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Highly Enantioselective Reduction of β,β-Disubstituted Aromatic Nitroalkenes Catalyzed by *Clostridium sporogenes*

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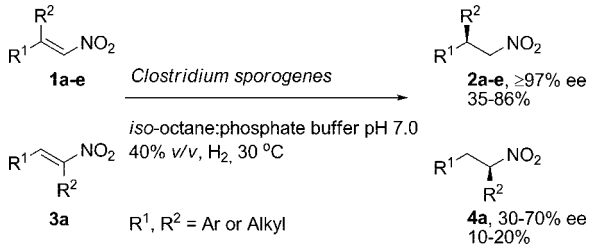
Received January 25, 2008



This is the first report of the use of *Clostridium sporogenes* extracts for enantioselective reduction of C=C double bonds of β,β-disubstituted (**1**) and α,β-disubstituted nitroalkenes (**3**). Crude enzyme preparations reduced aryl derivatives **1a**−**e** and **1h**, in 35−86% yield with ≥97% ee. Reduction of (*E*)- and (*Z*)-isomers of **1c** gave the same enantiomer of **2c** (≥99% ee). In contrast, α,β-disubstituted nitroalkene **3a** was a poor substrate, yielding (*S*)-**4a** in low yield (10−20%), and the ee (30−70% ee) depended on NADH concentration. An efficient synthesis of a library of nitroalkenes **1** is described.

Nitroalkanes are important intermediates in organic synthesis, mainly due to their easy conversion into the corresponding amines, aldehydes, carboxylic acids, or denitrated compounds.[1,2] Chiral nitroalkanes can be obtained by asymmetric conjugate addition to nitroalkenes[3−6] or enantioselective conjugate reduction of nitroalkenes using chiral transition metal catalysts[7,8] or Jacobsen-type organocatalysts.[9] The asymmetric reduction of C=C double bonds is especially attractive, as up to two stereogenic centers can be created. Unfortunately, the literature procedures suffer either from poor environmental acceptability due to the nature of the catalysts utilized or from the narrow substrate range that limits the synthesis of desirable products. As a result, there is considerable interest in developing alternative enzymatic procedures for the synthesis of optically active nitro-compounds[10] as starting blocks for chiral amines. Alkenes activated by electron-withdrawing groups, e.g., ketones, aldehydes, carboxylic acids, and amides, are reduced stereoselectively by various microorganisms, and the reductions are catalyzed by flavoenzyme oxidoreductases [EC 1.3.1.*x*].[10] The family of enoate reductases has received particular attention, and their potential value has provided the impetus to investigate several enoate reductases from various aerobic[11−13] and anaerobic[14] microorganisms and plants.[15,16]

Although the reduction of nitroalkenes by various microorganisms has been studied by several groups,[16−33] highly enantioselective examples of this reaction are limited to the reduction of β,β-disubstituted nitroalkenes by baker's yeast[17,18] and the recently reported reductases from *Lycopersicon esculentum*[16] and *Saccharomyces carlsbergensis*.[19] Contrarily, the reduction of α,β-disubstituted nitroalkenes has shown only poor enantio-

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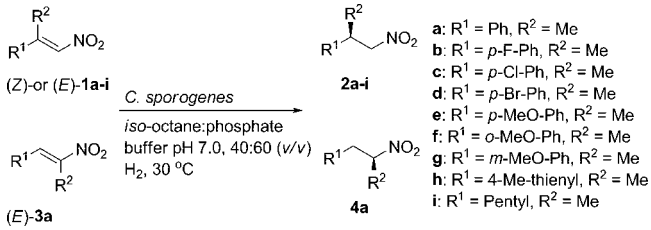
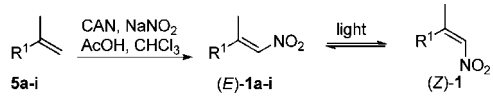
10.1021/jo800124v CCC: $40.75 © 2008 American Chemical Society
Published on Web 05/02/2008

