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Chemoenzymatic cascade for stilbene production from cinnamic acid catalyzed by ferulic acid decarboxylase and an artificial metathease†

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We report the preparation of symmetrical stilbene derivatives in a two-step one-pot cascade reaction based on enzymatic decarboxylation of cinnamic acid followed by olefin cross metathesis. Embedment of the metathesis catalyst in a protein scaffold enabled the cascade reaction to symmetric stilbenes and furthermore very efficient removal of metal impurities (<1 ppm in product fraction).

Chemoenzymatic cascade reactions are intriguing developments in synthetic organic chemistry. They combine the broad reaction scope of chemical catalysis with the unparalleled regio- and enantioselectivity of biocatalysts.[1–3] In such cascades, reactive intermediates are promptly converted to a desired product, suppressing side product formation or enzyme inhibition. Moreover, as the isolation of intermediates is not necessary, the process as a whole becomes more (cost-)efficient.[4,5] The types of catalysts employed in such cascades are manifold. Yet, challenges concerning compatibility are often initially faced.[6,7] Metal catalysts can be poisoned by protein- or whole-cell-borne functional groups, commonly resulting in mutual deactivation of the metal catalyst and the enzyme. Therefore, reaction conditions that are suitable for both catalysts need to be established. An example for a bioorthogonal chemical transformation that could be coupled to an enzymatic reaction[8–12] is offered by olefin metathesis, in which C=C double bonds are rearranged.[13]

Aqueous decarboxylation reactions are catalyzed by enzymes coined decarboxylases. Applied to cinnamic acid derivatives, this reaction yields styrene derivatives that could be further converted to stilbene derivatives *via* olefin cross metathesis.[14] Owing to the electron poor double bond in cinnamic acid derivatives, this conversion has hitherto not been efficiently achieved using common olefin metathesis catalysts. Stilbene derivatives are building blocks for many biologically active compounds (*i.e.*, pharmaceuticals). For instance, symmetrical stilbene derivatives were shown to effectively induce apoptosis in human cancer cell lines or act as modulators of hormone receptors.[15,16]

Kourist and co-workers developed a one-pot cascade reaction to convert hydroxycinnamic acids to 4,4′-dihydroxystilbene derivatives by employing a chemoenzymatic approach involving decarboxylation and olefin metathesis.[12] Compatibility between the decarboxylase (*Bs*PAD – *Bacillus subtilis* phenolic acid decarboxylase) and the olefin metathesis catalyst was ensured by compartmentalization (*i.e.*, encapsulation) of the enzyme into PVA/PEG beads (PVA = poly(vinyl alcohol); PEG = poly(ethylene glycol)).[12] Still, the best results were achieved when the solvent was exchanged by MTBE, followed by addition of anhydrous $MgSO_4$ to obtain a dry reaction medium for the olefin metathesis step. In a subsequent work, the Kourist group presented a similar reaction sequence involving decarboxylation of fatty acids by cytochrome P450 OleT from *Jeotgalicoccus* sp. ATCC 8456 ($OleT_{JE}$) followed by olefin cross metathesis.[8] Hartwig, Zhao and co-workers demonstrated the combination of a Grubbs–Hoveyda type olefin metathesis catalyst and a P450 monooxygenase for epoxidation.[9,10]

Ring-closing metathesis (RCM) in combination with enzymatic catalysis was shown by Gröger *et al.*[11] In a two-step one-pot reaction, diethyl malonates were converted *via* RCM to the corresponding cyclic alkenes followed by enzymatic saponification of the ester.[11] All studies mentioned above showed that it is in principle possible to combine olefin metathesis with biocatalysis. However, efficient conversion by both catalysts always required their spatial separation to avoid mutual inactivation.

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In all these examples, relatively high metal catalyst loadings (~5 mol%) were required, especially if the metathesis step succeeded the enzymatic step. It is assumed that this is based on the inactivation of the metal catalyst by unspecific coordination to proteins. High catalyst loadings entail the necessity for metal removal in a post-reaction step. The latter is of paramount importance if the desired product is used for pharmaceutical applications.[17]

Biohybrid catalysts (BHCs), also dubbed artificial metalloproteins, are attractive tools to achieve the spatial separation of chemoenzymatic reaction steps and to facilitate metal removal post-reaction. In BHCs, the transition metal catalyst is embedded in a protein scaffold that lacks an intrinsic catalytic activity.[18–25] In this way, organometallic catalysts can be immersed in an aqueous reaction medium and the reaction scope of biocatalysis can be enhanced by new-to-nature reactions. So far, the one-pot combination of a BHC and an enzyme was demonstrated only for the purpose of regenerating cofactors such as NAD(P)H either by the enzyme or the BHC.[26–28] The potential of BHC/enzyme combinations in sequential cascade reactions beyond the regeneration of cofactors has not been explored.

