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Synthesis of novel cyano-cyclitols and their stereoselective biotransformation catalyzed by *Rhodococcus erythropolis* A4

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ABSTRACT

A variety of novel cyano-cyclitols possessing complex stereochemistry have been synthesized. These compounds were subjected to the biocatalyzed hydrolysis of their nitrile groups. The bacterial strain *Rhodococcus erythropolis* A4, expressing a nitrile hydratase/amidase bienzymatic system, was able to recognize (1*R*,2*S*,3*S*,4*R*)/(1*S*,2*R*,3*R*,4*S*)-1-cyano-2,3,4-trihydroxy-cyclohex-5-ene and *trans*-3-cyanocyclohexa-3,5-diene-1,2-diol, and to catalyze their transformations into the corresponding amides and acids. The kinetic and stereochemical trends of these biotransformations, a rare example of the enantiorecognition of a rigid bulky aliphatic substrate, are discussed.

1. Introduction

The development of molecules with enhanced biological activity is a continuing primary goal in biomedical research. The need for compounds interacting with biological receptors characterized by a high grade of asymmetry leads researchers to synthesize molecules possessing well defined stereochemical patterns, with the declared aims of obtaining better substrate-receptor interactions, minimizing collateral effects due to different isomeric forms, and reducing the amount of drug administered. An important class of cyclitols[1] characterized by a high stereochemical complexity are conduritols,[2] 1,2,3,4-Cyclohexenetetrols possessing four stereogenic centers, and consequently existing as four different pairs of enantiomers, namely conduritol B, C, E, and F, and two *meso* forms, conduritol A and D. Only conduritol A and B are present in nature,[3] but all possible stereoisomers have been synthesized[4,5] and all of them have exhibited important biological activities,[6] making these cyclitols potentially key molecules in hit-to-lead drug discovery. The conduritol scaffold can also be found in many natural cells mediators such as inositols,[7] glycerophosphoinositols,[8] pseudosugars,[9] and antibiotics such as manumycin A[10] and tricholomenyn A,[11] or RNA mimics.[12] Possible key synthetic equivalents in the preparation of these valuable compounds are the nitrilic derivatives of conduritols, due to the great versatility of cyano group transformations.

Unfortunately, even if the conventional chemical hydrolysis of nitriles is a well known reaction,[13] it suffers from several disadvantages, including the requirement for highly acidic or basic reaction conditions, high temperatures, formation of undesirable by-products, low yields and the generation of large amounts of waste salts, making this route economically and environmentally inconvenient, and unsuitable, especially with unstable substrates such as polyhydroxylated compounds. A possible alternative is offered by biocatalysis, since the enzymatic hydrolysis of nitriles can be performed under milder conditions, for example, at approximately neutral pH.[14]

In Nature, the biotransformation of nitriles proceeds via two different pathways (Fig. 1): the direct nitrilase-catalyzed reaction to yield the corresponding carboxylic acids and ammonia, or a two-step indirect reaction, in which nitriles are first transformed to amides by nitrile hydratase, and then the amides are transformed to the corresponding carboxylic acids and ammonia by amidase.

In contrast with this definite synthetic potential, the use of nitrile hydrolyzing enzymes in organic synthesis has remained restricted until now and examples of their application in the hydrolysis of cyclic aliphatic substrates are negligible in number.[15] Moreover, although many of the enzymatic transformations of nitriles which have been reported thus far involve straightforward achiral substrates,[16] stereoselective transformations involving racemic or prochiral substrates are particularly interesting.[17] While in the first publications[18] to describe the hydrolysis of racemic nitriles, the resolution was assumed to only occur in the amide hydrolysis step under the influence of the amidase, subsequent work[19] showed that, with selected substrates, nitrile hydratases or nitrilases could also present their intrinsic enantioselectivity. Due to the unpredictable nature of enantioselective enzymatic nitrile hydrolysis, there is still great interest in investigating a variety of new enzymatic transformations of nitriles.

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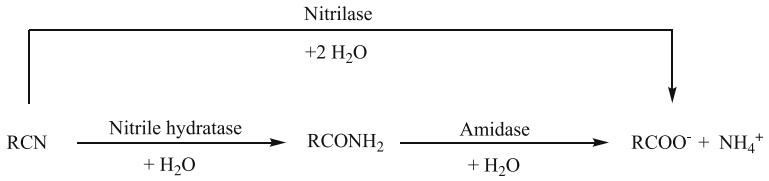


Figure 1. Different pathways of nitrile metabolism in microorganisms.

Herein, we present the synthesis of novel chiral nitrilic conduritol derivatives, and an investigation of their biotransformation reactions, aiming to obtain optically active carboxylic acids and amides for the preparation of valuable bioactive compounds.

2. Results and discussion

2.1. Synthesis of substrates

The first part of this work was concerned with the development of a synthetic strategy for the preparation of cyano-deoxyconduritols, novel molecules in which a hydroxyl group of the conduritol framework has been replaced by a nitrile group. Commercially available *p*-benzoquinone **1**, an inexpensive and highly versatile building block, was used as the starting material. Compound **1** was subjected to sequential bromination, reduction and epoxidation reactions (Scheme 1)[20], yielding the racemic diepoxide (±)-**4**.

In the presence of calcium oxide as a heterogeneous catalyst[21] and trimethylsilylcyanide as the nitrilating agent, compound (±)-**4** gave a quantitative yield of non-isolable intermediate ((±)-**5**), that upon treatment with trifluoroacetic acid produced two main compounds, which were chromatographically purified and spectroscopically identified as (1*R*, 2*S*, 3*S*, 4*R*)/(1*S*, 2*R*, 3*R*, 4*S*)-1-cyano-2,3,4-trihydroxy-cyclohex-5-ene (±)-**6** and *trans*-3-cyanocyclohexa-3,5-diene-1,2-diol (±)-**7** (Scheme 2).

