Determination of the Enantioselectivity for Kinetically Controlled Condensations Catalysed by Amidases and Peptidases

Boris Galunsky, Volker Kasche*

Biotechnology II, Technical University Hamburg-Harburg, Denickestr. 15, 21071 Hamburg, Germany
Fax: (+49)-40-42878-2127, e-mail: kasche@tuhh.de

Received: May 15, 2002; Accepted: August 2, 2002

Dedicated to Professor R. Sheldon on the occasion of his 60th birthday

Abstract: For kinetically controlled synthetic reactions catalysed by amidases and peptidases, the enantio- or stereoselectivity determined from initial rates with racemic mixtures ($E_{syn, rac}$) was found to differ from the enantioselectivity determined from measurements with isolated enantiomeric nucleophiles (E_{syn}). This was observed for kinetically controlled condensation of *R*-phenyglycineamide with *S*-, *R*- and racemic Phe and *S*-, *R*- and racemic Leu catalysed by penicillin amidase from *E. coli* and for kinetically controlled condensation of N^{α}-acetyl-*S*-tyrosine ethyl ester with *S*-, *R*- and racemic $AlaNH_2$ catalysed by bovine α-chymotrypsin. It is shown that only $E_{syn, rac}$ determined with racemic nucleophiles is an intrinsic enzyme property which should be used to study the influence of the primary structure, physicochemical parameters and immobilisation on biocatalyst enantioselectivity in kinetically controlled synthetic reactions catalysed by these enzymes.

Keywords: α-chymotrypsin; enantioselectivity; kinetically controlled synthesis; kinetic resolution; penicillin amidase; stereoselectivity

Introduction

Biocatalytic production of pure chiral substances is an expanding area in the pharmaceutical and fine chemical industry.[1] A large number of these substances (β-lactam antibiotics, biologically active peptides, oligosaccharides, fatty acid esters, oligonucleotides) can be synthesised in kinetically and equilibrium controlled reactions catalysed by hydrolytic enzymes. The maximum yield of product is reached faster and is larger in the kinetically than in equilibrium controlled synthesis. Contrary to the latter, the yield in the kinetically controlled synthesis depends also on the kinetic properties of the enzyme.[2] When these processes are used for kinetic racemate resolutions the steric purity of the products depends on the enantio- or stereoselectivity of the used hydrolase. It is quantified by the ratio of the rate constants for the competing enzymatic transformations of the enantiomeric substrates.[3]

$$E = k_S/k_R \quad (1)$$

To obtain high steric purity of the products E should be > 100 for an *S*-specific enzyme or < 0.01 for an *R*-specific enzyme. An enzyme generally has different binding sub-sites adjacent to the bond changed during the enzyme-catalysed reaction. For hydrolases such as amidases and peptidases they are conventionally designated S_1 for the residue with the carboxyl group and S'_1 for the residue with the amino group involved in the formation of the amide (peptide) bond.[4] Thus, quantitative measures for the enantioselectivity of the different binding sub-sites of an enzyme are required for the selection of the optimal enzyme for a specific racemate resolution. This has mainly been done for *equilibrium controlled* enzyme-catalysed reactions such as hydrolysis by hydrolases. For these it has been shown that E_{hyd} equals the ratio of the specificity constants for the enantiomeric substrates,[5]

$$E_{hyd} = \frac{(k_{cat}/K_m)_S}{(k_{cat}/K_m)_R} \quad (2)$$

i.e., it reflects an *intrinsic* enzyme property. E_{hyd} can be determined with isolated enantiomers or racemic mixtures from initial rate measurements.[6] When determined with isolated enantiomers in initial rate studies E_{hyd} gives fundamental information on how binding and activation energies are utilised by the enzyme to discriminate between the enantiomers and allows one to analyse the influence of primary structure, temperature and pH on biocatalyst stereoselectivity.[7] These studies also showed that the stereoselectivity of the different binding sub-sites of amidases and peptidases may differ considerably. Penicillin amidases have

  1615-4150/02/34410-1115 – 1119 $ 17.50+.50/0

an *R*-specific S_1- and an *S*-specific S'_1-binding sub-site. E_{hyd} determined from initial rates cannot, however, generally be used for practical racemate resolutions. In racemic mixtures each enantiomer is a competitive inhibitor for the other.[8] For determinations of the enantioselectivity with racemic mixtures mainly progress curves have been analysed using integrated rate equations.[6,9] These studies indicated that enzyme enantioselectivity can be modulated by product inhibition.[9]