SCHEME 1. Asymmetric Reduction of β-Alkyl-β-aryl- (1) and α-Alkyl-β-aryl-nitroalkenes (3) by *C. sporogenes*



SCHEME 2. Synthesis of (*E*)- and (*Z*)-β,β-Disubstituted Nitroalkenes 1a−i



selectivity (≤50% ee).[20–23] The enoate reductases from anaerobic *Clostridium* species have been extensively investigated by Simon et al.[14,34–36] These enzymes, including the enoate reductase from *C. sporogenes*,[37–39] proved to be versatile, stereoselective biocatalysts for the reduction of α,β-unsaturated carboxylic acids, ketones, and aldehydes.[34] However, these microorganisms have not been used for bioreduction of nitroalkenes, and therefore we wished to explore their potential for reduction of this class of compounds.

Here, we report the substrate specificity of *C. sporogenes* toward aromatic β-alkyl-β-aryl- (**1**) and α-alkyl-β-aryl-nitroalkenes (**3**), as depicted in Scheme 1. Using crude enzyme preparations, we were able to obtain stereoselective reduction of substrates **1a**−**e** in excellent yield.

Synthesis of Substrates. (*E*)-1-Aryl-1-nitroalkenes of structure **3** can be synthesized conveniently by base-catalyzed condensation of nitroalkanes with benzaldehyde under sonication conditions in very good yields (>80%).[40] However, one of the barriers to the study of the biotransformations of nitroalkenes is that the standard syntheses of substrates of structure **1** is relatively complicated or has structural limitations. They can be prepared from α-methylstyrenes via a two-step method involving addition of N_2O_4 and subsequent elimination[7] or in a similar synthetic approach, with a nitration reaction with HNO_3 as a first step.[9,16,17] The conditions for the latter reaction limit the synthesis to the derivatives with electron-withdrawing substituents at the phenyl ring. Therefore, we began by developing a more convenient, single-step method for the synthesis of β,β-disubstituted nitroalkenes **1** from their respective α-methylstyrenes (**5**) via a CAN-catalyzed nitration reaction,[41] as shown in Scheme 2. This procedure allowed the straightforward preparation of a library of nitropropenes (*E*)-**1a**−**i** in 14−52% yield, giving access to alkyl and aryl derivatives, with both electron-withdrawing and electron-donating substituents at the phenyl ring.

Under the reaction conditions employed, the (*Z*)-isomer was formed only as a minor product (<5% for R_1 = Ar by NMR and ∼30% for R_1 = pentyl). However, upon exposure to UV light, (*E*)-**1c** could be transformed to the respective stable (*Z*)-isomer, as observed previously.[42,43] Racemic nitroalkanes **2** and **4**, required as analytical standards, were obtained in a $NaBH_4$-catalyzed reduction, as described previously.[44]

Biotransformations. Because substrate delivery and product isolation from biotransformations in aqueous media can be troublesome, we used an anaerobic biphasic solvent system consisting of *iso*-octane/50 mM phosphate buffer (pH 7.0) at 20:80 v/v ratio. Initially, we employed whole cells of *C. sporogenes* DSM 795 with H_2 as the electron donor. The reduction of **1a** was enantioselective, yielding (*R*)-**2a** in >99% ee. In contrast, the enantioselectivity for reduction of nitroalkene **3a** was poor (≤30% ee).

Nitroalkene reductase activity was also retained with similar enantioselectivity after anaerobic preparation of crude cell-free extracts. The reaction time was shorter by a factor of 2, presumably because there was no requirement for the transport of substrates and products across the cell membrane. Like the whole cells, the enzyme preparation was sensitive to oxygen. Nitroalkene reduction could be driven using H_2 in the absence of added cofactors, most probably because an H_2-dependent system for recycling endogenous cofactors was present in the crude extract. However, the product yield was doubled and reaction time halved when $20\%_{mol}$ amount of exogenous NADH was also added, and this procedure was therefore used throughout.