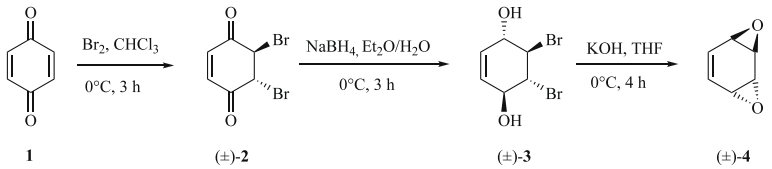
A study of the coupling constants J_{vic} observed in ^{1}H NMR spectra permitted the assignment of all the relative configurations of the chiral centres, indicating compound (±)-**6** as a derivative of conduritol B. Analogously, the relative configurations of the stereogenic centers of (±)-**7** were determined, and the results were in agreement with the hypothesis that it is a stable derivative of (±)-**6**, generated under acidic conditions, by the β-elimination of a water molecule driven by an increase in conjugation. Compound (±)-**7** was further subjected to *trans*-dihydroxylation via a regioselective epoxidation reaction by treatment with *m*-chloroperbenzoic acid (*m*CPBA), and subsequent stereoselective opening of the oxirane ring by the action of trifluoroacetic acid (Scheme 3), yielding (1*R*,2*S*,3*R*,4*S*)/(1*S*,2*R*,3*S*,4*R*)-5-cyano-1,2,3,4-tetrahydroxycyclohex-5-ene (±)-**8** as the sole product.

Again, the analysis of ^{1}H NMR coupling constants J_{vic} permitted the assignment of the relative configuration of the stereogenic centres of compound (±)-**8**, a direct derivative of conduritol A, in which the nitrile substituent is directly located on the alkenic bond. The spectroscopic data as well as elemental analysis data for the novel compounds are given in the Section 4.

2.2. Biotransformations

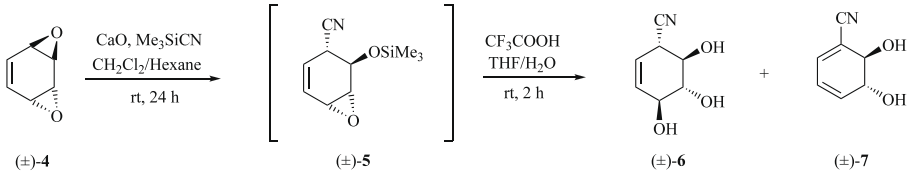
Compounds (±)-**6**, (±)-**7** and (±)-**8** were subjected to a preliminary general biotransformation protocol (see Section 4), which could give rise, depending on the catalyst used, to the corresponding amides and/or acids. Bacterial whole-cell preparations of *Rhodococcus erythropolis* A4 grown under conditions to express its bienzymatic nitrile hydratase/amidase system were used, as well as those of *Rhodococcus* sp. NDB 1165 and *Nocardia* sp. NHB-2 grown under conditions to express their nitrilase activities. A fungal strain of *Fusarium solani* O1 expressing nitrilase activity was also assayed. All substrates were dissolved in a Tris-HCl buffer (pH 8) containing up to 5% of methanol as a cosolvent, to increase their solubility. The nitrilase-producing strains *Rhodococcus* sp., *Nocardia* sp. and *F. solani* O1 did not give any reaction with the compounds tested. This observation was in accordance with the characterization of the nitrile-transforming enzymes in all these organisms as 'aromatic nitrilases' with a preference for benzonitrile analogues.[22] The nitrilases of this type generally exhibit no or low activities for aliphatic, arylaliphatic or alicyclic nitriles. On the other hand, whole cells from *R. erythropolis* A4 recognized substrates (±)-**6** and (±)-**7**, catalyzing their biotransformation to the corresponding amides and acids. This was in accordance with the previous finding that nitrile hydratases are largely less sensitive to steric hindrance than nitrilases and hence they also transform relatively bulky substrates.[23]

Since nitrile-hydrolyzing biocatalysts are intracellular enzymes, with substrates having to cross the cell wall to interact with them, the observed activity of *R. erythropolis* A4 could also be a result of the facilitated uptake of the aforementioned hydrophobic substrates by cells with long aliphatic chains of mycolic acids in their cell envelopes. In contrast, the more hydrophilic nature of substrate (±)-**8** could result in an incapacity to cross the cell wall, and explain its complete absence of recognition. To verify this hypothesis, the biotransformation of compound (±)-**8** was repeated in the presence of a cellular extract of *R. erythropolis* A4, again yielding no reaction product; this result, which goes against our previous hypothesis, would indicate that in this case the absence of reactivity is not due to the polarity/mobility features of the substrate but more generally to its structural properties. Using the



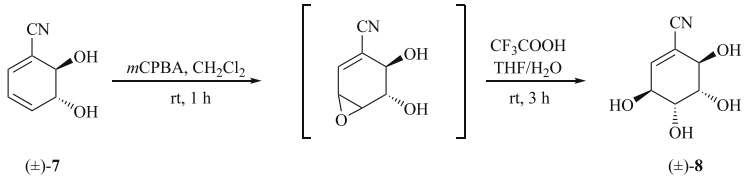
Only one isomer is drawn for every pair of enantiomers.

Scheme 1.



Only one isomer is drawn for every pair of enantiomers.

Scheme 2.



Only one isomer is drawn for every pair of enantiomers.

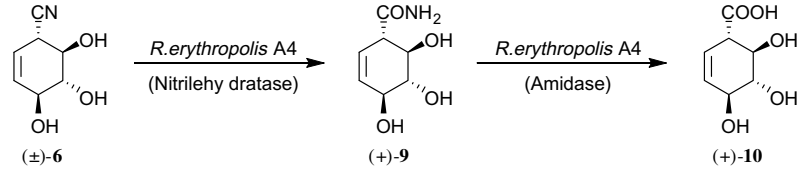
Scheme 3.

same cellular extract, compounds (±)-**6** and (±)-**7** continued to be recognized, even though the enzymes lost their activity after only 2 h, probably because of a greater instability of the catalytic system when not operating in its natural cellular environment.

