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Biotransformation of new racemic (R,S)-5-benzylhydantoin derivatives by D-hydantoinases from adzuki bean

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Abstract

In the present work the scope of D-hydantoinase enzyme application was increased towards new racemic (*R,S*)-5-benzylhydantoin derivatives. Five new substrates for the D-hydantoinase (*R,S*)-5-(3′-carboxybenzyl)hydantoin, (*R,S*)-5-(4′-carboxybenzyl)hydantoin, (*R,S*)-5-(2′-carbomethoxybenzyl)hydantoin, (*R,S*)-5-(3′-carbomethoxybenzyl)hydantoin and (*R,S*)-5-(4′(4-ethoxycarboxy)propoxybenzyl)hydantoin were synthesised and converted using a two-step hydantoinase process into their corresponding D-phenylalanine derivatives. In this study two D-hydantoinases from *Vigna angularis* (adzuki bean) obtained from commercial sources were used: pure, isolated directly from *Vigna angularis* (*V.a.*D-HYD) 494 U/g and immobilised, recombinant, cloned and expressed in *Escherichia coli* (*r*D-HYD) 53.1 U/g. The results obtained showed that the examined enzymes catalysed hydrolysis of all new substrates into their corresponding *N*-carbamoyl-D-phenylalanine derivatives. High enantiomeric purities of the resulting D-phenylalanine derivatives were also determined. However, very low conversion yields of (*R,S*)-5-(3′-carboxybenzyl)hydantoin and (*R,S*)-5-(2′-carbomethoxybenzyl)hydantoin to corresponding *N*-carbamoyl-D amino acid were observed. Three D-phenylalanine derivatives: 4-carboxy-D-phenylalanine, 3-carbomethoxy-D-phenylalanine and 4-carbopropoxy-D-phenylalanine were obtained and isolated from the reaction mixtures using ion-exchange chromatography.

Keywords: *D-amino acids, D-phenylalanine, Hydantoinase, Dihydropyrimidinase, Capillary electrophoresis*

Introduction

The D-hydantoinase enzyme, also known as dihydropyrimidinase or 5,6-dihydropyrimidine amidohydrolase (E.C 3.5.2.2), plays a very important role in pyrimidine catabolism (Syldatk et al. 1999). D-hydantoinase is commonly used for the two-step production of D-amino acids from racemic 5-monosubstituted hydantoins. In the first step of this process, the D-hydantoinase catalyses stereoselective hydrolysis of (*R*)-enantiomer of hydantoin ring yielding optically pure *N*-carbamoyl-D-amino acid. The racemisation of 5-monosubstituted hydantoin derivatives under alkaline conditions, or in the presence of a racemase, allows high-yield bioconversion of hydantoins by D-hydantoinase. In the second step, the *N*-carbamoyl-D-amino acid is converted into the corresponding D-amino acid using *N*-carbamoylase or a chemical procedure using a diazotisation reaction (Takahashi et al. 1979; Altenbuchner et al. 2001). However, the hydantoinase process is not only used for D-amino acid production, but also used for the production of natural and unnatural L-amino acids using modified D-hydantoinase with inverting enantioselectivity (May et al. 2000).

The industrial application of D-hydantoinase in the pharmaceutical field include biosynthesis of D-phenylglycine and its 4-hydroxy derivative, starting from (*R,S*)-phenylhydantoin or (*R,S*)-4-hydroxyphenylhydantoin (Takahashi 1986). Nowadays, the annual production of D-4-hydroxyphenylglycine (about 10 000 t), an important intermediate for the synthesis of β-lactam antibiotics, is carried out mostly using hydantoinase (Ogawa et al. 2012). Apart from an industrial process, hydantoinase has been used on a laboratory scale to produce unnatural D-amino acids for use as ligands of some classes of receptors or important chiral building blocks for producing biologically active compounds. For instance, a series

