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

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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-(2R,3S)-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 nitrilases and nitrile hydratases, are investigated for the enzymatic hydrolysis of two different sidechain precursors. Both sidechain precursors, an openchain α-hydroxy-β-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.1–4 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.6–8 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.9–11 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 (Fig. 1).13

Various sidechain precursors have been prepared in asymmetric chemical syntheses14 and in chemoenzymatic approaches15 using acylases,16 lipases17 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

Fig. 1. Structure of Paclitaxel (taxol), consisting of the baccatin III core structure and the (2R,3S)-N-benzoyl-3-phenylisoserine C-13 sidechain.
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. In the first synthetic step, benzaldehyde was transformed to a mixture of (±)-cis- and (±)-trans-3-phenoxylxirane-2-carbonitrile in a Darzens reaction. The epimers were separated by column chromatography and used separately to synthesise the dihydrooxazoles (±)-trans-1 and (±)-cis-1 in a Ritter-type reaction. Ring opening under acidic conditions gave the openchain precursors (±)-syn-2 and (±)-anti-2.

The sidechain precursors (±)-trans-1 and (±)-syn-2 and their epimers were investigated as substrates for a set of different nitrilases and nitrilases (Scheme 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. 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. Commercially available, easy to use nitrilases from Prozomix, Ltd (PRO-E0257 to PRO-E0259) were used. Nitrilases included 18 commercially available nitrilases from two different suppliers, namely Codexis, Inc. (NIT-101 to NIT-114) and Prozomix, Ltd (PRO-E0260 to PRO-E0264) and six fungal nitrilases overexpressed in E. coli.

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 nitrilases tested, as summarised in Table 1. Conversions for (±)-anti-2 were higher than for (±)-syn-2. Examination of the amino acid products from benzaldehyde as described in the ESI.

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

<table>
<thead>
<tr>
<th>Nitrile hydratase</th>
<th>Conversion of (±)-syn-2 to amide [%]</th>
<th>Conversion of (±)-anti-2 to amide [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRO-E0256</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PRO-E0257</td>
<td>5.8</td>
<td>15.8</td>
</tr>
<tr>
<td>PRO-E0258</td>
<td>12.5</td>
<td>42.0</td>
</tr>
<tr>
<td>PRO-E0259</td>
<td>37.1</td>
<td>53.7</td>
</tr>
</tbody>
</table>
sequences of the nitrile hydratases\textsuperscript{26} indicated that Co-type nitrile hydratases (PRO-E0257, PRO-E0258, PRO-E0259), but not the Fe-type nitrilase (PRO-E0256) catalysed this reaction.

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 Fig. 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.

(±)-anti-N-(2-Cyano-2-hydroxy-1-phenylethyl)benzamide, (±)-anti-2, was accepted by similar nitrilases as (±)-syn-2, as depicted in Fig. 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 Fig. 3. PRO-E0263 gave the best results for the hydrolysis of (±)-anti-2, yielding the desired 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 \textit{Bradyrhizobium japonicum}. Nitrilase PRO-E0260 is an arylaceto-nitrilase and exhibits its highest activity for (R,S)-mandelonitrile but did not show enantioselectivity.\textsuperscript{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 \textit{e.g.} hydrocinnamomnitrile and heptanenitrile.\textsuperscript{30} No activity of PRO-E0264 was observed towards (±)-syn-2 and (±)-anti-2.

The dihydrooxazoles (±)-trans-1, (±)-trans-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile, 0.4 mM, and its epimer (±)-cis-1 (0.4 mM) with commercially available nitrile hydratases

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Enzyme & Conversion of (±)-cis-1 to amide [%], ee-value\textsuperscript{a} [%] & Conversion of (±)-trans-1 to amide\textsuperscript{a} [%] \\
\hline
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 \\
\hline
\end{tabular}
\caption{Screening results of (±)-trans-1, (±)-trans-2,4-diphenyl-4,5-dihydrooxazole-5-carbonitrile.}
\end{table}

\textsuperscript{a}(±)-cis-1: 200 µL commercial enzyme preparation, reaction time 21 h, (±)-trans-1: 50 µL commercial enzyme preparation.\textsuperscript{26} Total volume 500 µL. Results for additional enzyme concentrations and reaction times are available in the ESI.

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 (Fig. 5–7).