To study both the kinetic behaviour of the bienzymatic system and its potential enantioselective features, substrate (±)-**6** was transformed by the whole cells of *R. erythropolis* A4 and samples withdrawn at regular intervals were appropriately derivatized and analyzed using gas chromatography. In the early stages of the reaction, it was possible to observe a fast conversion of nitrile (±)-**6** into the corresponding amide and, in turn, the conversion of the latter into the acid derivative, as demonstrated by the increase in the concentrations of the species 1-carboxamido-2,3,4-trihydroxy-cyclohex-5-ene **9** and 2,3,4-trihydroxy-cyclohex-5-en-1-carboxylic acid **10** in the solution (Fig. 2).

At a reaction time of 3 h and 20% conversion, the concentration of amide **9** tended to reach a plateau while the concentration of the acid continued to increase and that of the nitrile to decrease. At 8 h, the biotransformations were drastically slowed down, and at 24 h it virtually stopped with final concentrations of 40% both for compounds **6** and **10**; this can be mainly explained by the inactivation of the biocatalyst. The biotransformation displayed a notable level of enantiorecognition. The first step catalyzed by the nitrile hydratase displayed a low enantioselectivity ($E \approx 2.5$) as demonstrated by the maximum value of enantiomeric excess (40%, Fig. 3), of nitrile recovered after a reaction time of 24 h (approx. 60% conversion, Figure 2).

Nevertheless, this observation is noteworthy, since examples of enantioselective nitrile hydratases are scarce in the scientific literature and are almost non-existent for aliphatic bulky substrates. In



Only one isomer is drawn for the racemic mixtures.

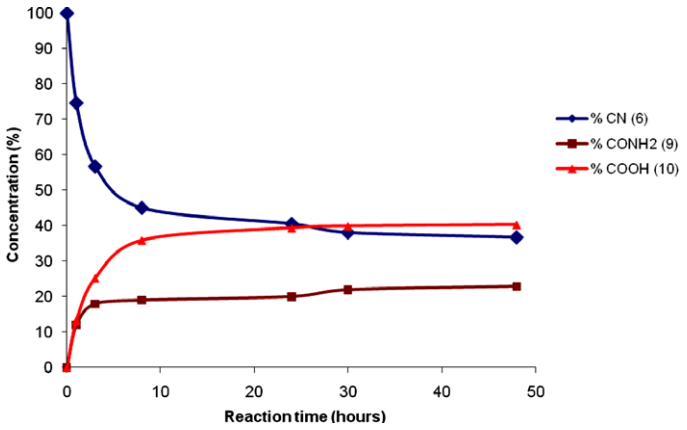


Figure 2. Kinetic trend of biotransformation reaction of (±)-**6** catalyzed by *Rhodococcus erythropolis* A4.

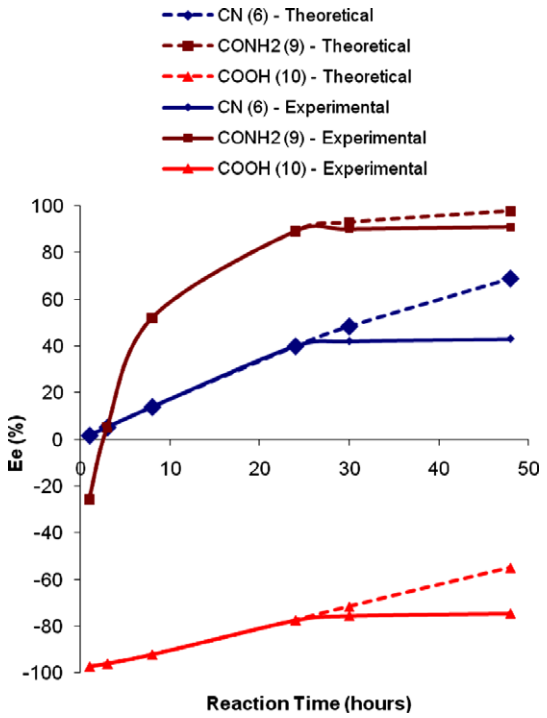


Figure 3. Stereochemical trend of biotransformation reaction of (±)-**6** catalyzed by *Rhodococcus erythropolis* A4.

contrast, the second step catalyzed by the amidase exhibited a moderate to good enantioselectivity, since it was possible to recover both the acid and the amide with an ee of 70–90%, depending on the reaction time. To better understand the potentialities of the enzymatic system, the theoretical trends of enantiomeric excesses in case the catalyst was not inactivated after 20 h were calculated, by using the simulation software 'SeKiRe' developed by Faber's group for the study of bienzymatic systems,[24] and are shown as dotted lines in Figure 3. To define the stereopreference displayed by the bienzymatic system, the absolute configuration of the enantiomerically enriched product **10** was determined after chemical derivatization (Scheme 4), and the specific rotation of the resulting compound **12** was measured.

By comparing the sign of the specific rotation determined and those reported in literature for the enantiomers of **12**,[25] it was possible to identify compound **10** as (1*S*,2*R*,3*R*,4*S*)-2,3,4-trihydroxy-cyclohex-5-en-1-carboxylic acid. This data demonstrated the stereopreference of the amidase towards the substrate with an (*S*)-configuration. Considering this information and the enantioselectivity trends shown in Figure 3, it was also possible to identify amide **9** as (1*R*,2*S*,3*S*,4*R*)-1-carboxamido-2,3,4-trihydroxy-cyclohex-5-ene, and, as in the previous case, the stereospecificity of the enzyme (in this case nitrile hydratase) towards a substrate with an (*S*)-configuration. The 'inversion' in the configuration of the recovered amide was not surprising, since it is a typical effect of a sequential double resolution with two enzymes having the same enantiopreference but differing levels of enantioselectivity (the second catalytic system being more selective than the first).

