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Communication

Dienes from Diacids

Enzymatic Oxidative Tandem Decarboxylation of Dioic Acids to Terminal Dienes

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Abstract: The biocatalytic oxidative tandem decarboxylation of C_7–C_{18} dicarboxylic acids to terminal C_5–C_{16} dienes was catalyzed by the P450 monooxygenase OleT with conversions up to 29 % for 1,11-dodecadiene (0.49 g L^{-1}). The sequential nature of the cascade was proven by the fact that decarboxylation of intermediate C_6–C_{11} ω-alkenoic acids and heptanedioic acid exclusively gave nonconjugated 1,4-pentadiene; scale-up allowed the isolation of 1,15-hexadecadiene and 1,11-dodecadiene; the system represents a short and green route to terminal dienes from renewable dicarboxylic acids.

Introduction

To reposition the chemical economy, novel metal-free synthetic routes to chemical building blocks from renewable C sources are demanded.[1,2] Among the primary platform chemicals, short- and medium-chain 1-alkenes (obtained by steam cracking of crude oil at >800 °C)[3] are of outstanding economic importance.[4] In contrast, terminal dienes (>C_4) cannot be obtained from steam cracking, the Shell higher olefin process (SHOP) process,[4b] or the chemical decarboxylation of fatty acids (FAs),[3,5] but they are synthesized by cross-metathesis cleavage of cycloalkenes or polyenes with lower alkenes, such as ethylene (ethenolysis).[6] As dienes represent essential building blocks for synthetic rubbers, co-crosslinkers, and starting materials for macrocycle synthesis, alternative routes are highly desired.[1b,2d] A sustainable synthetic route to terminal dienes from dicarboxylic acids has so far not been reported. In 2011, Rude et al. reported the first direct enzymatic oxidative decarboxylation of FAs by the P450 monooxygenase OleT to yield long-chain 1-alkenes[7] through a not-yet-elucidated mechanism.[8] By employing whole cells, cell-free extracts, or purified enzyme preparations,[7–9] the reaction proceeds either with H_2O_2 or O_2 as the oxidant. By using purified OleT in combination with the putidaredoxin electron-transfer system CamAB,[10] efficient NADH (NAD = nicotinamide adenine dinucleotide) regeneration and atmospheric O_2 as the oxidant allowed the synthesis of 1-alkenes ranging from C_3 to C_{21}[9c] with product titers close to 1 g L^{-1}. In contrast, in vivo production by using whole cells (e.g., *E. coli* and electron-transfer proteins Fdr/FldA) led to mixtures of 1-alkenes (4–48 mg L^{-1} d^{-1}),[9a,9e] and dienes were only detected in their terminal/internal forms (e.g., 1,10-heptadecadiene).[7,9e] Other promising 1-alkene-forming enzymes are the non-heme di-iron monooxygenase UndA,[11] which is limited by a narrow substrate scope and low total turnover numbers (TTNs), and ferulic acid decarboxylases,[12] which require α,β-unsaturated acids as substrates. On the other hand, aliphatic diacids are widely distributed in various metabolic pathways[13] and hence represent a renewable basis for the synthesis of terminal dienes. Alternatively, enzymatic ω-oxidation of FAs yields C_6–C_{22} diacids in industrially relevant quantities (>100 g L^{-1}).[14] Further sources of diacids (e.g., adipic acid) are polyester waste materials.[2c]

Results and Discussion

To explore the synthetic potential and substrate scope of the P450 monooxygenase OleT, we investigated the direct tandem decarboxylation of dicarboxylic acids to terminal dienes (Scheme 1). As saturated FAs are initially regarded as the natural substrates of OleT, it was unclear to what extent terminal modification of the substrate would influence the overall catalytic performance. Initial experiments with the use of **5a** (10 mM) and the OleT-CamAB-FDH reaction system[9c] and O_2 as the oxidant revealed the formation of **5c** with 12.4 % conversion. After product extraction and derivatization of the remaining acid(s) in the reaction medium, α- and β-hydroxy diacids were found as side products (in total 38 %, Table 1), whereas intermediate **5b** was not found (see the Supporting Information). However, α- and β-hydroxy derivatives of **5b** were detected. To explore the substrate scope, 10 dicarboxylic acids (i.e., compounds **1a**–**10a**, 10 mM) were subjected to the OleT-CamAB-FDH reaction system (Scheme 1, Route 1; Table 1).