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of D-phenylglycine derivatives, potential glutamate receptors effectors, were successfully produced from racemic-ring-substituted 5-phenylhydantoins (PHs) using pure enzyme or microorganisms with D-hydantoinase activity (Garcia & Azerad 1997). The range of racemic PHs, which can be efficiently converted by D-hydantoinase, were also extended to include fluorine and trifluoromethyl moieties (Arcuri et al. 2003). Silicon-containing D-configured *N*-carbamoyl amino acids were also successfully obtained using D-hydantoinase from racemic 5-silylmethylated hydantoins (Smith et al. 2001). Recently, stereoselective D-hydantoinase catalysed hydrolysis of aryl-substituted dihydropyrimidines to the corresponding *N*-carbamoyl-β-amino acids for conversion to optically pure β-amino acids was also investigated (Engel et al. 2012).

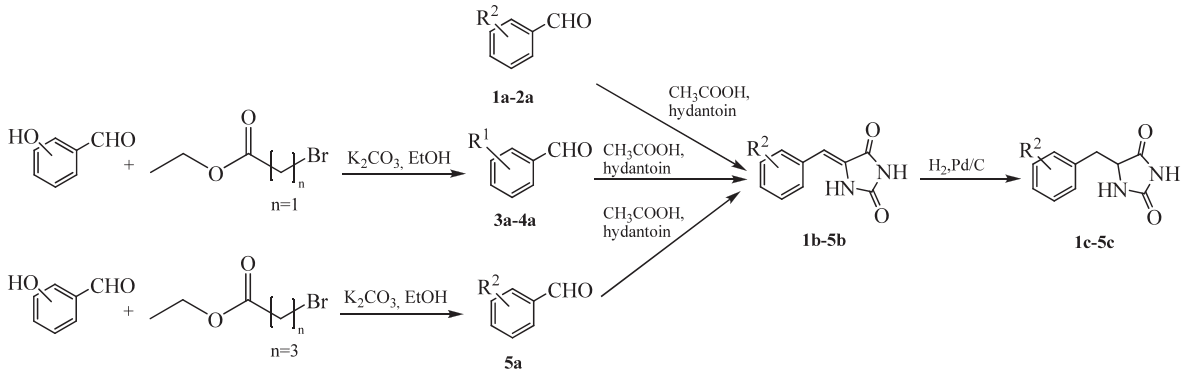
The occurrence of D-amino acids in nature has been described extensively in recent decades. An important D-amino acid, which was found in many naturally occurring, biologically active peptides, is D-phenylalanine. For instance, pharmaceutically important peptides with strong antibacterial activity from *Bacillus sp*. contain D-phenylalanine: Gramicidin S, Tyrocidine A–D (Katz & Demain 1977), Bacitracin A (Ikai et al. 1995), Polymyxin B (Banerjee & Bose 1963). D-phenylalanine has also been found in Fungisporin, a cyclooctapeptide isolated from spores of species of *Penicillium* and *Aspergillus* (Studer 1969; Wakayama et al. 2003). Several active peptides containing D-phenylalanine have also been isolated from animals: Conotoxins (Buczek et al. 2005), Ocp-1 (Iwakoshi et al. 2000), cHH (Soyez et al. 2000), Achatin I (Kim et al. 1991). Moreover, D-phenylalanine was reported to be important for the design new peptidomimetics because of their hydrophobic activity, which give better transport properties of peptidomimetics through cellular membranes (Wang et al. 2002). The other benefits include better resistance to hydrolysis by peptidases and proteases and antibody recognition of peptidomimetics containing D-amino acids (Pappenheimer et al. 1994; Chong et al. 1996). D-phenylalanine has been used for the synthesis of following important peptidomimetics: Ac-rfwink-NH2 (Dooley et al. 1994), Nateglinide (Tentolouris et al. 2007), Octreotide and Hexarelin (Dasgupta 2004; Melis et al. 2000). An important group of decapeptides containing not only D-phenylalanine but also their derivatives, 4-amino-D-phenylalanine and 4-chloro-D-phenylalanine, are the analogues of GnRH hormone (Herbst 2003; Martinez-Rodriguez et al. 2010). Additionally, D-phenylalanine was shown to possess antidepressant activity in *Parkinson's* disease treatment and was also described as an analgesic in warm-blooded animals (Heller 1980). Moreover, the enantiomers of phenylalanine derivatives were reported to be a potent ligands of metabotropic (Ma et al. 1998; Pin et al 1999) and ionotropic glutamate receptors (Szymańska et al. 2009, 2011; Venskutonyte et al. 2012).

