An investigation of nitrile transforming enzymes in the chemo-enzymatic synthesis of the taxol sidechain

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Complete List of Authors:	Klempier, Norbert; University of Technology, Institute of Organic Chemistry Wilding, Birgit; Institute of Cancer Research, Division of Cancer Therapeutics Vesela, Alicja; Academy of Sciences of the Czech Republic, Institute of Microbiology Perry, Justin; Northumbria University, Department of Applied Sciences Black, Gary; Northumbria University, Department of Applied Sciences Zhang, Meng; Northumbria University, Department of Applied Sciences Martinkova, Ludmila; Academy of Sciences of the Czech Republic,

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Birgit Wilding,[a] Alicja B. Veselá,[b] Justin J. B. Perry,[c] Gary W. Black,[c] Meng Zhang,[c] Ludmila Martínková[b] and Norbert Klempier*[a]

Paclitaxel (taxol) is an antimicrotubule agent widely used in the treatment of cancer. Taxol is prepared in a semisynthetic route by coupling the *N*-benzoyl-(2*R*,3*S*)-3-phenylisoserine sidechain to the baccatin III core structure. Precursors of the taxol sidechain have previously been prepared in chemoenzymatic approaches using acylases, lipases, and reductases, mostly featuring the enantioselective, enzymatic step early in the reaction pathway. Here, nitrile hydrolysing enzymes, namely nitrile hydratases and nitrilases, are investigated for the enzymatic hydrolysis of two different sidechain precursors. Both sidechain precursors, an openchain α-hydroxy-ß-amino nitrile and a cyanodihydrooxazole, are suitable for coupling to baccatin III directly after the enzymatic step. An extensive set of nitrilases and nitrile hydratases was screened towards their activity and selectivity in the hydrolysis of two taxol sidechain precursors and their epimers. A number of nitrilases and nitrile hydratases converted both sidechain precursors and their epimers.

Introduction

Paclitaxel (taxol) is a complex natural compound used in anticancer therapy against a variety of cancers, such as ovarian, gastric, head and neck, non-small lung, prostate and breast cancer.[1,2,3,4] Taxol was first isolated from the bark of the pacific yew *Taxus brevifolia*, following an initiative of the US National Cancer Institute (NCI), screening for antineoplastic activity of new substances from various origins.[1,5]

Taxol acts as microtubule stabilizer, binding to tubulin in polymerized microtubules, disrupting the cell cycle, ultimately leading to cell death.[6,7,8] Despite its known limitations, *e.g.*, poor solubility, toxicities and emerging drug resistance, taxol is still widely used in cancer therapy. New administration forms, formulations and taxane analogues have been designed to overcome selectivity, efficacy, toxicity and drug resistance issues.[9,10,11] Numerous efforts have been made to determine the structure activity relationship of taxol.[12] In recent years, the role of the (*2R,3S*)-*N*-benzoyl-3-phenylisoserine C-13 sidechain had been confirmed as essential for the biological activity of taxol (Figure 1).[13]

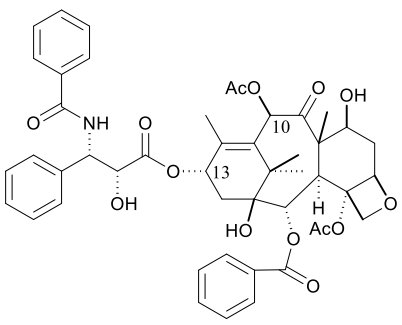


Figure 1 Structure of Paclitaxel (taxol), consisting of the baccatin III core structure and the (2*R*,3*S*)-*N*-benzoyl-3-phenylisoserine C-13 sidechain

[a] *acib GmbH (Austrian Centre of Industrial Biotechnology), c/o Institute of Organic Chemistry, Graz University of Technology, Stremayrgasse 9, A-8010 Graz, Austria. E-mail: klempier@tugraz.at, Tel: +43 316 873 32445, Fax: +43 316 873 32402.*

[b] *Institute of Microbiology of the Academy of Sciences of the Czech Republic, v.v.i., Vídeňská 1083, CZ-14220 Prague, Czech Republic.*

[c] *Industrial Biotechnology Research Group, Department of Applied Sciences, Faculty of Health and Life Sciences, Northumbria University, Newcastle upon Tyne NE1 8ST, United Kingdom.*

Electronic Supplementary Information (ESI) available: NMR spectra of all compounds, experimental procedures for acid and amide reference materials, representative HPLC chromatograms of biotransformation reactions from non-chiral and chiral HPLC measurements, biotransformations with cosolvents, information on commercial enzyme preparations. See DOI: 10.1039/b000000x/

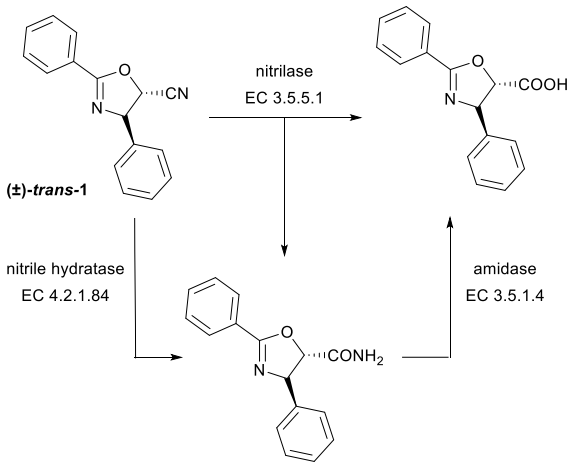
Various sidechain precursors have been prepared in asymmetric chemical syntheses[14] and in chemoenzymatic approaches[15] using acylases,[16] lipases[17] and reductases.[18]