For *kinetically controlled synthesis* with amidases and peptidases the apparent ratios of the transferase to hydrolase rate constants, used to quantitate the nucleophile specificity, determined for the *S*- and *R*-enantiomeric nucleophiles have been used to evaluate the stereoselectivity of the S'_1-binding sub-site.[10] It has not yet been analysed whether this is a suitable quantitative measure for the enantioselectivity in kinetically controlled synthesis. This is done here, where enantioselectivities were determined for both isolated enantiomers and racemic mixtures.

Results and Discussion

Enantioselectivity Measures in Kinetically Controlled Synthesis

To quantify the S_1'- or nucleophile enantioselectivity in kinetically controlled synthesis catalysed by amidases or peptidases the following ratio has been used[10]

$$E_{syn} = \frac{(k_T / k_H)_S}{(k_T / k_H)_R} \tag{3}$$

The ratios of apparent second-order rate constants for acyl transfer to nucleophile and water were determined from initial rate measurements with isolated enantiomers

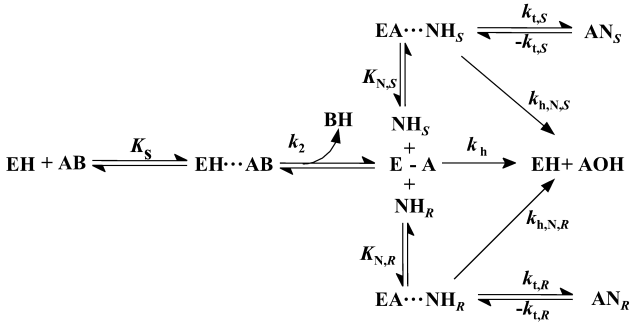
$$\left(\frac{k_T}{k_H}\right)_{app} = \frac{v_T}{v_H}\frac{[H_2O]}{[NH]} \tag{4}$$

where v_T and v_H are the initial rates for formation of the condensation and hydrolysis products respectively.[2] From the definition (Equation 1) and considering equal initial concentrations of the enantiomeric nucleophiles, E for a racemic mixture

$$E_{syn,\,rac} = \frac{v_{T,S}}{v_{T,R}} \tag{5}$$

Thus, only when $E_{syn} = E_{syn,\,rac}$ can the enantioselectivity be determined from measurements with isolated enantiomers.

Whether E_{syn} or $E_{syn,\,rac}$ are intrinsic properties of the enzyme can be analysed based on the following mechanism for the kinetically controlled synthesis that has been shown to apply for amidases, peptidases and glucosidases with covalent acyl- or glycosyl-enzyme intermediates.[2]



Scheme 1. Mechanism for the hydrolase-catalysed synthesis of a condensation product AN from an activated substrate AB and a racemic nucleophile NH. The mechanism implies a covalent acyl-enzyme intermediate and nucleophile binding to the acyl-enzyme before its deacylation.[2] The mechanism is simplified neglecting nucleophile binding to EH and EH ··· AB.

Assuming equilibrium in NH binding and negligible AN hydrolysis, $(k_T/k_H)_{app}$ for an isolated enantiomer determined from the initial rates of formation of condensation and hydrolysis products is given by the relation[2]

$$\left(\frac{k_T}{k_H}\right)_{app} = \frac{k_t}{(k_h K_N + k_{h,N}[NH])} \tag{6}$$

(which also covers the case where the acyl-enzyme can be formed from the enzyme-substrate complex with bound nucleophile) where K_N is the equilibrium constant for nucleophile binding to the acyl-enzyme, k_t is the deacylation rate constant of the acyl-enzyme-nucleophile complex, k_h is the deacylation rate constant of the acyl-enzyme by water, and $k_{h,N}$ is the deacylation rate constant of the acyl-enzyme-nucleophile complex by water. Then E is

$$E_{syn} = \frac{k_{t,S}(k_h K_{N,R} + k_{h,N,R}[NH_R])}{k_{t,R}(k_h K_{N,S} + k_{h,N,S}[NH_S])} \tag{7}$$

i.e., it is not an intrinsic property of the enzyme as it depends on the nucleophile concentration. When using racemic nucleophiles for determination of E both nucleophiles compete for the acyl-enzyme and from Scheme 1 can be derived