Further optimization of the reaction conditions was attempted by comparing various other nonpolar solvents (toluene, benzene, chloroform, dichloromethane, and *n*-alkanes from pentane to tetradecane and *iso*-octane) in 20:80 v/v phase ratio with phosphate buffer (see Supporting Information, Figure 2). No apparent loss of enzymatic activity with respect to the reduction of approximately 1.7 mM 1-nitro-2-phenyl-propene (**1a**) by 40 mg of crude extract was observed with any of the alkanes tested (data not shown). With all other solvents, lower yields of product **2a** were obtained, probably as a result of enzyme deactivation. We selected *iso*-octane as the solvent of choice because it possesses favorable physical properties, namely, a low boiling point and good substrate solubility. Increasing the *iso*-octane phase ratio to 60% v/v did not result in a decrease in product yield.

The best yields of (*R*)-**2a** were obtained when the reactions were performed in the pH range 6.5−8.0, and the enantioselectivity was unaffected by pH (see Supporting Information, Figure 3). In contrast, the enantioselectivity for reduction of nitroalkene **3a** was poor (≤30% ee, depending on pH). The optimum pH for the formation of nitroalkane **4a** was significantly lower at pH 5.5, but the enantioselectivity for the reduction of nitroalkene **3a** was optimal at pH 6.5−7.0 (30% ee). Therefore, subsequent reductions were conducted at pH 7.0. Having optimized the assay conditions at small scale, preparative-scale reactions with substrates **1a**−**i** and **3a** were conducted anaerobically using *C. sporogenes* crude extract preparations (Table 1).

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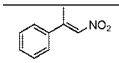
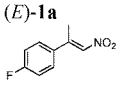
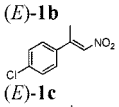
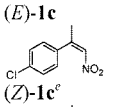
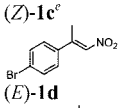
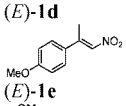
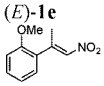
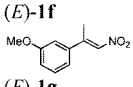
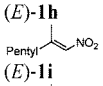
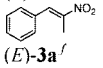
TABLE 1. Reduction of 2-Phenyl-1-nitropropenes 1a−h and 1-Phenyl-2-nitropropene 3a by *C. sporogenes* Crude Extract[I]

substrate	conv (%)[a]	*t* (d)	yield (%)[b]	product, ee (%)[c]	$[\alpha]_D$[d]
 (*E*)-**1a**	100	5	59	(*R*)-**2a**,[17] ≥99	+ 52.0 (*c* 0.56)
 (*E*)-**1b**	100	6	50	(*R*)-**2b**, ≥99	+ 48.4 (*c* 0.52)
 (*E*)-**1c**	60	7	53	(*R*)-**2c**,[17] ≥99	+ 51.3 (*c* 0.60)
 (*Z*)-**1c**[e]	100	10	nd	(*R*)-**2c**,[17] ≥99	nd
 (*E*)-**1d**	39	7	35	(*R*)-**2d**,[17] ≥99	+ 43.2 (*c* 0.58)
 (*E*)-**1e**	100	5	39	(*R*)-**2e**,[3] 97	+ 66.2 (*c* 0.99)
 (*E*)-**1f**	43	7	5	(*R*)-**2f**, nd	0 (*c* 0.44)
 (*E*)-**1g**	72	7	17	(*R*)-**2g**, 89	+ 43.2 (*c* 0.58)
 (*E*)-**1h**	100	5	86	(*S*)-**2h**, ≥98	+ 58.4 (*c* 1.10)
 (*E*)-**1i**	<5%	10	trace	nd	nd
 (*Z*)-**1i**	<5%	10	trace	nd	nd
 (*E*)-**3a**[f]	100	11	10	(*S*)-**4a**,[26] 70[g]	+ 34.0 (*c* 0.60)[h]