Herein, we report a one-pot cascade reaction to produce stilbene derivatives **4a–b** from cinnamic acids in aqueous solution by combining an enzyme and a BHC (Scheme 1). In our approach, the decarboxylation of cinnamic acids **1a–b** to styrene derivatives **5a–b** was catalyzed by ferulic acid decarboxylase (FDC1) from *Saccharomyces cerevisiae* (*S. cerevisiae*). The styrene intermediates were further converted by an olefin metathesis BHC that is composed of an engineered variant of the outer membrane protein Ferric hydroxamate uptake protein component: A (FhuA) of *Escherichia coli* (*E. coli*), equipped with a covalently anchored Grubbs–Hoveyda type catalyst (Fig. 1).[29–31] The resulting BHC is stable in aqueous solution for more than a week (confirmed by titration with ThioGlo) without detectable protein precipitation or any indication for a detachment of the metal catalyst.

Decarboxylation is an important and ubiquitous metabolic reaction in both prokaryotes and eukaryotes. Several decarboxylases have been characterized.[32] *S. cerevisiae* harbors a ferulic acid decarboxylase (FDC1) that requires a prenylated flavin mononucleotide (FMN) cofactor for catalytic activity. *In vivo* synthesis of the cofactor is accomplished by phenolic acid decarboxylase (PAD1).[33] In this study, the genes encoding FDC1 and tPAD1 were codon-optimized and co-expressed in *E. coli* to debottleneck the assembly of the functional recombinant enzyme. tPAD1 is a truncated version of PAD1 lacking a mitochondrial targeting sequence, which is redundant for expression in *E. coli*.[34] FDC1 converts cinnamic acids to styrenes at a pH range of 6–8[34–36]and at temperatures up to 45 °C.[35] This reaction requires no additional cofactors such as NAD(P)H, which obviates the need for cofactor regeneration systems. FDC1 converts a wide range of substrates carrying different substitutions at the substrate's aromatic moiety.[33] Taken together, these features make FDC1 an intriguing candidate for implementation in cascade reactions together with bioorthogonal olefin metathesis BHCs.

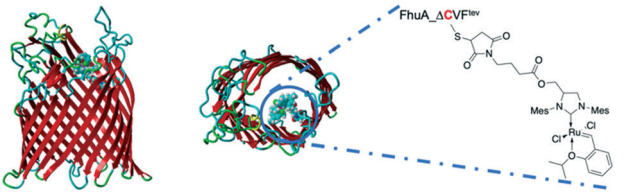
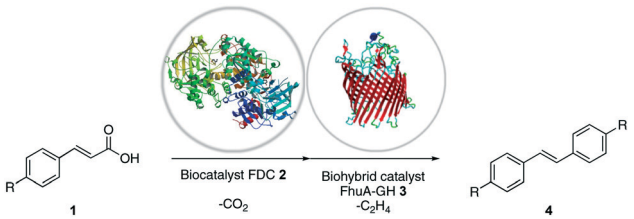


Fig. 1 Biohybrid catalyst FhuA-GH **3** consisting of the transmembrane protein FhuA and the immobilized Grubbs–Hoveyda type catalyst (right).

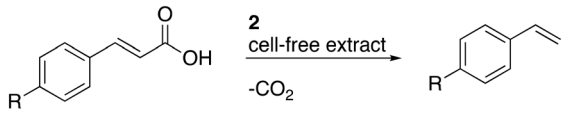
For olefin metathesis, we employed a previously introduced BHC consisting of an engineered variant of FhuA and a Grubbs–Hoveyda type catalyst.[29–31] FhuA is an outer-membrane β-barrel protein stable at temperatures up to 64 °C.[37] Moreover, the protein retains its structural integrity in the presence of organic co-solvents like THF (up to 40 (v/v)%).[38] The use of organic co-solvents enables the use of substrates exhibiting low water solubility. To solubilize the membrane protein, sodium dodecyl sulfate (SDS) was used as previously described.[29,30] First, biocatalyst FDC1 (2) was probed in the decarboxylation of substrates **1a** and **1b** (Table 1). At low pH values (pH < 6) that are beneficial for the subsequent olefin metathesis step, the substrates **1a** and **1b** are moderately soluble in aqueous buffer solution. Therefore, different co-solvents were tested for substrate solubilization. DMSO inhibited the Grubbs–Hoveyda catalyst and was therefore deemed unsuitable for the cascade.