On the other hand, the kinetic and enantioselective trends of biotransformation of substrate (±)-**7** by whole cells of *R. erythropolis* A4 were quite different. The first step, catalyzed by the nitrile hydratase, was very fast; complete conversion to the corresponding amide (±)-**13** was achieved within 15 min (Fig. 4); no enantiopreference being shown by the catalyst. By prolonging the reaction time, complete transformation of compound (±)-**13** into carboxylic acid (±)-**14** catalyzed by the amidase could be observed.

This second step was characterized by a very low value of the enantiomeric ratio ($E = 2.2$), both amide **13** and acid **14** being produced with poor enantiomeric excesses as determined by chiral HPLC (Figure 5). A comparison with literature data[26] relative to compound **14** enabled the absolute configuration of the preferentially synthesized enantiomer to be identified as (+)-(1*S*,2*S*) *trans*-3-carboxylic-cyclohexa-3,5-diene-1,2-dihydroxy acid.

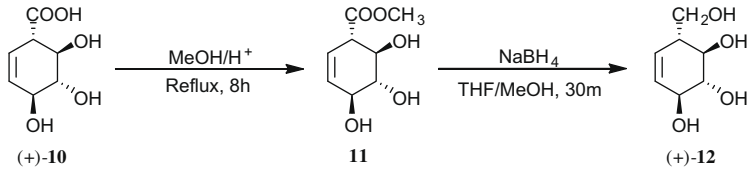
3. Conclusions

The chemical synthesis of several novel cyano-cyclitols has been achieved. Enzymatic hydrolysis of these racemic substrates was obtained for the first time using, under mild conditions (buffer solution pH 8, 35 °C), bacterial whole-cell preparations of *R. erythropolis* A4 expressing the bienzymatic system nitrile hydratase/amidase. However, both the efficiency and enantioselectivity of the biotransformations were strongly dependent upon the structures of the nitrile substrates. The best results were obtained when (1*R*,2*S*,3*S*,4*R*)/(1*S*,2*R*,3*R*,4*S*)-1-cyano-2,3,4-trihydroxy-cyclohex-5-ene (±)-**6** was used as the substrate, with a conversion value of 60% after 8 h, and enantiomeric excesses values in the range 70–90% both for the corresponding amide and acid, depending on the reaction time. While this observation shows an important instance of an (*S*) enantioselective amidase, even more noteworthy is that it represents the first case to our knowledge of a nitrile hydratase that is enantioselective towards bulky aliphatic substrates. The versatile utility of the resulting optically active novel carboxylic acids and amide derivatives renders this method very attractive and practical in organic synthesis. The extension of biotransformations for the synthesis of various chiral cyclitols and their applications in organic chemistry to the synthesis of enantiopure bioactive compounds are currently being investigated in our laboratories.

4. Experimental

4.1. General

^{1}H and ^{13}C NMR spectra were recorded in the indicated deuterated solvents in a Bruker Avance™ 400 spectrometer at 400.13 and 100.62 MHz, respectively. Chemical shifts (δ) are given as parts per million relative to the residual solvent peak and coupling constants (J) are in hertz. Column chromatography was performed on Silica Gel 60 (70–230 mesh) using the specified eluents. GC analyses were carried out in a Shimadzu® Fast-GC-17A equipped with a FID detector and a SUPELCO® Fast-SPB™-5, using *n*-undecane as the internal standard. Chiral GC analyses were carried out in a Perkin–Elmer® 8500 equipped with a FID detector and a MEGA® dimethyl-*tert*-butylsilyl-β-cyclodextrin (coated on OV 1701) column. Chiral HPLC analyses were carried out in a DIONEX® appa-



(+)-**10** **11** (+)-**12**

Scheme 4.

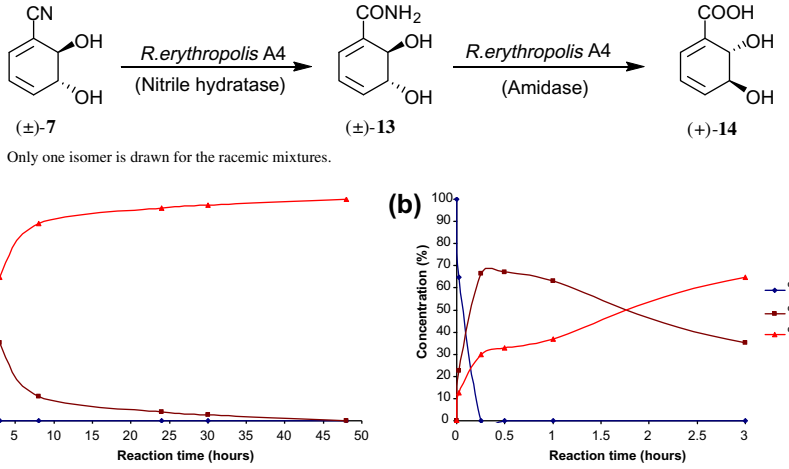


Figure 4. Kinetic trend of biotransformation reaction of (±)-**7** catalyzed by *Rhodococcus erythropolis* A4: (a) full reaction time range; (b) expansion in the time range 1–3 h.