In this work, nitrile transforming enzymes, namely nitrilases and nitrile hydratases, are investigated for the synthesis of the taxol sidechain. Nitrilases (EC 3.5.5.1) and nitrile hydratases (EC 4.2.1.84) are attractive biocatalysts for the fine chemicals and pharmaceutical industries. Nitrilases catalyse the cleavage of nitriles to the corresponding carboxylic acids and ammonia and have been shown to catalyse the hydrolysis of a variety of nitriles, including the enantioselective synthesis of ß-amino acids from ß-amino nitriles.[19] However, several nitrilases were reported to convert nitriles to both, acid and amide products (Scheme 1).[20] Nitrile hydratases are Fe- or Co-metalloenzymes that catalyse the hydration of nitriles to their corresponding amides.[21] To the best of our knowledge, nitrilases have not yet been investigated in the synthesis of the taxol sidechain. Previously, a nitrile hydratase from *Debaryomyces hansenii* DSM 3428 was used in whole cell experiments for the hydration of (±)-*trans*-3-phenyloxirane-2-carbonitrile. The reaction proceeded with low enantioselectivity (*ee*-value <36%), despite stopping the reaction shortly before 50% conversion was achieved.[15b]

Here, two different taxol sidechain precursors were prepared in chemical synthesis, an openchain α-hydroxy-ß-amino nitrile and a cyanodihydrooxazole. A set of 24 nitrilases and four nitrile hydratases was investigated for the biotransformation of these taxol sidechain precursors and their epimers. The enzymatic hydrolysis of these nitrile containing sidechain precursors applies the stereoselective, enzyme-catalysed reaction as the last step in the synthesis.

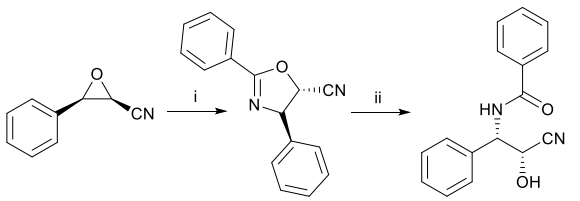
Results and Discussion

Two different taxol sidechain precursors were prepared in chemical synthesis, as depicted in Scheme 2. The corresponding acids and amides were prepared in chemical synthesis as reference materials for the biotransformation reactions.[22] In the first synthetic step, benzaldehyde was transformed to a mixture of (±)-*cis*- and (±)-*trans*-3-phenyloxirane-2-carbonitrile in a Darzens reaction.[23] The epimers were separated by column chromatography and used separately to synthesise the dihydrooxazoles **(±)-*trans*-1** and **(±)-*cis*-1** in a Ritter-type reaction.[24] Ring opening under acidic conditions gave the openchain precursors **(±)-*syn*-2** and **(±)-*anti*-2**.



Scheme 1 Enzymatic conversion of the taxol sidechain precursor **(±)-*trans*-1** to the corresponding carboxylic acid and/or carboxamide. Nitrilase and nitrile hydratase catalysed reactions were investigated for the taxol sidechain precursors **(±)-*trans*-1** and **(±)-*syn*-2** and their epimers **(±)-*cis*-1**, and **(±)-*anti*-2**.

The sidechain precursors **(±)-*trans*-1** and **(±)-*syn*-2** and their epimers were investigated as substrates for a set of different nitrile hydratases and nitrilases (Schemes 1 and 2). Both sidechain precursors, **(±)-*trans*-1** and the cyanohydrin **(±)-*syn*-2** were stable under the reaction conditions of the biotransformation reactions, as confirmed in blank reactions.[25] All biotransformation reactions (nitrilase and nitrile hydratase catalysed reactions) were evaluated in a HPLC-(MS) based screening, using acid and amide references prepared in chemical synthesis.[22] Commercially available, easy to use nitrile hydratases from Prozomix, Ltd. (PRO-E0257 to PRO-E0259)[26] were used. Nitrilases included 18 commercially available nitrilases from two different suppliers, namely Codexis, Inc. (NIT-101 to NIT-114)[27] and Prozomix, Ltd. (PRO-E0260 to PRO-E0264)[26] and six fungal nitrilases overexpressed in *E. coli*.[28]



Scheme 2 Synthesis of the taxol sidechain precursors **(±)-*trans*-1** and **(±)-*syn*-2**. The epimers **(±)-*cis*-1** and **(±)-*anti*-2** were prepared analogously; (i) benzonitrile, BF_3*Et_2O, (ii) 1M aq. HCl, methanol, 60°C. (±)-*cis*- and (±)-*trans*-3-Phenyloxirane-2-carbonitrile were prepared from benzaldehyde as described in the supporting information.

The openchain precursor (±)-*syn*-*N*-(2-cyano-2-hydroxy-1-phenylethyl)benzamide, **(±)-*syn*-2**, and its epimer *(±)-anti*-*N*-(2-cyano-2-hydroxy-1-phenylethyl)benzamide, **(±)-*anti*-2**, were readily hydrolysed by three out of four nitrile hydratases tested, as summarized in Table 1. Conversions for **(±)-*anti*-2** were higher than for **(±)-*syn*-2**. Examination of the amino acid sequences of the nitrile hydratases[26] indicated that Co-type nitrile hydratases (PRO-E0257, PRO-E0258, PRO-E0259), but

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not the Fe-type nitrile hydratase (PRO-E0256) catalysed this reaction.

Table 1 Screening results of **(±)-*syn*-2,** (±)-*syn*-*N*-(2-cyano-2-hydroxy-1-phenylethyl)benzamide, and its epimer **(±)-*anti*-2** with commercially available nitrile hydratases. Reaction time 22h, substrate concentration 0.4mM, 200µL commercial enzyme preparation, total volume 500µL.[26]

nitrile hydratase	conversion of **(±)-*syn*-2** to amide [%]	conversion of **(±)-*anti*-2** to amide [%]
PRO-E0256	0	0
PRO-E0257	5.8	15.8
PRO-E0258	12.5	42.0
PRO-E0259	37.1	53.7

Less than one third of the commercially available nitrilases catalysed the hydrolysis of (±)-*syn*-*N*-(2-cyano-2-hydroxy-1-phenylethyl)benzamide, **(±)-*syn*-2**. Of these five nitrilases, NIT-106 and NIT-114 gave carboxamide, 23% and 15% conversion (after 15 hours) respectively, as the only product. Two other nitrilases, NIT-111 and PRO-E0260, yielded the desired carboxylic acid as product, however also gave significant amounts of amide as the by-product, as depicted in Figure 2. A single nitrilase, PRO-E0263, yielded the desired carboxylic acid and only minor amounts (less than 10%) of the amide by-product. **(±)-*syn*-2** was not hydrolysed by any of the fungal nitrilases tested.