[a] By GC−MS and referenced to substrate recovered. [b] Isolated. [c] By HPLC using Chiralcel OD column. [d] In $CHCl_3$, 30 °C. [e] Reaction carried out at analytical scale. [f] Additional 150 mg of NADH was added after 5 days, and the reaction was continued until it reached full conversion. [g] On Chiralcel OJ. [h] +34.0 (*c* 0.60, $CHCl_3$) = +38.0 (*c* 0.44, MeOH).[26] [I] Conditions: substrate **1** or **3** (150 mg); *iso*-octane (400 mL); phosphate buffer pH 7.0 (800 mL, 50 mM); crude extract (2.2 g total protein equivalent to approximately 3.4 g whole cell dry weight), NADH (150 mg).

The reactions were allowed to proceed until full substrate conversion was obtained, as determined by GC−MS, or were stopped after a maximum of 7 days. The optical purity of products was analyzed by chiral HPLC, and the absolute configuration was assigned by comparing the optical rotation sign or HPLC data with literature.[3,6,17,26]

The bioreduction of aromatic nitroalkenes (*E*)-**1a**−**e** was highly enantioselective, giving the corresponding propane derivatives (*R*)-**2** in high optical purity (≥97% ee). Product yields ranged from 35−59%, but the substrates were consumed with greater efficiency than this. We assume that this was due to side reactions catalyzed by other enzymes in the cell-free extract, since the yields increased when the enzyme was partially purified (results not shown).

The presence of a substituent in the *para* position of the phenyl ring ((*E*)-**1b**−**e**) resulted in an increased reaction time for total substrate conversion. The efficiency of reduction of unsubstituted and *p*-halogen-substituted nitroalkenes **1a**−**d** followed the trend H > *F* > Cl > Br. *p*-Methoxy-substituted nitroalkene **1e** was completely converted after 5 days, similar to the reduction of **1a**; however, the isolated yield of the product was much lower due to formation of byproduct arising from possible alternative metabolic pathways. These compounds were tentatively identified as respective propionaldehyde derivatives (Nef reaction product) by GC−MS but were not fully characterized. When the methoxy substituent was in the *meta* position (**1g**), the reaction time increased and the optical purity of the product **2g** decreased (89% ee), while the reduction of the *ortho* derivative (**1f**) led to an even longer reaction time and a total loss of enantioselectivity, resulting in the formation of racemic product. *Clostridium sporogenes* crude extracts also catalyzed the highly enantioselective reduction of 2-(4′-methyl-thienyl)-1-nitropropene **1h** to its corresponding alkane (*S*)-**2h** in excellent optical purity (98% ee), which shows that the substrate range also extends to heterocyclic aromatic nitroalkenes. Interestingly, aliphatic nitroalkene derivatives were very poor substrates, since the reduction of (*E*)- and (*Z*)-2-methyl-1-nitro-octene **1i** gave only trace amounts of product **2i**, as monitored by GC−MS. Furthermore, none of the other aliphatic substrates tested, namely, 1-nitrocyclohexene and 1-cyclohexyl-2-nitro-propene, were reduced, as determined by spectrophotometric assays.