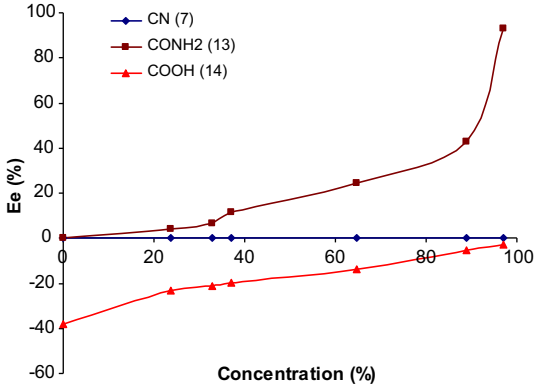


Figure 5. Stereochemical trend of biotransformation reaction of (±)-**7** catalyzed by *Rhodococcus erythropolis* A4.

ratus equipped with a Chiralcel® OD or OJ column (Daicel Chemical Industries) using *n*-hexane/*iso*-propanol mixtures as the mobile phase and detection by UV-vis detector at 220, 250, 266 and 275 nm. Optical densities (OD) were measured on a DAD-UV–visible Agilent® spectrophotometer at 610 nm. Optical rotations were measured on a DIP 135 JASCO® instrument at 25 °C. ESI-MS spectra were acquired in positive mode in a Waters® Micromass ZQ2000, using a 10 V cone voltage, 3 kV capillary voltage and 150 °C source temperature. Melting points are uncorrected. The simulation of kinetic and enantioselectivity trends were performed using the specific software 'Sequential Kinetic Resolution' (SeKiRe), developed by Professor K. Faber and his group.[24] The enantiomeric ratio (*E*) was calculated from the experimental values of conversion and enantiomeric excess using the equation reported in the literature.[27] The enantiomeric excess of compound **10** was calculated from the experimental values of concentrations and enantiomeric excesses of compounds **6** and **9**, using the aforementioned 'SeKiRe' software. The chemicals, solvents and materials for microbiological cultures were obtained from Sigma–Aldrich®, Fluka®, Carlo Erba®, Riedel-de Haen®, Difco® and used without further purification.

4.2. Synthesis of (5*R*,6*R*)/(5*S*,6*S*)-5,6-dibromocyclohex-2-ene-1,4-dione, (±)-2

Br_2 (12.5 ml, 0.24 mol) at 0 °C was added over 2 h to a solution of *p*-benzoquinone **1** (25 g, 0.23 mol) in 150 ml of $CHCl_3$. After 1 h, the reaction was stopped by evaporating the solution under vacuum at 40 °C. The mixture was purified by silica gel column chromatography using 100% CH_2Cl_2 as the eluant, and recovered as a white solid (54.64 g, 0.20 mol, 87% yield). ^1H and ^{13}C NMR spectra, and melting point range (not reported) are in accordance with literature data.[20]

4.3. Synthesis of (1*S*,4*S*,5*R*,6*R*)/(1*R*,4*R*,5*S*,6*S*)-5,6-dibromocyclohex-2-ene-1,4-diol, (±)-3

Compound (±)-**2** (54.64 g, 0.2 mol) was dissolved in 900 ml of distilled diethyl ether and cooled in an ice bath. After the addition of 300 ml of water, $NaBH_4$ (19.0 g, 0.50 mol) was added over 30 min with stirring. After 2.5 h, the clarified organic phase was extracted with water, dried over Na_2SO_4 and taken to dryness to yield compound (±)-**3** (47.3 g, 0.17 mol, 87% yield) as a white solid (mp 110–111 °C). MS (ESI+): *m*/*z* 295 $[M+Na]^+$. ^1H NMR ($(CD_3)_2CO$): 5.73 (2H, s, H-2, H-3); 4.85 (2H, d, $J = 6.5$, OH); 4.6–4.4 (2H, m, H-1, H-4); 4.4–4.3 (2H, m, H-5, H-6). ^{13}C NMR ($(CD_3)_2CO$): 131.1 (2C, C-2, C-3); 73.9 (2C, C-1, C-4); 61.9 (2C, C-5, C-6). Anal. Calcd for $C_6H_8Br_2O_2$: C, 26.50; H, 2.97. Found: C, 26.61; H, 2.99.

4.4. Synthesis of (3*S*,4*S*,5*S*,6*S*)/(3*R*,4*R*,5*R*,6*R*)-3,4:5,6-diepoxycyclohex-1-ene, (±)-4

Compound (±)-**3** (2.0 g, 7.35 mmol) was dissolved in 30 ml of anhydrous tetrahydrofuran, under an argon atmosphere at 0 °C, and KOH (4.7 g, 0.08 mol) was slowly added to the solution. After 2 h, another portion of KOH (1.7 g, 0.03 mol) was added, and the solution stirred continually for a further 2 h, centrifuged at 4000 rpm and taken to dryness to yield compound (±)-**4** (783 mg, 7.11 mmol, 96.8% yield) as a white solid. ^1H and ^{13}C NMR spectra, and melting point range (not reported) are in accordance with literature data.[20]

4.5. Synthesis of (1*R*,2*S*,3*S*,4*S*)/(1*S*,2*R*,3*R*,4*R*)-1-cyano-3,4-epoxy-2-trimethylsilylcyclohex-1-ene, (±)-5

CaO (2 g, 0.036 mol) and Me_3SiCN (2 ml, 15.9 mmol) were added to a solution of (±)-**4** (485 mg, 4.40 mmol) in 14 ml of CH_2Cl_2/hexane 1:1, and the mixture was stirred for 24 h at room temperature. The solution was filtered and concentrated under a nitrogen stream, producing a mixture containing compound (±)-**5** that could not be isolated because of its instability.

MS (ESI+): *m*/*z* 216 $[M+Na]^+$.

4.6. Synthesis of (1*R*,2*S*,3*S*,4*R*)/(1*S*,2*R*,3*R*,4*S*)-1-cyano-2,3,4-trihydroxy-cyclohex-5-ene (±)-6 and *trans*-3-cyanocyclohexa-3,5-diene-1,2-diol (±)-7

A mixture containing compound (±)-**5** was sequentially dissolved in 500 μl of THF, and 10 ml of H_2O. An initial volume of CF_3COOH (600 μl) was added dropwise to the solution and a second amount (500 μl) was added 1 h later, with the mixture continually stirred. After 1 h, the reaction was stopped by evaporating the solution under vacuum. The mixture, after being purified by silica gel column chromatography using $MeOH/CH_2Cl_2$ 1:9 as the eluents, yielded compound (±)-**6** (171.4 mg, 1.10 mmol, 25% yield), and compound (±)-**7** (259.2 mg, 1.89 mmol, 43% yield), both in the form of oils.