Terminal dienes **1c**–**10c** were detected with up to 29 % conversion at a 0.06 mol-% catalyst loading. The highest conversion was obtained with **3a**, which yielded up to 29 % of **3c**; this

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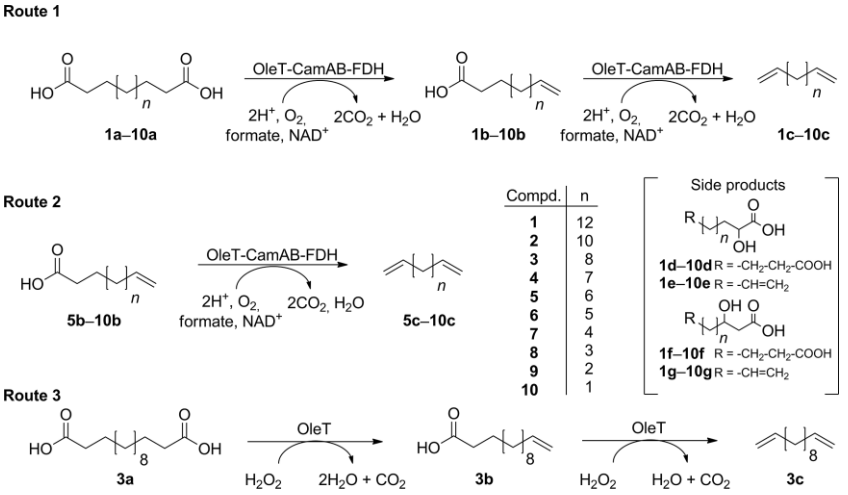

Scheme 1. Biocatalytic routes to terminal dienes through oxidative decarboxylation of (di)carboxylic acids by using P450 monooxygenase OleT. Route 1: Tandem decarboxylation of dicarboxylic acids (C_{18}–C_7) to dienes with the OleT-CamAB-FDH cascade[9c] and O_2 as oxidant. Route 2: Decarboxylation of ω-alkenoic acids (C_{11}–C_6) with the OleT-CamAB-FDH cascade and O_2 as oxidant. Route 3: Tandem decarboxylation of C_{14} dioic acid with OleT and H_2O_2 as oxidant; 2- and 3-hydroxy diacid/ω-alkenoic acid side products are shown in brackets.

Table 1. Decarboxylation of dicarboxylic acids with the OleT-CamAB-FDH cascade at room temperature and at 4 °C.[a]

Entry	Substrate	Diene [mM] r.t./4 °C	TOF [h^{-1}][b]	TTN r.t./4 °C	Diene [%] r.t./4 °C	α-OH + β-OH diacids [%][c] r.t./4 °C	α-OH + β-OH ω-enoic acids [%][c] r.t./4 °C
1	**1a**	n.d./n.d.	n.d.	n.d./n.d.	66/70	9/9[d]	25/21[d]
2	**2a**	1.12/0.74	18 ± 1	373/245	54/69	46/31[d]	0/0[d]
3	**3a**	2.93/1.63	73 ± 2	978/542	48/51	41/43	11/6
4	**4a**	0.31/0.95	36 ± 2	104/317	24/44	51/37	25/19
5	**5a**	1.24/1.22	91 ± 9	413/406	44/43	38/37	18/20
6	**6a**	0/2.05	n.d.	n.d./684	0/48	74[e]/39	26[e]/13
7	**7a**	0.06/<0.05[f]	n.d.	20/13	≥99[g]/≥99[g]	n.d./n.d.	n.d./n.d.
8	**8a**	<0.05[f]/0.09	n.d.	4/29	≥99[g]/≥99	n.d./0	n.d./0
9	**9a**	0.12/0.1	n.d.	38/34	≥99[g]/≥99[g]	n.d./n.d.	n.d./n.d.
10	**10a**	<0.05/0.4	n.d.	4/146	≥99[g,h]/≥99[g,h]	n.d./n.d.	n.d./n.d.

