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New Hydantoinases from Thermophilic Microorganisms - Synthesis of Enantiomerically Pure D-Amino acids

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Abstract: *A series of 14 D-α-amino acids were prepared in high chemical and optical yields from the corresponding racemic hydantoins by employing two novel hydantoinases from thermophilic microorganisms.*

Enantiomerically pure D-α-amino acids are important building blocks for a variety of biologically active pharmaceuticals like peptides[1], semisynthetic β-lactam antibiotics[2] and ACE inhibitors[3]. On a technical scale several enzymatic processes for their preparation are known[4]. Of particular interest is the highly efficient preparation of D-amino acids *via* the enantioselective hydrolytic ring opening of hydantoins (5-monosubstituted imidazolidin-2,4-dions) by D-specific hydantoinases EC 3.5.2.2 (Fig.1).

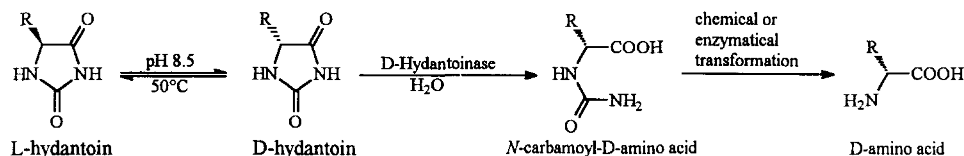

Fig. 1. Enzymatic preparation of enantiomerically pure D-amino acids from racemic 5-monosubstituted hydantoins

The thus produced *N*-carbamoyl-D-amino acids are transformed chemically or enzymatically into the corresponding amino acids with complete retention of the configuration. Due to the spontaneous racemization of the hydantoins under the reaction conditions (pH> 8), the method allows a quantitative conversion of the racemic starting materials into the desired D-amino acids. In contrast to other hydrolases, D-hydantoinases were not readily available, also most of the described hydantoinases are instable at elevated temperatures.

In the present paper we report the use of two new, commercially available and thermally highly stable hydantoinases from thermophilic microorganisms. The required racemic 5-substituted hydantoins (±)-**1-14** were synthesized according to the procedure of Bucherer and Bergs[5] by simple condensation of the corresponding aliphatic or aromatic aldehydes with KCN and $(NH_4)_2CO_3$.

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In typical experiments, 7 mmol of (±)-**1-14** were added to 70 ml of a thermostated (50°C) buffer solution [0.1 M glycine/NaOH, pH 8.5,1 mM Mn^{2+}] under nitrogen, followed by the addition of 70 µl D-HYD 1 or 14 µl D-HYD 2 solution. The beginning enzymatic hydrolysis is indicated by a decrease of pH which is kept constant at pH 8.5 by continuous addition of 1 N NaOH-solution using an autotitrator. The specific activity of the enzymes[6] was calculated from the initial rate of the transformation. After completion the obtained reaction mixtures were filtered over Celite®, concentrated (vacuum) to 10 -20 ml and then acidified with HCl to pH 2-3. With the exception of the products resulting from (±)-**9** and (±)-**10**, the *N*-carbamoyl-D-amino acids were isolated by filtration after ice cooling, washed with a small amount of water and then dried. They were further converted into the corresponding D-amino acids by diazotation. For this 1-3 mmol of the corresponding carbamoylate were dissolved in 50 ml 3.5 N HCl to which at 0°C an equimolar quantity of $NaNO_2$, dissolved in 10 ml H_2O was added under vigorous stirring. The resulting D-amino acids were purified by ion exchange chromatography (25 g DOWEX® 50). Their enantiomeric purities were determined *via* derivatisation with 2,3,4,6-tetra-O-benzoyl-β-D-glucopyranosylisothiocyanate BGIT[7] followed by HPLC analysis [LiChrospher 100 RP-18; acetonitrile/0.1% trifluoroacetic acid] of the resulting diastereomeric thiourethanes. The optical purities of D-**23-27** were determined by HPLC using a chiral column [Chrownpack® CR(+), Daicel]. The results, also showing the broad substrate tolerance, are summarized in the table.

