(*S,S*)-2,3-Dihydroxy-2,3-dihydrobenzoic Acid: Microbial Access with Engineered Cells of *Escherichia coli* and Application as Starting Material in Natural-Product Synthesis

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Abstract: Cyclohexadiene-*trans*-5,6-diols such as (*S,S*)-2,3-dihydroxy-2,3-dihydrobenzoic acid (2,3-*trans*-CHD) have been shown to be of importance as chiral starting materials for the syntheses of bioactive substances, especially for the syntheses of carbasugars. By using methods of metabolic-pathway engineering, the *Escherichia coli* genes *entB* and *entC*, which encode isochorismatase and isochorismate synthase, were cloned and over-expressed in *E. coli* strains with a deficiency of *entA*, which encodes 2,3-dihydroxybenzoate synthase. A 30-fold increase in the corresponding EntB/EntC enzyme activities affects the accumulation of 2,3-*trans*-CHD in the cultivation medium. Although the strains did not contain deletions in chorismate-utilising pathways towards aromatic amino acids, neither chorismate nor any other metabolic intermediates were found as by-products. Fermentation of these strains in a 30 L pH-controlled stirred tank reactor showed that 2,3-*trans*-CHD could be obtained in concentrations of up to 4.6 gL^{-1}. This demonstrates that post-chorismate metabolites are accessible on a preparative scale by using techniques of metabolic-pathway engineering. Isolation and separation from fermentation salts could be performed economically in one step through anion-exchange chromatography or, alternatively, by reactive extraction. Starting from 2,3-*trans*-CHD as an example, we established short syntheses towards new carbasugar derivatives.

Keywords: arene diols · bioorganic chemistry · carbohydrate mimetics · chorismate · enzyme catalysis · shikimate

Introduction

Intermediates in the common biosynthetic pathway of aromatic amino acids (AAA pathway) like shikimate[1] and quinate[1a–c, 2] have emerged as valuable precursors in the syntheses of natural products and pharmacologically active substances. Starting from quinic acid, a synthesis towards, say, the immunosuppressant FK506[3] has been established. Additionally, shikimic acid has been used as a starting material for the generation of a large combinatorial compound library.[4] The neuraminidase inhibitor GS-4104[1a–c] and its derivatives[1d, 5] have recently been synthesised starting from quinic acid and shikimic acid.

Much effort has been concentrated on the molecular characterisation of enzymes and their mechanisms that are involved in the AAA pathway. This has finally led to substantial progress in microbially producing many new chiral pool substances on an industrial scale, including, for example,

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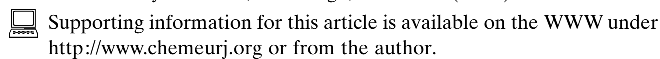
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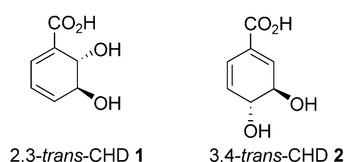
dehydroshikimate,[6] shikimate,[7] dehydroquinate[8] and quinate.[7b, 9]

Nevertheless, metabolites derived from chorismate, the metabolic branch point in the biosynthesis of ubiquinones, menaquinones, folates and aromatic amino acids, have not been examined in the same way. This may be due to the fact that chorismate itself has not yet been microbially produced in high concentrations.[10]

Recently, increasing attention has been paid to substituted cyclohexadiene-*trans*-diols (*trans*-CHDs). Bearing in mind that they are small and highly functionalised chiral molecules,



it is obvious that they can be regarded as valuable building blocks for a wide variety of target molecules. Substances such as plant metabolites of the cyclohexane epoxide class and valienamine have already been synthesised starting from *trans*-CHDs.[11]

trans-CHDs are also found in bacteria, plants and fungi, as well as in yeast, as intermediates of the post-chorismate pathway towards enterobactin and menaquinones[12] or are biosynthesised from shikimate.[13] Therefore, they should also, in principle, be accessible by techniques of metabolic-pathway engineering.

As has been shown by Leistner and co-workers,[14] strains of *Klebsiella pneumoniae* with deficiencies in the AAA pathway excrete two different *trans*-CHDs, the (*S*,*S*)-2,3-dihydroxy-2,3-dihydrobenzoic acid (2,3-*trans*-CHD, **1**) and the (*R*,*R*)-3,4-dihydroxy-3,4-dihydrobenzoic acid (3,4-*trans*-CHD, **2**) when enzymes catalysing the reaction of chorismate (**3**) towards these metabolites are overproduced. However, the applicability of these microbial systems for preparative purposes has been limited by the low final product concentrations (up to 200 $mg L^{-1}$) and by the occurrence of chorismate as an ubiquitous by-product, necessitating further purification processes.[14]

In this article we describe the construction and characterisation of recombinant *Escherichia coli* strains for the efficient microbial production of 2,3-*trans*-CHD **1**, which has been published in a preliminary form.[15] The microbial production could easily be scaled up, and **1** was obtained on a higher decagram scale. Efficient isolation from the fermentation broth was achieved by ion-exchange chromatography or, alternatively, by reactive extraction. The potential of enantiopure **1** as a valuable synthetic building block in the chemistry of carbohydrate mimetics is demonstrated through the straightforward synthesis of a carbohydrate analogue.

Abstract in German: *Cyclohexadien-*trans*-diole wie beispielsweise (5S,6S)-Dihydroxycyclohexa-1,3-diencarbonsäure (2,3-*trans*-CHD) haben sich als wichtige chirale Ausgangsverbindungen zur Synthese pharmakologisch aktiver Substanzen, insbesondere zur Synthese von Carbazuckern und Aminocarbazuckern erwiesen. Unter Verwendung von Techniken der Stoffflussderegulation wurden die* Escherichia coli-*Gene* entB *und* entC*, kodierend für Isochorismatase und Isochorismatsynthase, in* E. coli-*Stämmen überexprimiert, welche eine Defizienz von entA, kodierend für 2,3-Dihydroxybenzoatsynthase, hatten. Eine 30-fache Steigerung der entsprechenden Enzymaktivitäten EntB/EntC bewirkte die Bildung von 2,3-*trans-*CHD im Kultivierungsmedium. Obwohl die Stämme keine Mutationen in den chorismatverwendenden Biosynthesewegen zu den aromatischen Aminosäuren besaßen, wurde weder Chorismat noch ein anderes metabolisches Intermediat als Nebenprodukt gefunden. Fermentationen mit diesen Stämmen in einem pH-geregelten 30 L Rührkesselreaktor zeigten, dass 2,3-*trans-*CHD in Konzentrationen bis zu 4.6 g L^{-1} erhalten werden kann. Dies demonstriert zum ersten Mal, dass Metabolite des Enterobactin-Biosyntheseweges durch Techniken der Stoffflussderegulation in präparativem Maßstab erhalten werden können. Aufreinigung und Separation von den Fermentationssalzen konnte auf effiziente Weise in einem Schritt unter Verwendung von Anionentauscherharzen oder alternativ durch Reaktivextraktion durchgeführt werden. Ausgehend von 2,3-*trans-*CHD haben wir exemplarisch eine kurze Synthese zu* ent-*Streptol (ent-Valienol) durchgeführt.*

Results and Discussion

Our strategic approach to strains for the production of 2,3-*trans*-CHD **1** is in analogy to work with *K. pneumoniae* strains (Scheme 1).[14]

Nonpathogenic *E. coli* was selected as a host strain for genetic modification because of the availability of potent mutants and well-established fermentation conditions.

An increase in the metabolic production of **1** was achieved by over-expression of *entC*, which encodes isochorismate synthase, and *entB*, which encodes isochorismatase. In order to prevent product loss due to metabolic processing of **1**, an *entA*-negative mutant was used as the production host.