[a] General reaction conditions: Unless stated otherwise, all reactions were performed in 4 mL glass vials closed with a polytetrafluoroethylene (PTFE) septum by using potassium phosphate buffer (KPi, pH 7.5, 0.1 M) containing 6 μM purified OleT, 0.05 U CamAB, 2 U FDH, 1200 U catalase (for H_2O_2 removal), 100 mM ammonium formate, 0.4 mM NAD^+, 10 mM substrate, and 5 % EtOH (v/v) in a final volume of 1 mL at r.t. (24 h and 170 rpm shaking) or at 4 °C (72 h and 100 rpm stirring). Reference data for the assignment of side products can be found in the Supporting Information; n.d.: not determined. [b] Turnover frequency (TOF) calculated from the product amount after 1 h reaction time. [c] % GC area of products (α,ω-diene + α-/β-hydroxy acids = 100 % GC area). [d] Additional minor side products were detected during conversion of diacids **1a** and **2a**; 5 % DMSO (v/v) was used as co-solvent. [e] No diene was formed; therefore, the ratio between hydroxy diacid/hydroxy enoic acid peak areas is given. [f] Detection limit 50 μM. [g] Volatile fraction analyzed. [h] 1-Heptene was detected in the volatile fraction; TOF (product extraction and quantification after 1 h reaction time) and TTN (product extraction and quantification after 24 or 72 h reaction time) were calculated on the basis of quantified diene products. Volatile dienes **6c**–**10c** were quantified by headspace GC–MS analysis by using commercial standards for calibration (see the Supporting Information).

corresponds to a product titer of 0.49 mg mL^{-1} (or 0.49 g L^{-1}) and a TTN of 978. Remarkably, the corresponding saturated FA with identical chain length (C_{14}:0) gave only 5.5 % conversion under similar reaction conditions.[9c] Lowering the concentration of **3a** to 5 and 2 mM decreased the TTN of OleT by 3.1- and 8.9-fold, respectively (5 mM: 18 % conversion; TTN 312; 2 mM: 19 % conversion, TTN 110), which indicated a strong dependence of OleT activity/productivity on the concentration of the diacid substrate. To address the low solubility of long-chain fatty acid/diacid substrates (>C_{14}), we performed a co-solvent study by using 10 mM stearic acid as the model substrate and six water-miscible organic solvents (Figure 1). OleT displayed the highest TTN (1028 to 1817) in the presence of EtOH, DMF, and dioxane with an optimal concentration of 5 % (v/v). In contrast, THF and propan-2-ol led to a significant decrease in productivity at very low concentrations (>2.5 %). Interestingly, DMSO was tolerated from 0 to 20 % (v/v), albeit at significantly decreased productivities (TTN max. 445) relative to the other co-solvents. The selectivity for the formation of dienes (defined as the amount of diene formed relative to that of all reaction products) ranged between 0 and 99 %: >99 % of **7c**–**10c** was identified in the volatile fraction starting from **7a**–**10a**. Besides α- and β-hydroxy diacids (7–74 % of total products), minor amounts of α- and β-hydroxy ω-alkenoic acids, presumably arising from hydroxylation of the ω-alkenoic acids, were detected (6–26 % of total products; Tables 1 and 2, also see the Supporting Information). In the case of **6a**, only hydroxy acid products were detected in a ratio of 74:26 [(α/β-hydroxy diacid)/(α/β-hydroxy ω-alkenoic acid); Table 1, entry 6; r.t. value],[15] which indicated that mono-decarboxylation had occurred.

Table 2. Decarboxylation of intermediate ω-alkenoic acids to terminal dienes at room temperature and at 4 °C.

Substrate	Concentration [mM]	Diene [μM] r.t./4 °C	TTN r.t./4 °C	Diene [%] r.t./4 °C	α-OH + β-OH ω-enoic acids[c] [%] r.t./4 °C
5b	4	141/n.d.	24/n.d.	70/n.d.	30/n.d.
5b	10	0/0	0/0	0/0	0/13*
6b	10	21/0	4/0	45/0	55/3*
6b	2	267/n.d.	53/n.d.	47/n.d.	53/n.d.
7b	10	345/669	57/111	≥99[a]/≥99[a]	n.d./10*
8b	10	77/192	13/32	≥99[a]/≥99[a]	n.d./0
9b	10	71/34	12/7	≥99[a]/≥99[a]	n.d./0
10b	10	59/1776[b]	10/296	≥99[a]/≥99[a]	n.d./n.d.