4.6.1. (1*R*,2*S*,3*S*,4*R*)/(1*S*,2*R*,3*R*,4*S*)-1-Cyano-2,3,4-trihydroxy-cyclohex-5-ene (±)-6

MS (ESI+): *m*/*z* 178 $[M+Na]^+$. ^1H NMR ($(CD_3)_2SO$): 5.69 (1H, d, OH), 5.65 (1H, ddd, *J* = 10.0, 2.3, 2.7 Hz, H-5), 5.47 (1H, ddd, *J* = 10.0, 2.0, 2.1 Hz, H-6), 5.23 (1H, d, OH), 5.14 (1H, d, OH), 3.88 (1H, dd, J = 2.1, 7.9 Hz, H-1), 3.53 (1H, t, *J* = 9.7 Hz, H-3), 3.42 (1H, ddd, *J* = 9.7, 2.9, 2.2 Hz, H-4), 3.17 (1H, dd, *J* = 9.7, 8.0 Hz, H-2). ^{13}C NMR ($(CD_3)_2SO$): 134.4 (1C, C-5), 120.9 (1C, CN), 119.5 (1C, C-6), 76.6 (1C, C-2), 71.4 (2C, C-1, C-3), 36.6 (1C, C-4). Anal. Calcd for $C_7H_9NO_3$: C, 54.19; H, 5.85; N, 9.03. Found: C, 54.00; H, 5.91; N, 9.05.

4.6.2. *trans*-3-Cyanocyclohexa-3,5-diene-1,2-diol (±)-7

MS (ESI+): *m*/*z* 160 $[M+Na]^+$. ^1H NMR (CD_3OD): 6.77 (1H, dd, *J* = 5.60, 1.21 Hz, H-4), 6.23 (1H, ddd, *J* = 9.8, 3.1, 1.0 Hz, H-6), 6.07 (1H, ddd, *J* = 9.7, 5.5, 1.6 Hz, H-5), 4.34 (1H, ddd, *J* = 9.6, 3.1, 1.6 Hz, H-1), 4.30 (1H, dd, *J* = 9.6, 1.2 Hz, H-2). ^{13}C NMR (CD_3OD): 138.2 (1C, C-4), 136.8 (1C, C-6), 122.5 (1C, C-5), 117.9 (1C, C-3), 114.9 (1C, CN), 71.8 (1C, C-1), 71.2 (1C, C-2). Anal. Calcd for $C_7H_7NO_2$: C, 61.31; H, 5.14; N, 10.21. Found: C, 61.57; H, 5.19; N, 10.16.

4.7. Synthesis of (1*R*,2*S*,3*R*,4*S*)/(1*S*,2*R*,3*S*,4*R*)-5-cyano-1,2,3,4-tetrahydroxycyclohex-5-ene (±)-8

At first, *m*-CPBA (90 mg, 0.52 mmol) was added to a solution of (±)-**7** (43.7 mg, 0.32 mmol) in 5 ml of CH_2Cl_2, and the mixture stirred at room temperature for 1 h. After evaporating the solvent under vacuum, the mixture was redissolved in 50 μl of THF and 10 ml of H_2O, and CF_3COOH (200 μl) was added. After 3 h of continuous stirring, the mixture was filtered, evaporated under vacuum and purified by silica gel column chromatography using $MeOH/CH_2Cl_2$ 1:4 as the eluents, yielding compound (±)-**8** as an oil (27 mg, 0.16 mmol, 50% yield). MS (ESI+): *m*/*z* 194 $[M+Na]^+$. ^1H NMR (CD_3OD): 6.56 (1H, d, *J* = 2.9 Hz, H-6), 4.24 (1H, dd, *J* = 6.4, 2.7 Hz, H-1), 4.15 (1H, d, *J* = 4.6 Hz, H-4), 3.84 (1H, dd, *J* = 4.6, 2.3 Hz, H-3), 3.77 (1H, dd, *J* = 6.4, 2.2 Hz, H-2). ^{13}C NMR (CD_3OD): 147.4 (1C, C-6), 118.8 (1C, C-5), 116.8 (1C, CN), 73.9 (1C, C-4), 72.4 (1C, C-1), 70.7 (1C, C-3), 69.6 (1C, C-2). Anal. Calcd for $C_7H_9NO_4$: C, 49.12; H, 5.30; N, 8.18. Found: C, 48.90; H, 5.31; N, 8.12.

4.8. Microorganisms and cultures

The bacterial strains were maintained at 4 °C on meat peptone agar (in g/L, Bacto beef extract 3, peptone 10, NaCl 5, agar 15). *R. erythropolis* A4[28] was grown for 2 days at 28 °C in shaken 500-mL Erlenmeyer flasks containing 100 mL of basal salts broth according to Di Geronimo and Antoine,[29] supplemented with 10 g/L of glycerol and 3 g/L of yeast extract. *Nocardia globerula* NHB-2[22a] and *Rhodococcus* sp. NDB 1165[22b] were grown in the presence of isobutyronitrile (nitrilase inducer) as described previously.[30] *F. solani* O1 was maintained at 4 °C on a modified Czapek-Dox agar[31] and grown in the presence of 2-cyanopyridine (nitrilase inducer) as described previously.[32]

4.9. Preparation of whole-cell catalysts

Whole cells of bacteria were harvested by centrifugation and washed with Tris–HCl buffer (50 mM, pH 8). The *F. solani* mycelium was harvested by filtration, washed with the same buffer and lyophilized. Bacterial biomass was immediately used for biotransformations. The lyophilized fungal mycelia were stored at −20 °C until use, the nitrilase activity being stable for several months under these conditions.

