A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

www.angewandte.org

Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201900926
Angew. Chem. 10.1002/ange.201900926

Link to VoR: http://dx.doi.org/10.1002/anie.201900926
http://dx.doi.org/10.1002/ange.201900926

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10.1002/anie.201900926

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Chemoenzymatic Total Synthesis of deoxy-, *epi*- and Podophyllotoxin and a Biocatalytic Kinetic Resolution of Dibenzylbutyrolactones

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This study is dedicated to Prof. Kurt Faber on the occasion of his retirement.

Abstract: Podophyllotoxin (**1**) is probably the most prominent representative of lignan natural products. Deoxy-, *epi*- and podophyllotoxin – all precursors to frequently used chemotherapeutic agents – were prepared via a stereodivergent biotransformation and a biocatalytic kinetic resolution of the corresponding dibenzylbutyrolactones by the same 2-oxoglutarate dependent dioxygenase. The reaction can be upscaled to two grams and the enzyme allows tailoring of the initial "natural" structure transforming various non-natural derivatives. Depending on the substitution, the enzyme performs an oxidative C-C bond formation via C-H activation or hydroxylation at the benzylic position prone to the ring closure.

The selective activation of inert C-H bonds represents one of the most remarkable reactions in modern organic chemistry.[1] At the same time it can be considered as one of the biggest challenges as well. Organometallic chemistry has provided an impressive assembly of examples requiring in most cases adjacent directing groups to achieve selectivity.[1] In contrast to these endeavours, nature provides selectivity for C-H activation by the enzyme's active site, which aligns the substrate(s) properly towards the active center. The most prominent representatives are cytochrome P450 monooxygenases (CYPs), which activate the C-H bond via an iron-heme center at the expense of oxygen.[2] While significant efforts have been dedicated to CYPs,[3] 2-oxoglutarate dependent dioxygenases (2-ODDs) have gained rather moderate attention thus far.[4] Predominantly, the hydroxylation of aminoacids has been studied in a biocatalytic context (e.g. hydroxylation of proline[5] and leucine/isoleucine[6]). Nevertheless, recent reports demonstrate the high biocatalytic potenital of these proteins (even on gram scale).[7] Besides these studies, the portfolio of reactivity is far beyond "simple" hydroxylation reactions: Many natural products' biosyntheses rely on 2-ODDs and reactions such as selective oxidation of sugar moieties,[8] endo-peroxide formation,[9] ring expansion[9] and contraction,[10] selective halogenations[11] and multi-step oxidations[12] have been attributed to 2-ODDs. Recently, the reactivity has been extended to the ring closure of the C-ring in the podophyllotoxin (**1**) biosynthesis by *Podophyllum hexandrum*[13] and *Sinopodophyllum hexandrum*.[14] The 2-ODD enzyme – further referred to as 2-ODD-PH – catalyzes the cyclization of yatein (**2a**) and yields deoxypodophyllotoxin (**12a**) at the expense of 2-oxoglutaric acid and oxygen (see Figure 1).

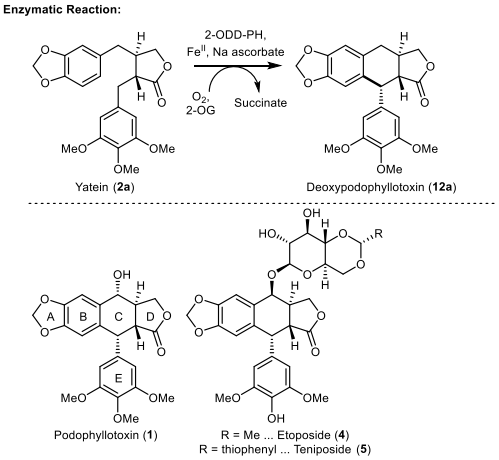

Figure 1. Reaction of 2-ODD-PH in nature and structures of pharmaceutical compounds.

Podophyllotoxin (**1**) is the precursor of etoposide (**4**) and teniposide (**5**), two chemotherapeutic agents used in several clinical therapies. Both compounds inhibit the topoisomerase II, a key enzyme in cell mitosis, by forming a ternary complex with the DNA and isomerase enzyme. This prevents religation of the DNA strands and ends up in cell apoptosis. As cancer cells proliferate more rapidly, their cell death occurs preferentially.[15]

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CCDC 1889727 contains the supplementary crystallographic data for product **12h**. These data are provided free of charge by The Cambridge Crystallographic Data Centre.

