had to be used for cloning of two of the variants for which purpose a mutation N2D was introduced through the N-terminal cloning primers as reported previously.[7] In order to generate the wild type sequence of the construct containing an N-terminal His_6-Tag, which showed the highest levels of soluble protein expression, the mutation originating from the cloning primers was mutated back by QuikChange-PCR using the primers SMO_D2N_sense (5'-GCGCGGCAGCCATATGAACGGCCAGC AT-3') and SMO_D2N_anti (5'-ATGCTGGCCGTTCATATGGCTGCCG CGC-3') thereby generating the plasmid pHisSTMO-WT. Expression level and activity of the wild type enzyme showed no significant differences compared to the N2D mutant (data not shown) and the wild type enzyme fused to an N-terminal His_6-Tag was used for all subsequent experiments. Thus, the final vector encoding the BVMO used for expression was pHisSTMO-WT and carried a N-terminal His_6-Tag coding sequence followed by a recognition site for thrombin protease resulting in a fusion protein of 569 amino acids, which differs in 20 amino acids at the N-terminus from native STMO from *R. rhodochrous*.

For protein production, pHisSTMO-WT containing the BVMO gene was transformed into *E. coli* BL21 (DE3) and cultivation was routinely carried out in 500 ml of the TB medium (2.4% (w/v) yeast extract, 1.2% (w/v) tryptone, 4% (v/v) glycerol, 10% (v/v) potassium phosphate buffer (pH 7.4, 1 M)) containing 20 μg/ml kanamycin in shaking flasks. The cells were grown in a shaking incubator at 37 °C

and 200 rpm to an optical density at 600 nm (OD_{600nm}) of 0.6–0.8, then induced by the addition of IPTG to a final concentration of 0.2 mM and cultivated at 20 °C for further 15 h. Cells were harvested by centrifugation (4 °C, 15 min, 4500*g*) and washed twice with sodium phosphate buffer (50 mM, pH 8.0). Cell disruption was carried out using glass beads (0.1–0.11 mm) with FastPrep24 homogenizer (MP Biomedicals, Solon, OH, USA) three times (4 m/s for 20 s) and crude extract was obtained by centrifugation (4 °C, 20 min, 10,000*g*).

4.4. Protein purification, concentration and purity

Overexpressed BVMO was purified using His_6-Tag based metal ion affinity chromatography. The cell pellet from 100 ml cell culture was disrupted in a sodium phosphate buffer (50 mM, pH 8.0) containing 300 mM NaCl and 30 mM imidazole as described in Section 4.3. The filtered crude extract was loaded on a HisTrap™ FF crude column (5 ml) integrated into an Äkta purifier (GE Healthcare) at a flow of 1.5 ml/min. The column was washed with 3 column volumes of the same buffer and one column volume of sodium phosphate buffer (50 mM, pH 8.0) containing 300 mM NaCl and 60 mM imidazole thereafter at a flow of 5 ml/min. The elution of the His_6-Tagged protein was performed with 4 column volumes of sodium phosphate buffer (50 mM, pH 8.0) containing 300 mM NaCl and 300 mM imidazole. Subsequently, the active fractions were pooled and desalted into a sodium phosphate buffer (50 mM, pH 8.0) using a centrifugal filter device (cut off 10 kDa, Amicon).

Protein concentration was determined in triplicate using the bicinchoninic acid (BCA) assay[12] with the protein quantification kit (Uptima, Montluçon Cedex, France) using bovine serum albumin as a standard in sodium phosphate buffer (50 mM, pH 8.0). Protein size and purity were determined by SDS–PAGE carried out according to the method described by Laemmli.[14] The gels were stained with 1% (w/v) Coomassie brilliant blue R250 in 10% (v/v) acetic acid, 30% (v/v) ethanol in water.

4.5. Enzyme assay and kinetics

The STMO activity was determined spectrophotometrically by monitoring the decrease of NADPH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) at 25 °C. Reaction mixtures (1 ml) contained sodium phosphate buffer (50 mM, pH 8.0), 160 μM NADPH, 10–150 mg/l of pure enzyme depending on the activity of the enzyme toward the respective substrate and 1% (v/v) dimethylformamide. The reaction was started by adding the enzyme to the mixture. One unit of BVMO is defined as the amount of protein that oxidizes 1 μmol of NADPH per min. Substrate specificity and kinetic parameters were determined using a purified enzyme. For the comparison of the overall activity, substrate concentrations were 0.2 mM for steroids and 1 mM for all other substrates unless otherwise stated. Oxygenation activity was determined photometrically for the steroids progesterone, pregnenolone, 4-androstene-3,17-dione, prasterone, 11-α-hydroxyprogesterone, 11-ketoprogesterone, hydrocortisone, and corticosterone. Furthermore, the (bi)cyclic ketones cyclobutanone, 2-hydroxy cyclobutanone, cyclopentanone, cyclohexanone, 2-phenyl cyclohexanone, cycloheptanone, cyclooctanone, bicyclo[3.2.0]-hept-2-ene-6-one, and 1-indanone were tested. Activity was also investigated for the open-chain ketones cyclohexyl methyl ketone, cyclopentyl methyl ketone, 2-acetyl cyclopentanone, cyclobutyl methyl ketone, cyclopropyl methyl ketone, dicyclopropyl ketone, 3-acetylindole, acetophenone, 2-butanone, 2-hexanone, 2-octanone, 2-decanone, 3-decanone, 4-decanone, and 2-dodecanone. Kinetic data were analyzed by non-linear regression analysis based on Michaelis–Menten kinetics using the program Kaleidagraph.