The activities of nitrile-transforming enzymes and amidase were determined with benzonitrile and benzamide, respectively, using the previously described assays.[33,34] The nitrile hydratase and amidase activities in whole cells of *R. erythropolis* A4 were approx. 1.4 and 0.3 U mg^{-1} of dry cell weight, respectively. The nitrilase activities of *Nocardia globerula* NHB-2, *Rhodococcus* sp. NDB 1165 and *F. solani* were approx. 1.9, 1.1 and 0.5 U mg^{-1} of dry cell weight, respectively.

4.10. Preparation of cell-free extract of *R. erythropolis* A4

Whole cells of *R. erythropolis* A4 suspended in Tris/HCl buffer (50 mM, pH 8) were disrupted using a Retsch MM-200 oscillation mill as described previously.[33] The cell extracts were immediately used for biotransformations. The activities of nitrile hydratase and amidase were approx. 3 and 0.7 U mg^{-1} of protein, respectively.

4.11. General procedure for biotransformations

A 10 mM solution of substrate was prepared using a suspension of biomass in Tris–HCl buffer (50 mM, pH 8), having an optical density (OD) value of approx. 15. A percentage of MeOH up to 5% was previously used to solubilise the substrate. The suspension was shaken (200 rpm) at 35 °C; aliquots were drawn at regular time intervals and analysed by TLC ($MeOH/CH_2Cl_2$ 1:1).

4.12. Biotransformation of (±)-6 catalysed by whole cells from *R. erythropolis* A4

Compound (±)-**6** (14 mg, 0.09 mmol) and undecane (14 μl, internal standard for gas chromatography analysis) were dissolved in 500 μl of MeOH, and added to a suspension containing whole cells from *R. erythropolis* A4 (OD = 16; approx. 3.7 mg dry cell weight mL^{-1}) in Tris–HCl buffer (50 mM). The mixture was shaken (200 rpm) at 35 °C for 2 days; the reaction was stopped by centrifugation of the solution, and evaporation under vacuum of the supernatant at 40 °C. The mixture was purified by silica gel column chromatography using $MeOH/CH_2Cl_2$ 1:1 as the eluents, and yielded compound (−)-**9** (3.0 mg, 0.017 mmol, 20% yield, ee = 91.0%), compound (+)-**10** (5.3 mg, 0.030 mmol, 34% yield, ee = 74.5%), and (−)-**6** as an unreacted substrate (4.2 mg, 0.027 mmol, 30% yield, ee = 43%).

The course of the reaction, including the concentration and enantiomeric excesses values, was monitored by withdrawing aliquots at regular time intervals that, after centrifugation, lyophilisation and derivatization (see below), were analyzed by gas chromatography.

Derivatization protocol: a 200 μl sample was added to 100 μl of a sylilating mixture (hexamethyldisilazane/trimethylchlorosilane/pyridine, 3:1:9), and stirred at 40 °C for 30 min.

Fast-GC conditions: injector temperature 250 °C; detector temperature 280 °C; oven 100 °C for 1 min then 10 °C/min up to 280 °C for 1 min. Chiral-GC conditions: injector temperature 250 °C;

detector temperature 250 °C; oven 100 °C for 1 min then 0.4 °C/min up to 200 °C for 1 min.

4.12.1. (−)-(1*R*,2*S*,3*S*,4*R*)-1-Carboxamido-2,3,4-trihydroxy-cyclohex-5-ene, (−)-9

MS (ESI+): *m*/*z* 174 $[M+Na]^+$; ^1H NMR (CD_3OD): 5.66 (1H, dt, *J* = 10.0, 2.4 Hz, H-6), 5.55 (1H, dt, J = 10.4, 2.4 Hz, H-5), 4.10–4.05 (1H, m, H-1), 3.82 (1H, t, *J* = 9.7 Hz, H-3), 3.44 (1H, dd, *J* = 10.1, 8.0 Hz, H-2), 3.15–3.06 (1H, m, H-4). ^{13}C NMR (CD_3OD): 176.0 (1C, -$CONH_2$), 130.8 (1C, C-6), 123.9 (1C, C-5), 76.9 (1C, C-2), 71.8 (1C, C-1), 70.9 (1C, C-3), 50.9 (1C, C-4). $[\alpha]_D = -180.3$ (*c* 0.150, MeOH, ee = 91.0%). Anal. Calcd for $C_7H_{11}NO_4$: C, 48.55; H, 6.40; N, 8.09. Found: C, 48.67; H, 6.46; N, 8.17.

4.12.2. (+)-(1*S*,2*R*,3*R*,4*S*)-2,3,4-trihydroxy-cyclohex-5-en-1-carboxylic acid, (+)-10

MS (ESI-): *m*/*z* 173 $[M-H]^-$; ^1H NMR (CD_3OD): 5.76 (1H, d, *J* = 10.1, 2.0 Hz, H-6), 5.55 (1H, d, *J* = 10.4, 2.3 Hz, H-5), 4.10–4.05 (1H, m, H-1), 3.87 (1H, t, *J* = 9.6 Hz, H-3), 3.47 (1H, dd, *J* = 10.1, 7.6 Hz, H-2), 3.00–2.90 (1H, m, H-4). ^{13}C NMR (CD_3OD): 178.6 (1C, –COOH), 128.3 (1C, C-5), 126.3 (1C, C-6), 76.9 (1C, C-2), 72.2 (1C, C-4), 71.7 (1C, C-3), 52.9 (1C, C-1). $[\alpha]_D = +128.2$ (*c* 0.265, MeOH, ee = 74.5%). Anal. Calcd for $C_7H_{10}O_5$: C, 48.29; H, 5.79. Found: C, 48.48; H, 5.74.