Within this study we determined the substrate specifity and stereoselectivity of two D-hydantoinases from adzuki bean towards several new (*R,S*)-5-benzylhydantoin derivatives with different substituents. We also increased the scope of D-hydantoinase application through production of a new series of D-phenylalanine derivatives. Additionally, a simple method for monitoring the biotransformation by capillary electrophoresis (CE) is also proposed.

Materials and methods

Chemicals

Unless otherwise stated, all materials were obtained from commercial sources and used without further purification. The benzaldehydes **1a** (3-carboxybenzaldehyde) and **2a** (4-carboxybenzaldehyde) were obtained from Alfa Aesar. The benzaldehydes **3a**, **4a** and **5a** were synthesised using 2-, 3- or 4- hydroxybenzaldehydes and corresponding bromoesters in the presence of K_2CO_3/EtOH (Figure 1). The respective hydroxybenzaldehyde (0.05 mol) and appropriate bromo ester (0.05 mol) and K_2CO_3 (0.025 mol) were stirred for 8 h in EtOH (50 ml). After removing K_2CO_3 by filtration, EtOH was evaporated *in vacuo*. Next, to the remaining material 2% NaOH was added and extracted with CH_2Cl_2. Evaporation of the organic layer (dried with Na_2SO_4) gave crude benzaldehydes **3a**–**5a** used for further reactions. The substrates for the enzymatic reactions **1c**–**5c**: (*R,S*)-5-(3′-carboxybenzyl) hydantoin (**1c**), (*R,S*)-5-(4′-carboxybenzyl)hydantoin (**2c**), (*R,S*)-5-(2′-carbomethoxybenzyl)hydantoin (**3c**), (*R,S*)-5-(3′-carbomethoxybenzyl)hydantoin (**4c**) and (*R,S*)-5-(4′(4-ethoxycarboxy)propoxybenzyl)hydantoin (**5c**) were synthesised from **1a**–**5a** initially using Knoevenagel's condensation to afford 5-arylidenehydantoins as we described previously (Latacz et al. 2006). Next, catalytic hydrogenation of the unsaturated bond in 5-position of the hydantoin ring in the presence of Pd/C catalyst was performed (Figure 1). For this reaction the 5-arylidenehydantoins (0.5 mM) (**1b**–**5b**) were dissolved in methanol (250 ml) and, after addition of 100 mg Pd/C, treated with hydrogen (40 ml/min) at 55°C for 8 h. Pure (*R,S*)-5-benzylhydantoins (**1c**–**5c**) were recovered by evaporation of the methanol *in vacuo*. The reactions were monitored using CE at 20 kV, pH 8.9 of the background electrolyte. ^{1}H NMR spectra, CE and LC/MS analysis confirmed slight (compound **5c**) or almost complete (compounds **3c** and **4c**) hydrolysis of the ester moiety from the



1a, 1b, 1c: R^2 = 3-COOH
1a, 2b, 2c: R^2 = 4-COOH
3b, 3c: R^2 = 2-OCH_2COOH
4b, 4c: R^2 = 3-OCH_2COOH
5a, 5b, 5c: R^2 = 4-[$O(CH_2)_3COOCH_2CH_3$]

3a: R^1 = 2-$OCH_2COOCH_2CH_3$
4a: R^1 = 3-$OCH_2COOCH_2CH_3$

Figure 1. Synthesis of (*R,S*)-5-benzylhydantoins.

obtained alkoxybenzaldehyde derivatives after the Knoevenagel reaction. Complete ester hydrolysis occurred under conditions of diazotisation (Figure 2).