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 Fig. 5, while reactions giving carboxylic acid as main product are summarised in Fig. 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.

(±)-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 Fig. 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 characterised 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 disulfide 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 er37 of 1/1.6 (4S,5R)-2,4-diphenyl-4,5-dihydro-1,3-dihydro1,3-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 (A. benhamiae, A. niger, N. crassa, A. oryzae) gave less than 20% of amide by-product. Four of the six fungal nitrilases (NIT-105, NIT-111, PRO-E0260) gave less than 10% of amide by-product. The same nitrilases also hydrolysed (±)-trans-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile (±)-trans-1, when run. HPLC-MS analysis was carried out on an Agilent 1100 series using a Chiralpak AD-H (Daicel Chemical Industries, Ltd, 0.46 cm × 25 cm) column, ethanol as eluent, flow 0.55 mL min⁻¹, column oven temperature 40 °C or a Chiralpak APG column (Daicel Chemical Industries, Ltd, 150 × 4 mm, 5 µm) and 90% acetonitrile buffer (100 mM, pH 4.4), 10% acetonitrile as eluents, flow 0.9 mL min⁻¹, column oven temperature 25 °C or a Chiralpak AGP column (Daicel Chemical Industries, Ltd, 150 × 4 mm, 5 µm) 10% iso-propanol and 90% citrate buffer (50 mM, pH 4.4), flow 0.9 mL min⁻¹, column oven 22 °C.

Substrate synthesis

(±)-trans-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile, (±)-trans-1, was prepared from (±)-Phenyloxirane-2-carbonitrile (6.12 g, 42.2 mmol) was dissolved in benzonitrile (129 mL, 1.25 mol) and cooled to −10 °C. The reaction mixture was stirred under nitrogen atmosphere. Freshly distilled boron trifluoride diethyl etherate (5.72 mL, 46.4 mmol) was added and the resulting mixture was stirred overnight in an ice bath. Subsequently, the solution was basified by addition of saturated Na2CO3 solution followed by solid NaCl. 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 isolated as a white solid (8.37 g, 80.0%). m.p. 81 °C; EI-HRMS m/z 248.0952 ([M]+, C16H12N2O, calc. 248.0950); 1H NMR (DMSO-d6) δ 7.15 (1H, d, J = 7.4 Hz, H-2), 7.34–7.46 (5H, m, H-2″, H-3″, H-4″), 5.86 (1H, d, J = 6.0 Hz, H-4), 7.33–7.46 (5H, m, H-2″, H-3″, H-4″), 5.78 (2H, t, J = 7.5 Hz, H-3), 7.68 (1H, t, J = 7.4 Hz, H-4″), 8.00 (2H, d, J = 7.8 Hz, H-2″); 13C NMR (DMSO-d6) 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, 129.99 (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-1. (±)-cis-1 was prepared from (±)-trans-phenyloxirane-2-carbonitrile (500 mg, 3.44 mmol) analogous to the (±)-trans-compound. (±)-cis-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile was isolated as a white solid (580 mg, 67.8%). m.p. 104 °C; 1H NMR (DMSO-d6) δ 5.88 (1H, d, J = 10.0 Hz, H-4), 6.22 (1H, d, H-4, J = 10.0 Hz, H-5), 7.34–7.50 (5H, m, H-2″, H-3″, H-4″), 7.58 (2H, t, J = 7.5 Hz, H-3″, H-4″), 7.68 (1H, t, J = 7.4 Hz, H-4″), 8.00 (2H, d, J = 7.8 Hz, H-2″, H-6″); 13C NMR (DMSO-d6) δ 71.58 (C-5″), 74.90 (C-4″), 115.63 (CN), 125.66 (C-1″), 126.74 (C-4″), 128.31, 128.43, 128.84, 129.99 (C-2″, C-3″, C-2″, C-3″), 132.65 (C-4″), 138.90 (C-1″), 161.86 (C-2″).