The reactions described above were all done using the (*E*)-nitroalkenes. The outcome with the corresponding (*Z*)-isomers was thus of interest, and we therefore tested the reduction of (*Z*)-**1c** on analytical scale with crude extract, using the same general conditions. This resulted in the formation of the same enantiomer as obtained from reduction of (*E*)-**1c**, namely, (*R*)-**2c**, with the same high optical purity (≥99%), allowing enantioconvergent reduction. The time required for full conversion of (*Z*)-**1c** was approximately 1.5 times longer than for the (*E*)-isomer with crude extract, but preliminary experiments with partially purifed enzyme showed that the initial rates of reduction were the same for (*Z*)-**1c** and (*E*)-**1c**. Similar behavior has been observed from studies with yeast as a biocatalyst[17,20] but is in striking contrast to the reduction of enoates and enals, where different enantiomers are obtained using the (*E*)- and (*Z*)-isomers of substrates.[10,11,13,15,19] Furthermore, there is still a possibility that only one isomer is stereoselectively reduced since pure isomers of **1c** equilibrated very slowly to form a mixture of isomers *E*/*Z*: substrate purity of 98% for (*E*)-**1c** and 97% for (*Z*)-**1c**, after 5 days in the absence of enzyme under the same reaction conditions. Thus, the selective reduction of only one of the isomers would perturb the equilibrium for isomerization, ensuring a continuing supply of the preferred isomer as a substrate for the stereoselective reduction. At present, it is not possible to determine which of these hypotheses explains the apparent lack of discrimination between the geometrical isomers, and therefore we are attempting to obtain enzyme of sufficient purity for crystallographic studies of substrate binding. Nevertheless, the ability to reduce a mixture of geometrical isomers to the same stereoisomer with no loss of enantioselectivity is of important practical value because the preparation of a single isomer is then not required. While this is extremely advantageous, it does mean that it was not possible to control the absolute configuration of the product (*R* or *S*) by the stereochemistry of the alkene.

In contrast to β,β-disubstituted nitroalkenes **1**, the reduction of the α,β-disubstituted nitroalkene, 1-nitro-2-phenyl-nitropropene **3a**, was 5−10 times slower and gave product (*S*)-**4a** in low yield (20%). At preparative scale, the ee was higher (50%

ee) after 5 days than in the small-scale experiments. However, the conversion was incomplete, so we added additional NADH (150 mg), and full conversion was obtained after 11 days. This also improved the optical purity of the product (70% ee), demonstrating that that enantioselectivity is influenced by the NADH concentration. Interestingly, during the reduction of **3a**, phenylacetone was also formed in approximately 30% yield. The difference in enantioselectivity of the bioreduction of nitroalkenes, high for β,β-disubstituted[16,17] and low for α,β-disubstituted nitroalkenes,[20–23] has been observed for many microorganisms. It has been suggested that the low optical purity of products possessing substituents at the α-carbon atom (e.g., **3a**) is due to product racemization under the conditions in the reaction.[10,17] However, Kawai et al. showed that the rate of spontaneous epimerization at the α-carbon of nitroalkanes was actually very low.[20] We found the deuterium exchange of the α-proton of (±)-**4a** was negligible (<5%) if incubated in D_2O/*iso*-octane (40:60, v/v) at 30 °C for 7 days. This suggests that the stereochemical outcome is due to the mechanism of enzymatic reduction rather than racemization, and further studies with purified protein are needed to explain this phenomenon.

In summary, the nitroalkene reductase from *C. sporogenes* is a versatile biocatalyst for stereoselective reduction of aromatic and heteroaromatic nitroalkenes, although it does not reduce alkyl derivatives. Thus, reduction of 2-aryl-1-nitro-propenes **1a**−**e** and **1h** was efficient and highly enantioselective, providing single enantiomers of the respective products (*R*)-**2a**−**e** and (*S*)-**2h** (≥97% ee) in 35−86% yield. The absolute configuration of product was independent of which geometric isomer of substrate was used. Contrary to the substrates of structure **1**, the bioreduction of 1-nitro-2-phenyl-propene **3a** by crude extract resulted in (*S*)-**4a** in low yield, and the optical purity depended on NADH concentration. The low enantiopurity could not be explained by racemization.

This is the first report of the use of enzymes from Clostridia for the highly enantioselective reduction of nitroalkenes with good yields, thus demonstrating that their substrate range extends beyond enoates and enals.[35,36,38] The mechanism and scope of the reduction of nitroalkenes and other unsaturated compounds by *C. sporogenes* are currently under investigation in our laboratory.