Enzymes

Two D-hydantoinases were used in this study: pure, isolated directly from (adzuki bean) *Vigna angularis* (*V.a*.D-HYD, 494 U/g using hydantoin as a substrate) and immobilised, recombinant, cloned and expressed in *Escherichia coli* (*r*D-HYD, 53.1 U/g using hydantoin as a substrate), both obtained from Sigma-Aldrich.

Analysis

Chemical syntheses were monitored using thin-layer chromatography (TLC) carried out on 0.25 mm Kieselgel 60F254 plates (MERCK) and visualised using UV light. Melting points were determined on a MEL-TEMP II apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Mercury VX 300 MHz PFG instrument (Varian Inc., Palo Alto, CA, USA) in [d6]-DMSO at ambient temperature using the solvent signal as an internal standard. Chemical shifts are shown in δ. J values are in Hertz (Hz) and splitting patterns are designated as follows: s (singlet), d (doublet), t (triplet), q (quartet), qu (quintet), m (multiplet). Mass spectra were recorded on LC/MS system consisted of a Waters Acquity UPLC, coupled to a Waters TQD mass spectrometer (electrospray ionisation mode ESI-tandem quadrupole). A Beckman CE system (P/ACE MDQ) equipped with diode-array detector was used for all separations. It was controlled using 32 Karat Software version 8.0. An uncoated fused-silica capillary with total length of 60 cm (50.2 cm to detection window) and internal diameter 75 μm was purchased from Beckman. The separations were carried out in sodium borate buffer pH 8.9 or pH 9.0 (Beckman). The CE conditions were 25–30 kV, and the

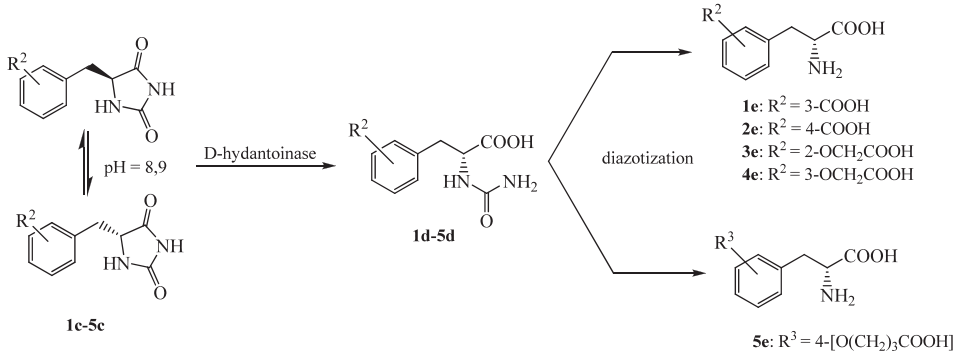


Figure 2. Two-step D-amino acid production using D-hydantoinase and diazotisation.

temperature was 25°C. For the chiral analysis of 4-carboxypropoxy-D-phenylalanine a Beckman Chiral Methods Development Kit was used. The Kit contained the following: uncoated fused-silica capillary with total length of 30 cm (20 cm to detection window), phosphate buffer 50 mM pH 2.5, 20% HS-α-CD and PTS (1,3,6,8-pyrene tetrasulfonate) as an internal standard. The enantioseparation was performed in 5% solution of HS-α-CD in phosphate buffer at pH 2.5. The CE conditions were 15 kV, and the temperature was 25°C. The enantiomeric purities of all D-phenylalanine derivatives were analysed using Dionex P580 HPLC instrument equipped with a ChiroSil (RCA+) column containing the chiral stationary phase prepared by a covalent trifunctional bonding of (+) or (-)-(18-Crown-6)-tetracarboxylic acid. The mobile phase was prepared according to the manufacturer's instructions dedicated for the enantioseparation of D,L-phenylalanine and was composed of 70% methanol/30% 10 mM CH_3COOH. The flow rate was 1.5 ml/min, the temperature 25°C and the detection wavelength 225 nm. Ion-exchange chromatography was performed using Amberlite 120 IR/H^+ cation-exchange resin obtained from Alfa Aesar. Optical rotations were measured on a Jasco DIP-1000 spectropolarimeter at the sodium D-line with a 1 dm path-length analytical cell.

