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Selective Enzymatic Synthesis of the Grapefruit Flavor (+)-Nootkatone

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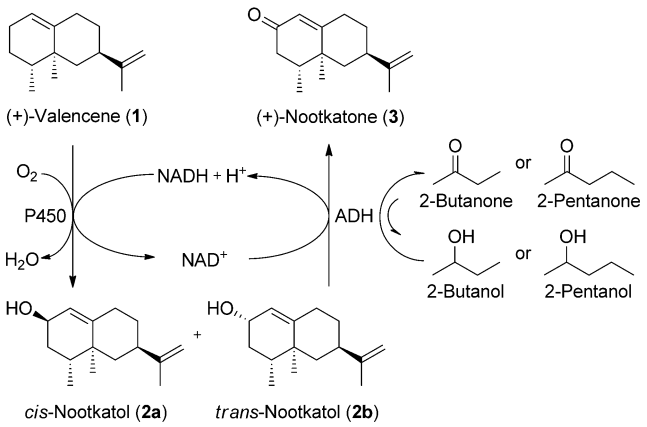
(+)-Nootkatone is a high-value sesquiterpenoid known for its grapefruit-odor impression. Its isolation from natural plant sources suffers from low yields, and chemical syntheses involve carcinogenic or hazardous compounds. Herein, a biocatalytic route for the synthesis of (+)-nootkatone that combines two enzymes in one pot is presented. In the first step, a cytochrome P450 monooxygenase catalyzes the selective allylic hydroxylation of the sesquiterpene (+)-valencene to the intermediate alcohol nootkatol. In the second step, nootkatol is further oxidized to (+)-nootkatone by an alcohol dehydrogenase (ADH). The challenging task of finding a suitable cofactor regeneration system was solved by careful selection of an appropriate cosubstrate for the ADH, which works in a dual-functional mode. After reaction optimization, involving cosolvent and cosubstrate screening, (+)-nootkatone concentrations of up to 360 mg L^{-1} and a space-time yield of 18 mg L^{-1} h^{-1} were achieved.

Selective (enzymatic) oxyfunctionalizations of readily available terpene molecules have attracted attention of chemists and biotechnologists because the resulting products are often sought-after compounds with high market values.[1] One example is the sesquiterpenoid (+)-nootkatone, a high-price constituent of grapefruit with applications in the flavor, fragrance, and pharmaceutical industries. Isolation of (+)-nootkatone from natural sources suffers from low yields.[2] Its chemical synthesis by means of (+)-valencene oxidation involves carcinogenic *tert*-butyl chromate and sodium dichromate, or hazardous compounds, such as *tert*-butyl peracetate and *tert*-butyl hydroperoxide.[2–3] Hence, biotechnological routes for (+)-nootkatone synthesis have become important.[2] Cytochrome P450 monooxygenases (EC 1.14.–.–; P450) represent an attractive enzymatic alternative for the allylic oxidation of (+)-valencene. P450s catalyze the direct insertion of one atom of molecular oxygen into (non-)activated C–H bonds upon formation of water as byproduct.[4] The existing biocatalytic approaches for (+)-valencene oxidation based on P450s are hampered by incomplete conversion of nootkatol to (+)-nootkatone.[5] The odor threshold of (+)-nootkatone is approximately 50 times lower (≈1 μg L^{-1}) compared to that of nootkatol,[2,6] therefore, complete conversion to (+)-nootkatone is desired.

We have developed a two-enzyme reaction sequence for the synthesis of (+)-nootkatone, operating in a one-pot mode in aqueous solution. The reaction includes P450-catalyzed regioselective allylic C2-hydroxylation of (+)-valencene (**1**) to yield the intermediate alcohols *cis*- (**2a**) and *trans*-nootkatol (**2b**), which are both further oxidized to (+)-nootkatone (**3**) by an unselective alcohol dehydrogenase (ADH) (Scheme 1). The



Scheme 1. Cosubstrate-supported two-enzyme cascade for selective allylic oxidation of (+)-valencene (**1**). P450 catalyzes the hydroxylation step to *cis*-(**2a**) and *trans*-nootkatol (**2b**). An unselective ADH oxidizes both nootkatol (**2**) isomers to (+)-nootkatone (**3**) and simultaneously converts cosubstrates to regenerate the cofactor NADH.

same ADH converts an appropriate cosubstrate to ensure effective regeneration of the cofactor NADH.