[a] Volatile fraction analyzed. [b] Trace amounts of 1-heptene (derived from trace amounts of octanoic acid in the starting material). [c] % GC area of products (α,ω-diene + α-/β-hydroxy acids = 100 % GC area). *GC area compared to GC area of internal standard (0.1 % v/v 1-decanol); n.d.: not determined.

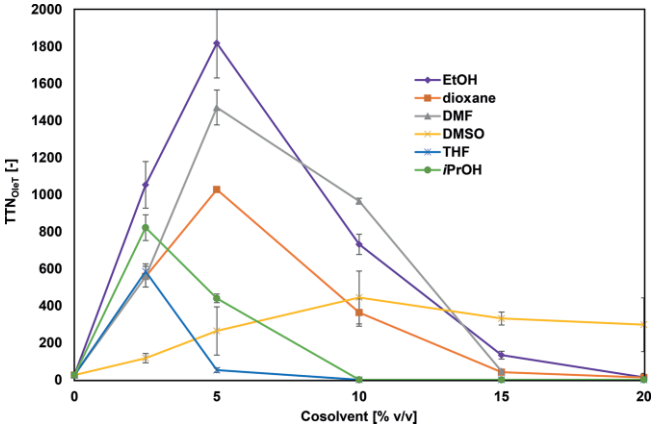

Figure 1. Influence of co-solvents on the catalytic activity of OleT for the conversion of 10 mM stearic acid. TTN values were calculated on the basis of the formation of 1-heptadecene by using 6 μM of OleT. Experiments were performed as described in the general reaction setup for decarboxylation with the OleT-CamAB-FDH system (see the Supporting Information). Reaction buffer volume was replaced by a given amount of co-solvent.

Identification of α/β-hydroxy ω-enoic acid side products during conversion of **1a**–**6a** indicate that dicarboxylic acids undergo tandem decarboxylation via ω-alkenoic acid intermediates to yield the corresponding terminal dienes (Scheme 1, Route 1). To prove the practical applicability of the method, reactions were scaled up to 20 mL to isolate dienes **1c** (4.9 mg, 5 % isolated yield) and **3c** (6 mg, 7.2 % isolated yield).[16]