Supporting information for this article is given via a link at the end of the document.

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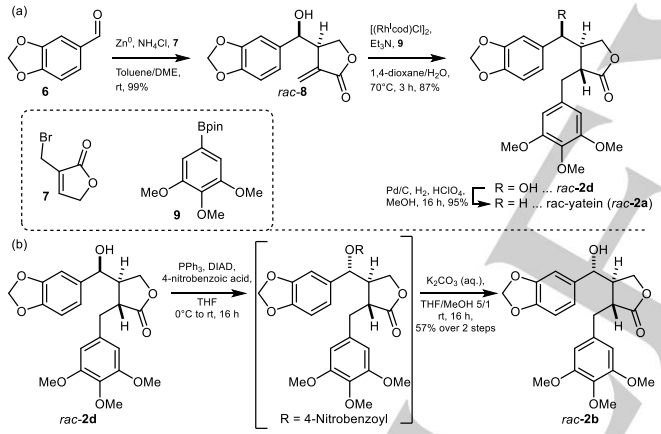
Etoposide (**5**) therefore became a member of the WHO's list of essential medicines.[16] Since other closely related compounds exhibit interesting bioactivities too (e.g. cytotoxic, insecticidal, antifungal, antiviral, anti-inflammatory, neurotoxic, immunosuppressive, antirheumatic, antispasmogenic and hypolipidemic properties),[17] the structural motif has been the subject of several synthetic research endeavours. Nevertheless, the asymmetric construction of the yatein precursor (**2a**) and the stereoselective ring closure of ring C still represent major bottlenecks in the synthesis. Especially the construction of the southern stereocenter with the appropriate configuration has been remarkably difficult via the Friedel-Crafts approach, both chemically[18] and via biotechnologic methods.[19] Some of these challenges have been recently resolved, but require expensive reagents or catalysts.[20] Therefore, a chemoenzymatic, scalable route to the target compound podophyllotoxin (**1**) and the biocatalyst's substrate scope demonstrate an extremely tempting scientific target.

Aiming for an efficient synthesis by integrating biocatalysis[21] en route towards podophyllotoxin, the racemic yatein precursor (*rac*-**2a**) was prepared following a slightly modified literature protocol (see Scheme 1a).[22] A simple switch to less polar solvents for the allylation step gave better diastereomeric ratios in our hands compared to literature and the target compound was obtained in good yields (82% overall yield).



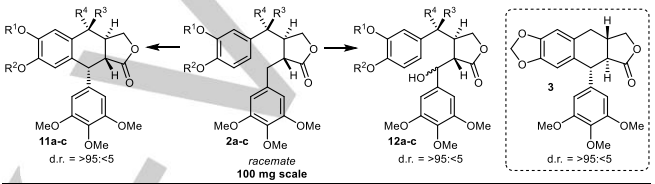
Scheme 1. (a) Preparation of *rac*-yatein (*rac*-**2a**); (b) Preparation of substrate *rac*-**2b**: installation of the "natural" stereoconfiguration. Bold and dashed lines refer to relative stereochemistry.

For getting access to a sufficient amount of the enzyme 2-ODD-PH, the gene was expressed in *E. coli* BL21(DE3) using a pET21(a)+ vector system. Since first ca. 80% of the catalyst remained insoluble and inactive, the expression was improved by late induction (at OD_{600} = 1.2 - 1.4), yielding about fifty percent of the enzyme in the soluble fraction at a high expression level (33 mg of soluble enzyme per 1.0 g of cells).

When performing the reaction with 2-ODD-PH (see supporting information for further details) substrate *rac*-**2a** was recovered with an ee of 10% (26% recovered material; see Table 1, entry 1), while two enantiopure products were observed. We were able to identify these two products as the diastereoisomers deoxypodophyllotoxin (**12a,** 19% isolated yield) and isodeoxypodophyllotoxin (**3**, 20% isolated yield). Thus, the biocatalyst is non-stereoselective concerning the two chiral centers present in the substrate **2a** and forms the new C-C bond within both substrate enantiomers. However, the new chiral center formed during C-C bond formation possesses the same configuration for both substrate enantiomers leading to an enantiodivergent reaction (two diastereomers are formed). Consequentially, the enzyme overrides the substrate controlled stereopreference for the ring formation.[18a] The diastereomeric mixture complicated the purification and pure products were finally obtained via preparative HPLC only.