Isolation and cloning of *entB* and *entC*: *entB* and *entC* were isolated from *E. coli* W3110[16] (wild-type). Both fragments were inserted into plasmid pJF119EH1,[17] either separately or in tandem, to give plasmids pDF1, pDF2 and pDF3.

DNA sequencing of the cloned fragments proved to be in conformity with literature data,[18] except for a point mutation in *entB* at bp 525 (gcg → gct), which should not result in any change of the protein sequence (quiet mutation).

Cells of *E. coli* cloning strain DH5α,[19] *E. coli* wild-type strain W3110,[16] *E. coli entA*$^-$ mutants AN193[20, 21] and H1882[22] and *E. coli entC*$^-$/*menF*$^-$ mutant PBB8[23] were transformed with the plasmids pDF1, pDF2 or pDF3. Additionally, transformations were performed with the "empty" plasmid pJF119EH1. Transformants were grown on LB medium in the presence of IPTG (100 μM). SDS-PAGE analysis of the crude cell extracts showed expression of the inserted gene fragments in all cases.

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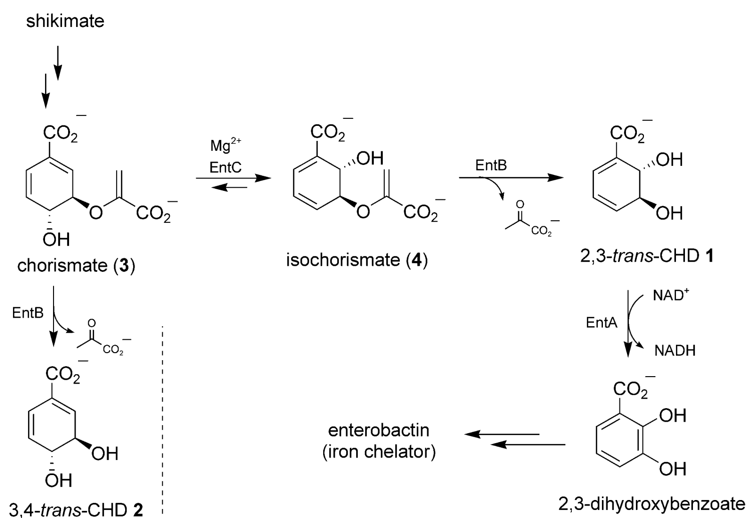

Scheme 1. Biosynthesis of *trans*-CHD starting from chorismate (**3**). The reaction towards 2,3-*trans*-CHD **1** via isochorismate (**4**) is catalysed by isochorismate synthase EntC and isochorismatase EntB. *trans*-CHD **1** is metabolised to 2,3-dihydroxybenzoate by 2,3-dihydroxybenzoate synthase EntA. 3,4-*trans*-CHD **2** is a non-natural metabolite and is formed out of chorismate under a large excess of EntB.

In extracts of cells containing the empty plasmid pJF119EH1 or cells without any plasmids, only a very slow reaction of chorismate (**3**) to isochorismate (**4**) and 2,3-*trans*-CHD **1** was observed (Figure 1A). Nevertheless, an almost linear conversion of **3** to other undetectable products was observed ($t_{1/2} = 15$ h). Eliminating the thermal decomposition of **3** ($t_{1/2} = 28$ h; applying the same conditions) in enzyme balancing, a total enzymatic chorismate-degradation activity of 21 mU per mg of protein was calculated.

Crude cell extract of strains with plasmid pDF1, harbouring *entB*, showed a linear increase of the concentration of 3,4-*trans*-CHD **2** as a major product and 2,3-*trans*-CHD **1** as a minor product (Figure 1B). Isochorismate (**4**) could not be detected at any time. The formation of 3,4-*trans*-CHD **2** verifies that EntB is overproduced in a catalytically active form. The production of small amounts of 2,3-*trans*-CHD **1** indicates the weak activity of chromosomally encoded EntC in *E. coli* strains AN193, H1882, DH5α and W3110.

Enzyme-activity test: The enzyme activities of EntC and EntB were determined in cell-free extracts of IPTG-induced cells by monitoring the conversion of chorismate (**3**) to either 3,4-*trans*-CHD **2** (EntB) or to isochorismate (**4**) and 2,3-*trans*-CHD **1** (EntC + EntB). This was performed in a buffered aqueous solution of the cell-free extracts for a period of 400 minutes. Time-course data of the analysis of the *entA*$^-$ strain AN193 are shown in Figure 1.

Cell-free extracts of strains containing plasmid pDF2, harbouring *entC*, catalysed the reaction of chorismate (**3**) to isochorismate (**4**, major product) and 2,3-*trans*-CHD **1** (minor product) (Figure 1C). The slow and nearly linear increase of the concentration of **1** can be attributed to the wild-type activity of EntB.

Analysis of crude cell extract of strains in which *entB* and *entC* were over-expressed in parallel (pDF3) verified the conversion of chorismate (**3**) to 2,3-*trans*-CHD **1** via intermediate isochorismate (**4**) (Figure 1D). The linearity of increase of the concentration of **1** and the formation of **4** as an intermediate results from the catalysis by EntB as the rate-limiting step.

Analogous experiments with cell-free extract of strains H1882, W3110 or DH5α showed, within the limits of measurement accuracy, approximately the same enzyme activities for natural and over-expressed EntB and EntC. No product loss of 2,3-*trans*-CHD **1** or 3,4-*trans*-CHD **2** due to metabolic processing in strains

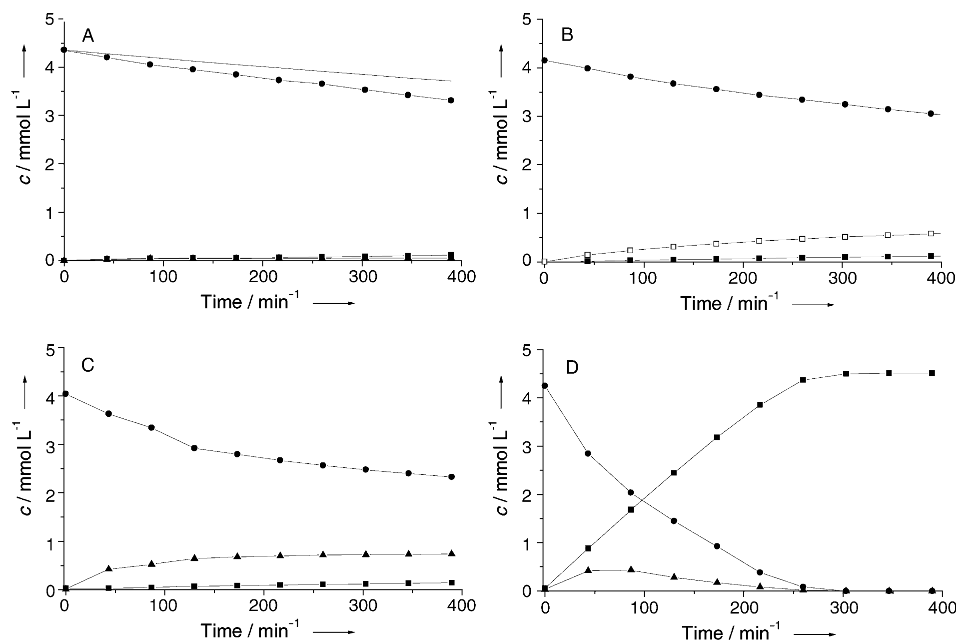

Figure 1. Determination of enzyme activities in the crude cell extract of transformants of *E. coli* strain AN193 at 20°C (chorismate 8 mM). The change of concentration of reactants and products was monitored by HPLC. A) Transformants with plasmid pJF119EH1 catalyse the degradation of chorismate (**3**, ●) slowly. The products are 2,3-*trans*-CHD **1** (■) and isochorismate (**4**, ▲) in equal parts. As a reference, time-course studies of the nonenzymatic decomposition of chorismate in buffered solution showed slower degradation under the same conditions (——). B) Transformants with plasmid pDF1 (EntB is over-expressed) mainly catalyse the reaction of **3** to 3,4-*trans*-CHD **2** (□) and small amounts of **1**. C) Transformants with pDF2 (EntC is over-expressed) catalyse the reaction of **3** to **4** and small amounts of **1**. D) Transformants with plasmid pDF3 containing *entB* and *entC* completely convert **3** to **1** with **4** as a temporarily formed intermediate.

