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MICROBIAL REDUCTION OF N-ALLYLHYDROXYLAMINES TO N-ALLYL-AMINES USING CLOSTRIDIA

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Abstract: Chiral organic hydroxylamines can be reduced to the corresponding chiral amines in high yield without racemization using resting cells of *C. kluyveri, C. tyrobutyricum or C. thermoaceticum* in aqueous buffer with H_2, CO or Na-formate as reducing agents. These reduction systems are unselective with respect to substrate chirality and leave C-C double bonds and aliphatic carbon-chlorine bonds unharmed. Considering the product inhibition by the amines optimized reaction conditions were elaborated to employ formate, and 60-120 mg wet packed cells of *C. thermoaceticum* for reducing 0.1 mmol substrate in 1 hour.

Introduction

In spite of the great variety of methods available to reduce organic hydroxylamino compounds to the amine stage (see preceeding paper [1]), problems might arise in these reactions, if chiral polyfunctional substrates are involved. Very frequently biocatalysts in form of isolated enzymes or whole microbial cells can be used to an advantage in these instances due to their selectivity and the generally mild reaction conditions. With respect to the reduction of chiral allylhydroxylamines, which are readily obtained from alkenes and chloronitroso sugar derivatives (see preceding paper [1]), biological systems consisting of nonpathogenic clostridia might prove preparatively useful. These obligatory anaerobic microorganisms possess a rather negative electrochemical potential. In fact it has been shown that crude extracts of clostridia (*C. kluyveri, C. tyrobutyricum, C. sporogenes, C. pasteurianum*) are able to reduce aromatic and aliphatic nitro compounds to the amines passing the hydroxylamine stage as intermediates. [2] It has also been demonstrated that these clostridia reductively split the N-O bond of dihydroxyoxazines, a reaction used for preparative purposes. [3]
Here we report on the utility of suspensions of resting clostridia cells to convert chiral hydroxylamines containing unsaturated residues to chiral allylic amines.
In order to see whether chemical reduction procedures lead to partial racemization in some cases the biologically reduced products were compared with those obtained by chemical reduction procedures.

Results and Discussion

Whole cells of clostridia can use external reducing agents to maintain an intracellular supply of biological reductants like reduced ferredoxins or certain abiotic electron carriers as i.e. reduced viologens. [4] The latter often function as reversible redox mediators connecting an electron transport chain from the enzymes processing the primary reducing agents hydrogen, carbon monoxide or formate (i. e. hydrogenase, CO-dehydrogenase or formate dehydrogenase) to some target redox enzyme which performs the

reduction of an administered non natural substrate. This concept has been worked out successfully in a number of cases.[5] Thus, no difficulty was encountered to use cell suspensions of *C. kluyveri or C. tyrobutyricum* under an atmosphere of hydrogen gas for the conversion of the hydroxylamines **1**, **5** and **7** to the corresponding unsaturated amines **8**, **12** and **14**, respectively (scheme 1). Though the productivity numbers PN [300 - 950 mmol/kg * biocatalyst(dryweight) * h] were quite respectable using methylviologen (MV) as mediator between hydrogenase and the yet not characterized hydroxylamines reducing enzymes, we assumed that employing *C. thermoaceticum* and carbon monoxide might serve the applicability of this biological reduction, since this organism furnishes higher enzymatic activities and is easier to cultivate to high cell yields.[6]

Whereas reduced methylviologen ($MV^{+\cdot}$) reacts spontaneously with nitro compounds [2] that is not the case with hydroxylamines. This was carefully checked in cuvettes under strictly anaerobic conditions.[7]

Under our standard reaction conditions (phosphate buffer pH 8.5, 40°C, 1 mM MV) using *C. thermoaceticum* with H_2 as reducing agent the reductions of **1**, **2** or **3** were rather sluggish. Manometric and gas chromatographic monitoring revealed that the substrates slowly deteriorated, causing mediocre PNs (30 - 100) on the prolonged incubations that were necessary. Switching to carbon monoxide as the electron donor improved the reaction pace, but the solution progressively acidified. Complete substrate conversion was then brought about by continuous addition of NaOH maintaining mildly alkaline conditions. The PNs were distinctly higher (up to PN 2000), but we observed the initially dark blue suspension (coloured by the MV radical cation) to decolourize on addition of the substrate. This suggested that electron delivery from the CO to the oxidized methylviologen is slower than the electron delivery from the reduced viologen via the reductase to the hydroxylamine. Since the carbon monoxide reductase tested by the reaction

$$CO + 2\,MV^{++} + H_2O \dashrightarrow CO_2 + 2\,V^{+\cdot} + 2\,H^+$$

is rather high in C. *thermoaceticum* (5 - 10 units/mg protein) we assume that the diffusion of CO from the gas phase into the solution is rate limiting. (One unit converts 1 umol of substrate/min.)