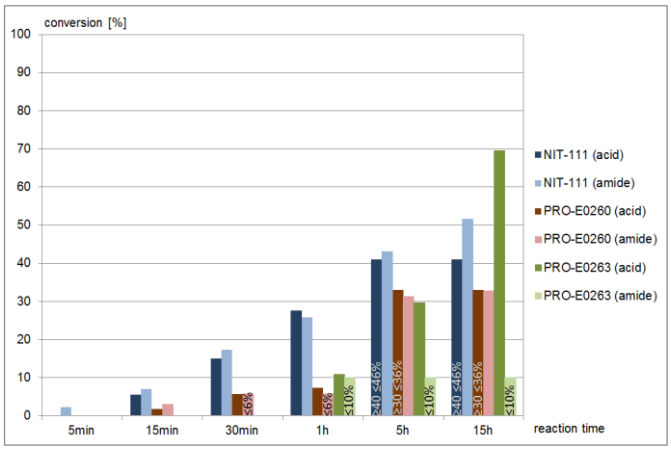

Figure 2 Screening results of **(±)-*syn*-2,** *(±)-syn-N*-(2-cyano-2-hydroxy-1-phenylethyl)benzamide, 0.4mM, with commercially available nitrilases (4mg/mL commercial enzyme preparation in buffer). NIT-101 to NIT-105, NIT-108, NIT-112, NIT-113, PRO-E0261, PRO-E0262 and PRO-E0264 gave no conversion, NIT-106 and NIT-114 gave amide as only product. None of the fungal nitrilases tested hydrolysed **(±)-*syn*-2**.

(±)-anti-N-(2-cyano-2-hydroxy-1-phenylethyl)benzamide, **(±)-*anti*-2,** was accepted by similar nitrilases as **(±)-*syn*-2**, as depicted in Figure 3. NIT-106 gave the amide as the main product, NIT-111 and PRO-E0260 gave almost equal amounts of the acid and amide throughout the reaction time, as depicted in Figure 3. PRO-E0263 gave the best results for the hydrolysis of **(±)-*anti*-2**, yielding the carboxylic acid as the only product. **(±)-*anti*-2** was not hydrolysed by any of the fungal nitrilases tested.

Nitrilases PRO-E0260 and PRO-E0264 are nitrilases from *Bradyrhizobium japonicum*. Nitrilase PRO-E0260 is an arylacetonitrilase and exhibits its highest activity for (*R,S*)-mandelonitrile but did not show enantioselectivity.[29] PRO-E0260 hydrolysed **(±)-*syn*-2** and **(±)-*anti*-2**, however, gave almost equal amounts of the acid and amide as products. PRO-E0264 has little activity towards branched nitriles, such as mandelonitrile, its preferential substrates being, *e.g.* hydrocinnamonitrile and heptanenitrile.[30] No activity of PRO-E0264 was observed towards **(±)-*syn*-2** and **(±)-*anti*-2.**

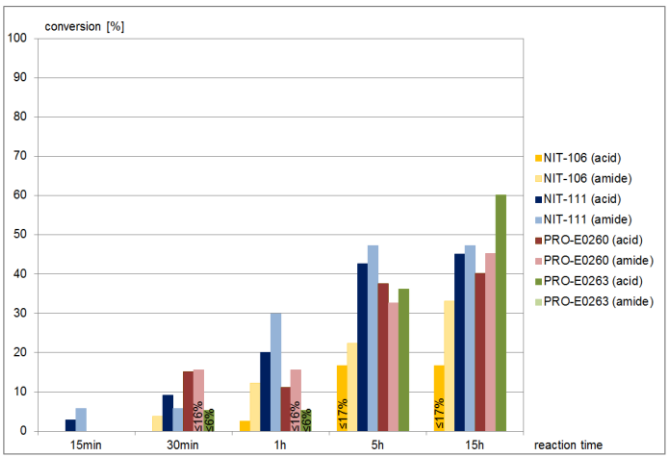

Figure 3 Screening results of **(±)-*anti*-2,** *(±)-anti-N*-(2-cyano-2-hydroxy-1-phenylethyl)benzamide, 0.4mM, with commercially available nitrilases. NIT-101–NIT-105, NIT-108, NIT-112 to NIT-114, PRO-E0261, PRO-E0262 and PRO-E0264 gave no conversion. None of the fungal nitrilases tested hydrolysed **(±)-*anti*-2.**

The dihydrooxazole **(±)-*trans*-1**, (±)-*trans*-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile, was chosen as the second sidechain precursor. Here, the enantioselective α-hydroxy-ß-amino nitrile moiety is protected in a cyclic precursor. The corresponding carboxylic acid of **(±)-*trans*-1** can be directly coupled to baccatin III. The final cleavage of the dihydrooxazole ring occurs subsequently under defined acidic conditions.[31]

Table 3 Screening results of **(±)-*trans*-1,** (±)-*trans*-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile, 0.4mM, and its epimer **(±)-*cis*-1** (0.4mM) with commercially available nitrile hydratases.

enzyme	conversion of **(±)-*cis*-1** to amide [%]; *ee*-value [%][a]	conversion of **(±)-*trans*-1** to amide [%][a]
PRO-E0256	31.7; 65.2	91.7
PRO-E0257	51.6; 83.4	93.7
PRO-E0258	63.2; 87.6	96.9
PRO-E0259	74.4; 90.9	100

[a] **(±)-*cis*-1**: 200µL commercial enzyme preparation, reaction time 21h, **(±)-*trans*-1**: 50µL commercial enzyme preparation.[26] Total volume 500µL. Results for additional enzyme concentrations and reaction times are available in the supporting information.

