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Enantioseparation of 5-Monosubstituted Hydantoins by Capillary Gas Chromatography - Investigation of Chemical and Enzymatic Racemization

Holger Lickefett*[1], Karsten Krohn[2], Wilfried A. König[3], Bärbel Gehrcke[3] and Christoph Syldatk[1]

[1]Institut f. Biochemie u. Biotechnologie der Technischen Universität Braunschweig
Konstantin Uhde Str.5, W-3300 Braunschweig, Germany

[2] Fachbereich Chemie der Universitäts-GH-Paderborn
Warburger Str.100, W-4790 Paderborn, Germany

[3] Institut f. organische Chemie der Universität Hamburg
Martin-Luther-King-Platz 6, W-2000 Hamburg 13, Germany

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Abstract: The chemical as well as the enzymatic racemization of *R*- and *S*-5-monosubstituted hydantoin derivatives play an important role in the chemoenzymatic synthesis of optically pure *D*- or *L*-amino acids. To study these reactions, specifically with 5-alkylhydantoins enantiomer-separation by capillary gas chromatography, octakis(2,6-di-O-methyl-3-O-pentyl)-γ-cyclodextrin as a chiral stationary phase was used. It will be shown that this method allows the sensitive and reproducible detection of a wide range of substrates in a short time . Examples are given for the chemical racemization of different 5-alkylhydantoins as well as for their enzymatic racemization by a hydantoin racemase of *Arthrobacter* sp. DSM 3745.

INTRODUCTION

R,S-5-monosubstituted hydantoins (imidazolidin-2,4-diones) play an important role in the chemoenzymatic production of optically pure *D*- and *L*-α-amino acids (for detailed reviews see [1,2]). Up to three enzymes may be involved in this bioconversion process, as shown in Figure 1.

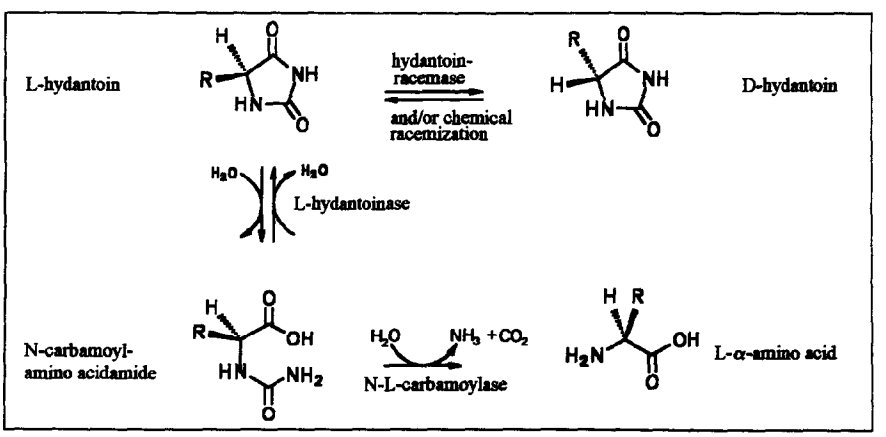

Figure 1: Enzymatic conversion of hydantoins into L-α-amino acids

The major advantage of bioconversion processes starting from *R,S*-5-monosubstituted hydantoins as substrates is the total conversion of a racemic precursor into an optically pure product, because chemical as well as enzymatic racemization processes can be observed [2]. It has been discussed that the velocity of the chemical racemization of hydantoin derivatives is influenced by the electronic nature of the substituent in 5-position. Its electronegativity, as well as other effects stabilizing the enolate structure due to participation of mesomeric structures, are responsible for the rate of racemization [2,3].

Reaction rates may be studied by polarimetry [4], however, this method is not very sensitive, requires relatively high substrate concentrations in comparison with HPLC- or GC- methods and is only useful for studying the chemical racemization. It does not work in the presence of other optically active compounds in the reaction solution, as in the case of the reaction shown in Fig. 1 or with crude enzyme extracts.