In order to circumvent problems of CO-diffusion, we made use of the formate dehydrogenase reaction. According to the literature and as checked by us this enzyme activity catalyzing the reaction

$$HCOO^- + 2MV^{++} \dashrightarrow CO_2 + 2MV^{+\cdot} + H^+$$

occurs up to 5 units/mg protein in crude extracts of *C. thermoaceticum.*[8] Therefore we applied 0.3 M sodium formate for electron donation which guaranteed substrate saturation of this enzyme (K_M (formate) = $2.3 * 10^{-4}$M) even if several electron consuming reactions would proceed simultaneously.

The system was optimized using the allylhydroxylamine **1** as reducible substrate. Product formation was monitored by quantitative glc-analysis and supplemented by glc-MS peak identification. The unsaturated amine was the only reduction product formed and yields approached 100% with no indication of C-C-double bond reduction. Under otherwise identical conditions the pH value exerted a marked influence on the enzymic activity: At pH 8.5 we observed 68% conversion after 1 h which decreased on lowering the pH to 8.0 (55 %), 7.0 (11 %) and 6.0 (< 2 %), reflecting at least in part the pH dependence of formate dehydrogenase.

With the use of whole cell suspensions it was no surprise that hydroxylamine reduction was brought about in the absence of artificial redox mediators. However, addition of 0.5 mM methylviologen increased the productivity number from 440 to 2000, but remained invariant on further doubling the mediator concentration.

In order to economize on educt conversion the dependence on initial substrate concentration was studied. Our observation (table 1) that productivity numbers decrease with increasing initial substrate concentration suggested some inhibition to take place either due to the substrate itself or to the product formed. To distinguish between substrate and/or product inhibition substrate was added consecutively in

hydroxylamine	amine	R^3	R^2	R^1
1	8	CH_3	H	$^tC_4H_9$
2	9	H	H	$^tC_4H_9$
3	10	H		$-(CH_2)_2-$
4	11	H		$-(CH_2)_4-$
5	12	H		$-(CH_2)_5-$
6	13	H		$-CH_2CH=CH(CH_2)_2-$
7	14	Cl		$-(CH_2)_2-$

Scheme 1.

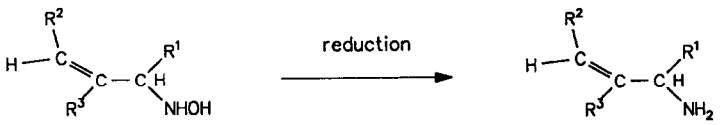

Table 1. Dependence of the rate of reduction of 1 on initial substrate concentration. 400 mg *C. thermoaceticum* wet cells in 3 ml 0.1 M phospate buffer pH 8.5, 0.3 M Na formate, 0.001 M methylviologen, 40°C.

substrate concentr.[M]	reaction time [h]	conversion [%]	PN
0.033	5	80	1380
0.150	19.6	70	880
0.225	19.8	75	850
0.40	18.7	33	20

portions to a standard run always waiting until the preceding portion had been transformed into product completely. The initial rates of each step progressively decreased much more than could be accounted for by some unspecific enzyme deactivation. This indicated product inhibition. This was further confirmed in an experiment, in which amine product was added at the beginning. Increasing product concentrations slowed down the hydroxylamine consumption severely, so that we set the substrate concentration to 33 mM as a compromise for all subsequent experiments.

Table 2. Efficiency of the reduction of chiral hydroxylamines by cell suspensions of *C.thermoaceticum*; conditions as given in table 1

substrate	(+)**1**	(-)**1**	(±)**1**	(+)**2**	(-)**2**	(±)**2**	(+)**3**	(-)**3**	(±)**3**	(-)**4**	(+)**5**	(+)**6**
product	(+)**8**	(-)**8**	(±)**8**	(+)**9**	(-)**9**	(±)**9**	(+)**10**	(-)**10**	(±)**10**	(-)**11**	(+)**12**	(+)**13**
PN	890	930	980	3130	3130	3250	2380	3050	3300	1250	1880	1700

Table 3. Preparative reduction of selected chiral hydroxylamines with cells of *C. thermoaceticum* and characterization of the enantiomeric purity of the products

substrate amount [mg]		weigth wet cells[g]	volume ml]	reaction time [h]	chemical[a] yield	optical yield[b] % ee
(+)**1**;	600	13	50	5	86	85 (84)[b)]
(-) **1**;	100	2	30	3	81	50 (52)[b)]
(±)**1**;	100	2.5	30	3.2	75	0
(+)**2**;	1000	17	180	0.8	75	83 (90)
(-) **2**;	140	3	26	1.2	80	80 (83)
(-) **3**;	1000	20	300	2.0	92	94 (94)
(+)**3**;	375	14	120	0.5	75	75 (82)
(±)**3**;	500	10	150	1.5	81	0

[a] isolated material; [b] Determined by the methods described in ref. 1; [c] The values in paren thesis refer to the optical yields obtained in the chemical reduction starting from the same batch of chiral hydroxylamines.