Two previously developed P450 BM3 (CYP102A1) mutants, F87A/A328I (BM3-AI) and F87V/A328V (BM3-VV),[5d] were applied for the initial hydroxylation of **1**. BM3-AI produced a mixture of **2a** and **3** (and minor amounts of **2b**), whereas BM3-VV generated almost exclusively **2b** and minor amounts of **3** (Supporting Information, section 2.7), as described previously.[5d] Thus, in contrast to the common demand for stereoselective ADHs, we were interested in an enzyme with no pronounced stereoselectivity for either of the two isomers, **2a** and **2b**.

To find a nootkatol-oxidizing ADH, we screened the proprietary enzyme collection at c-LEcta (c-LEcta GmbH, Leipzig, Ger-

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many), which included wild-type enzymes from biodiversity screenings and well-studied ADHs,[7] as well as mutants thereof. Remarkably, only two active evolved ADH variants could be identified for the desired reaction, whereas none of the screened wild-type ADHs showed detectable activity. Based on conversion experiments and kinetic data with **2** (Supporting Information, section 2.1), ADH-21 was chosen for the P450–ADH cascade.

ADH-21 showed a clear preference for the cofactor NAD^+; activity with $NADP^+$ was $<1\%$ of the activity with NAD^+. Although P450 BM3 prefers NADPH, it has been reported to also accept NADH.[8] Similar levels of conversion of **1** with P450 BM3 mutants, supported by a glucose dehydrogenase (GDH) for cofactor regeneration, could be achieved regardless of whether NADPH or NADH was applied (Supporting Information, section 2.5). Based on this finding we combined the P450 BM3 mutants and ADH-21 in one pot with NADH as the cofactor. Additionally, we intended to further optimize BM3-AI for NADH acceptance by introduction of two previously described amino acid substitutions in the reductase domain (R966D/W1046S).[8b] However, the resulting mutant was approximately 50% less productive than BM3-AI when using NADH (Supporting Information, section 2.3).

Initial attempts to develop the two-enzyme cascade led, as expected, to the formation of **3**, however, the reaction stopped after 30 min. Uncoupling reactions in which NAD(P)H is consumed by the P450, but the target substrate is not oxidized, often occur in reactions with non-natural substrates.[9] This was also observed for the oxidation of **1** by BM3-AI (coupling efficiency of 33%). We concluded that the cascade reaction stopped as the NADH was depleted. Indeed, when doses of NADH were added during the reaction course, conversion of **1** continued (Supporting Information, section 2.2).

The addition of stoichiometric amounts of costly NADH is not economically feasible. Addition of GDH for cofactor regeneration along with BM3-AI and ADH-21 resulted in incomplete conversion of **2** to **3** (data not shown), which can be explained by the reduced availability of NAD^+ for ADH-21 owing to GDH. To address this issue we screened for an ADH-21 cosubstrate to effectively regenerate NADH while ensuring simultaneous complete oxidation of **2** to **3**. Consequently, ADH-21 should serve as a dual-functional enzyme, which performs both the oxidation of **2** and cofactor regeneration by cosubstrate conversion (Scheme 1). Several alcohols were identified as potential cosubstrates for ADH-21, which displayed an apparent preference for secondary alcohols (Supporting Information, section 2.4). All measured volumetric activities were lower compared with that of **2**; an important factor to achieve complete conversion of **2**. 2-Butanol and 2-pentanol, for which ADH-21 displayed 9 and 42% activity relative to the activity for **2**, respectively, were chosen to establish a cosubstrate-supported P450–ADH cascade on an analytical scale. Indeed, addition of either of the cosubstrates to the reaction resulted in double the concentration of C2-oxidized products (sum of **2** and **3**) in comparison with the control reaction without cosubstrate (Figure 1A and Supporting Information, section 2.5). In reactions with either BM3-AI or BM3-VV intermediate alcohol **2** was completely oxidized to **3** in the 2-butanol system, and was detectable only in very low amounts in the 2-pentanol system (Supporting Information, section 2.7).