Biotransformations

To prepare the substrate solutions each (*R,S*)-5-benzylhydantoin derivative was dissolved in 100 µl of DMSO and then sodium borate buffer pH 9 (MERCK) up to a concentration of 1 mM was added. The calibration curves for compounds **1c**–**5c** were estimated using CE with the following substrate concentrations: 1, 0.7, 0.5, 0.1 and 0.02 mM using DMSO as the internal standard. The biotransformation was carried out on the orbital shaker 120 rpm, at 37°C for 96 h and monitored using CE. All experiments were carried out with 10 mg of D-hydantoinase suspended in a 2 ml solution of 1 mM 5-benzylhydantoin derivative in sodium borate buffer pH 9. The course of reaction was followed by CE determination of concentration of the respective (*R,S*)-5-benzylhydantoin derivative (**1c**–**5c**). Biotransformation data were collected after 24, 48, 72 and 96 h of the reaction (Figures 3 and 4). The electrophoreses were performed at room temperature applying a voltage of 20 kV and at pH 8.9 of the background electrolyte for reaction mixtures with *rD*-HYD and applying a voltage of 30 kV and at pH 9 of the background electrolyte for reaction mixtures with *V.a.*D-HYD. In the next step, the D-hydantoinase was removed using a 0.2 µm membrane filter

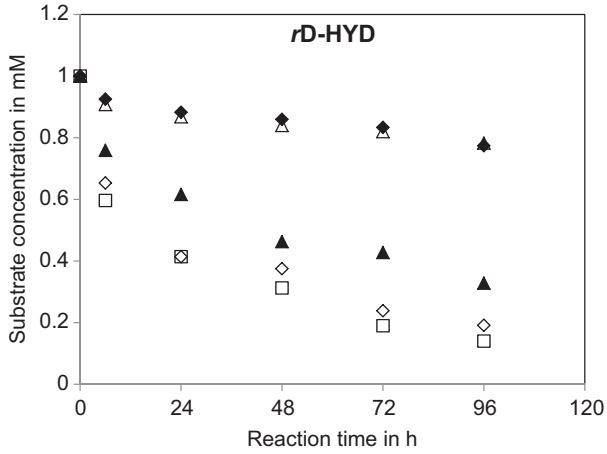


Figure 3. The bioconversion of (*R,S*)-5-benzylhydantoin derivatives with *r*D-HYD in sodium borate buffer pH 8.9, at 37°C for 96 h: (◆) 1c, (□) 2c, (△) 3c, (▲) 4c, (◊) 5c.

and the reaction mixture was acidified with 3.5 M HCl to pH 1. Then, 5 µl of 3.5 M $NaNO_2$ was added and the reaction mixture was stored on ice for 6 h. The reaction was stopped by alkalisation to pH 9.0 with 3.5 M NaOH (Figure 2). The D-amino acids were not isolated. Enantiomeric excess (ee) of the obtained D-amino acid was measured using HPLC and CE (Table I).

*Preparation of D-phenylalanine derivatives **1e, 2e, 3e, 4e, 5e** by rD-HYD*

For the larger-scale reaction (*R,S*)-5-benzylhydantoin derivatives (0.2 mmol) were dissolved in 200 ml sodium borate buffer pH 8.9 and *r*D-HYD (1 g) was added and incubated with shaking (120 rpm) for 144 h in 37°C. The reaction was monitored using