To investigate the substrate selectivity we took the open chain- and cyclic aliphatic hydroxylamines listed in scheme 1. As can be seen from the results collected in table 2, this biological reduction system does not differentiate between optical antipodes possessing their chiral centre adjacent to the reducible hydroxylamino function. Either enantiomer as well as the racemate are processed with identical rates. Thus, this system represents a case, where the interaction of a chiral catalyst with a chiral compound does not at all give rise to some kinetic resolution.

With the help of the chiral hydroxylamines **1**, **2**, and **3** we could tackle the problem whether the substrates undergo racemization on reduction by chemical (see preceding paper [1]) or biocatalytic means. The corresponding amino compounds had been obtained by chemical reduction and their optical purities were characterized by formation of diastereomeric Mosher acid or camphansulfonic acid amides followed by chromatographic or NMR analysis. The adaptation of this sequence for the present case required to conduct the biological reduction on a semipreparative scale. Doing this we could demonstrate the preparative utility of this process. The results given in table 3 evidence that gram quantities of chiral hydroxylamines can be reduced to chiral amines in good to excellent yields within hours by simple stirring cells of *C. thermoaceticum* in an aqueous cocktail of buffer, a commercial redox mediator and sodium formate. Starting with material from the same batch of chiral hydroxylamines the chemical and the biological reduction method yield the same optical purity in the product within experimental error rendering it improbable that racemizations take place in one of the methods.

We consider the catalytic reduction of chiral organic hydroxylamines to the corresponding chiral amines with the help of *C. thermoaceticum* cell suspensions a competitive alternative to the chemical method with respect to yield, optical purity, experimental simplicity, ease of product isolation and risk of chemical hazards.

EXPERIMENTAL

The microorganisms were obtained from DSM (*C. kluyveri: DSM 555; C. tyrobutyricum: DSM 1460; C. thermoaceticum: DSM 521*) and cultivated according to published procedures [6,7]. A typical 10 l batch fermentation yielded 80 g wet packed cells of *C. thermoaceticum* after centrifugation, which were stored under N_2 at -15° C. Tests in cuvettes under strictly anaerobic conditions were conducted as described. [7]

Reduction procedure

The wet packed cells were thawed and 400 mg (wet weight corresponding to 80 mg dry weight) suspended in 2.6 ml 0.1 M potassium phospate buffer pH 8.5 and transferred under N_2 to a ~ 17 ml vessel immersed into a thermostate and equipped with a mercury manometer to utilize Warburg manometric techniques. After addition of 60 ul 0.05 M methylviologen solution, the gas phase either was replaced by H_2 or CO, respectively, or 300 ul 3 M sodium formate solution was added. This mixture was shaken at 100 strokes per min at 40° C until the gas pressure indicated by the manometer stayed constant. The solution containing the substrate was added than, and the time course of the reduction was followed manometrically and/or by glc [25 m OV-1 crossbond fused silica capillary column, helium carrier gas, temp. program: 50° (1 min)/50-270 °(5°/min)/270 °(5 min)].

Finally the bioreduction was stopped by addition of concentrated HCl, the cells were spun down, the clear supernatant was decanted and the pellet was washed several times with water until no more product could be extracted. The combined aqueous phases were brought to dryness and the residual

amine hydrochlorides were recrystallized or converted to the acetamide or trifluoroacetamide derivatives.

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REFERENCES AND NOTES

1. Braun, H.; Felber, H.; Ritter, A.; Schmidtchen, F.P.; Schneider, A.; Simon, H. *Tetrahedron* ▪▪ ▪▪ ▪▪ ▪▪, preceding paper.

2. a) Angermaier, L.; Simon, H. *Hoppe Seyler'sZ. Physiol. Chem.* **1983**, *364*, 961-975. b) Angermaier, L.; Simon, H. *Hoppe Seyler'sZ. Physiol. Chem.* **1983**, *364*, 1653-1663.

3. Klier, K.; Kresze, G.; Werbitzky, O.; Simon, H. *Tetrahedron Lett.* **1987**, *28*, 2677-2680.

4. Simon, H.; Bader, J.; Günther, H.; Neumann, S.; Thanos. J. *Angew. Chem.* **1985**, *97*, 541-555; *Angew. Chem.* Int. Ed. Engl. *24*, 539-553.

5. Simon, H. in: *Biocatalysis*, Ed. Abramowicz, D.A.; Van Nostrand Reinhold Catalysis Series **1990**, 217-242.

6. White, H.; Strobl, G.; Feicht, R.; Simon, H. *Eur. J. Biochem.* **1989**, *184*, 89-96.

7. Thanos, J.; Bader, J.; Günther, H.; Neumann, S.; Krauss, F.; Simon, H. *Meth. Enzymol.* **1987**, *136*, 302-317.

8. Yamamoto, I.; Saiki, T.; Lin, S.-M.; Ljungdahl, L.G. *J. Biol. Chem.* **1983**, *258*, 1826-1832.