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W3110 or DH5α was observed. Cell-free extract of *E. coli* strain PBB8 (*entC*⁻/*menF*⁻-mutation) is characterised by the absence of natural isochorismate synthase activity (below quantification limit). Catalytic activities for plasmid-encoded EntB and/or EntC were much lower in this strain; however, this observation was examined not in detail.

From the decrease in chorismate concentration (**3**) and increase of secondary-metabolite concentrations, absolute activities of EntB and EntC were quantified relative to total protein concentrations (Table 1).

Table 1. Enzyme activities of cell extracts in Tris-HCl buffer (100 mM, pH 8.5) and at 20 °C depending on the genes over-expressed in strains AN193 and PBB8.

Strains		Plasmid-borne genes	Enzyme activity [mU per mg protein] EntC	EntB (c)[b]	EntB (isoc)[c]
AN193	/pJF119EH1	–	5	< 0.25	3
	/pDF1	*entB*	7	77	n.d.[a]
	/pDF2	*entC*	224	n.d.	< 7
	/pDF3	*entB*, *entC*	212	n.d.	442
PBB8	/pJF119EH1	–	< 0.25	< 0.25	n.d.
	/pDF1	*entB*	< 0.25	40	n.d.
	/pDF2	*entC*	34	< 0.25	< 0.25
	/pDF3	*entB*, *entC*	74	32	> 74

[a] n.d. = not determined. [b] chorismate. [c] isochorismate.

In summary, EntC activity in all the tested *E. coli* strains was amplified at least 30-fold by plasmidic over-production relative to strains in which EntC is not overproduced, amounting to more than 210 mU per mg of protein (strains DH5α, W3110, AN193 and H1882). The amplification of EntB activity was even higher (almost 150-fold) amounting to 442 mU per mg of protein. Similar amplification factors were observed in strains H1882, DH5α and W3110.

Fermentation experiments for the production of 1: Cultivation of the *E. coli* strains in shaking flasks proved that **1** was excreted into the medium by strains containing pDF3 (over-expressing *entB*/*entC*), whereas **1** was not detected in suspensions of any other transformants, plasmid-free strains or uninduced strains. Interestingly, neither 3,4-*trans*-CHD **2** nor isochorismate (**4**) was detected in suspensions of any transformants or in strains containing pDF1 or pDF2.

Production rates of **1** in mineral-salt media were best with *entA*⁻ mutants AN193 (29 mg h^{-1} per g of dry cell mass (DCM) and H1882, moderate in *entC*⁻/*menF*⁻ strain PBB8 and low in wild-type strains W3110 and DH5α (Figure 2). With regard to production rates, the excretion of **1** was threefold higher in *entA*⁻ strains than in strains without *entA* defect.

The final product concentration of **1** after 14 h was significantly higher in strains PBB8, AN193 and HI1882 than in wild-type strains. Maximum concentrations of 550 mg L^{-1} (3.5 mM) were achieved in shaking-flask experiments with strain AN193.

Several attempts to determine intracellular metabolite concentrations by analysing crude extracts of induced cells with RP-HPLC failed, presumably because the metabolite

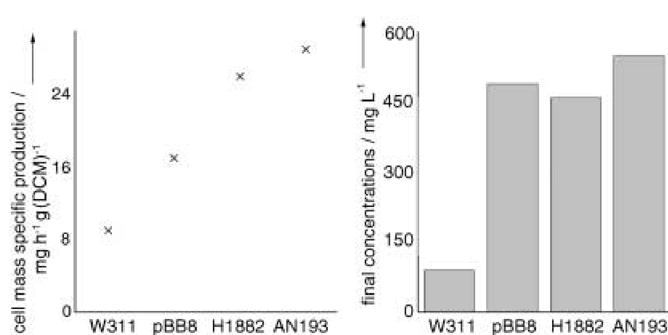

Figure 2. Production rates and final concentrations of **1** for cultivation of strain AN193/pDF3 in mineral salts medium.

concentrations were below the detection limit (7 μM). Even in extracts of highly concentrated cell suspensions (DCM > 21 g L^{-1}) no metabolites **1**–**4** were detected. Concentrations of 2,3-*trans*-CHD **1** in the cell-free extracts were estimated to be below 45 μg per g of DCM in any case, corresponding to an intracellular concentration of less than 86 μmol L^{-1} (cell volume).[24]

Other experiments in shake flasks showed that optimal production conditions, with regard to production rate, growth rate and long-term stability, were 37 °C and a pH between 6 and 8. Alternative carbon sources like galactose, fructose, glycerol, acetate or lactate could also be used for production. The use of glycerol or galactose allows even better cell-mass-specific product-formation rates of up to 48 mg h^{-1} per g of DCM.

Various experiments in which the inductor IPTG was added at different cell growth phases showed that induction only temporarily reduced growth rate for 4 to 5 hours and only after the first addition. If IPTG was added directly at the very beginning of incubation, no reduction of growth rate or final cell density was observed. Formation of **1** started at the very beginning of induction, and was found in both growing and resting cells. Almost maximal induction was achieved with inductor concentrations as low as 50 μM (IPTG).

In order to obtain **1** on a preparative scale, strain AN193/pDF3 was cultivated in a 30 L pH-controlled stirred tank reactor (STR, process volume 20 L) in a mineral-salts medium with glucose as the carbon source. Production was monitored over a period of 50 h. Maximal cell-mass-specific product-formation rates of 58 mg h^{-1} per g of DCM were found 3 hours after induction. A molar yield of 17 % was calculated with respect to glucose. Final product concentrations of more than 4.6 g L^{-1} (**1**) were achieved.

Methods for isolation and purification of 1: One major challenge for separating **1** from fermentation salts, proteins and other organic compounds is its instability under strongly acidic or strongly basic conditions or if heated. On the other hand, **1** was found to be stable in aqueous solution at moderate pH ($3 \leq pH \leq 11$) and room temperature in the absence of oxidising agents.

All attempts to extract **1** from acidified fermentation permeate (pH 3) by using alkanes, chloroalkanes, diphenyl ether and similar solvents were characterised by low extraction coefficients ($\varepsilon \leq 7\,\%$). Nevertheless, using more polar

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extraction solvents like short-chain alcohols significantly improves the extraction coefficient ($\varepsilon = 30\%$, butan-1-ol); however, at the cost of simultaneously extracting other polar compounds as well.

Additionally, two separation methods were established that make use of selectively binding the carboxylic acid functionality of **1** to a dispersed or solid-phase surface. Both methods work on the laboratory scale as well as on a higher gram scale.