In the presence of 7.5 (v/v)% THF at 30 °C, **1a** was converted to **5a** at 86% yield (Table 1, entry 1). When the temperature was elevated to 35 °C, the yield dropped to 73% (Table 1, entry 2). A similar behavior was observed for substrate **1b**, resulting in yields of 99% and 58% at 30 °C and 35 °C, respectively (Table 1, entries 3 and 4). Decreasing the THF concentration to 5 (v/v)% afforded an increased conversion of **1a** to **5a** with 91% and 87% at 30 °C and 35 °C, respectively (Table 1, entry 5 and 6). Substrate **1b** was quantitatively converted to **5b** at 30 °C or 35 °C (Table 1, entry 7 and 8), suggesting a preferred acceptance of this substrate by FDC1 under the investigated conditions. It is surmised that



Scheme 1 Chemoenzymatic cascade reaction starting from cinnamic acid derivatives **1** that are decarboxylated by biocatalyst **2** to yield styrene derivatives which are transformed into stilbene derivatives **4** by the biohybrid catalyst FhuA-GH **3**.

Table 1 Decarboxylation of cinnamic acid derivatives **1** by cell-free extracts containing the recombinant biocatalyst **2** (FDC1)



R = H - **1a**
R = Cl - **1b**

R = H - **5a**
R = Cl - **5b**

Entry	Substrate	THF [(v/v)%]	T [°C]	[%] **1**	[%] **5**
1	**1a**	7.5	30	13	86
2	**1a**	7.5	35	26	73
3	**1b**	7.5	30	<1	>99
4	**1b**	7.5	35	41	58
5	**1a**	5	30	8	91
6	**1a**	5	35	12	87
7	**1b**	5	30	<1	>99
8	**1b**	5	35	<1	>99

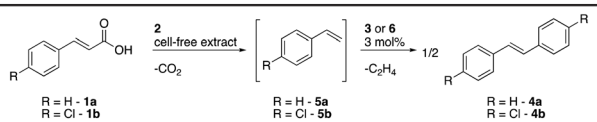
Conversion were determined by GC-FID. The conversions of 12 mM **1a–b** were performed with 15 μM FDC1 (cell-free extract) in sodium phosphate buffer (100 mM, 137 mM NaCl, pH 6.0). The reactions were run for 1 h. Δconv. = ±2%.

the chloro-substituent at the styrene mimics the natural substrate of the FDC1.[36] In summary, 5 (v/v)% THF was determined as the most suitable co-solvent concentration tolerable for FDC1 in the cascade reaction (Table 1, entries 5–8). Next, we aimed to determine conditions under which both catalysts would be active. Increasing the temperature slightly lowered the conversion efficiency of FDC1 (Table 1, entries 2, 4, 6 and 8). In turn, slightly acidic conditions (pH 6) that are necessary for the subsequent olefin metathesis step still afforded reasonable conversions despite the enzyme's pH-optimum being at pH 7–8.[39]

With suitable conditions for the first conversion step in hand, the two catalysts to produce stilbene derivatives from cinnamic acid derivatives were combined. As expected, in the absence of the catalysts, no conversion of substrates **1a** or **1b** was detected (Table 2, entry 1). Likewise, the corresponding stilbene derivative was not formed when only the biocatalyst FDC1 was present.

BHC **3** and AquaMet[40] **6** (a commercially available and water-soluble Grubbs–Hoveyda type catalyst; for chemical structure see ESI† Fig. S4) were probed in the conversion of the cinnamic acid derivatives to obtain stilbene derivatives. Expectedly, reactions with both catalysts yielded only trace amounts (<1%) of the respective stilbene derivatives as determined by GC-MS (Table 2, entries 2 and 3). Upon combining biocatalyst **2** with either BHC **3** or AquaMet **6** directly at reaction start ('concurrent mode'), less than 5% of products **5a** or **5b** were formed (Table 2, entries 4 and 5). The low conversion efficiency indicates a deactivation of the decarboxylase **2** by the catalyst AquaMet **6** (Table 2, entry 4), or by the SDS detergent used for solubilization of BHC **3** (Table 2, entry 5).[41] Therefore, we employed an alternative approach, in which catalyst **3** or **6** were added subsequently after completion of the decarboxylation reaction ('sequential mode'). Again, the combination of biocatalyst **2** and AquaMet **6** yielded only traces of the corresponding stilbene derivatives