$$E_{syn,\,rac} = \frac{k_{t,S}K_{N,R}}{k_{t,R}K_{N,S}} \tag{8}$$

that it is an intrinsic property of the enzyme. From Equations (7) and (8) it follows that $E_{syn} = E_{syn,\,rac}$ only when $k_{h,N,R} = k_{h,N,S} = 0$, i.e., the acyl-enzyme cannot be deacylated by water when it has bound a nucleophile. When this is not the case, only the enantioselectivity $E_{syn,\,rac}$ determined with a racemic mixture is an intrinsic molecular property, i.e., independent on the nucleophile concentration (Equation 8).

Experimental Determination of the Enantioselectivity in Kinetically Controlled Synthesis

The S_1'-enantioselectivity in kinetically controlled synthetic reactions was determined for two hydrolytic enzymes: *E. coli* penicillin amidase (PA) and bovine α-chymotrypsin (α-CT). The enantiomeric ratios were determined in reactions with isolated enantiomeric and racemic nucleophiles. PA was used to catalyse the condensation of *R*-phenylglycineamide (*R*-PhgNH$_2$) with *S*-, *R*- and racemic Phe and with *S*-, *R*- and racemic Leu. α-CT was used as a catalyst for the kinetically controlled condensation of N^{α}-acetyl-*S*-tyrosine ethyl ester (ATEE) and *S*-, *R*- and racemic AlaNH$_2$. In both cases the use of chiral activated substrate allowed resolution of the diastereomeric products without chiral chromatography.

The results obtained with the two enzymes are presented in Table 1. For all studied reactions the enantioselectivity determined with isolated enantiomeric nucleophiles was considerably lower than the one determined with racemic nucleophiles. For the reactions catalysed by α-chymotrypsin they differ by about 6-fold. For the PA-catalysed transformations the observed difference is about an order of magnitude. When racemic Phe was used as a nucleophile only one diastereomeric product (*R*-*S*) was observed. These results show that $E_{syn} \neq E_{syn,\,rac}$ and that the acyl-enzyme-nucleophile complex can be deacylated by water. Therefore, only $E_{syn,\,rac}$ can be used as a measure for enzyme S_1'-enantioselectivity in kinetically controlled synthesis. The $E_{syn,\,rac}$-values apply for initial rate measurements. Of practical importance, however, is the enantiomeric or diastereomeric excess (ee, de) at the kinetically controlled maximal concentration of the desired product. Analytical expressions or numerical solutions for this concentration have not yet been developed. It was, however, found that the observed diastereomeric excess (de) with racemic nucleophiles at the kinetically controlled maximal concentration of the product with the more specific enantiomeric nucleophile $[AN_{max}]_{R\text{-}S}$ corresponds to the enantioselectivity[11] $E_{syn,\,rac}$ derived from the initial rates $v_{T,S}$ and $v_{T,R}$ of the same reaction. Thus, $E_{syn,\,rac}$ is relevant to estimate the enantiomeric or diastereomeric excess at the maximal product concentration. For very high enantiomeric ratios, however, the difference between E_{syn} and $E_{syn,\,rac}$ becomes less important in the calculation of ee or de.

Conclusions

For the S_1'-enantioselectivity in kinetically controlled condensation reactions catalysed by amidases and peptidases only the enantiomeric ratio $E_{syn,\,rac}$ determined with racemic mixtures of the nucleophile is an

Table 1. The S'$_1$- enantioselectivities E_{syn} (Equation 7) and $E_{syn,\,rac}$ (Equation 8) for penicillin amidase- and α-chymotrypsin-catalysed kinetically controlled syntheses determined with enantiomeric and racemic nucleophiles.