The dihydrooxazoles **(±)-*cis*-1** and **(±)-*trans*-1** were hydrolysed by all nitrile hydratases tested. Good conversions and *ee*-values were achieved for the hydration of **(±)-*cis*-1** (Table 3). Biotransformations of **(±)-*trans*-1** gave higher, almost quantitative, conversions with all nitrile hydratases, even when reaction time and concentration of enzyme were

significantly decreased. Quantitative conversions, however, indicate poor enantioselectivity of these nitrile hydratase catalysed reactions.

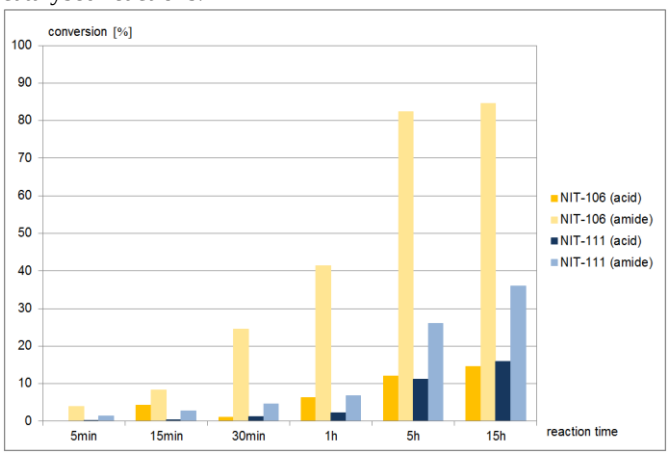

Figure 4 Screening results of **(±)-*cis*-1,** *(±)-cis*-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile, 0.4mM, with commercially available nitrilases (4mg/mL commercial enzyme preparation in buffer). None of the fungal nitrilases tested hydrolysed **(±)-*cis*-1.**

Only two nitrilases, of the 15 commercial nitrilases and six fungal nitrilases tested, hydrolysed **(±)-*cis*-1**, and both gave amide as the major product (Figure 4). In contrast to **(±)-*cis*-1**, the sidechain precursor **(±)-*trans*-1** was hydrolysed by half of the commercially available nitrilases and all the fungal nitrilases tested (Figures 5, 6, 7).

Nine different commercially available nitrilases accepted *(±)-trans*-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile, **(±)-*trans*-1** as the substrate. Reactions giving similar amounts of acid and amide are summarised in Figure 5, while reactions giving carboxylic acid as main product are summarised in Figure 6. NIT-106, NIT-108 and NIT114 gave 15-25% of amide by-product after 15 hours reaction time, NIT-105, NIT-111 and PRO-E0260 gave only minor amounts of less than 10% of amide by-product.

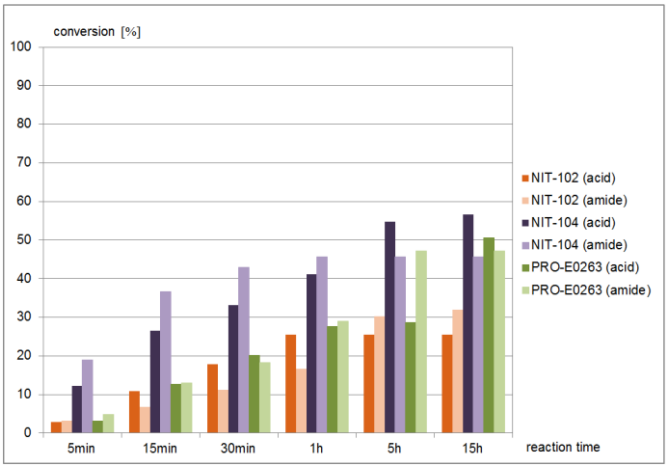

Figure 5 Screening results of **(±)-*trans*-1,** *(±)-trans*-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile, 0.4mM, with commercially available nitrilases (4mg/mL commercial enzyme preparation in buffer). NIT-102, NIT-104, PRO-E0263, giving similar amounts of acid and amide as products.