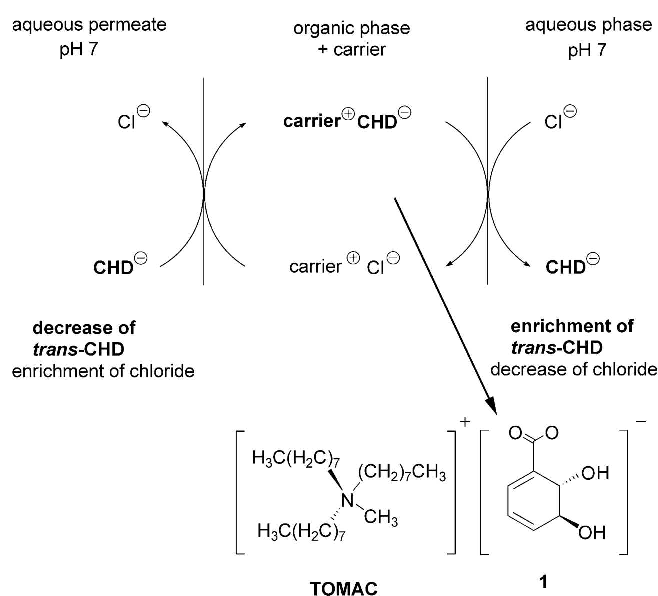
A reactive extraction process for separation: The extraction coefficients in organic solvents are substantially improved by adding ionic carriers that bind the product.[25] Ion-pair formation of the anion of **1** with the cationic carrier trioctylmethylammonium chloride (TOMAC) results in uncharged, less hydrophilic species that can be extracted with organic solvents with a higher extraction coefficient ($\varepsilon = 23\%$, octan-1-ol with 5% carrier). The selective re-extraction of **1** and separation of the remaining nonpolar impurities can be done by using aqueous phases with a high anion concentration, such as brine, at neutral pH ($\varepsilon > 90\%$) (Scheme 2).

After concentration in vacuo, separation from salts can easily be done in a third extraction step with butan-1-ol ($\varepsilon = 30\%$) to afford **1** with 90% purity after lyophilisation (HPLC analysis).

Although reactive extraction and re-extraction normally work most efficiently in a multicycle, continuously operating separation process that is integrated into the production process, all the tested cationic carriers were characterised as being bacteriostatic or even partially bacteriocidal. Therefore, integration of the extraction method into the production process (for technical application) affords further improvement in preventing carrier contact with production cells.

Ion exchange chromatography was used advantageously to selectively bind **1** to DOWEX 1×8 (Cl^-) anion-exchange resin[26] at pH 7 with a dynamic capacity of 15 to 16 $g L^{-1}$ (96 to 102 mM) sedimented resin. In contrast, binding of proteins or other by-products was not observed. Elution of the product occurs under moderate acidic conditions at pH 2.8 by protonation of the carboxylic acid functionality; this allows isolation without any aromatisation. By using 2 kg of resin 27 g (0.17 mol) of **1** could be isolated and purified in a single operation step with 95% purity and 75% yield after lyophilisation.

Application in organic synthesis: a short synthesis of the enantiomer of the plant-growth inhibitor streptol:[27] The synthesis of unsaturated carbasugars requires stereoselective oxidation, for example at the C3,C4 positions. Previously, we have shown regio- and stereoselective epoxidation of **1**, and the application of the resultant structures in the synthesis of *iso*-crotepoxide and *ent*-senepoxide.[28] Nucleophilic opening results in the introduction of a new functionality in *trans*-orientation to the simultaneously formed alcohol function. Introduction of a *cis*-diol functionality is possible by the OsO_4-catalysed dihydroxylation presented here.

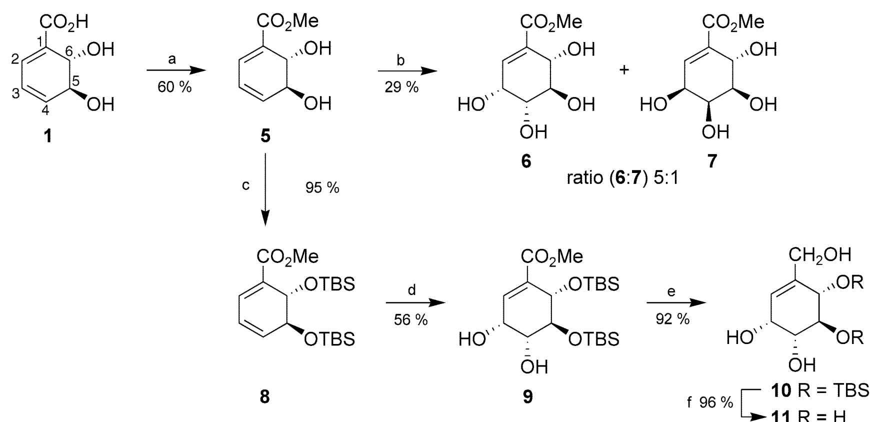


Scheme 2. Isolation of **1** by reactive extraction with cationic carriers (TOMAC) as extraction mediators in organic solvents like octan-1-ol.

Compound **1** was transformed into its methyl ester **5** with 60% yield by using a solution of hydrogen chloride in methanol (Scheme 3).

Regioselective dihydroxylation at the C3,C4 positions followed by OsO_4-catalysed oxidation with *N*-methylmorpholine *N*-oxide (NMO) gave two diastereomeric vicinal alcohols **6** and **7** in a ratio of 5:1, which could not be separated chromatographically. No oxidation of the electron-poor double bond at the C1,C2 positions was observed.

On increasing the steric demand of the alcohol functionalities by introduction of *tert*-butyldimethylsilyl (TBS) pro-



Scheme 3. Esterification of **1** and subsequent transformations of the methyl ester **5**. Reagents and conditions: a) HCl in MeOH, RT, 48 h; b) NMO, cat. OsO_4, tBuOH/H_2O (1:1), RT, 72 h; c) TBS-OTf, NEt_3, CH_2Cl_2, RT, 18 h; d) NMO, $MeSO_3NH_2$, cat. K_2OsO_4, acetone/nBuOH/H_2O (10:9:1), RT, 16 h; e) DIBAL-H, CH_2Cl_2, 0°C, 3 h; f) tris(dimethylamino)sulfoniumdifluorotrismethylsilicate, CH_2Cl_2, −78°C to RT, 16 h.

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tecting groups, the OsO_4-catalysed dihydroxylation results in **9** as the main product in 56% yield. Only traces of the product resulting from oxidation of the C1,C2 double bond were found.

While *cis*-dihydroxylation starting from the BOC-protected 2-amino analogue of **5** investigated by Steel et al. occurs in a comparable regio- and stereoselective manner,[29] oxidation of the corresponding *cis*-diastereomer of **8** by Sutherland et al. gave a 10:1 mixture of oxidation product at the C1,C2 positions over oxidation product at the C3,C4 positions;[30] this means that any implication from the chemical behaviour of *cis*-CHD to *trans*-CHD should be handled with care.

The stereochemistry of **9** was proven by synthesis of *ent*-streptol (*ent*-valienol) **11**. Reduction of the ester function with diisopropylaluminium hydride (DIBAL-H) and deprotection with tris(dimethylamino)sulfonium difluorotrismethylsilicate as a fluoride source gave *ent*-streptol **11**. The spectroscopic data of **11** are identical to those published by Sakuda et al.[27]

Streptol was isolated from a culture of a *Streptomyces* sp. by Sakuda et al. and inhibited the growth of lettuce seedlings at a concentration above 13 ppm.[27] Valienol, synonymous with streptol, was isolated from *Actinoplanes* sp. in the context of acarbose biosynthesis studies.[31] *rac*-**11** was synthesised by Suami et al.[32a] and Block.[32b]

We have shown that the dihydroxylation of *trans*-CHD can be carried out in a regio- and stereoselective manner. In combination with stereoselective epoxidation and subsequent nucleophilic opening, this will enable straight-forward diversity-oriented access to regio- and stereoisomeric (amino)-carbasugars[33] of potential biological activity.