4.13. Synthesis of (1*S*,2*R* 3*R*,4*S*)-2,3,4-trihydroxy-cyclohex-5-en-1-methylcarboxylate, 11

At first, HCl (100 μl) was added to a solution of (+)-**10** (4.5 mg, 0.025 mmol) in 2 ml of MeOH, and the mixture stirred under refluxing conditions for 8 h. After the addition of water, the reaction mixture was repeatedly partitioned with AcOEt; the final organic phase was dried over anhydrous Na_2SO_4 and taken to dryness to yield compound (1*S*,2*R*,3*R*,4*S*)-2,3,4-trihydroxy-cyclohex-5-en-1-methylcarboxylate, **11** (3.7 mg, 0.019 mmol, 76% yield) in the form of an oil. MS (ESI+): *m*/*z* 211 $[M+Na]^+$; ^1H NMR (CD_3OD): 5.75–5.70 (1H, m, H-6), 5.60–5.50 (1H, m, H-5), 4.03–3.97 (1H, m, H-1), 3.75–3.70 (1H, m, H-3), 3.48–3.42 (1H, m, H-2), 2.99–2.94 (1H, m, H-4). ^{13}C NMR (CD_3OD): 173.0 (1C, -CO), 127.3 (1C, C-6), 125.3 (1C, C-5), 76.9 (1C, C-2), 72.7 (1C, C-3), 70.2 (1C, C-4), 53.9 (1C, C-1), 52.2 (1C, -OCH_3). Anal. Calcd for $C_8H_{12}O_5$: C, 51.06; H, 6.43. Found: C, 50.87; H, 6.50.

4.14. Synthesis of (−)-(1*R*,2*R*,3*R*,4*S*)-1-(hydroxymethyl)cyclohex-5-en-2,3,4-triol (−)-12

At first, $NaBH_4$ (4.3 mg, 0.114 mmol) was added to a solution of (1*S*,2*R*,3*R*,4*S*)-2,3,4-trihydroxy-cyclohex-5-en-1-methylcarboxylate, **11** (3.7 mg, 0.019 mmol) in 2 ml of distilled THF/MeOH (1:3), and the mixture kept agitated for 30 min. After the addition of water, the reaction mixture was extracted with AcOEt; the final organic phase was dried over anhydrous Na_2SO_4 and taken to dryness to yield compound (−)-**12** (3.0 mg, 0.018 mmol, 96% yield) in the form of an oil. MS (ESI+): *m*/*z* 183 $[M+Na]^+$; ^1H NMR data (not reported) are in accordance with literature data.[25] $[\alpha]_D = -11.94$ (*c* 0.3, MeOH, ee = 74.5%), lit. $[\alpha]_D = -13.4$ (*c* 1.1, MeOH).[25] Anal. Calcd for $C_7H_{12}O_4$: C, 52.49; H, 7.55. Found: C, 52.62; H, 7.61.

4.15. Biotransformation of (±)-7 catalysed by whole cells from *R. erythropolis* A4

Compound (±)-**7** (15 mg, 0.11 mmol) was dissolved in 550 μl of MeOH, and added to a suspension containing whole cells from *R. erythropolis* A4 (OD = 17; approx. 3.9 mg dry cell weight mL^{-1}) in Tris–HCl buffer (50 mM, pH 8). The mixture was shaken (200 rpm) at 35 °C for 2 h, then stopped by centrifugation of the solution, and evaporation under vacuum of the supernatant at 40 °C. The mixture was purified by silica gel column chromatography using MeOH/CH_2Cl_2 1:1 as the eluants, and yielded compound (−)-**13** (7.7 mg, 0.050 mmol, 45% yield, ee = 27.0%), and compound (+)-**14** (8.3 mg, 0.053 mmol, 48.6% yield, ee = 25.0%). The course of the reaction was monitored by Fast-GC using the same protocols and device conditions as described above for the biotransformation of compound (±)-**6**. The enantiomeric excesses values were obtained by chiral HPLC analysis using a Chiralcel® OJ column and hexane/ethanol 9:1 as the mobile phase for compound **7**, and a Chiralcel® OD column and hexane/*i*-propanol 85:15 as the mobile phase for compound **13**.

4.15.1. (−)-(1*R*,2*R*)-*trans*-3-Carboxyamidocyclohexa-3,5-diene-1,2-diol, (−)-13

MS (ESI+): *m*/*z* 178 $[M+Na]^+$; ^1H NMR (CD_3OD): 6.91–6.88 (1H, m, H-4), 6.19 (1H, dd, *J* = 3.6 Hz, H-6), 6.09 (1H, dd, *J* = 1.6, 4.0 Hz, H-5), 4.62–4.58 (1H, m, H-1), 4.32–4.27 (1H, m, H-2). ^{13}C NMR (CD_3OD): 171.9 (1C, $CONH_2$), 134.7 (1C, C-4), 133.6 (1C, C-3), 130.5 (1C, C-6), 124.6 (1C, C-5), 73.8 (1C, C-2), 71.1 (1C, C-1). $[\alpha]_D = -0.8$ (*c* 0.385, MeOH, ee = 27.0%). Anal. Calcd for $C_7H_9NO_3$: C, 54.19; H, 5.85; N, 9.03. Found: C, 53.92; H, 5.79; N, 9.06.

4.15.2. (+)-(1*S*,2*S*)-*trans*-3-Carboxylic-cyclohexa-3,5-diene-1,2-dihydroxy acid, (+)-14

MS (ESI-): *m*/*z* 155 $[M-H]^-$. ^1H NMR (CD_3OD): 6.90–6.86 (1H, m, H-4), 6.39–6.33 (1H, m, H-6), 6.29–6.24 (1H, m, H-5), 4.60–4.54 (1H, m, H-1), 4.30–4.23 (1H, m, H-2). ^{13}C NMR (CD_3OD): 171.3 (1C, COOH), 134.4 (1C, C-4), 130.5 (1C, C-3), 124.5 (1C, C-5), 73.9 (1C, C-2), 71.2 (1C, C-1). $[\alpha]_D = +1.0$ (*c* 0.415, H_2O, ee = 25.0%); lit. $[\alpha]_D = +3.9$ (*c* 0.1, H_2O).[26] Anal. calcd for $C_7H_8O_4$: C, 53.85; H, 5.16. Found: C, 53.65; H, 5.22.

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