Using (±)-**2** and (±)-**3** as model substrates the dependence of the specific activities from (a) buffer, (b) pH (Fig.2) and temperature (Fig.3) were determined for both enzymes. Glycine /NaOH buffer proved to be superior to TRIS/HCl, a pH 8.5 resulting in the highest activity for both D-HYD 1 (85 U/mg protein) and D-HYD 2 (200 U/mg protein) (Fig.2). A ten fold increase was observed in changing the temperature from 37°C to 80°C (Fig.3). Both enzymes display a remarkable temperature stability and can be used for several hours at temperatures as high as 70°C, while at T>80°C increasing loss of activity is observed. A similar stability was reported previously only in one case (hydantoinase from *Agrobacterium* sp. IP-671[8]).

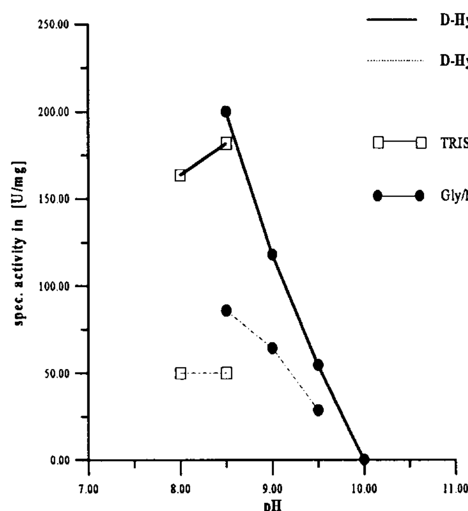

Fig.2: Effect of the pH on the specific activity in 0.1 N glycine/NaOH and 0.1 N TRIS/HCl buffer using DL-n-butylhydantoin (±)-**2** as substrate

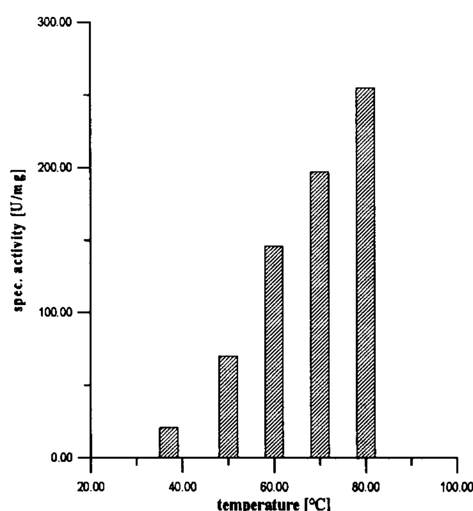

Fig.3: Effect of temperature on the specific activity of hydantoinase catalysed hydrolysis of (±)-5-(β-phenylethyl)hydantoin (±)-**3** with D-HYD 2

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Table Specific activity, reaction time, isolated yield, optical rotation of the *N*-carbamoyl-amino acids and the enantiomeric purities of the isolated amino acids