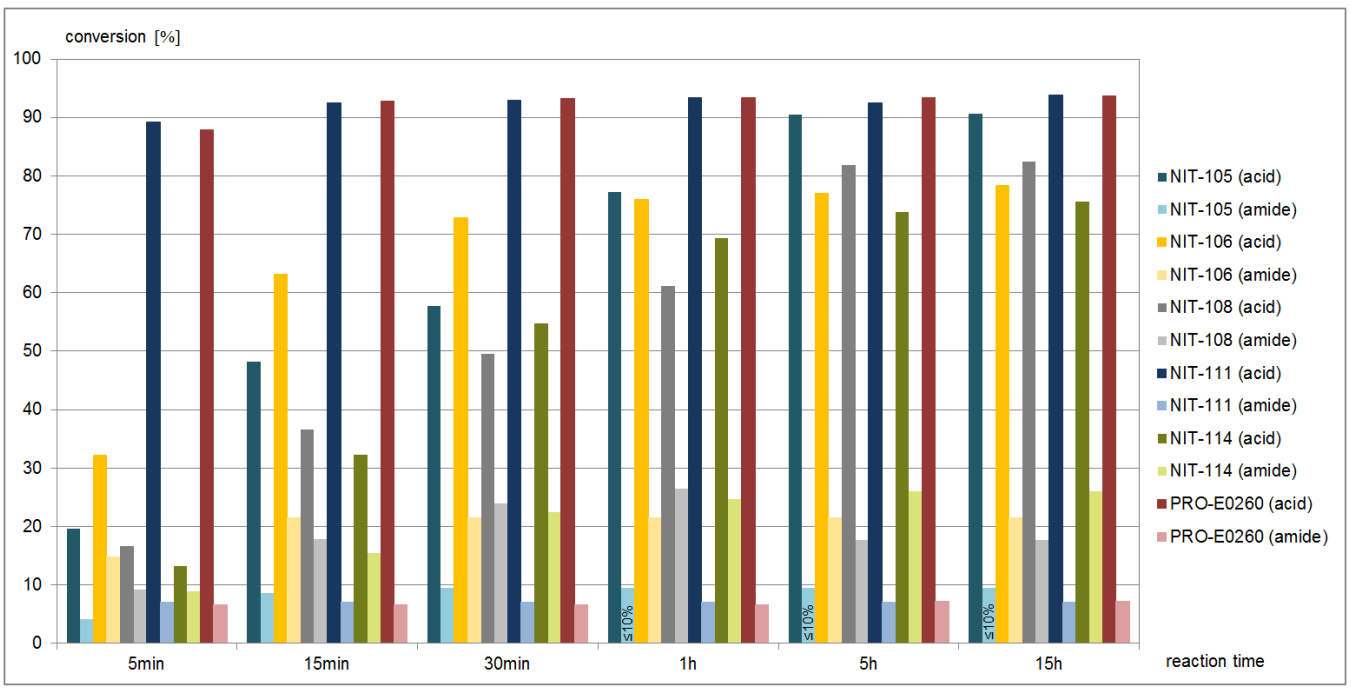

Figure 6 Screening results of **(±)-*trans*-1,** (±)-*trans*-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile, 0.4mM, with commercially available nitrilases giving the acid as the main product (4mg/mL commercial enzyme preparation in buffer). NIT-101, NIT-103, NIT-109, NIT-110, NIT-112, NIT-113, PRO-E0261, PRO-E0262 and PRO-E0264 gave no conversion, NIT-102, NIT-104 and PRO-E0263 gave similar amounts of acid and amide products, as summarized in Figure 5.

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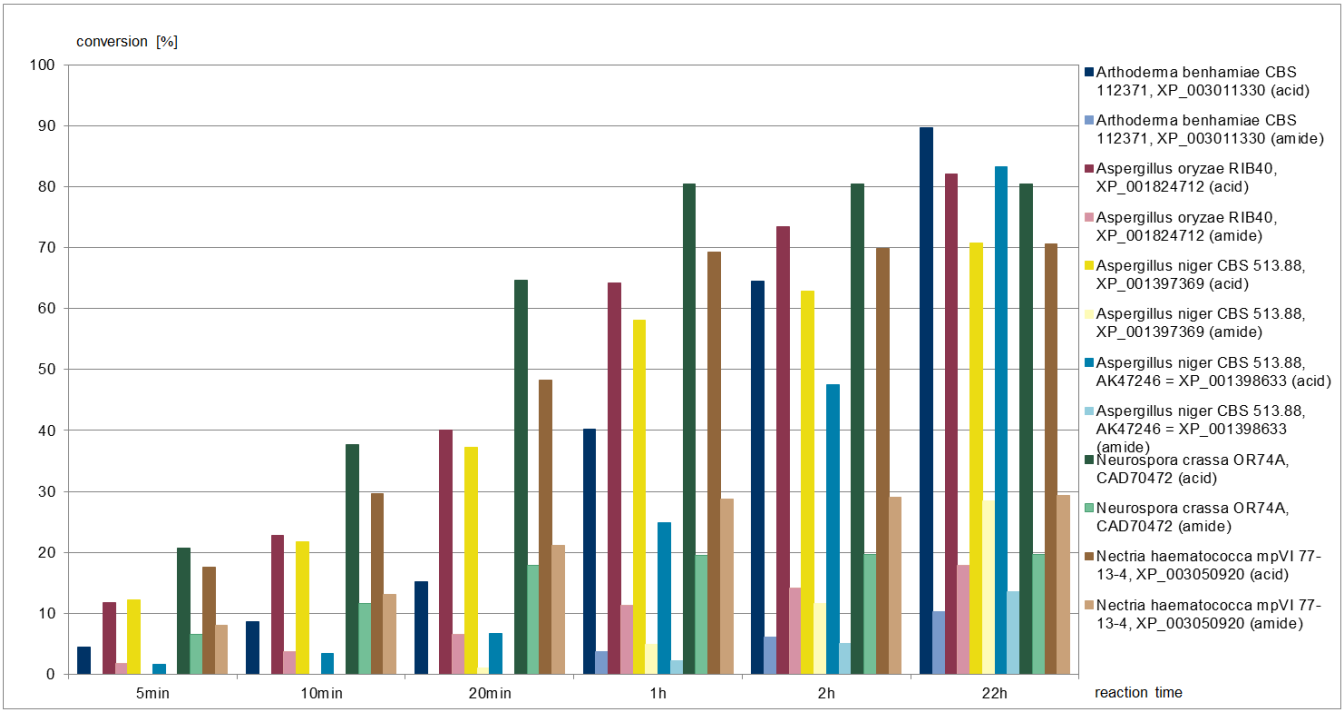

Figure 7 Screening results of *(±)-trans*-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile (1.0mM) with fungal nitrilases (9.5mL cell suspension of appropriate optical density in 100mL buffer).

(±)-trans-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile, **(±)-*trans*-1**, was hydrolysed to the carboxylic acid by all six fungal nitrilases, as depicted in Figure 7. The nitrilases from *Aspergillus oryzae*, *Neurospora crassa*, and *Nectria haematococca* produced both carboxylic acid and amide throughout the reaction time. The nitrilases from *Arthoderma benhamiae* and *Aspergillus niger* hydrolysed **(±)-*trans*-1** to the carboxylic acid at the beginning of the reaction, amide was only formed after approximately 25% conversion to the acid.