4.6. Biocatalysis using resting cells

Biotransformations were carried out at 25 °C in Tris–HCl buffer (50 mM, pH 8.0) using resting cells of *E. coli* BL21 (DE3) expressing STMO at a final $OD_{600\ nm}$ of 15 in 24-well deep well plates covered with a breathable AeraSeal™ sealing film (Excel Scientific, Victorville, USA). The reaction mixtures of a total volume of 2.5 ml contained 5 mM 3-phenylcyclobutanone, 10 mM β-cyclodextrine, 100 mM glucose, and 2% (v/v) of dimethylformamide. Samples were taken after 0, 3, and 20 h, extracted with ethyl acetate and analyzed by GC–MS.

Further biotransformations were carried out in order to verify the conversion of substrates established through a spectrophotometric assay, using a series of substances against which the enzyme was shown active alongside with other typical BVMO substrates, which were not shown to be converted in spectrophotometric studies. The substances were assayed at a final concentration of 2 mM together with 50 mM glucose for cofactor regeneration and included progesterone, 11-ketoprogesterone, 4-androstene-3,17-dione, prasterone, corticosterone, cyclopentanone, cyclohexanone, 2-phenylcyclohexanone, bicyclo[3.2.0]-hept-2-en-6-one, 1-indanone, norcamphor, cyclohexyl methyl ketone, cyclopentyl methyl ketone, 3-acetylindole, acetophenone, and 2-decanone.

4.7. GC analysis

GC–MS analysis for the conversion of 3-phenylcyclobutanone was carried out on a GCMS-QP 2010 (Shimadzu Europa GmbH, Duisburg, Germany) with a Hydrodex®-β-TBDac column (Macherey-Nagel, Düren, Germany). Injection temperature was set to 220 °C, detection temperature was initially set to 80 °C followed by a ramp of 2 °C/min to 220 °C. The enantiomeric configuration of the ester products formed in biotransformation was established through comparison to the products of a biotransformation using the CHMO from *A. calcoaceticus* which have been reported previously.[9] The substrate was eluted after 32.1 min whereas the peaks for the (*S*)- and (*R*)-lactone products were measured after 54.1 and 54.5 min, respectively.

Conversions for all of the other substrates from the biocatalysis using resting cells were measured using a BPX5 column (5% phe-

Table 4
GC conditions for measurement/achiral analyses of BVMO substrates

Substrate	Temperature program	Retention time substrate (min)	Retention time product (min)
Cyclohexanone	Program A	4.71	19.64
Cyclopentanone	Program B	6.20	14.71
2-Phenyl cyclohexanone	Program A	26.66	30.35
Cycloheptanone	Program A	10.68	16.56
Bicyclo[3.2.0]hept-2-en-6-one	Program A	6.37	20.77
1-Indanone	Program A	22.65	–
Norcamphor	Program C	8.71	31.65
Cyclohexyl methyl ketone	Program A	12.05	12.33
Cyclopentyl methyl ketone	Program A	5.71	6.05
2-Decanone	Program D	7.40	7.58
Acetophenone	Program A	15.54	–
3-Acetylindole	Program A	25.67	29.30
Progesterone	Program E	16.86	17.96
11-Ketoprogesterone	Program E	19.95	17.96
4-Androstene-3,17-dione	Program E	12.94	–
Prasterone	Program E	10.71	–

Program A: 15 min 60 °C, 10 °C/min to 180 °C, maintain 5 min.
Program B: 10 min 32 °C, 20 °C/min to 140 °C, maintain 2 min.
Program C: 25 min 60 °C, 10 °C/min to 180 °C, maintain 10 min.
Program D: 5 min 85 °C, 20 °C/min to 180 °C, maintain 5 min.
Program E: 5 min 240 °C, 2 °C/min to 270 °C, maintain 5 min.

nyl/95% methylpolysilphenylene siloxane, SGE GmbH, Darmstadt, Germany) as described previously.[11] The injection temperature was set to 220 °C or 250 °C and detection temperatures for all substances measured are shown in Table 4. Identification of the product esters formed was performed through similarity analysis of the mass spectra obtained by GC/MS.

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