These results encouraged us to challenge the biocatalyst by a substrate congener with an additional chiral center like the hydroxyl group present in podophyllotoxin's northern benzylic position (see Figure 1). In order to obtain the "natural" relative configuration of the OH group as present in podophyllotoxin (**1**), we performed a Mitsunobu inversion on intermediate *rac*-**2d**, and cleaved off the ester moiety in a two-step protocol yielding substrate *rac*-**2b** (see Scheme 1b).

Table 1. Biotransformations with 2-ODD-PH.



Entry	Substrate #	R^1 = R^2 =	R^3 = R^4 =	HPLC area [%][a] **11 a-c**	HPLC area [%][a] **12 a-c**	Yield [%][b] (ee) **11 a-c**	Yield [%][b] (ee) **12 a-c**	ee of **2a-c** [%][c]
1	*rac*-**2a**	–CH_2–	H H	27[d]	-	19[e]	-	10
2[f]	*rac*-**2b**	–CH_2–	H OH	-	29	-	15	46
3	*rac*-**2c**	Me Me	OH H	12	5	7	4	26

Reaction conditions: Cell-free extract (CFE, 44 vol-%), 20 mM substrate, 2-oxoglutarate (1.75 eq.), sodium ascorbate (3 eq.), 23% DMSO as cosolvent; [a] determined via peak area integration of the HPLC-UV chromatogram (at 215 nm); [b] Isolated yields of chromatographically pure and fully characterized products are reported; [c] ee was determined via HPLC-UV on a chiral stationary phase (for details see supporting information); [d] plus 37% of diastereoisomer **3**; [e] 20% of compound **3** were isolated from the same batch; $[\alpha]_D^{20}$ values for **11a** and **3** are in full consistency with literature values;[24] [f] experiment was conducted on 50 mg substrate scale. Bold and dashed lines refer to relative stereochemistry, bold and dashed wedges refer to absolute stereochemistry.

When transforming substrate *rac*-**2b** the biocatalyst turned out to be enantioselective leading to a kinetic resolution (see Table 1, entry 2). Only one enantiomer was transformed, but instead of the expected ring-closed aryltetralin structure, product **12b** was obtained with the benzylic position – formerly prone to cyclization – hydroxylated. Probably substrate **2b** may not be positioned properly in the enzyme preventing the C-C bond formation and favouring hydroxylation instead. Interestingly, substrate **2c** (Table 1, entry 3), which bears two methoxy groups on the western aromatic ring system, led to a mixture of products obtained from ring-closure (**11c**) and hydroxylation (**12c**). These findings are in consistency with a recent mechanistic study, which observes related hydroxylated products.[23] In agreement with this report, a cationic mechanism involving a Friedel Crafts alkylation scenario is the most likely mechanistic pathway of the reaction. Nevertheless, a radical pathway cannot be ruled out completely (for a detailed outline of the catalytic mechanism please consult

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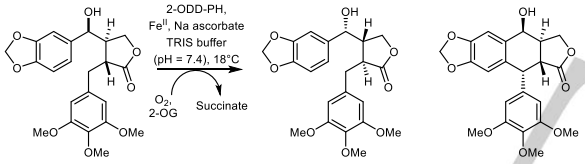
the ESI provided) and structural information of the enzyme is required to provide further arguments for either way.
As substrate *rac*-**2b** was transformed via a kinetic resolution, the hydroxyl function at the northern benzylic position seems to play a fundamental role in recognition. *rac*-**2d** possesses the opposite relative configuration for this hydroxy group and therefore we tested this compound too (see Table 2, top scheme). The results clearly show, that with this stereoconfiguration the aromatic moiety seems to be aligned in the proper way and the ring formation was observed exclusively in a kinetic resolution of substrate *rac*-**2d**. Only the enantiomer possessing the same absolute configuration for the northern hydroxyl group as in etoposide (**4**) was transformed. Consequently, product **11d** bears the same stereoconfiguration as the APIs etoposide (**4**) and teniposide (**5**, see Figure 1) which may facilitate the follow up steps towards these compounds.
Performing preparative reactions, the measured conversion was confirmed by the isolated yield. We were able to obtain 770 mg (39% isolated yield) of the target compound **11d** from 235 mL reaction mixture within 18 h. This corresponds to a space time yield of 200 mg L^{-1} h^{-1} and an overall yield of 32% over the four steps (note that a late stage kinetic resolution was performed, which is limited to 50% yield by theory). The discrepancy in conversion between the small scale and the upscale experiments (see Table 2) results most likely from different surface to volume ratios leading to a lower oxygen input as well as from deactivation of the enzyme over time. Additionally, kinetics imply that the reaction is actually performed within two to four hours only (see Tables SI13a and SI13b in the supporting information; the upscale experiment was performed for 16 h). This sums up to a space time yield of 1.6 – 0.8 g L^{-1} h^{-1} for the analytical scale and we assume that similar numbers can be obtained in upscale experiments.