Another suitable method for investigating chemical and enzymatic racemization of hydantoins is HPLC on chiral supports, as originally used for the enantioseparation of amino acids [3,4] with UV-detection This method can even be used for resting cell experiments, if there are no overlapping peaks from other reaction products [2,3] Unfortunately this method is only sensitive in the case of hydantoins with aromatic side-chains. A pre- or post-column derivatization used for the detection of amino acids is of no use for hydantoins

The aim of this work was to develop a highly sensitive and reproducible method for the enantioseparation of 5-monosubstituted hydantoins using modified cyclodextrins as a new type of chiral stationary phases for enantiomer separation by capillary gas chromatography. These have been applied very succesfully in recent years [5].This method should allow not only to study chemical, but also enzymatic racemization processes of these compounds catalyzed by a hydantoin racemase from *Arthrobacter* sp. DSM 3745 in the presence of other optically active compounds and crude enzyme extracts.

RESULTS AND DISCUSSION

Chemical synthesis of optically active 5-monosubstituted hydantoin derivatives. Bioconversion processes for the production of optically pure α-amino acids catalyzed by hydantoinases start from racemic 5-monosubstituted hydantoin derivatives as substrates. These can be synthesized either by the method of Bucherer and Bergs from aldehydes, KCN and $(NH_4)_2SO_4$ or from unsubstituted hydantoin and aldehydes [6]. To investigate the chemical and enzymatic racemization it was necessary to prepare optically pure 5-monosubstituted hydantoins. The optically active hydantoins **3a-f** were prepared from the corresponding amino acids **1a-f** by condensation with potassium isocyanate. The intermediate open chain α-ureido amino acids **2a-e** were then cyclized with hot, diluted hydrochloric acid. A typical procedure is shown below in Figure 2 in the conversion of *tert*-leucine (**1e**) to 5-*tert*-butyl-imidazolidin-2,4-dione (**3e**).

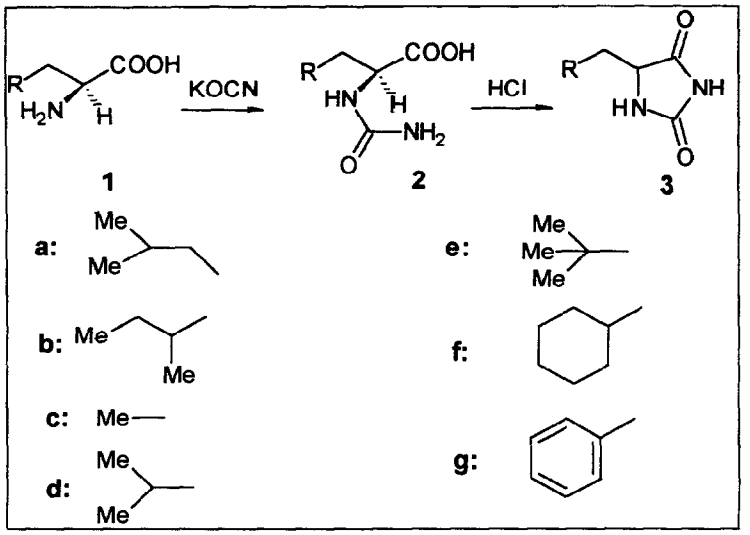

Figure 2: Chemical synthesis of optically active 5-monosubstituted hydantoins from the corresponding α-amino acid

The low solubility of some (notably aromatic) amino acids can be overcome by the addition of 30 % of acetic acid. The use of pyridine as recommended by Suzuki et al.[6] was avoided, because it leads to partial racemization in some cases. The hydantoins **3a-f** thus obtained after recrystallization from acetic acid or water were optically pure and could be used as substrates for racemization studies, whereas the stereochemical integrity was lost in the condensation with urea.

Enantioseparation of 5-monosubstituted hydantoins. For the resolution of 5-substituted chiral hydantoins, octakis(2,6-di O-methyl-3-O-pentyl)-γ-cyclodextrin [7] proved to be a highly selective stationary phase. Most of the hydantoins could be resolved using a capillary column of only 4.5 m length at moderate operating temperatures, thus taking advantage of the increase in enantioselectivity of the cyclodextrin derivative at lower temperatures [8]. The results in terms of separation factors for the enantiomers are given in Table 1. The preparation of the cyclodextrin derivative [7] and the coating procedure for fused silica capillary columns has been previously described [9]. S-enantiomers were eluted before R-enantiomers. Typical gas chromatographic separations are shown in Figure 3. The method is applicable to enzymatic investigation including microbial conversions, for studies of the kinetics of chemical racemization and for analysis of the purity of substrates.