All fungal nitrilases used here were previously characterized as arylacetonitrilases with preference for phenylacetonitrile and (*R*,*S*)-mandelonitrile as substrates. Their similar substrate specificities correspond with considerable identities of their amino acid sequences (mostly over 50%). Nevertheless, differences have previously been observed between their specific activities, enantioselectivities and chemoselectivities for (*R*,*S*)-mandelonitrile.[28,32]

The *ee*-values obtained in the nitrilase catalysed reactions of **(±)-*trans*-1** were below 80%.[33] The moderate *ee*-values might be explained by a number of reasons. Racemisation and/or epimerisation of the compounds might occur during or after the biotransformation. Stopping the reaction by precipitating the enzyme might not have been efficient enough, as the protein might not have been quantitatively precipitated. Reactions could be most efficiently stopped by using immobilized enzyme which can be easily removed from the reaction mixture. In commercially available enzymes, additives in the enzyme preparation might influence enantioselectivity and ratio of the acid and amide products. The additive dithiothreitol (DTT) is added to nitrilases to prevent disulphide bond formation of the catalytically active cysteine, though it has been previously proven to catalyse the non-stereoselective hydrolysis of nitriles to amides.[34] In recent examples, the presence of organic solvents has been shown to enhance activity and stereoselectivity in nitrilase catalysed biotransformations.[35] The influence of organic solvents on enzymatic nitrile hydrolysis is poorly studied so far, especially compared to other hydrolytic enzymes, such as lipases and esterases.[36] Influences on the stereoselectivity of the nitrilase and nitrile hydratase catalysed reactions need to be further investigated to achieve a reliable nitrile transforming biocatalyst for the synthesis of the taxol sidechain.

A preparative scale biotransformation of **(±)-*trans*-1** was carried out with with whole cells of *E. coli* expressing the nitrilase from *Neurospora crassa* OR74A. Approximately 50% conversion of **(±)-*trans*-1** were achieved after three hours. However, the enantioselectivity of the reaction was not satisfactory, with an *er*[37] of 1/1.6 (4*S*,5*R*)-2,4-diphenyl-4,5-dihydro-1,3-dihydro-1,3-oxazole-5-carboxylic acid to enantiomer.

Conclusions

In this work, an extensive set of nitrilases and nitrile hydratases was screened towards their activity and selectivity in the hydrolysis of two taxol sidechain precursors and their epimers.

Both sidechain precursors were designed to utilize the enzymatic step as final step in the synthesis. A number of nitrilases and nitrile hydratases catalysed the biotransformation of both sidechain precursors and their epimers.

All nitrilases and nitrile hydratases tested showed similar substrate specificity towards the taxol sidechain precursor *(±)-syn-N*-(2-cyano-2-hydroxy-1-phenylethyl)benzamide **(±)-*syn*-2**, and its epimer **(±)-*anti*-2**. The openchain compounds **(±)-*syn*-2** and **(±)-*anti*-2** were converted by all Co-type nitrile hydratases tested, while the Fe-type nitrile hydratase PRO-E0256 did not convert **(±)-*syn*-2** or **(±)-*anti*-2**. Three commercially available nitrilases were found that hydrolyse the openchain precursor **(±)-*syn*-2**. Nitrilase PRO-E0263 gave less than 10% of amide by-product. The same nitrilases also hydrolysed **(±)-*anti*-2**. Here, PRO-E0263 produced the desired carboxylic acid as only product from **(±)-*anti*-2**.

(±)-*cis*- and (±)-*trans*-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile, **(±)-*cis*-1** and **(±)-*trans*-1**, were excellent substrates for all Co- and Fe-nitrile hydratases tested, giving the corresponding amides in 30-100% yield. The substrate specificity of nitrilases towards **(±)-*trans*-1** and its epimer **(±)-*cis*-1** differed quite drastically. Only two of 23 nitrilases accepted **(±)-*cis*-1** as substrate, the amide was the major product formed. The epimer **(±)-*trans*-1**, however, was converted by 18 different nitrilases, where three nitrilases gave similar amounts of acid and amide as product, and the remaining 15 gave the desired carboxylic acid as main product. Three commercially available nitrilases (NIT-105, NIT-111, PRO-E0260) gave less than 10% of amide by-product, four of the six fungal nitrilases (*Arthoderma benhamiae, Aspergillus niger, Neurospora crassa, Aspergillus oryzae*) gave less than 20% of amide by-product.

Fungal nitrilases, first of all arylacetonitrilases, have almost been neglected until recently due to their low production in wild-type strains, but genome mining has enabled the discovery of the first enzymes of this type. Their biocatalytic potential was confirmed with a new type of nitrile substrates in this work, and indicated further screening of this enzyme group would be promising.

This work presents the first investigation of nitrilases as tools for the chemo-enzymatic synthesis of the taxol sidechain. Nitrile hydratases (especially Co-type nitrile hydratases) and arylacetonitrilases (both, bacterial and fungal) were found to be suitable catalysts for one or both taxol sidechain precursors tested. The stereoselectivity of the enzyme catalysed reactions will need to be further investigated and improved for a possible application of these nitrile transforming enzymes in an enantioselective, chemo-enzymatic synthesis of the taxol sidechain.