Table 2 Two-step one-pot reaction for stilbene production



R = H - **1a**
R = Cl - **1b**

R = H - **5a**
R = Cl - **5b**

R = H - **4a**
R = Cl - **4b**

Entry	Substrate	Catalyst	**1** [%]	**5** [%]	**4** [%]
1	**1a/b**	—	>99	—	—
2	**1a**	**3**	>98	—	<1
3	**1a**	**6**	>98	—	<1
4[a]	**1a**	**2, 6**	>95	<5	—
5[a]	**1a**	**2, 3**	>95	<5	—
6[b]	**1a**	**2, 6**	<1	>98	<1
7[b]	**1b**	**2, 6**	<1	>98	<1
8[b]	**1a**	**2, 3**	<1	25	74
9[b]	**1b**	**2, 3**	<1	35	64

Conversions were determined by GC-MS. The conversions of 12 mM **1a–b** were performed with 15 μM FDC1 (cell-free extract) in sodium phosphate buffer (100 mM, 137 mM NaCl, 5 (v/v)% THF, pH 6.0) at 35 °C. Addition of 3 mol% biohybrid catalyst **3** or AquaMet **6** in sodium phosphate buffer (100 mM, pH 6.0, final 0.04 (w/v)% SDS, 50 mM NaCl and 2 (v/v)% THF) initiated the subsequent olefin metathesis step. [a] Directly (concurrent mode, 6 h reaction time). [b] After 1 h (sequential mode) and further conversion for additional 4 h at 35 °C. Δ1/5/4 = ±3%.

from substrates **1a**/**b** *via* **5a**/**b** (Table 2, entries 6 and 7). This finding is in agreement with previous reports showing that 'unprotected' metal catalysts are prone to inactivation by cell lysate contents like glutathione.[7] Finally, when biocatalyst **2** and BHC **3** were combined in a sequential cascade reaction, 74% of the product **4a** and 64% of **4b** were detected (Table 2, entry 8 and 9). This suggests that by embedding the metal catalyst into the protein scaffold provided by FhuA, the metal site is shielded from inhibition.

Furthermore, unspecific coordination of the protein-free metal catalyst to proteins present in the reaction mixture appears to be circumvented by immobilizing (and embedding) the GH-type catalyst in the protein scaffold. Compared to previously reported cascade reactions combining decarboxylation and olefin metathesis, the cascade reaction reported here does not require the use of PVA/PEG beads in MTBE.[12] Through immobilization of the metal catalyst into a protein scaffold, the cascade reaction is enabled in aqueous solution.

An important challenge surrounding Ru-based catalysts (*e.g.*, for olefin metathesis) is the removal of metal contaminations upon product workup. This is particularly important in the case of pharmaceutical compounds, for which the transition metal content should be typically below 10 ppm.[17] A possible solution to this challenge could be offered by adding a Ru scavenger after the reaction and performing column chromatography, which has been successfully applied, previously.[42] It is already known from the work by Hoveyda *et al.* that metal leaching from Grubbs–Hoveyda type catalysts is relatively low.[43] In the present work, we hypothesized that BHC **3** (bearing the metal catalyst by covalent attachment) could be readily separated from the product fraction by performing a simple extraction step. Indeed, ICP analysis of the product fractions gave Ru contents as low as 1 ppm, whereas in the product fraction of the same reaction setup with the water-soluble catalyst AquaMet **6**, 36 ppm Ru were detected (see ESI† for details). This highlights the feasibility of BHCs for facile catalyst removal.

In conclusion, the combination of biocatalysis employing cell-free extracts – a cheap and easily obtainable source of the recombinant biocatalyst – as well as organometallic catalysts embedded in proteins provides a versatile tool to produce valuable compounds from renewable resources. Stilbene derivatives were produced in a one-pot cascade reaction starting from cinnamic acid derivatives. Biocatalyst FDC1 (**2**) in combination with a BHC for olefin metathesis (**3**) performed the stepwise conversion of cinnamic acid derivatives (**1**) to stilbenes (**4**) under mild reaction conditions in aqueous solution. Intermediate workup steps to isolate the styrene derivatives were not necessary, since both reactions proceeded in the same aqueous medium. Moreover, we have shown that by using the BHC instead of 'free' metal catalysts for olefin metathesis, changing the reaction conditions can be circumvented, since the olefin metathesis step can be performed in aqueous solution. Furthermore, metal contamination in the product fraction was remarkably low after performing one simple extraction step with dichloromethane. This shows that by immobilizing the catalyst in a protein cavity, we can not only combine aqueous biocatalysis with organic synthesis, but also facilitate sample workup and product isolation.

Conflicts of interest

There are no conflicts to declare.

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