In addition to the CamAB electron-transfer system, the decarboxylation of **3a** was tested with a crude cell-free lysate of Fdr/FldA from *E. coli*[9e,17] (not optimized), which resulted in a fivefold lower conversion to **3c** (0.56 mM; TTN 185). This indicates that the class I/ferredoxin-based electron-transfer system CamAB is more compatible with OleT.[9c,18] With H_2O_2 as the oxidant under reaction conditions (supplementation of 1.6 mM h^{-1}) identical to those used for the P450 peroxygenases $P450_{Cla}$ and $CYP_{BS\beta}$[15] at elevated catalyst loading (6 μM OleT), only trace amounts of **3c** (1.4 %, 140 μM; TTN 32) were detected; the amount was 31-fold lower than that obtained with the CamAB system (Table 1), which supports the redefinition of OleT as a monooxygenase rather than a peroxygenase.[8,9,9c,9e] As the catalytic performance (TTN and conversion) of OleT depended not only on the substrate chain length, type of oxidant, and source of electrons but also, in particular, on the reaction temperature,[9c] **1a**–**10a** were also converted at 4 °C. Overall, a lower temperature did not influence the product distribution, except for **4a**. Whereas the conversion of **6a** did not show any diene formation at room temperature (Table 1, entry 6), at 4 °C it turned out to be the best accepted substrate, which led to a remarkable conversion of 20.5 % (TTN 684; 0.25 g L^{-1} of **6c**; Table 1). The shortest diacids converted by OleT were **9a** and **10a** to yield **9c** and **10c** (Table 1). Although the conversion remained low (≈1 %; TTN 34 to 38 for **9a**), the selectivity for diene formation was very high (>99 %). Owing to a radical enzyme mechanism, we initially assumed that the C_5 diene formed from heptanedioic acid (**10a**) and 5-hexenenoic acid (**10b**) would be the more stable conjugated 1,3-pentadiene rather than the nonconjugated 1,4-analogue (see the Supporting Information). Much to our surprise, only nonconjugated 1,4-pentadiene was obtained as the major product. This indicates that OleT exerts strict control over β-radical formation followed by specific α/β-carbon bond cleavage. The TTN values for **4a**, **6a**, **8a**, and **10a** converted at 4 °C were, on average, higher than those at room temperature (Table 1), which indicates increased stability of OleT with certain substrates at 4 °C.[9c] The stability and productivity of P450 monooxygenases are strongly related to electron-transfer efficiency and O_2 activation during formation of the reactive oxygenating species (compound I),[19] which determines substrate turnover and catalyst lifetime.[20] Lowering the reaction temperature can significantly alter the productivity and selectivity of OleT, which was the most prominent for the conversions of **4a** and **6a** (Table 1). One explanation could be that a change in the temperature impacts the redox potential of the mediator CamB (5 °C = –0.195 V; 25 °C = –0.242 V),[21] which thereby affects the productivity of OleT.[18–20] Given that attempts to decarboxylate medium- and short-chain diacids (i.e., compounds **7a**–**10a**) generated only small amounts of dienes (max. ≤4 % conversion, 0.4 mM, TTN 146), the decarboxylation of ω-enoic acids was investigated as an alternative route to terminal dienes. The conversion of six ω-enoic acids (i.e., compounds **5b**–**10b**; Scheme 1, Route 2) allowed the synthesis of **5c**–**10c** (Table 2), which confirmed that ω-alkenoic acids are intermediates during the tandem decarboxylation of diacids (Route 1). In addition, the conversions and TTNs for **7b**, **8b**, and **10b** at 4 °C were higher (up to 17 %, TTN 296) than the conver-

sions of shorter diacids (i.e., compounds **7a**–**10a**; Table 1 vs. 2), which can be explained by the similarity of the lipophilic ω-olefinic terminus with that of the saturated FAs. The highest conversion and TTN values were achieved for **10b** (17 %; TTN 296 at 4 °C), which yielded **10c** with >99 % selectivity in the nonconjugated form. Again, lowering the reaction temperature allowed an improved conversion and a higher TTN, in particular for **10b** and **7b** (6.7 % conversion, TTN 111; Table 2, entry 5). Nonetheless, the conversion of **7b** was still 2 to 2.7-fold lower than that for the decarboxylation of nonanoic acid at 10 mM (18 % 1-octene),[9c] which is indicative of a strong influence of the ω terminus on substrate conversion by OleT. Decarboxylation was the main reaction with **5b** (70 % diene; 30 % hydroxylated product); however, in the case of **6b** (one carbon less), the selectivity shifted towards hydroxylation (45 % diene, 55 % hydroxylated products: Table 2). One explanation for this unpredictable change in chemoselectivity (depending on substrate chain length) is variations in the hydrogen-bonding interactions in the active site of OleT, which can alter energy barriers for decarboxylation/hydroxylation pathways.[8b] Under the standard reaction conditions, various diacids were converted into terminal dienes without any detectable formation of ω-enoic acid intermediates (Table 1), whereas decarboxylation of **6a** and **5b** to the respective dienes failed at room temperature (Tables 1 and 2). The ω-enoic acid intermediate either undergoes secondary decarboxylation to yield the terminal diene or is subject to hydroxylation to yield the corresponding hydroxy ω-enoic acid (Table 1, entry 6; Scheme 1, Route 1).