Experimental

General Methods

Thin layer chromatography was carried out with precoated aluminium silica gel 60 F_{254} plates, column chromatography with Merck Silica Gel 60 (0.040-0.063mm). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE III with autosampler (^{1}H NMR 300.36MHz, ^{13}C NMR 75.53MHz). Chemical shifts for ^{1}H NMR are reported in ppm relative to Me_4Si as internal standard. Assignments were supported by HSQC and/or HMBC measurements. For all screening reactions, including blank reactions, multiple parallel determinations (at least two) were run. HPLC-MS analysis was carried out on an Agilent 1200 series using a Merck Purospher STAR® (RP-18e, 5µm, LiChroCART® 250-4) column (flow 0.9mL/min, injection 10µL) or on a Waters 600 with a PDA detector 996 using a Chromolith® SpeedROD RP-18 endcapped 50-4.6 column (flow 2mL/min, injection 10 µL), and 0.1% v/v acetic acid in water and acetonitrile as eluents. Determination of *ee*-values was performed by HPLC analysis on an Agilent 1100 series using a Chiralpak AD-H (Daicel Chemical Industries, Ltd., 0.46cm x 25cm) column, ethanol as eluent, flow 0.55mL/min, column oven temperature 40°C or a Chiralpak AGP column (Daicel Chemical Industries, Ltd., 150x4mm, 5µm) and 90% acetate buffer (100mM, pH4.4), 10% acetonitrile as eluents, flow 0.9mL/min, column oven temperature 25°C or a Chiralpak AGP column (Daicel Chemical Industries, Ltd., 150x4mm, 5µm) 10% iso-propanol and 90% citrate buffer (50mM, pH 4.4), flow 0.9mL/min, column oven 22°C.[25,33]

Substrate synthesis

(±)-*trans*-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile, (±)-*trans*-2.[3434] (±)-*cis*-Phenyloxirane-2-carbonitrile (6.12g, 42.2mmol) was dissolved in benzonitrile (129mL, 1.25mol) and cooled to -10°C. The reaction mixture was stirred under nitrogen atmosphere. Freshly distilled boron trifluoride diethyl etherate (5.72mL, 46.4mmol) was added and the resulting mixture was stirred overnight in an ice bath. Subsequently, the solution was basified by addition of saturated Na_2CO_3 solution followed by solid Na_2CO_3. The solution was then extracted with ethyl acetate three times and the combined organic layers were washed with brine. The solvent and excess benzonitrile were removed in vacuum. The product was purified by column chromatography using cyclohexane/ethyl acetate 4/1 as eluent. *cis*-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile was found as side product of the reaction. *(±)-trans*-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile was isolated as a white solid (8.37g, 80.0%). m.p. 81°C; ^{1}H NMR (DMSO-d_6) δ 5.75 (1H, d, *J*=6.0Hz, H-5), 5.86 (1H, d, *J*=6.0Hz, H-4), 7.33-7.46 (5H, m, H-2'', H-3'', H-4''), 7.58 (2H, t, *J*=7.5Hz, H-3'), 7.68 1H, (t, *J*=7.4Hz, H-4'), 8.00 (2H, d, *J*=7.8Hz, H-2'); ^{13}C NMR (DMSO-d_6) δ 71.57 (C-5), 74.32 (C-4), 117.76 (CN), 125.50 (C-1'), 126.74 (C-4''), 128.31, 128.43, 128.84, 128.99 (C-2', C-3', C-2'', C-3''), 132.65 (C-4'), 138.90 (C-1''), 161.86 (C-2).

***(±)-cis*-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile, (±)-*cis*-2. *(±)-cis*-2** was prepared from (±)-*trans*-phenyloxirane-2-carbonitrile (500mg, 3.44mmol) analogously to the (±)-*trans*-compound. (±)-*cis*-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile was isolated as a white solid (580mg, 67.8%). m.p.

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104°C; ^{1}H NMR (DMSO-d_6) δ 5.88 (1H, d, *J*=10.0Hz, H-4), 6.22 (1H, d, *J*=10.0Hz, H-5), 7.34-7.50 (5H, m, H-2'', H-3'', H-4'', H-5'', H-6''), 7.58 (2H, t, *J*=7.5Hz, H-3', H-5'), 7.68 (1H, t, *J*=7.4Hz, H-4'), 8.00 (2H, d, *J*=7.8Hz, H-2', H-6'); ^{13}C NMR (DMSO-d_6) δ 71.58 (C-5), 74.90 (C-4), 115.63 (CN), 125.66 (C-1'), 127.31 (C-4''), 128.23, 128.41, 128.55, 128.97 (C-2', C-3', C-5', C-6', C-2'', C-3'', C-5'', C-6''), 132.57 (C-4'), 137.31 (C-1''), 162.34 (C-2).

***(±)-syn-N*-(2-cyano-2-hydroxy-1-phenylethyl)benzamide, (±)-*syn*-1.** *(±)-trans*-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile (506mg, 2.05mmol) was dissolved in methanol (21mL) and aqueous HCl (1M, 9mL) was added. The reaction mixture was stirred for 3.5 hours at 60°C. Subsequently, the solvent was reduced in vacuum. The remaining aqueous residue was diluted with dichloromethane (20mL). The layers were separated and the aqueous layer was twice extracted with dichloromethane. The combined organic layers were washed with water and dried over Na_2SO_4. *(±)-syn-N*-(2-cyano-2-hydroxy-1-phenylethyl)benzamide was purified by recrystallization from cyclohexane/ethyl acetate and isolated as a white solid (292mg, 53.8%). ^{1}H NMR (DMSO-d_6) δ 5.02 (1H t, *J*=7.3Hz, H-2), 5.45 (1H, t, *J*=8.0Hz, H-3), 6.93 (1H, d, *J*=7.2Hz, OH), 7.35-7.50 (3H, m, H-3'', H-4'', H-5''), 7.52-7.67 (5H, m, H-3', H-4', H-5', H-2'', H-6''), 7.96 (2H, d, *J*=7.1Hz, H-2', H-6'), 9.06 (1H, d, *J*=8.6Hz, NH), ^{13}C NMR (DMSO-d_6) δ 56.62 (C-3), 63.75 (C-2), 119.53 (C-1), 127.46, 127.81, 127.90, 128.28, 128.32 (C-2', C-3', C-5', C-6', C-2'', C-3'', C-4'', C-5'', C-6''), 131.47 (C-4'), 134.09 (C-1'), 138.09 (C-1''), 166.34 ($CONH_2$).