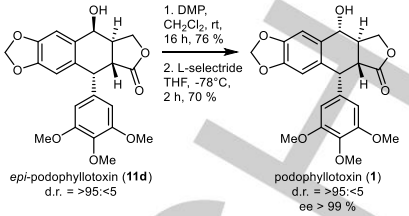
Table 2. Biocatalytic transformation of substrate **2d** by 2-ODD-PH.



Entry	Scale	Conv. [%][a]	**11d** [%]		(+)-**2d** [%]	
			Yield[b]	ee[d]	Yield[b]	ee[c]
1	1 mg	50	-[e]	99	-[e]	99
2	100 mg	43	38	95	50	72
3	2.0 g	41	39	95(>99[f])	45	66

Reaction conditions: CFE (44 vol-%), 20 mM substrate, 2-oxoglutarate (1.75 eq.), sodium ascorbate (3 eq.), 23% DMSO as cosolvent, 18 h; absolute configurations were determined via comparison to literature values;[24] [a] the conversion was determined via calibrated HPLC-UV spectra. [b] Isolated yields of chromatographically pure and fully characterized products are reported; [c] ee was determined via HPLC-UV on a chiral stationary phase (for details see supporting information); [d] ee was determined from conversion and ee of the remaining substrate;[25] [e] no isolated yield was determined; [f] ee determined via HPLC-UV analysis of the follow-up product **1** (see Scheme 2).

Consequently, the actual natural product podophyllotoxin (**1**) was prepared from **11d** according to a literature protocol within two additional steps (17 % overall yield over five steps, see Scheme 2).
Next, we focused on the substrate scope of the 2-ODD-PH enzyme: The results are depicted in Figure 2. Whereas the southern aromatic ring requires the trimethoxy motif for high enzyme activity, the substituents on the western aromatic system can be altered. Nevertheless, at high enzyme concentrations (enzyme:substrate 1:1.2) a phenyl substituent in the southern position was shown to be accepted by the enzyme recently.[23]



Scheme 2. Preparation of podophyllotoxin (**1**) from its *epi*-congener **10b**.

Despite the suitable configuration of the northern hydroxyl function (*vide supra*), almost all substrates tested yield the hydroxylated product type **12e-m** (see Figure 2). Only substrate **2l**, which has the methoxy substituent in *para*-position towards the ring closing site, provided the aryltetraline product **11l** as the major product. It is worth to mention, that it could be shown that the hydroxylated product **12l** does not spontaneously cyclize under these reaction conditions. A schematic overview about the substrate alterations and their consequences can be found in Figure 2 (for list of substrates, which were not accepted, please consult the ESI provided):

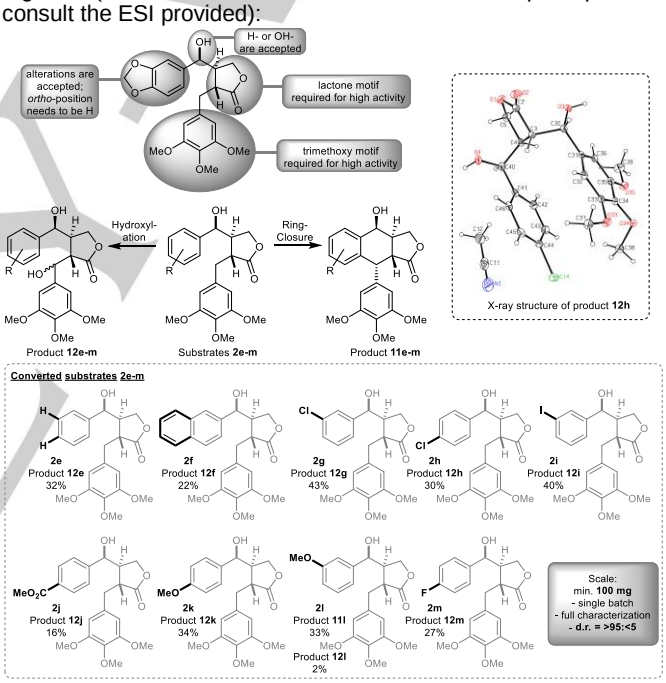


Figure 2. Substrate Scope of the 2-ODD-PH enzyme; schematic overview of the variable positions (top); two product structures (middle); substrate scope - alterations to substrate **2d** are indicated with black bold lines and letters (bottom).