Conclusion

Production of 2,3-*trans*-CHD **1**, an essential metabolite in bacteria, plants and fungi, was efficiently accomplished by methods of deregulating the metabolic flux in *E. coli* strains. Advantageously, no metabolic by-products such as chorismate were excreted even though the chorismate-utilising pathways are not totally blocked. A deficiency of EntA activity significantly increased the cell-mass-specific product-formation rate by a factor of 3. It was demonstrated that the microbial production of **1** can be scaled up, thus preparing the way for a preparative access to **1**. By using a 30 L stirred tank reactor, a final concentration of **1** of 4.6 gL^{-1} (0.17 M) was obtained within a 50 h process time. The molar yield relative to glucose consumption was calculated to be 17%. Two methods for separating **1** from medium ingredients were established, the use of reactive extraction or anion exchange chromatography. Starting from the purified product as an example, we established short and straight-forward syntheses of new carbasugar derivatives.

We are presently working on an enantioselective access to 3,4-*trans*-CHD **2** through methods of metabolic engineering in recombinant microorganisms.[34] We are convinced that *trans*-CHD **1** and **2** and derivatives thereof can be of similar value for preparative organic chemistry as has been described for the well-examined and commercially available *cis* analogues.

Experimental Section

General methods: All reagents used were of analytical grade. Solvents were dried by standard methods if necessary. TLC was carried out on aluminium sheets precoated with silica gel $60F_{254}$ (Merck). Detection was accomplished by UV light ($\lambda = 254$ nm). Preparative column chromatography was carried out on silica gel 60 (Merck, 40–63 µm). ^1H NMR spectra were recorded on an AMX 300 (Bruker Physik AG, Germany) with CD_3OH ($\delta = 4.87$ ppm), $CHCl_3$ ($\delta = 7.27$ ppm) or HDO ($\delta = 4.81$ ppm) as internal standard, ^{13}C NMR spectra were calibrated with $^{13}CD_3OD$ ($\delta = 49.15$ ppm), $^{13}CDCl_3$ ($\delta = 77.23$ ppm) or sodium trimethylsilylpropansulfonate (TSP) as internal standard. GCMS spectra were determined on an HP 6890 series GC system fitted with an HP 5973 mass-selective detector (Hewlett–Packard; column HP-5MS), 30 m × 250 µm; T_{GC} (injector) = 250 °C, TMS (ion source) = 200 °C, time program (oven): $T_{0min} = 60$ °C, $T_{3min} = 60$ °C, $T_{14min} = 280$ °C (heating rate 20 °C min^{-1}), $T_{19min} = 280$ °C. HR-MS (EI) was performed on an A.E.I. MS50 and elemental analysis on a Vario EL (Heraeus) at the analytical department of the Kekulé Institut für Organische Chemie und Biochemie (University of Bonn). Melting points were measured on a Büchi B-540 heating unit. UV spectra were recorded with an Ultrospec 2000 UV/Vis spectrophotometer (Pharmacia Biotech, Sweden). Identification and quantification of metabolites was performed by using an HPLC (HP series 1100, Hewlett–Packard), fitted with a diode-array detector, and equipped with a LiChrospher® C8 column (25 cm × 3 mm, 5 µm particle size, CS Chromatographie Service GmbH, Langerwehe, Germany). The metabolites were quantified by integration of peaks at a wavelength of 275 nm. The injection volume was 5 µL. The initial mobile phase was water (0.1% trifluoroacetic acid) at a flow rate of 0.45 mL min^{-1}. 5 min after injection the eluent was changed linearly to a 50% ratio of methanol/water at 40 min. Afterwards the column was washed, maintaining this composition for 10 min, and regenerated for 15 min. Retention times are as follows: chorismate 25.0 min, isochorismate 21.2 min, 2,3-*trans*-CHD 4.4 min and 3,4-*trans*-CHD 6.7 min. The limit of quantification was ≤16 µM for all metabolites.

Bacterial strains, cloning material and growth media: The strains, plasmids and primers used in this study are shown in Table 2. Primers were synthesised at MWG-Biotech AG, Ebersberg, Germany. *E. coli* strains were grown on Luria–Bertani (LB) medium.[35] Ampicillin (100 mg L^{-1}) was added when required.

The synthetic medium for analyses of product distribution was a slightly modified medium of Pan and co-workers,[36] containing per L: KH_2PO_4 (13 g), K_2HPO_4 (10 g), $NaH_2PO_4 \cdot 2H_2O$ (6 g), $(NH_4)_2SO_4$ (2 g), $MgSO_4 \cdot 7H_2O$ (3 g), NaCl (5 g), $FeSO_4 \cdot 7H_2O$ (40 mg), $CaCl_2 \cdot 2H_2O$ (40 mg), $MnSO_4 \cdot 2H_2O$ (10 mg), $ZnSO_4 \cdot 7H_2O$ (2 mg), $AlCl_3 \cdot 6H_2O$ (10 mg), $CoCl_2 \cdot 6H_2O$ (4 mg), $Na_2MoO_4 \cdot 2H_2O$ (2 mg), $CuCl_2 \cdot 2H_2O$ (1 mg), H_3BO_3 (0.5 mg), leucine (200 mg), proline (200 mg), adenine (200 mg), tryptophan (200 mg), thiamine (20 mg) and 2,3-dihydroxybenzoic acid (20 mg).

For long-term storage, cells in the mid-exponential phase of growth were harvested and shock-frozen in 50% glycerol suspension at −30 °C.

PCR amplification: EntB and EntC were amplified from chromosomal DNA of *E. coli* strain W3110. PCR was performed by using a *Pwo* DNA polymerase kit (Roche Diagnostics, Mannheim, Germany) and a Peltier Thermal Cycler PTC 200 (MJ Research, Waltham, MA, USA) in 100 µL volume. The concentration of chromosomal DNA was 0.1 ng μL^{-1}. EntB was amplified with 1 pmol μL^{-1} of primers BBAM and BPST. After an initial denaturation step of 5 min at 94 °C, 34 cycles of 4 min at 94 °C, 2 min at 57 °C and 90 s at 75 °C were performed. A final extension step of 7 min at 75 °C and cooling to 4 °C completed the reaction. EntB was obtained as a single product.

EntC was generated with 0.5 pmol μL^{-1} of primers CSAC and CBAM. The reaction was subjected to an initial denaturation step of 2 min at 94 °C, followed by 10 cycles of 30 s at 94 °C, 45 s at 40 °C, 1 min at 72 °C. Finally, 20 cycles of 30 s at 94 °C, 45 s at 40 °C and 1 min + 10 s per cycle at 72 °C finished the reaction before the mixture was cooled to 4 °C. Three products of approximately 500, 900 and 2500 bp were obtained from PCR amplification. The one with 900 bp (EntC) was isolated, purified on preparative agarose gel and used for further cloning procedures.

PCR products were analysed by electrophoresis in 0.8% (*w*/*v*) agarose gel in 1 × TAE buffer (Tris (40 mM), acetic acid (20 mM), EDTA (1 mM),

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Table 2. Strains, plasmids and primers used in this study.