Table 1: Separation factors (α) and elution temperature for the enantiomers of 5-substituted hydantoins on a 4.5 m / 0.25 mm (i. d.) capillary column with octakis(2,6-di-O-methyl-3-O-pentyl)-γ-cyclodextrin.

Substituent in 5-position	Separation factor	Temp [°C]
Methyl	1 045	135
Isopropyl	1.090	140
Isobutyl	1.402	140
tert. Butyl	1.093	135
Cyclohexylmethyl	1 085	175
Phenyl	1.287	175
Methylthioethyl	1 052	175
Methoxycarbonylmethyl	1.071	160

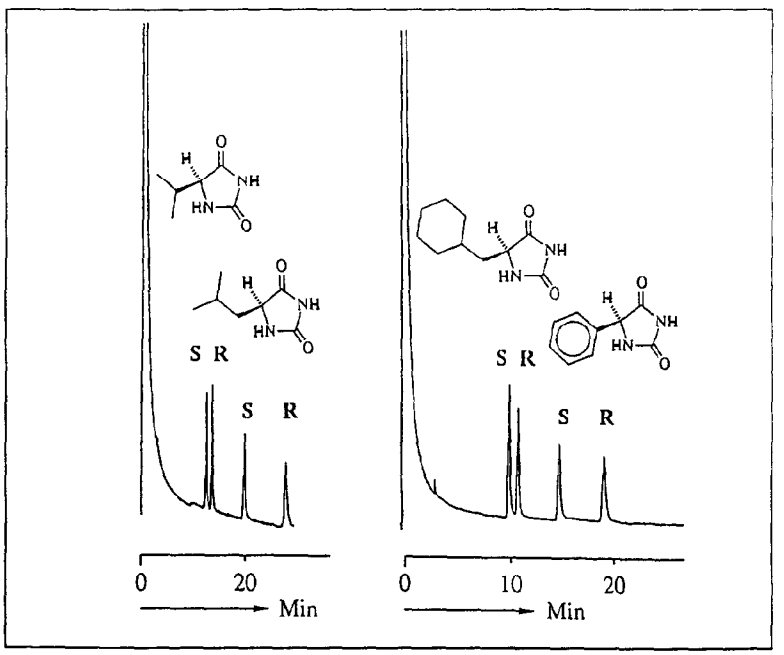

Figure 3: Gas chromatographic separations of 5-monosubstituted hydantoins on a 4.5 m fused silica capillary column with octakis(2,6-di-O-methyl-3-O-pentyl)-γ-cyclodextrin. Column temperature (left) 140°C and (right) 175°C.

Chemical and enzymatic racemization of 5-substituted hydantoins. The enzymatic or chemical racemization of the hydantoins **3a-g** (see Figure 2) was carried out in an aqueous system. The reactions were stopped by the addition of n-butanol which, at the same time, was used for extracting the highly polar products from the aqueous phase. After removal of n-butanol in a stream of dry nitrogen, the sample was taken up in dichloromethane and redissolved by the addition of methanol.

Figure 4 shows examples for the determination of both the chemical and enzymatic racemization over a period of 120 minutes. Under reaction conditions, no chemical racemization was observed. After an incubation period of 72 hours, about 10 % of chemical racemization was detected (data not shown). Contrary to the slow chemical racemization in the case of **3a** or **3d** , monosubstituted hydantoins with an aromatic substituent in the 5-position (for example phenylhydantoin) racemized spontaneously under alkaline conditions [10].

As shown in Figure 4, after addition of enzyme solution to the reaction mixture, a complete enzymatic racemization of both **3a** and **3d** was observed in a short incubation time. This confirms the theory of Battilotti *et al.*, who investigated the chemical racemization of **3d**, which proved to be rather slow. The fast and total conversion of the D,L-isopropylhydantoin to D-valine led to the assumption, that a hydantoin racemase must be responsible for the racemization of the L-enantiomer [11]. Until now, very little has been published on the subject of hydantoin racemases (for details see [2,3]). It would be of interest to compare these enzymes to those of *Arthrobacter* sp DSM 3745.