The failed decarboxylation of **5b** (Table 2, entry 2) prompted us to investigate the effect of substrate concentration on the decarboxylation of ω-enoic acids by OleT, which is important for process design. Conversions were repeated at varying concentrations of **5b** and **6b** (0.1 to 10 mM) at room temperature. Interestingly, the decarboxylation of **5b** to **5c** became feasible if the substrate concentration was decreased to 6 mM (≤1 % conversion, Figure 2). The optimum productivity was reached at 4 mM **5b**, which yielded 0.14 mM of **5c** (3.5 % conversion; Figure 2 and Table 2). Similarly, for **6b** a higher conversion was obtained at a 2 mM substrate concentration (13.3 %, Figure 2; Table 2, entry 4), whereas no substrate was recovered (Figure 2) and α/β-hydroxy ω-decenoic acids were identified as major products (55 %). This indicates a dramatic switch from decarboxylation to hydroxylation activity by modification of the ω terminus of the substrate. The results indicate that the conversion of dioic acids into terminal dienes by OleT proceeds in a stepwise fashion (Scheme 1, Route 1). For this, the ω-enoic acid intermediate formed during the first step leaves the substrate channel and rebinds in an inverted (u-turn) orientation. Substrate concentration, co-solvents, and reaction temperature all had a significant influence on catalyst productivity and conversions. Inhibition of OleT by unsaturated FAs[9d] has been suggested but has not been investigated in detail. This constitutes a key for future strategies with the use of whole-cell terminal alkene production, which is complicated by the hosts own FA metabolism.[22] So far, free enzyme (purified or cell-free lysates) proved to be the most productive reaching up to 1 g L^{-1} for 1-alkenes[9c] and 0.49 g L^{-1} for terminal dienes (Table 1, entry 3), which is (at least) one order of magnitude higher than the 1-alkene titers reported for whole-cell systems: Detailed studies on reaction/expression optimization and metabolic engineering to improve whole-cell conversions with OleT have (so far) not exceeded 48 mg L^{-1} d^{-1} in *E. coli* and have reached only 4 mg L^{-1} d^{-1} in *S. cerevisiae*.[9a,9e] A product titer of up 0.49 g L^{-1} for **3c** can be regarded as a good value for a P450-catalyzed reaction,[19,22] in particular considering that the ω-carboxylic acid functionality of a diacid substrate is likely to impair with the hydrophobic nature of the substrate channel of OleT.[9b]

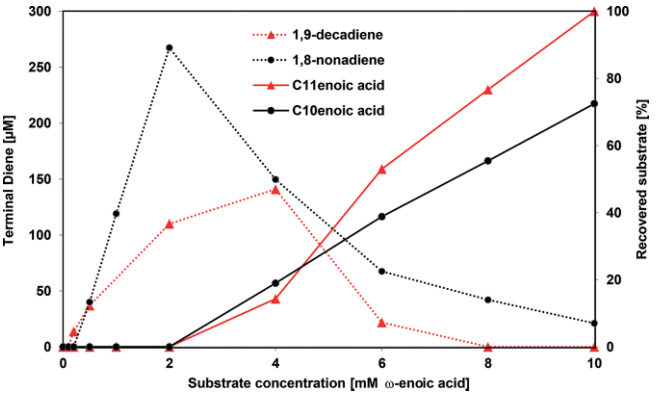

Figure 2. Substrate-concentration-dependent productivity of OleT for the conversions of **5b** and **6b**. Experiments were performed in triplicate according to the standard reaction protocol at room temperature and 1 mL scale (see legend of Table 1). Reactions went to completion at ≤2 mM of **5b** and **6b** (no substrate recovered). For both substrates, control reactions without OleT were performed as reference to calculate the recovery of the unconverted substrate; α-OH and β-OH ω-enoic acids were detected as side products.

Conclusions

For the first time, the production of terminal dienes with chain lengths of C_{16} to C_5 was achieved by sequential oxidative decarboxylation of dicarboxylic acids or ω-enoic acids by using O_2 as the sole oxidant catalyzed by the P450 monooxygenase OleT. The highest productivity of OleT was obtained for tetradecanedioic acid (**3a**), which yielded 1,11-dodecadiene (**3c**) after optimization of the substrate concentration, type of oxidant, and reaction temperature (TTN 978, 29 % conversion, 0.49 g L^{-1}). OleT showed the best performance as a cell-free catalyst with O_2 as the oxidant, and the highly selective formation of nonconjugated 1,4-pentadiene (rather than the more stable 1,3-isomer) underlines the advantages of the mild reaction conditions.

Supporting Information (see footnote on the first page of this article): Experimental details, including catalyst preparation and analytical data (GC-FID/GC–MS traces and ^{1}H NMR spectra) for isolated and purified compounds.

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Keywords: Biocatalysis · Enzyme catalysis · Carboxylic acids · Decarboxylation · Dienes

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