	Relevant characteristic(s)	Origin and refs.
Escherichia coli strains		
DH5α	F^- *endA1 hsdR17*($r_k^- m_k^+$) *recA1 supE44 thi-1* Δ(*lacZYA-argF*)*U169 Φ80lacZΔM15*	[19]
W3110	F^- λ IN(*rrnD-rrnE*) prototroph	[16]
AN193	*trpE38 leuB6 proC14 lacY1 fhuA23 rpsL109*(str^R) $λ^-$ *entA403*	[20, 21][a]
H1882	*araD139* Δ(*argF-lac*)*169* $λ^-$ *flhD5301 rpsL150*(str^R) Δ(*fepA-ent*)	[22][a]
PBB8	F^- *lacIq* Δ(*Ion*) *hflA150:Tn10* Δ(*argF-lac*)*169 proA*$^+$ *araD139 rpsL* $λ^-$ *entC*$^-$ *menF*$^-$	[23][b]
Plasmids		
pJF119EH1	amp^R, cloning vector	[17]
pDF1	amp^R (*entB* obtained with primers BBAM and BPST cloned into pJF119EH1 cut with *Bam*HI and *Pst*I)	this study
pDF2	amp^R (*entC* obtained with primers CSAC and CBAM cloned into pJF119EH1 cut with *Sac*I and *Bam*HI)	this study
pDF3	amp^R (*entC* obtained with primers CSAC and CBAM cloned into pDF1 cut with *Sac*I and *Bam*HI)	this study
Primers		
BBAM	(*Bam*HI) 5′-TATGGATCCACGCGCATCAGCCTGAA-3′	this study
BPST	(*Pst*I) 5′-GGGCTGCAGACATTTTTACCGCTG-3′	this study
CSAC	(*Sac*I) 5′-GGCGAGCTCATTATTAAAGCCTTT-3′	this study
CBAM	(*Bam*HI) 5′-TGCGGATCCTCGCTCCTTAATGC-3′	this study

[a] Obtained from Professor Dr. Klaus Hantke, University of Tübingen, Germany. [b] Obtained from Professor Dr. Eckhard Leistner, University of Bonn, Germany.

pH 8), stained with ethidium bromide. GeneRuler™ 1 kb DNA Ladder (MBI Fermentas, Vilnius, Lithuania) was used as molecular size marker.

Construction of plasmids/transformation of strains: Plasmids were constructed by using standard recombinant techniques.[35] Plasmid pJF119EH1 and the PCR product of EntB were cut with *Bam*HI and *Pst*I and afterwards ligated with T4 DNA ligase. Transformation of *E. coli* DH5α with the resulting pDF1 gave the recombinant strain which shows ampicillin resistance. Correct ligation and transformation were verified by restriction site analyses and sequence analysis of the polylinker region and the EntB insert.

The PCR product of EntC was restricted with *Pst*I and *Sac*I and analogously cloned into the vectors pJF119EH1 and pDF1 to give vectors pDF2 and pDF3, respectively. Transformation of *E. coli* DH5α with these constructs, followed by restriction analysis and sequencing of the isolated plasmid, verified the correct insertions of EntC.

Strains W3110, AN193, H1882 and PBB8 were also transformed with plasmids pJF119EH1, pDF1, pDF2 and pDF3.

Enzyme expression analyses: For verification of enzyme expression, cell extracts of all *E. coli* strains (host DH5α W3110, AN193, H1882, PBB8 with plasmids pJF119EH1, pDF1, pDF2 or pDF3) were analysed with sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* precultures (100 μL) from LB medium (5 mL, 150 rpm, 37 °C, overnight) were used as inoculum for 100 mL volume of LB medium containing IPTG (100 μM) as inductor. Incubation was done in 1 L shake flasks (150 rpm, 37 °C, 10 h). Cells from 30 mL suspension were harvested by centrifugation (5000 g, 5 °C, 10 min) and resuspended in distilled water (1 mL). Cell disruption was done with an Ultrasonic Cell Disruptor "Sonifier 250" (Branson Inc., Danbury, USA) at 20 % duty cycle and output 2 for 4 min on ice. The cell homogenate was centrifuged (19 000 g, 5 °C, 10 min) to remove cell debris, and the supernatant was analysed. Total protein concentrations were determined with protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) by using bovine serum albumin as standard.[37] SDS-PAGE was performed with a Xcell II Mini-Cell electrophoresis apparatus (NOVEX Experimental Technology, San Diego, CA, USA). Proteins were separated in NuPAGE 4 – 12 % (*w/v*) Bis-Tris polyacrylamide gels and stained with Coomassie brilliant blue R-250 (Serva Electrophoresis GmbH, Heidelberg, Germany). Sigmamarker Low Range (Sigma Chemical Co., St. Louis, USA) was used as standard.

Enzyme activity test: Enzyme activities were determined by monitoring the degradation of chorismate (**3**). Solution A contained chorismic acid (8 mM), $MgCl_2 \cdot 6H_2O$ (10 mM) and Tris-HCl (200 mM) in aqueous solution at pH 8.5. Solution B contained crude cell extract at a total protein concentration of 85 μg mL^{-1}. For analyses 50 μL of each solution A and B were gently mixed, and the degradation of **3** towards isochorismate (**4**), 2,3-*trans*-CHD **1** or 3,4-*trans*-CHD **2** was monitored at 20 °C by HPLC.

Examination of excretion of 1: For the examination of product excretion, 100 mL cell suspensions from LB medium were prepared as described above. At an optical density of 3.0 (λ = 600 nm), cells were harvested (5 000 g, 5 °C, 5 min) and resuspended in 100 mL synthetic medium (pH 7 with 1M NaOH) that contained IPTG (100 μM) and glucose (15 g L^{-1}). Incubation was done in 1 L shake flasks at 37 °C and 150 rpm. Aliquots were taken at 1 h intervals. Maximal excretion rates (**1**) were found 2 h after incubation.

For the determination of optimal fermentation conditions the following parameters were varied. The pH was regulated (1N HCl or 1N NaOH) from pH 3 to pH 9 (steps of 1 pH unit). The cultivation temperature was tested at 20 °C, 30 °C and 37 °C. Production of **1** was maximal under physiological conditions at 37 °C and pH 7. Glucose was substituted by mass-equivalent quantities of the following other C sources: galactose, glycerol, fructose, yeast extract, acetic acid and lactic acid. For the induction experiments IPTG was added to the preculture and production media to the following final concentrations: 0, 5, 10, 20, 40, 60, 80, 100, 200, 500 μM.

Metabolite quantification: For the quantification of metabolites, aliquots of cell suspension were centrifuged (5 000 g, 4 °C, 10 min), and afterwards the supernatant was diluted with water until maximum concentrations of 500 mg L^{-1} of 2,3-*trans*-CHD were achieved. The diluted samples were analysed by HPLC.

Fed-batch fermentation in a 30 L stirred tank bioreactor: Fermentations in a 30 L stirred tank bioreactor (Chemap, Switzerland, 20 L working volume) were done at pH 6.8 (controlled) and 37 °C with a mineral-salts medium. 1 L preculture from LB medium (150 rpm, 37 °C, overnight) served as inoculum. The initial concentration of glucose was 30 g L^{-1} (0.17 M). Glucose was added in portions so that the concentration was maintained in the range of 5 to 10 g L^{-1} (28 to 56 mM). Induction by addition of IPTG (100 μM final concentration) was done at a cell concentration of 5 g L^{-1} (dry cell mass). The aeration rate was regulated to 25 L min^{-1} (air). Antifoam AF298 (Sigma Co., St. Louis, USA) was added in a sterile manner as required. Fermentation was stopped after 50 h. Cells and insoluble components were removed by centrifugation (4000 rpm, 4 °C, 15 min) to give a brown solution.

Preparative ion-exchange chromatography: Preparative ion-exchange chromatography was done with a IndEX Tm Column 100, inner diameter 100 mm, 3.5 L maximal bed volume, from Amersham Pharmacia Biotech (Uppsala, Sweden), by using DOWEX® 1 × 8 (2 kg, 100 – 200 mesh, Cl^- form) from Merck (Darmstadt, Germany). The resin was packed to a bed volume of 2.6 L with the aid of a hydraulic stamp. Elution of the product was monitored at a wavelength of 280 nm by using a UV/Vis detector K-2001 from Knauer (Berlin, Germany). The flow rate during the whole process was set to 70 mL min^{-1}.