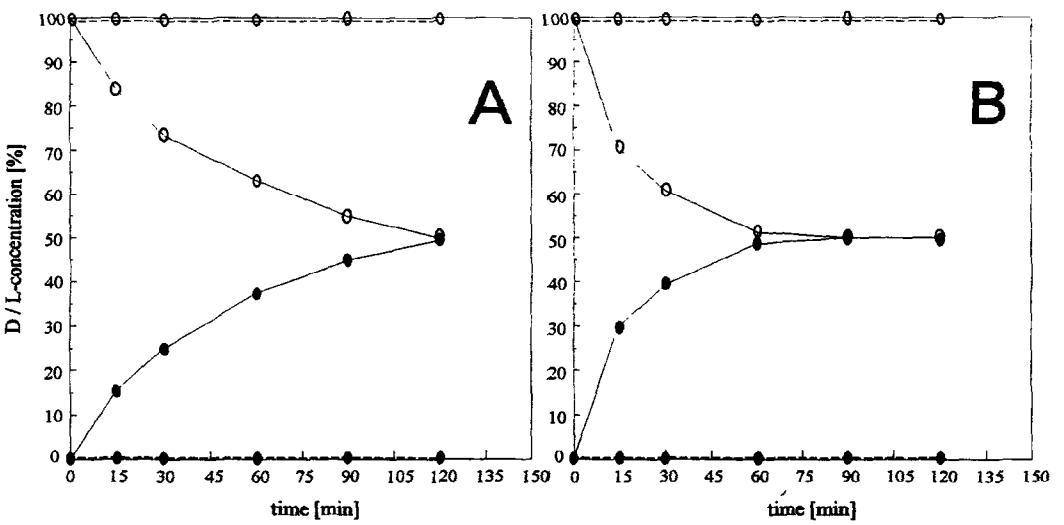

Figure 4: Chemical and enzymatic racemization of different 5-substituted hydantoins. Standard-assay: 800 µl 0.1 M triethanolamine buffer pH 8.5, was incubated with 0.5 g/l of either the D-isomer (open circles) or the L-isomer (closed circles) in an Eppendorf®-system at 37°C for various lengths of time. In the case of the enzymatic racemization (solid lines) 50 µl enzyme solution was added to the standard assay. Chemical racemization (broken lines). (A) 5-isopropylhydantoin; (B) 5-isobutylhydantoin;

CONCLUSIONS

The method described is useful for a rapid and convenient investigation of the chemical or enzymatic racemization of 5-monosubstituted alkyl-hydantoins. Unlike other possible methods, enantioselective gas chromatography has the advantage of high precision and speed and a minimum of sample handling. The extraction of the reaction solution with butanol includes a separation of the enzyme protein, which is necessary for example, when analyzing aqueous samples by HPLC. Further experiments will be carried out to investigate a new substrate spectrum e.g. 5,5-disubstituted hydantoins, which are of pharmacological interest.

EXPERIMENTAL SECTION

MATERIALS AND METHODS

For gas chromatographic studies the hydantoins **3a-g** were used. The enzymatic or chemical racemization was carried out in an aqueous system containing a 0.1 M triethanolamine buffer (pH 8.5) at 37°C for various times of incubation, depending on the reaction. The reaction was stopped with n-butanol (HPLC-solvent) and the sample was extracted for about 2 minutes. After centrifugation for 1 minute, the organic supernatant was directly used for the analysis by capillary gas chromatography The enzyme solution for the enzymatic investigations was prepared as follows: *Arthrobacter* sp DSM 3745 was cultivated as described previously [12] and then disrupted with a glas bead mill. The crude enzyme extract was precipitated with at least 2.5 M $(NH_4)_2SO_4$. The resolved pellet was separated by FPLC using a phenyl-sepharose chromatography column Active fractions were sampled and than concentrated by ultrafiltration. The concentrated enzyme solution, free of both hydantoinase and carbamoylase activity, was used for enzymatic studies

(*RS*)-5-*tert*-butyl-imidazolidin-2,4-dione (3e). A solution of *tert*-leucine (**1e**) (5.00 g, 38 mmol) and potassium cyanide (4.00 g, 48 mmol) in water/30 % acetic acid (25 ml) was heated for 1 hour at 70 °C 6 N hydrochloric acid (15 ml) was then added and the precipitate collected (α-ureido-*tert*-leucine, 4.50 g, 68 %, m. p. 227 °C). The α-ureido-*tert*-leucine was then refluxed for 1 hour with a mixture of water (20 ml), acetic acid (10 ml), and 6 N HCl (15 ml). The solution was cooled and the precipitated crystals collected and recrystallized from acetic acid to afford **3e** (3.05 g, 52 %), m.p. 232 °C $[\alpha]_D^{20}$ +63.9 (c = 0.27, ethanol). ^{1}H-NMR (d^6-DMSO, 400 MHz). δ = 0.92 (s, 9 H, H-6), 3.66 (d, J = 1 Hz, H-5), 7.94 (s, 1 H, H-1), 10.53 (s, 1 H, H-3).
^{13}C-NMR (d^6-DMSO, 100 MHz): δ = 26.73 (q, C-6), 33 66 (d, C-5), 60 78 (s), 158.67 (s), 173 76 (s).
IR (KBr): = 3228 cm^{-1} (N-H), 3107 (N-H), 1769 (C=O).