(±)-syn-N-(2-cyano-2-hydroxy-1-phenylethyl)benzamide, (±)-syn-2. (±)-trans-2,4-Diphenyl-4,5-dihydrooxazole-5-carbonitrile (506 mg, 2.05 mmol) was dissolved in methanol (21 mL) and aqueous HCl (1 M, 9 mL) 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 (20 mL). The layers were
separated and the aqueous layer was twice extracted with dichloromethane. The combined organic layers were washed with water and dried over Na2SO4. (+)-syn-N-2-(cyano-2-hydroxy-1-phenylethyl)benzamide was purified by recrystallisation from cyclohexane/ethyl acetate and isolated as a white solid (292 mg, 53.8%). 1H NMR (DMSO-d6) δ 5.02 (1H, t, J = 7.3 Hz, H-2), 5.45 (1H, t, J = 8.0 Hz, H-3), 6.93 (1H, d, J = 7.2 Hz, 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-6′, H-6″), 9.06 (1H, d, J = 8.6 Hz, NH). 13C NMR (DMSO-d6) δ 56.62 (C-3), 63.75 (C-2), 119.53 (C-1), 127.46, 127.81, 127.90, 128.28, 128.32, 128.35 (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 (CONH2).

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 1.5 mL microcentrifuge tubes using the following conditions and concentrations: nitrile hydratase suspension (490 µL), and substrate in DMSO (10 µL of a 20 mM stock solution, end concentration of substrate 0.4 mM, 2% v/v DMSO), total volume 500 µL. Blank reactions contained substrate in DMSO (0.4 mM, 2% v/v DMSO), and buffer (50 mM K2HPO4, pH 8). The screening reactions were incubated on a shaker at 30 °C and 800 rpm. The reactions were stopped by adding methanol (290 µL). The protein was precipitated by centrifugation and the supernatant was analysed by HPLC-MS.

Biotransformations with fungal nitrilases. The nitrilase genes from Arthrobacter benhamiae CBS112371 (XP_003113303) (NitAb), Aspergillus oryzae RIB40 (XP_001824712) (NitAo), Aspergillus niger CBS513.88 (XP_0013973369) (NitAn), Aspergillus niger CBS5513.88 (XP_001398633) (NitAn2), Neurospora crassa OR74A (CAD70472) (NitNc), and Nectria haematococca mpV17713-4 (XP0030_50920) (NitNc) were overexpressed in E. coli BL21 Gold DE3 and the cells were grown as previously described. 4.75 mL of cell suspension of appropriate optical density (NitAb 66, NitAn 15, NitAn2 21, NitAo 22, NitNc and NitNc 38) in 50 mM Tris buuffer with 150 mM NaCl, pH 8.0, in 5 mL tubes was preincubated for ten minutes at 25 °C and 600 rpm. The reaction was started by addition of 250 µL of 20 mM stock solution of (+)-trans-1 in methanol (end concentrations: (+)-trans-1 1 mM, methanol 5% v/v). Blank reactions were performed in buffer without cells under the same conditions. Samples (500 µL of the reaction mixture) were withdrawn after 5, 10, 20, 40, 60, 90, 120 minutes and 22 hours, mixed with 500 µL of methanol and then centrifuged for 10 minutes at 13000 rpm. 500 µL 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 250 mL Erlenmeyer flask, containing 100 mL of 50 mM Tris/HCl buffer with 150 mM NaCl, pH 8.0 and (+)-trans-2,4-diphenyl-4,5-dihydroxazole-5-carbonitrile, (+)-trans-2 (100 mg, 0.40 mmol) in DMSO (30% v/v), optical density of the cells approximately 2. The reactions were stopped after three hours reaction time by addition of 0.5 M 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 (4 × 50 mL). The combined organic layers were washed with brine (2 × 100 mL), 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) 70 mg. 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 (15.6 mg, 10%, purity 71%, er37 1/1.6 (4S,5R)-acid/enantiomer). 1H NMR and 13C NMR data were found in accordance with the reference acid.

Acknowledgements

The authors wish to thank Jasmin Resch and Carina Hasenöhrl for skilful assistance in substrate synthesis and screening reactions. Financial support from project P504/11/0394 (Czech Science Foundation) is gratefully acknowledged.
Notes and references


22 Procedures for the synthesis of the acid and amide reference materials and characterization data are available in the ESL†


25 Representative HPLC chromatograms are available in the ESL†

26 Additional information on the commercial enzyme preparations by Prozomix, Ltd. can be found in the ESL† or online at http://www.prozomix.com/.

27 Additional information on the commercial enzyme preparations by Codexis, Inc. can be found in the ESL† or online at http://www.codexis.com/.


33 ee-values/er-values (see ref. 37) were determined by chiral HPLC, methods and representative chromatograms are available in the ESL†