For one separation cycle the resin was equilibrated with dipotassium hydrogen phosphate (50 mM) at pH 8 and 9.5 mS cm^{-1} (phosphoric acid, potassium hydroxide). Cell-free fermentation supernatant (8 L, pH 7) containing product **1** (4.6 g L^{-1}) was then passed through the column, allowing the adsorption of the product. Elution was performed with formic

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acid (50 mM, pH 2.3, 1.2 mS cm^{-1}). Compound **1** was generally eluted at pH 2.8 and 0.6 mS cm^{-1}. Regeneration of the DOWEX resin was done with brine (2 M).

Without neutralisation the eluate (7.8 L) was concentrated in vacuo to give a yellow oil (500 mL). The syrup was lyophilised at 5×10^{-2} mbar to give a yellow-white solid (27.5 g). This corresponds to 75% yield for the separation step. The purity was determined with HPLC analysis and ^{1}H NMR to be 95%.

(*S*,*S*)-5,6-Dihydroxycyclohexa-1,3-dienecarboxylic acid [(*S*,*S*)-5,6-Dihydroxy-5,6-dihydrobenzoic acid] (1): $[\alpha]_D^{20} = +3.8$ ($c = 0.6$ in ethanol); ^{1}H NMR (300 MHz, [D$_4$]MeOH, 23 °C): $\delta = 4.10$ (d, $J = 2.5$ Hz, 1H; H-5), 4.50 (d, $J = 2.5$ Hz, 1H; H-6), 6.20 (m, 2H; H-3,4), 7.06 (dd, $J = 3.3$, 3.2 Hz, 1H; H-2); ^{13}C NMR (75 MHz, [D$_4$]MeOH, 23 °C): $\delta = 68.8$, 70.4, 125.1 (CH), 130.8 (C-1), 134.3, 134.4 (CH), 170.2 (CO); IR(KBr): $\tilde{\nu} = 1699$, 1644, 1586, 1258, 1075, 1008 cm^{-1}; UV/Vis (H_2O): $\lambda_{max}(\varepsilon) = 279$ nm (4900); MS (70 eV, EI): *m*/*z* (%): 156 (6) [M^+], 138 (100) [$M^+ - H_2O$], 110 (63), 93 (10), 82 (29), 65 (13); HR-MS: calcd for $C_7H_8O_4$: 156.0423; found: 156.0424.

Methyl (*S*,*S*)-5,6-dihydroxycyclohexa-1,3-dienecarboxylate [Methyl (*S*,*S*)-5,6-dihydroxy-5,6-dihydrobenzoate] (5): A solution of **1** (3.11 g, 96% purity, 19 mmol) in anhydrous methanol (200 mL) was treated with a solution of hydrogen chloride in methanol (~1.25 M) and stirred for 48 h at RT. The solution was neutralised by addition of sodium hydrogen carbonate (10.5 g) and concentrated in vacuo. The crude product was purified by flash chromatography (EtOAc) to give **5** as yellow crystals (2.02 g, 60%). M.p. 79–80 °C; $R_f = 0.35$ (EtOAc); $[\alpha]_D^{20} = +4.6$ ($c = 0.45$ in ethanol); ^{1}H NMR (300 MHz, CDCl$_3$, 23 °C): $\delta = 2.50$ (s, 1H; OH), 3.82 (s, 3H; OCH$_3$), 4.05 (s, 1H; OH), 4.57 (ddd, $J = 9.3$, 3.3, 1.9 Hz, 1H; H-5), 4.76 (dd, $J = 9.3$, 1.3 Hz, 1H; H-6), 6.08 (ddd, $J = 9.6$, 5.4 Hz, 1.9 Hz, 1H; H-3), 6.29 (ddd, $J = 9.6$, 3.3, 0.9 Hz, 1H; H-4), 6.99 (d, $J = 5.4$ Hz, 1H; H-2); ^{13}C NMR (75 MHz, CDCl$_3$, 23 °C): $\delta = 52.3$ (OCH$_3$), 72.5, 72.7, 122.9 (CH), 129.4 (C-1), 133.4, 136.4 (CH), 167.9 (CO); IR(KBr): $\tilde{\nu} = 2971$, 2931, 1725, 1638, 1454, 1372, 1276, 1109 cm^{-1}; UV/Vis (H_2O): $\lambda_{max} = 282$ nm; MS (70 eV, EI): *m*/*z* (%): 170 (27) [M]$^+$, 152 (54) [$M^+ - H_2O$], 138 (91), 128 (2), 121 (89), 110 (100), 93 (38), 82 (69), 65 (50), 53 (38); HR-MS: calcd for $C_8H_{10}O_4$: 170.0579; found: 170.0580.

Methyl (3*R*,4*R*,5*R*,6*S*)-3,4,5,6-tetrahydroxycyclohex-1-enecarboxylate (6) and methyl (3*S*,4*S*,5*R*,6*S*)-3,4,5,6-tetrahydroxycyclohex-1-enecarboxylate (7):

a) OsO_4 (2 mg, 8 μmol) was added to a solution of **5** (400 mg, 2.3 mmol) and *N*-methylmorpholine *N*-oxide (653 mg, 4.8 mmol) in *tert*-butanol (5 mL) and water (5 mL). The solution was stirred at RT for 72 h, during which time it turned black. The reaction was quenched by addition of aqueous $NaHSO_3$ (1 mL). All volatiles were removed in vacuo, and the crude product was purified by flash chromatography (EtOAc/MeOH 4:1) to give a chromatographically inseparable 5:1 mixture of **6** and **7** as a white solid (139 mg, 29%).

b) A solution of **9** (50 mg, 0.1 mmol) in CH_3CN (2 mL) was treated with aqueous HF (50 μL, 40%) and the mixture was stirred at 0 °C for 3 h. Water (5 mL) was added, and the solution was extracted with EtOAc (3 × 15 mL). The combined organic layers were dried over $MgSO_4$, filtrated and concentrated in vacuo to give **6** as a white wax without further purification (7 mg, 30%). $R_f = 0.18$ (CH_3CN); **6**: ^{1}H NMR (300 MHz, [D$_4$]MeOH, 23 °C): $\delta = 3.76$ (dd, $J = 7.5$, 4 Hz, 1H; H-3), 3.82 (s, 3H; OCH$_3$), 4.05 (dd, $J = 7.5$, 5 Hz, 1H; H-4), 4.32 (d, $J = 5$ Hz, 1H; H-6), 4.40 (pt, $J = 5$ Hz, 1H; H-5), 6.78 (d, $J = 4$ Hz, 1H; H-2); ^{13}C NMR (75 MHz, [D$_4$]MeOH, 23 °C): $\delta = 52.5$ (OCH$_3$), 67.3, 70.4, 71.4, 73.0 (CH), 134.1 (C-1), 139.3 (C-2), 168.4 (CO). **7**: ^{1}H NMR (300 MHz, [D$_4$]MeOH, 23 °C): $\delta = 6.73$ (dt, $J = 3$, 0.9 Hz, 1H; H-2), all other signals are superposed; ^{13}C NMR (75 MHz, [D$_4$]MeOH, 23 °C): $\delta = 52.5$ (OCH$_3$), 68.6, 69.9, 72.2, 75.7 (CH), 133.2 (C-1), 141.1 (C-2), 168.3 (CO).