***(±)-anti-N*-(2-cyano-2-hydroxy-1-phenylethyl)benzamide, (±)-*anti*-1.** *(±)-anti-N*-(2-cyano-2-hydroxy-1-phenylethyl)benzamide was prepared analogously from *(±)-cis*-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile (295mg, 1.19mmol). ***(±)-anti*-1** was isolated as a white solid (201mg, 63.5%). ^{1}H NMR (DMSO-d_6) δ 4.90 (1H, t, *J*=8.8Hz, H-2), 5.39 (1H, t, *J*=9.5Hz, H-3), 6.87 (1H, d, *J*=7.9Hz, OH), 7.32-7.47 (3H, m, H-3'', H-4'', H-5''), 7.51-7.67 (5H, m, H-3', H-4', H-5', H-2'', H-6''), 7.93 (2H, d, *J*=7.2Hz, H-2', H-6'), 9.15 (1H, d, *J*=9.0Hz, NH), ^{13}C NMR DMSO-d_6) δ 55.58 (C-3), 63.59 (C-2), 120.15 (C-1), 127.35, 127.74, 127.94, 128.20, 128.38 (C-2', C-3', C-5', C-6', C-1'', C-2'', C-3'', C-4'', C-5'', C-6''), 131.58 (C-4'), 134.01 (C-1'), 138.76 (C-1''), 165.92 ($CONH_2$).

Biotransformation reactions

Biotransformation reactions with commercially available nitrile hydratases. Nitrile hydratases were obtained from Prozomix Ltd. (PRO-E0256 to PRO-E0259). Screening reactions were done in 1.5mL microcentrifuge tubes using the following conditions and concentrations: nitrile hydratase suspension (50µL-200µL), and substrate in DMSO (10µL of a 20mM stock solution, end concentration of substrate 0.4mM, 2%v/v DMSO), buffer (50mM K_2HPO_4, pH 8) to achieve a total volume of 500µL. Blank reactions contained substrate in DMSO (0.4mM, 2%v/v DMSO), and buffer (50mM K_2HPO_4, pH 8). The screening reactions were incubated on a thermomixer at 22°C and 500rpm, unless stated otherwise. The reactions were stopped by adding methanol (290µL). The protein was precipitated by centrifugation and the supernatant was analysed by HPLC-MS.

Biotransformation reactions with commercially available nitrilases. Nitrilases from Codexis Inc. (NIT-101 to NIT-106, NIT-108 to NIT-114) and Prozomix Ltd. (PRO-E0260 to PRO-E0264) were used. Nitrilases were resuspended in buffer (50mM K_2HPO_4, pH 8, 4mg nitrilase in 1mL buffer). Screening reactions were done in 1.5mL microcentrifuge tubes using the following conditions and concentrations: nitrilase suspension (490µL), and substrate in DMSO (10µL of a 20mM stock solution, end concentration of substrate 0.4mM, 2%v/v DMSO), total volume 500µL. Blank reactions contained substrate in DMSO (0.4mM, 2%v/v DMSO), and buffer (50mM K_2HPO_4, pH 8). The screening reactions were incubated on a thermomixer at 30°C and 800rpm. The reactions were stopped by adding methanol (290µL). The protein was precipitated by centrifugation and the supernatant was analysed by HPLC-MS.

Biotransformations with fungal nitrilases. The nitrilase genes from *Arthoderma benhamiae* CBS112371 (XP_003011330) (NitAb), *Aspergillus oryzae* RIB40 (XP_001824712) (NitAo), *Aspergillus niger* CBS513.88 (XP_0013973369) (NitAn), *Aspergillus niger* CBS513.88 (XP_001398633) (NitAn2), *Neurospora crassa* OR74A (CAD70472) (NitNc), and *Nectria haematococca* mpVI77-13-4 (XP0030_50920) (NitNc) were overexpressed in *E. coli* BL21 Gold DE3 and the cells were grown as previously described.[28] 4.75mL of cell suspension of appropriate optical density (NitAb 66, NitAn 15, NitAn2 21, NitAo 22, NitNc 2 and NitNh 38) in 50mM Tris buffer with 150mM NaCl, pH 8.0, in 5mL tubes was preincubated for ten minutes at 25°C and 600rpm. The reaction was started by addition of 250µL of 20mM stock solution of **(±)-*trans*-1** in methanol (end concentrations: **(±)-*trans*-1** 1mM, methanol 5%v/v). Blank reactions were performed in buffer without cells under the same conditions. Samples (500µL of the reaction mixture) were withdrawn after 5, 10, 20, 40, 60, 90, 120 minutes and 22 hours, mixed with 600µL of methanol and then centrifuged for 10 minutes at 13,000 rpm. 500µL of the sample was transferred into a vial and analysed by HPLC.

Preparative scale biotransformations. Preparative scale biotransformations were carried out with whole cells of *E. coli* expressing the nitrilase from *Neurospora crassa* OR74A. Five parallel biotransformations were run, each in a 250mL Erlenmeyer flask, containing 100mL of 50mM Tris/HCl buffer with 150mM NaCl, pH 8.0 and (±)-*trans*-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile, **(±)-*trans*-2** (100mg, 0.40mmol) in DMSO (30%v/v), optical density of the cells approximately 2. The reactions were stopped after three hours reaction time by addition of 0.5M HCl (dropwise addition until pH 3), representing approximately 50% conversion of the nitrile substrate. The cells were removed by centrifugation. The supernatant was extracted with DCM (4x 50mL). The combined organic layers were washed with brine (2x 100mL), dried over sodium sulphate and reduced in vacuum until

dryness. Residual DMSO was removed by lyophilisation overnight. Yield (crude product, containing a mixture of acid product and unreacted starting material) 70mg. The crude product was purified by column chromatography using a gradient of chloroform to 15%v/v methanol in chloroform as eluents. The product was isolated as an off-white solid (15.6mg, 10%, purity 71%, *er*[37] 1/1.6 (4*S*,5*R*)-acid/enantiomer).[33] ^{1}H NMR and ^{13}C NMR data were found in accordance with the reference acid.

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