Substrate (5-substituted hydantoin)	Enzyme	Specific Activity [U/mg]	Reaction time [h]	Isolated yield of carbamoylate	$[\alpha]_D^{20}$ (c=1 in MeOH)	enantiomeric excess of the amino acid [%]	
DL-5-Methyl (±)-**1**	D-HYD-1	133	22.8	71%	-4.0	Alanine	94
	D-HYD-2	769	7.6	73%	-1.8	D-**15**	34
DL-5-n-Butyl (±)-**2**	D-HYD-1	82	23.3	67%	-14.0	Norleucine	>99
	D-HYD-2	127	21.5	70%	-11.5	D-**16**	83
DL-5-(β-Phenylethyl)	D-HYD-1	63	41.2	53%	-19.0	Homophenyl-	>99
(±)-**3**	D-HYD-2	70	27.2	77%	-17.8	alanine D-**17**	>99
DL-5-Isobutyl (±)-**4**	D-HYD-1	94	19.2	89%	+5.8[a)]	Leucine	>99
	D-HYD-2	156	18.0	93%	+4.7[a)]	D-**18**	>99
DL-5-Benzyl (±)-**5**	D-HYD-1	47	39.2	67%	-46.7	Phenylalanine	>99
	D-HYD-2	5	29.2	12%	-43.7	D-**19**	>99
DL-5-Isopropyl (±)-**6**	D-HYD-1	61	45.0	66%	-15.6	Valine	>99
	D-HYD-2	65	43.7	71%	-16.0	D-**20**	>99
DL-5-sec-Butyl (±)-**7**	D-HYD-1	29	24.2	65%	-23.1	Isoleucine	>99, >99[b)]
	D-HYD-2	23	41.0	71%	-23.0	D-**21a**,**b**[b)]	>99, >99[b)]
DL-5-Methylthioethyl	D-HYD-1	71	12.7	81%	-1.7	Methionine	>99
(±)-**8**	D-HYD-2	358	14.1	75%	-1.4	D-**22**	99
DL-5-Hydroxymethyl	D-HYD-1	27	16.6	-	-	Serine	98
(±)-**9**	D-HYD-2	156	10.2			D-**23**	97
DL-5-(2-Hydroxyethyl),	D-HYD-1	61	21.0	-	-	Threonine	>98
(±)-**10** (*allo*-free)	D-HYD-2	78	21.2			D-**24a**,**b**[b)]	>98
DL-5-(2-Thienyl)	D-HYD-1	612	0.5	42%	-110.4	(2-Thienyl)glycine	97
(±)-**11**	D-HYD-2	1351	0.3	50%	-111.9	D-**25**	96
DL-5-Phenyl	D-HYD-1	620	0.75	95%	-143.3	Phenylglycine	96
(±)-**12**	D-HYD-2	1221	0.6	90%	-152.5	D-**26**	>99
DL-5-(p-Hydroxyphenyl)	D-HYD-1	302	5.8	65%	-172.0	(p-Hydroxyphenyl)	>99
(±)-**13**	D-HYD-2	52	11.0	78%	-167.5	glycine D-**27**	>99
DL-5-Methyl-5-phenyl	D-HYD-1	<1	72	3	-	2-Phenylalanine	>99
(±)-**14**	D-HYD-2	<1	72	7		D-**28**	97

a) c=0.5 in MeOH b) *allo*-configuration; enantiomeric purity of *allo*-threonine not determined

D-HYD 2 usually displays higher activities with aliphatic substituted hydantoins (±)-**1-7** than D-HYD 1. Considerable activity differences were observed for compounds (±)-**1,2,4** which have two or more protons at C(1) next to the ring system, and also for the aliphatic heterosubstituted hydantoins (±)-**8-10**. The specific activity of both D-hydantoinases seems to decrease with increasing steric bulk in (±)-**1-7**. In the hydrolysis of the aromatic substituted compounds (±)-**11-13** remarkably high specific activities were observed, most likely due to the high acidity of the proton on C(5) of the hydantoin ring.

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In contrast to this, both hydantoinases display only a very small activity towards the 5,5-disubstituted hydantoin (±)-**14**. The resulting *N*-carbamoyl-D-amino acids were isolated in high yields (≥75%) and further converted into the corresponding D-amino acids, which were obtained with excellent enantiomeric purities. D-HYD 1 usually leads to higher enantiomeric purities for aliphatic amino acids D-**15-24** as compared to D-HYD 2, which in turn is the preferred enzyme for the produced D-amino acids with aromatic substituents D-**25-27**. Both enzymes display no diastereoselectivity, hydrolysis of (±)-**7** and (±)-**10** produces diastereomeric product mixtures.

In summary, the described thermostable D-hydantoinases catalyze the highly enantioselective hydrolysis of hydantoins with great structural variety. They thus allow the synthesis of numerous α-D-amino acids in high chemical and optical yields.

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