Methyl (*S*,*S*)-5,6-bis(*tert*-butyldimethylsilanyloxy)cyclohexa-1,3-dienecarboxylate (8): A solution of methyl ester **5** (18 mg, 0.11 mmol) and triethylamine (20 mg, 0.2 mmol) in dry methylene chloride (10 mL) was treated with *tert*-butyldimethylsilyltriflate (34 mg, 1.3 mmol). After being stirred for 18 h at RT, the remaining reactant was hydrolysed by quenching with saturated aqueous Na_2CO_3 (4 mL). The solution was diluted with diethyl ether and dried ($MgSO_4$). Volatiles were removed in vacuo, and the crude product was purified by flash chromatography (isohexane/EtOAc 20:1 to 9:1) to give **8** as colourless oil (42 mg, 95%). All analytical data were identical to the data obtained by Trost et al.[11a] for the (−)-enantiomer. $[\alpha]_D^{20} = +322.1$ ($c = 1.0$ in CHCl$_3$).

Methyl (3*R*,4*R*,5*S*,6*S*)-5,6-bis(*tert*-butyldimethylsilanyloxy)-3,4-dihydroxycyclohex-1-enecarboxylate (9): A solution of K_2OsO_4 (7 mg, 19 μmol) and LiOH (3 mg, 0.1 mmol) in water (1 mL) was added to a solution of **8** (775 mg, 1.9 mmol), *N*-methylmorpholine *N*-oxide (395 mg, 2.9 mmol) and methanesulfonamide (278 mg, 2.9 mmol) in acetone (10 mL) and butan-1-ol (9 mL). The resulting solution was stirred vigorously at RT for 16 h, during which time it turned black. The reaction was quenched with saturated aqueous $NaHSO_3$ (5 mL). All volatiles were removed in vacuo, and the crude product was purified by flash chromatography (isohexane/EtOAc 10:1) to obtain **9** (467 mg, 56%) as a colourless oil. $R_f = 0.42$ (isohexane/EtOAc 6:1); $[\alpha]_D^{20} = -9.9$ ($c = 0.9$ in CHCl$_3$); ^{1}H NMR (300 MHz, CDCl$_3$, 23 °C): $\delta = 0.09$ (s, 3H; CH$_3$), 0.10 (s, 3H; CH$_3$), 0.11 (s, 3H; CH$_3$), 0.24 (s, 3H; CH$_3$), 0.84 (s, 9H; *t*Bu), 0.87 (s, 9H; *t*Bu), 2.94 (d, $J = 10$ Hz, 1H), 3.78 (s, 3H; OCH$_3$), 3.93 (m, 1H), 4.04 (d, $J = 9.5$ Hz, 1H), 4.21 (dd, $J = 4.2$, 2.9 Hz, 1H), 4.37 (m, 1H), 4.46 (m, 1H), 6.89 (dd, $J = 2.2$, 1.6 Hz, 1H); ^{13}C NMR (75 MHz, CDCl$_3$, 23 °C): $\delta = -4.85$, −4.83, −4.81, −4.7 (SiCH$_3$), 18.0, 18.2 (C$_q$), 25.7, 25.8 (CH$_3$), 52.1 (OCH$_3$), 66.8, 68.1, 70.1, 70.9 (CH), 129.3 (C-1), 141.6 (C-2), 166.5 (CO); MS (70 eV, EI): *m*/*z* (%): 432 (0.5) [M^+], 417 (2) [$M^+ - CH_3$], 399 (2), 375 (80) [$M^+ - C(CH_3)_3$], 357 (100) [$M^+ - C(CH_3)_3, H_2O$], 271 (20), 243 (20), 225 (15), 215 (16), 197 (14), 147 (15); IR(KBr): $\tilde{\nu} = 3457$, 2954, 2931, 2897, 2858, 1721, 1472, 1389, 1361, 1256, 1111.

(1*R*,2*R*,5*S*,6*S*)-5,6-Bis-(*tert*-butyl-dimethylsilanyloxy)-4-hydroxymethylcyclohex-3-ene-1,2-diol (10): An aliquot (1.5 mL) of a solution of DIBAL-H in methylene chloride (1 M) was added dropwise to a solution of **9** (200 mg, 0.45 mmol) in dry methylene chloride (5 mL) at 0 °C. The resulting pale yellow solution was stirred for 3 h. The reaction was quenched by addition of MeOH (2 mL) and hydrochloric acid (2 mL, 2 M). The mixture was dissolved in hydrochloric acid (10 mL, 2 M), then brine (10 mL) and diethyl ether (30 mL) were added. The aqueous layer was extracted with EtOAc (3 × 10 mL). The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. The crude product was recrystallised (isohexane/EtOAc 20:1) to obtain **10** as white needles (173 mg, 92%). M.p. = 110–112 °C; $R_f = 0.38$ (isohexane/EtOAc 4:1); $[\alpha]_D^{20} = +2.4$ ($c = 0.5$ in CHCl$_3$); ^{1}H NMR (300 MHz, CDCl$_3$, 23 °C): $\delta = 0.11$ (s, 6H; 2 × CH$_3$), 0.17 (s, 3H; CH$_3$), 0.21 (s, 3H; CH$_3$), 0.85 (s, 9H; *t*Bu), 0.90 (s, 9H; *t*Bu), 2.80 (d, $J = 11$ Hz, 1H), 3.75 (dd, $J = 9.9$, 4.7 Hz, 1H), 3.92 (m, 1H), 4.05 (m, 1H), 4.15 (m, 3H), 4.29 (d, $J = 11$ Hz, 1H), 5.73 (d, $J = 1.4$ Hz, 1H); ^{13}C NMR (75 MHz, CDCl$_3$, 23 °C): $\delta = -5.0$, −4.64, −4.62, −4.3 (SiCH$_3$), 18.0, 18.1 (C$_q$), 25.8, 25.9 (CH$_3$), 64.4 (CH$_2$), 66.4, 68.9, 70.7, 71.0 (CH), 127.5 (C-3), 136.3 (C-4); IR(KBr): $\tilde{\nu} = 3516$, 3334, 2953, 2930, 2896, 2859, 1472, 1463, 1385, 1361, 1254, 1108, 1075, 1056 cm^{-1}; elemental analysis calcd (%) for $C_{19}H_{40}O_5Si_2$: C 56.39, H 9.96, found C 56.10, H 9.84.

(1*R*,2*R*,3*R*,4*S*)-5-hydroxymethylcyclohex-5-ene-1,2,3,4-tetraol (*ent*-streptol) (11): Compound **10** (110 mg, 0.27 mmol) was dissolved in dry methylene chloride (5 mL) and tris(dimethylamino)sulfonium difluorotrismethylsilicate (280 mg, 1.01 mmol) was added at −78 °C. The solution was stirred for 16 h and allowed to warm to RT. All volatiles were removed in vacuo, and the crude product was purified by flash chromatography (MeOH/EtOAc 1:2) to obtain **11** as a white solid (46 mg, 96%). $R_f = 0.45$ (EtOAc, MeOH 2:1); $[\alpha]_D^{20} = -92.5$ ($c = 0.2$ in H_2O); ^{1}H NMR (300 MHz, D_2O, 23 °C): $\delta = 3.60$ (dd, $J = 11$, 4 Hz, 1H; H-2), 3.69 (dd, $J = 11$, 8 Hz, 1H; H-3), 4.13 (d, 14 Hz, 1H; CH$_2$), 4.16 (m, 1H; H-4), 4.20 (d, 14 Hz, 1H; CH$_2$), 4.27 (d, 5 Hz, 1H; H-1), 5.83 (d, 5 Hz, 1H; H-6); ^{13}C NMR (75 MHz, D_2O, 23 °C, TSP): $\delta = 64.1$ (CH$_2$), 68.9, 73.5, 75.0, 75.3 (CH), 124.9 (C-5), 144.9 (C-6); IR(KBr): $\tilde{\nu} = 3331$, 2913, 1420, 1099, 1064, 1019, 994.

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