Experimental Section

General Procedure for Preparative-Scale Bioreduction of Nitroalkenes 1 or 3. Bioreductions were performed in a 2-L round-bottom flasks containing 800 mL of anaerobic 50 mM phosphate buffer pH 7.0 with approximately 2 g (total protein) of crude extract of *C. sporogenes* (DSM 795 strain; see Supporting Information), 150 mg of NADH, and 150 mg of substrate **1** or **3a** dissolved in 400 mL of anaerobic *iso*-octane. The vessel was sealed with a suba-seal stopper and flushed with H_2 for 2 min prior to continual stirring in an anaerobic cabinet. Reactions were monitored daily by GC−MS until they reached full conversion or were stopped after a maximum of 7 days. If reactions were observed to slow considerably, e.g., during the reduction of **3a**, then an additional 150 mg of NADH was added. Upon termination of the reaction, the phases were separated and the aqueous layer was extracted with $CHCl_3$. Combined organic layers were dried ($MgSO_4$) and evaporated to dryness. Column chromatography (hexane → hexane/Et_2O, 95:5) gave pure nitroalkane **2** or **4**.

(*R*)-1-Nitro-2-phenyl-propane ((*R*)-2a). 59% yield as transparent oil; purified by chromatography (hexane/Et_2O, 95:5); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.39 (d, J = 6.9 Hz, 3H), 3.64 (q, J = 7.4 Hz, 1H), 4.45−4.59 (m, 2H), 7.23−7.46 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.6, 38.6, 81.9, 126.8, 127.5, 128.9, 140.8; MS (EI) m/z 165 (1%, M^+), 91 (100%); IR (neat) 768, 1382, 1542 cm^{-1}; ee > 99% (Chiralcel OD, hexane/*i*-PrOH, 9:1, t_R = 9.6 min., t_S = 10.3 min, lit. chromatography data available for (*S*)-enantiomer[8]); $[\alpha]_D$ = +52.0 (*c* 0.56, $CHCl_3$, 30 °C), lit. +44.3 (*c* 3.4, $CHCl_3$, 27 °C, 98% ee[17]) or $[\alpha]_D$ = −48.8 (*c* 0.97, $CHCl_3$, 20 °C, (*S*)-enantiomer, 92% ee[3]). Anal. Calcd for $C_9H_{11}NO_2$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.48; H, 6.93; N, 8.28. All spectral data are consistent with the literature values.[3,8]

General Procedure for Synthesis of β,β-Disubstituted Nitroalkenes (*E*)-1a−i in a CAN-Catalyzed Reaction. To a suspension of α-methylstyrene **5** (1 mmol), $NaNO_2$ (10 mmol), and CAN (1 mmol) in $CHCl_3$ (10 mL) was added acetic acid (12 mmol) dropwise. The mixture was sonicated in a sealed flask connected to a bubbler until the reaction reached completion, as judged by TLC (5−60 min). $CHCl_3$ (20 mL) was added and solution was washed with saturated $NaHCO_3$ and water and then dried ($MgSO_4$). The solvent was evaporated and the product (*E*)-**1** was purified by flash chromatography (hexane/Et_2O, 9:1).

(*E*)-1-Nitro-2-phenyl-propene (1a). 42% yield as yellow oil; purified by chromatography (hexane/Et_2O, 95:5); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.66 (s, 3H), 7.32 (s, 1H), 7.46 (s, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.6, 126.8, 129.0, 130.3, 136.2, 138.2, 150.0; MS (EI) m/z 163 (1%, M^+), 115 (100%); IR (neat) 1320, 1518 cm^{-1}. Anal. Calcd for $C_9H_9NO_2$: C, 66.25; H, 5.56; N, 8.58. Found: C, 66.35; H, 5.64; N, 8.54. All spectral data are consistent with the literature values.[6,8,17]

Acknowledgment. Financial support from the Biotechnology and Biological Sciences Research Council (grant BBD0028261) is gratefully acknowledged. We also thank the EPSRC Mass Spectrometry Service at Swansea for mass spectral analyses.

Supporting Information Available: Full characterization data for the prepared compounds, anaerobic methodology, cell growth and enzyme preparation. This material is available free of charge via the Internet at http://pubs.acs.org.

JO800124V