Enzyme	Condensation reaction AB + NH	$(k_T/k_H)_{app}$[d]	$E_{syn} = \frac{(k_T/k_H)_S}{(k_T/k_H)_R}$	$E_{syn,\,rac} = \frac{v_{T,S}}{v_{T,R}}$	$\frac{[AN_{max}]_{R-S}}{[AN]_{R-R}}$	de (*R*-*S*), %
E. coli PA	*R*-PhgNH$_2$ + *S*-Phe[a]	10000		–	–	–
			2000			
	R-PhgNH$_2$ + *R*-Phe[a]	5		–	–	–
	R-PhgNH$_2$ + *rac* Phe[a]	–	–	>10000[e]	n.o.[e]	>99.9
	R-PhgNH$_2$ + *S*-Leu[a]	4000		–	–	–
			200			
	R-PhgNH$_2$ + *R*-Leu[a]	20		–	–	–
	R-PhgNH$_2$ + *rac* Leu[b]	–		3000	2900	99.9
Bovine α-CT	ATEE + *S*-Ala NH$_2$[a]	1550		–	–	–
			26			
	ATEE + *R*-Ala NH$_2$[a]	60		–	–	–
	ATEE + *rac* Ala NH$_2$[c]	–	–	160	163	99.0

[a] 10 mM AB, 100 mM NH.
[b] 20 mM AB, 100 mM NH.
[c] 10 mM AB, 200 mM NH.
[d] Determined from initial rate measurements at less than 10% conversion of AB. Standard deviation less than 20%.
[e] Only one diastereomer (*R*-*S*) was observed.

intrinsic molecular property (Equation 8). It should be used as a measure:

for the stereoselectivity of different enzymes used as biocatalysts for the same process, and

to study the influence of temperature, pressure, pH and immobilisation on enantioselectivity in kinetically controlled condensations with the same enzyme.[7]

The difference between $E_{syn,\,rac}$ and E_{syn} (determined with isolated enantiomers) implies that the acyl-enzyme with bound nucleophile can be deacylated by water (Scheme 1). This should also apply for other hydrolases (such as glucosidases) that have been shown to catalyse kinetically controlled synthetic reactions according to Scheme 1.

Experimental Section

General Remarks

R-PhgNH$_2$ was kindly provided by Röhm (Darmstadt, Germany). ATEE, *R*-phenylglycine (*R*-Phg), N^{α}-acetyl-*S*-tyrosine (AT), racemic and enantiomeric pure *S*- and *R*-Phe, Leu and enantiomeric pure *S*- and *R*-AlaNH$_2$ and *R*-Phg-Leu were from Sigma and Bachem. Racemic AlaNH$_2$ was prepared by mixing equimolar amounts of *S*- and *R*-enantiomers. The chiral purity of the enantiomeric substrates given by the manufacturer was $> 99\%$. All other chemicals were analytical grade. Penicillin amidase (EC 3.5.1.11) from *E. coli* was purchased from Sigma and purified by ion-exchange chromatography using a Mono Q column HR10/10 (Pharmacia, Sweden) as previously described.[12] Only the proteolytically processed form with isoelectric point 7.0 was used. The purity and the homogeneity of the enzyme was analysed by activity and protein stains on isoelectric focusing gels.[12] The molar concentration of PA was determined by active site titration with phenymethanesulfonyl fluoride[13] using 6-nitro-3-phenylacetamidobenzoic acid as a substrate. Bovine α-chymotrypsin (EC 3.4.21.1) was purchased from Sigma and was purified by affinity chromatography using soybean trypsin inhibitor (Sigma) immobilised[14] on Eupergit C250-L (Röhm, Darmstadt). The purity of the enzyme was determined by isoelectric focusing. The purified α-chymotrypsin was homogeneous and its concentration was determined by the absorbance at 280 nm, using an ε_{280} value of $4.9 \times 10^4\ M^{-1}\ cm^{-1}$.

Kinetically Controlled Condensations

In a typical experiment for enzyme-catalysed kinetically controlled synthesis of diastereomeric dipeptides, 2 mL reaction mixture containing 10 or 20 mM activated substrate (*R*-PhgNH$_2$ or ATEE) and 100 or 200 mM enantiomeric or racemic nucleophile (Phe, Leu, AlaNH$_2$; each concentration specified in Table 1) in bicarbonate buffer $I = 0.2$ with pH adjusted to 9.0 was preincubated at 25 °C. The reaction was initiated by addition of 10 μL enzyme solution preincubated at the same temperature in the same buffer. The final enzyme concentration in the reaction mixture was (depending on the nucleophile and the concentrations) in the range $1 \times 10^{-5} - 2 \times 10^{-5}$ for PA and $1 \times 10^{-6} - 2 \times 10^{-6}$ for α-CT. Periodically aliquots were withdrawn, diluted (1:10) and immediately analysed by HPLC. Each experiment was paralleled by a reference experiment without enzyme for a reading of the spontaneous hydrolysis of the activated substrate which was used to correct the input for calculation of k_T/k_H. The transferase to hydrolase ratio was calculated from the initial rates (less than 10% activated substrate exhausting) of condensation (v_T) and hydrolysis (v_H) product formation using Equation 4.[7c] The enantiomeric ratio for isolated enantiomeric nucleophiles was calculated using Equation 3, for racemic nucleophiles Equation 5 was used, respectively. The reactions with racemic nucleophiles were followed up to the maximal concentration of the specific diastereomeric product.