$C_7H_{12}N_2O_2$ (156.2)	Calc.	C 53.83	H 7 74	N 17.94
	Found	C 54.03	H 7.87	N 17.87

Similarly L-cyclohexylalanine was converted to the hydantoin; m. p. 235-245 °C. $[\alpha]_D^{20}$ +62.4 (c = 1.27, ethanol).
^{1}H-NMR (d^6-DMSO, 400 MHz): δ = 0.80 - 1 74 (m, 13 H, 6-H u. -CH_2), 4.00 - 4.03 (m, 1 H, 5-H), 7.96 (s, 1 H, 1-H), 10.54 (s, 1 H, 3-H)
^{13}C-NMR (d^6-DMSO, 100 MHz): δ = 25.51 (t), 25.75 (t), 25.95 (t), 31.75 (t), 33.23 (d), 33.35 (t), 39.27 (t), 55.56 (d), 157.43 (s), 176.64 (s).
IR (KBr): = 3443 cm^{-1} (N-H), 3200 (N-H), 2925 (C-H), 2853 (C-H), 1692 (C=O), 1655 (C=O).

$C_{10}H_{15}N_2O_2$ (196.2)	Calc.	C 61.20	H 8.22	N 14.27
	Found	C 60.91	H 8.21	N 13.96

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REFERENCES AND NOTES

1 Syldatk, C.; Müller, R.; Siemann, M.; Krohn, K.; Wagner, F.; Microbial and Enzymatic Production of D-Amino Acids from D,L-5-Monosubstituted Hydantoins. In *Biocatalytic Production of Amino Acids and Derivatives;* Rozell, J.D ; Wagner, F., Hanser Publishers: New York, 1992; pp. 75-128

2 Syldatk, C.; Müller, R.; Pietzsch, M., Wagner, F., Microbial and Enzymatic Production of L-Amino Acids from D,L-5-Monosubstituted Hydantoins. In *Biocatalytic Production of Amino Acids and Derivatives*; Rozell, J.D.; Wagner, F.; Hanser Publishers: New York, 1992; pp. 129-176.

3. Pietzsch, M.; Syldatk, C.; Wagner, F.; *Enzyme Engineering* **1992**, XI, 672, 478-483

4. Watabe, K.; Ishikawa, T.; Mukohara, Y., Nakamura, H.; *Journal of Bacteriology* **1992**, 11, 174, 3461-3466

5. König, W.A.; "*Gas Chromatographic Enantiomer Separation with Modified Cyclodextrins*", Huethig-Buchverlag: Heidelberg, 1992.

6 Suzuki, T.; Igarashi, K.; Hase, K.; Tuzimura, K.; *Agric. Biol. Chem.* **1973**, 37, 411

7 König, W.A; Gehrcke, B.; Icheln, D.; Evers, P ; Dönnecke,J.; Wang, W.; *J.High Res. Chromatogr.* **1992**, 15, 367

8. Hardt, I.; König, W.A.; *J.Microcol. Sep.*, **1993**, 5, 35

9. Pietruszka, J.; Hochmuth, D.H.; Gehrcke, B.; Icheln, D.; Runge, T.; König, W.A.; *Tetrahedron Asymmetry* **1992**, 5, 661

10. Yamada, H.; Shimizu, S.; Shimada, H.; Tani, Y.; Takahashi, S.; Ohashi, T.; *BIOCHIMIE* **1980**, 62, 395-399

11. Battilotti, M.; Barberini, U.; *J. Mol. Cat.* **1988**, 43, 343

12. Syldatk, C.; Mackowiak, V; Höke, H.; Gross, C.; Dombach, G.; Wagner, F.; *J. Biotechnol.* **1990**, 14, 345-362