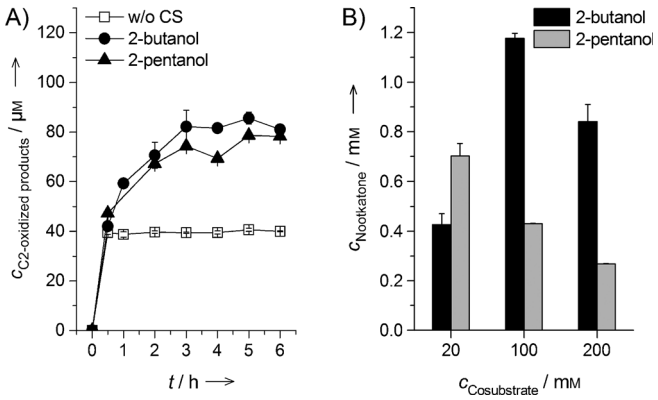

Figure 1. Cosubstrate-supported BM3-AI–ADH-21 cascade for (+)-nootkatone synthesis. A) Formation of C2-oxidized products (sum of **2** and **3**) versus time. Conditions: 500 μL, 25 °C, Tris-HCl buffer (50 mM, pH 7.5 with 2 mM $MgCl_2$), DMSO (2% v/v), substrate **1** (200 μM), cosubstrate (20 mM), NADH (400 μM), BM3-AI (1 μM), ADH-21 (100 mU mL^{-1}), catalase (1200 U mL^{-1}). Reaction without cosubstrate (w/o CS) served as control. B) Influence of cosubstrate concentration on formation of **3**. Conditions: 1 mL, 25 °C, 20 h, Tris-HCl buffer (50 mM, pH 7.5 with 2 mM $MgCl_2$), DMSO (2% v/v), substrate **1** (10 mM), cosubstrate (as indicated), NADH (400 μM), BM3-AI (5 μM), ADH-21 (500 mU mL^{-1}), catalase (600 U mL^{-1}). Where error bars are not recognizable, they are smaller than symbols or bar lines.

As the C2 selectivity of BM3-AI (up to 97%) was higher than that of BM3-VV (up to 86%) in the systems with cosubstrates (Supporting Information, section 2.7), BM3-AI was selected for reaction scale-up and optimization experiments at higher substrate concentrations (up to second phase formation at 10 mM **1**).

Monitoring of the NADH concentration during the reactions revealed that the presence of a cosubstrate clearly induced cofactor regeneration compared to the reactions without cosubstrate (Supporting Information, section 2.6). As expected from the determined activities of ADH-21, the cofactor regeneration was higher with 2-pentanol than with 2-butanol.

Unexpectedly, during optimization of the individual cosubstrate concentrations the highest concentration of **3** amounting to 1.2 mM was achieved with 100 mM 2-butanol, whereas at an optimized 2-pentanol concentration (20 mM) only 0.7 mM of **3** was produced (Figure 1B). Further increase of 2-butanol or 2-pentanol concentrations led to reduced amounts of **3**. Although 2-pentanol initially seemed to be the better cosubstrate for ADH-21 (Supporting Information, sections 2.4 and 2.6), conversions with 2-butanol yielded higher concentrations of **3** (Figure 1B). This could be explained by a negative effect of 2-pentanol on either protein stability or activity, demonstrating the importance of careful cosubstrate choice in the development of P450–ADH cascades.

The developed P450–ADH cascade with 2-butanol as cosubstrate was scaled up linearly (Table 1). Similarly to the results from using a reaction volume of 1 mL, 1.0 mM (221 mg L^{-1}) of **3** was obtained in 20 mL (Table 1, entry 3). Strikingly, in any of the reactions, **2a** and **2b** were detected in traces only, again

Table 1. (+)-Nootkatone titers achieved with the BM3-AI–ADH-21 enzyme cascade under optimized reaction conditions.