The lactone moiety and the southern aromatic fragment are required for high enzyme activity. The northern hydroxyl function can be removed, but may interfere with the stereorecognition and the product outcome (*vide supra*). Lager substituents – namely acetoxy moieties – gave no conversion (see ESI). The most flexible part of the structural motif is the western aromatic moiety, which accepts several substitution patterns in *meta*- and *para*-position. Substitution in *ortho*-position ceases the catalytic activity below the detection limit. In *meta*- and *para*-position electron withdrawing as well as electron donating substituents are accepted with similar yields of the obtained products. Even bulkier substrates (e.g. naphthyl, substrate **2f**) are accepted, whereas heteroaromatic residues (in detail a furyl substituent) led to no catalytic activity (see ESI).

The crystal structure, which we obtained from product **12h** (isolated from the biotransformation of compound **2h**), delivers information of the accepted enantiomer and the stereoselectivity of the hydroxylation (see Figure 2).
Summarizing, the chemoenzymatic, target oriented synthesis of podophyllotoxin (**1**) and its *epi*-congener **11d** was successfully achieved employing a steroselective biocatalytic C-C bond formation as the key step. This enzymatic transformation lacks analogies in conventional organic chemistry as it overrides the stereopreference of the ring closure and provides a short way to the precursor of etoposide (**4**) and teniposide (**5**). The latter are key compounds in chemotherapies. The substrate screening delivered predominantly products **12e-m**, and thus dibenzylbutyrolactones – another subclass of lignan natural products – in enantiopure form. The biocatalytic C-C bond formation was incorporated into a target oriented synthesis of *epi*-podophyllotoxin (**11d**) and the kinetic resolution was performed on a two gram substrate scale. The study opens a new avenue into the synthesis of key therapeutic agents and homologues using a biocatalytic C-C bond formation and represents one of the rare examples of the target oriented application of this class of enzymes.

Acknowledgements

M. Fuchs and M. Lazzarotto are grateful for an FWF grant (P 31276-B21). L. Hammerer was financed by the Austrian FFG, BMWFJ, BMVIT, SFG, Standortagentur Tirol and ZIT through the Austrian FFG-COMET-Funding Program. Philipp Neu is highly acknowledged for the HRMS measurements and Bernd Werner and Prof. Klaus Zangger are thanked for assistance with the recording of the NMR and IR spectra.

Keywords: 2-Oxoglutarate Dependent Dioxygenase • Podophyllotoxin • Lignans • Biocatalysis • Total Synthesis

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10.1002/anie.201900926

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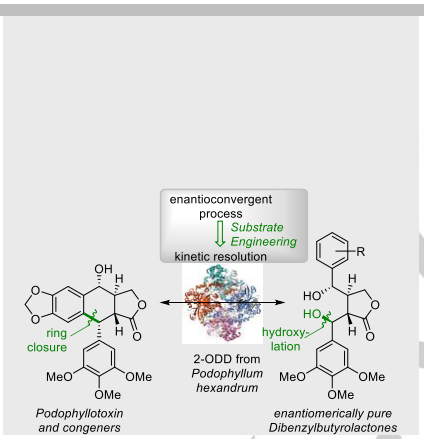
Layout 1:

COMMUNICATION

Taming the iron: Podophyllotoxin and congeners were prepared via a chemoenzymatic total synthesis employing the 2-oxoglutarate dependent iron enzyme from *Podophyllum hexandrum*. Via substrate engineering the reaction outcome could be changed from an enantiodivergent to a kinetic resolution process on a two gram scale. Modifications of the substrate allowed the kinetic resolution of dibenzylbutyrolactones.



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Chemoenzymatic Total Synthesis of *deoxy*-, *epi*- and Podophyllotoxin and a Biocatalytic Kinetic Resolution of Dibenzylbutyrolactones