HPLC Analysis

Products and reactants were identified and analysed by HPLC using a Pharmacia LKB 2249 solvent delivery system and LKB Bromma 2151 variable wavelength detector with two sequentially connected analytical columns. For the PA-catalysed condensation between *R*-PhgNH$_2$ and Phe the substances were quantified using two RP-8, 5 μm, 10 cm (Merck) columns thermostatted at 50 °C and calibration curves for the peak areas at 225 nm obtained with reference compounds of known concentrations. For all measurements the peak integration was done with Chrom Star software 1994 (Bruker). The response factor for the diastereomeric *R*-Phg-*S*-Phe and *R*-Phg-*R*-Phe was inferred from the response factors for *R*-PhgNH$_2$, *R*-Phg and assuming a constant sum of the concentrations of the activated substrate, condensation and hydrolysis products during a reaction. The elution system for resolution of *R*-Phg, *R*-PhgNH$_2$, *S*- or *R*-Phe, and the diastereomeric *R*-Phg-*S*-Phe and *R*-Phg-*R*-Phe was: 67 mM KH_2PO_4 (pH 4.5) for 3 minutes followed by a step gradient methanol/67 mM KH_2PO_4 (pH 4.5) (35:65 v/v) and elution for further 5 minutes. At flow rate 2 mL/min the retention times for *R*-Phg, *R*-PhgNH$_2$, *S*- or *R*-Phe, *R*-Phg-*S*-Phe and *R*-Phg-*R*-Phe were 0.9, 1.8, 2.4, 5.3 and 6.5, respectively, at good base-line resolution. For determination of k_T/k_H only the peaks for the hydrolysis product (*R*-Phg) and the condensation product were integrated. For determination of $v_{T,S}/v_{T,R}$ only the two diastereomeric products were considered. For the PA-catalysed condensation between *R*-PhgNH$_2$ and Leu the substances were quantified using two RP-18, 5 μm, 10 cm (Merck) columns thermostatted at 60 °C and calibration curves for the peak areas at 225 nm obtained as described above. The elution system for resolution of *R*-Phg, *R*-PhgNH$_2$, *S*- or *R*-Leu and the diastereomeric *R*-Phg-*S*-Leu and *R*-Phg-*R*-Leu was: methanol/67 mM KH_2PO_4 (pH 6.5) (10:90 v/v) for 2.5 minutes followed by a step gradient methanol/67 mM KH_2PO_4 pH 6.5 (35:65 v/v) and elution for further 7 minutes. At flow rate 2.2 mL/min the retention times for *R*-Phg, *S*- or *R*-Leu, *R*-PhgNH$_2$, *R*-Phg-*R*-Leu and *R*-Phg-*S*-Leu were 1.2, 1.7, 2.9, 5.6 and 7.5, respectively, at good base-line resolution. For the α-CT-catalysed condensation of ATEE and AlaNH$_2$ the substances were quantified using two RP-18, 5 μm, 10 cm (Merck) columns thermostatted at 60 °C and calibration curves for the peak areas at 280 nm obtained with reference compounds of known concentrations. For quantification of the diastereomeric dipeptides, the calibration curve for ATEE was

used since for the absorption at 280 nm the Tyr moiety is dominant (the difference of the peak areas for 1 mM ATEE and 1 mM AT is < 1%). The elution system for resolution of AT, ATEE and the diastereomeric N^{α}-acetyl-*S*-Tyr-*S*-$AlaNH_2$, and N^{α}-acetyl-*S*-Tyr-*R*-$AlaNH_2$ ($AlaNH_2$ was not detectable at 280 nm) was: methanol/5 mM KH_2PO_4 (pH 4.5) (6:94 v/v) for 5 minutes, followed by a step gradient methanol/5 mM KH_2PO_4 (pH 4.5) (35:65 v/v) and elution for further 8 minutes. At flow rate 2 mL/min the retention times of AT, N^{α}-acetyl-*S*-Tyr-*S*-$AlaNH_2$, N^{α}-acetyl-*S*-Tyr-*R*-$AlaNH_2$ and ATEE were 1.8, 4.7, 5.8 and 11.9, respectively.