Entry	Scale[a] [mL]	Cosolvent/additive	(+)-Nootkatone (**3**) [mM]	[mg L^{-1}]	Nootkatol (**2**)[b] [%]
1[c]	control	DMSO (2%)	0.22	48	28
2[d]	1	DMSO (2%)	1.03	225	<2
3	20	DMSO (2%)	1.01	221	<1
4[e]	1	methyl-β-CD (4%)	1.54[f]	336	17

[a] Conditions: 25 °C, 20–21 h, Tris-HCl buffer (50 mM, pH 7.5 with 2 mM $MgCl_2$), DMSO (2% v/v) or methyl-β-CD (4% w/v), substrate **1** (10 mM), 2-butanol (100 mM), NADH (400 μM), BM3-AI (5 μM), ADH-21 (500 mU mL^{-1}), catalase (600 U mL^{-1}). [b] Percentage of **2** of formed C2-oxidized products (estimated from GC peak areas). [c] Control (1 mL) without ADH-21. [d] Reactions with either NADH or NAD^+ showed similar results. [e] NAD^+ was applied as cofactor. [f] Value represents the average of three independent experiments. The maximal value observed in a single experiment was 1.65 mM (360 mg L^{-1}). Errors are <10%.

demonstrating a very efficient applicability of ADH-21 for the oxidation of both nootkatol (**2**) isomers.

To increase the solubility of **1**, a cyclodextrin (CD) was added instead of DMSO. CDs enhance the solubility of hydrophobic compounds in aqueous solution and have been applied for biocatalytic conversions of steroids,[10] lipophilic ketones,[11] fatty acids,[12] and others. Indeed, in the presence of methyl-β-CD, the concentration of **3** could be increased up to 1.65 mM (360 mg L^{-1}; Table 1, entry 4). It should be noted, however, that reactions containing methyl-β-CD displayed a residual 17% of the intermediate **2**, which is presumably because of complexation of **2** by CD.

The space-time yields of 17–18 mg L^{-1} h^{-1} achieved with the optimized cascade were higher than those previously reported for oxygenase systems (13 mg L^{-1} h^{-1}),[13] whereas the achieved (+)-nootkatone concentrations were comparable (317–320 mg L^{-1} for *Pleurotus sapidus* cells or recombinant *Pichia pastoris* cells in 24 h).[13] The presented cascade offers the clear advantage of rapid production of the biocatalysts in *E. coli* (1–2 days), and their direct application as cleared cell lysates without further purification. Future work will be focused on optimization of P450 BM3 by protein engineering with the goal to obtain higher product concentrations in shorter reaction times. In this regard, preliminary data indicate that the coupling efficiency seems to be a more important factor than high enzyme activity alone for the successful establishment of P450–ADH cascades.

The described dual-functional mode of an ADH that catalyzes the second oxidation step and simultaneously utilizes a cosubstrate for cofactor regeneration represents a valuable concept that generally could extend the applicability of P450–ADH cascades.[14] Thereby, the careful choice of a "low activity" cosubstrate was demonstrated to be key for the successful development of such a system.

Experimental Section

Chemicals were purchased from Sigma–Aldrich, AppliChem, GERBU, and VWR (all Germany) with a purity of ≥98%, except for **1**, which was obtained in technical grade (>70%) from Sigma–Aldrich. Catalase (from bovine liver) was from Sigma–Aldrich. Lyophilized enzyme preparations of ADH-21 are commercially available from c-LEcta GmbH (Germany). Enzyme production in *E. coli* BL21 (DE3), measurements of enzyme concentrations and activity assays are described in the Supporting Information. All enzymes were applied as cleared cell lysates without any further purification steps (except for coupling measurements). Chemical synthesis and analysis of **2** was performed as described previously.[5c] Reaction conditions are specified in Figure and Table captions. Analytical procedures are described in the Supporting Information. Data are given as means with errors representing absolute deviations ($n \geq 2$).

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