References

[1] a) R. A. Sheldon, *Chirotechnology. Industrial Synthesis of Optically Active Compounds*, Marcel Dekker, New York, **1993**; b) A. L. Margolin, *Enzyme Microb. Technol.* **1993**, *15*, 266 – 280; c) S. C. Stinson, *Chem. Eng. News* **1994**, 38 – 72; d) M. J. Van der Werf, W. J. J. van den Tweel, J. Kamphuis, S. Hartmans, J. A. M. de Bont, *TIBTECH* **1995**, *12*, 95 – 103.

[2] a) V. Kasche, *Enzyme Microb. Technol.* **1986**, *8*, 4 – 16; b) V. Kasche, in *Proteases a Practical Approach*, (Eds.: J. Bond, R. Beynon), 2nd edn., IRL-Press, Oxford, **2001**, pp. 265 – 292.

[3] C.-S. Chen, Y. Fujimoto, G. Girdaukas, C. J. Sih, *J. Am. Chem. Soc.* **1982**, *104*, 7294 – 7299.

[4] I. Schechter, A. Berger, *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157 – 162.

[5] a) V. K. Svedas, I. Y. Galaev, *Biochemistry (A translation of Biokhimia)* **1986**, *51*, 1283 – 1287; b) C.-S. Chen, C. J. Sih, *Angew. Chem. Int. Ed. Engl.* **1989**, *28*, 695 – 707.

[6] A. J. J. Straathof, J. A. Jongejan, *Enzyme Microb. Technol.* **1997**, *21*, 559 – 571.

[7] a) R. S. Phillips, *Trends Biotechnol.* **1996**, 13 – 16; b) V. Kasche, B. Galunsky, A. Nurk, E. Piotraschke, A. Rieks, *Biotechnol. Lett.* **1996**, *18*, 455 – 460; c) B. Galunsky, S. Ignatova, V. Kasche, *Biochim. Biophys. Acta* **1997**, *1343*, 130 – 138; d) P. L. A. Overbeeke, J. Ottosson, K. Hult, J. A. Jongejan, J. A. Duine, *Biocatal. Biotransform.* **1999**, *17*, 61 – 79; e) L. Lummer, A. Rieks, B. Galunsky, V. Kasche, *Biochim. Biophys. Acta* **1999**, *1433*, 327 – 334.

[8] a) D. A. Mironenko, E. V. Kozlova, V. K. Svedas, V. A. Solodenko, T. N. Kasheva, V. P. Kukhar, *Biokhimia* **1990**, *55*, 1124 – 1131; b) Z.-W. Guo, C. J. Sih, *J. Am. Chem. Soc.* **1989**, *111*, 6836 – 6841.

[9] J. L. L. Rakels, B. Romein, A. J. J. Straathof, J. J. Heijnen, *Biotechnol. Bioeng.* **1994**, *43*, 411 – 422.

[10] a) H.-D. Jakubke, P. Kuhl, A. Könnecke, *Angew. Chem. Int. Ed. Engl.* **1985**, *24*, 85 – 93; b) L. Riechmann, V. Kasche, *Biochem. Biophys. Acta* **1985**, *830*, 164 – 172; c) V. Kasche, G. Michaelis, B. Galunsky, *Biotechnol. Lett.* **1991**, *13*, 75 – 80.

[11] Y. F. Wang, C.-S. Chen, G. Girdaukas, C. S. Sih, *Enzymes in Organic Synthesis*, (Eds.: R. Porter, S. Klark), Pitman, London, **1985**, pp. 128 – 145.

[12] V. Kasche, U. Haufler, D. Markowski, A. Zeich, B. Galunsky, *Ann. N. Y. Acad. Sci.* **1987**, *501*, 97 – 102.

[13] V. K. Svedas, A. L. Margolin, C. F. Sherestyuk, A. A. Klesov, I. V. Berezin, *Bioorg. Khim.* **1977**, *3*, 545 – 553.

[14] N. Gottschlich, S. Weidgen, V. Kasche, *J. Chromatogr. A* **1996**, *719*, 267 – 274.