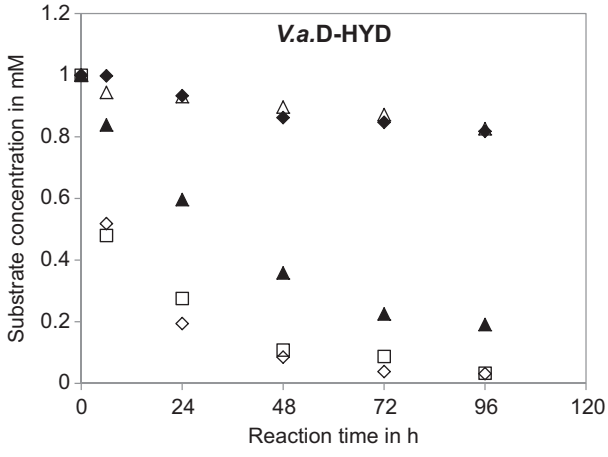


Figure 4. The bioconversion of (*R,S*)-5-benzylhydantoin derivatives with *V.a.*D-HYD in sodium borate buffer pH 8.9, at 37°C for 96 h: (◆) 1c, (□) 2c, (△) 3c, (▲) 4c, (◊) 5c.

Table I. Biotransformation efficiency and enantioselectivity.

	*r*D-HYD		*Va.*D-HYD	
Substrate	ee[a] (%)	Conversion (%)	ee[a] (%)	Conversion (%)
1c	83.0	23	76.0	18
2c	98.5	86	97.5	97
3c	94.5	22	98.7	17
4c	91.9	67	99.4	81
5c	98.5[b]			
	97.3[c]	81	99.4	97

[a]ee (%) estimated using HPLC for 1e–5e or CE for 5e.
[b]HPLC.
[c]CE.

CE every 24 h. The reaction was stopped after 144 h by removing *r*D-HYD using a 0.2 µm membrane filter. Next, the reaction mixture was acidified with 3.5 M HCl to pH 1 and 250 µl of 3.5 M $NaNO_2$ was added. After 6 h of cooling on ice 3.5 M NaOH was added to pH 9.0 and the D-phenylalanine derivatives were deposited on Amberlite 120 IR/H^+ cation-exchange resin. The D-amino acids were next eluted with 0.9 M NH_4OH and recovered by lyophilisation. The ^{1}H NMR analyses were performed for the compounds **2e**, **4e** and **5e**. The presence of all obtained D-amino acids was confirmed using CE, HPLC and LC/MS. Due to the low conversion yield of compounds **1c** and **3c** to the corresponding *N*-carbamoyl-D-phenylalanine derivatives and low ion-exchange chromatography efficiency, no ^{1}H NMR data of 3-carboxy-D-phenylalanine (**1e**) and 2-carbomethoxy-D-phenylalanine (**3e**) were obtained.

*3-carboxy-D-phenylalanine (**1e**)*. Prepared from **1c**. HPLC: tL = 3.34 min [minor, (L)-isomer], tD = 4.68 min [major, (D)-isomer], 83.03% ee; CE: t_m = 4.42 min; MS calculated for $[M+H]^+$: $C_{10}H_{11}NO_4$ *m/z*: 210.07 found 210.19;

*4-carboxy-D-phenylalanine (**2e**)*. Prepared from **2c** as brown powder. mp. 315–317°C; HPLC: tL = 3.16 min [minor, (L)-isomer], tD = 3.76 min [major, (D)-isomer], 98.5% ee; CE: t_m = 4.02 min; MS calculated for $[M+H]^+$: $C_{10}H_{11}NO_4$ *m/z*: 210.07 found 210.12; ^{1}H-NMR [D_2O] δ 2.96–3.03 (dd, J_1 = 6.15 Hz, J_2 = 8.46 Hz, 1H, H-C-H), 3.18–3.25 (dd, J_1 = 9.75 Hz, J_2 = 4.87 Hz, 1H, H-C-H); 3.78–3.83 (q, J = 4.45 Hz, 1H, C<u>H</u>-NH_2); 6.86 (d, J = 8.21 Hz, 2H, Ph-2-H, Ph-6-H); 7.70 (d, J = 8.46 Hz, 2H, Ph-3-H, Ph-5-H); $[\alpha]_D$ = + 17.8 (*c* = 0.0005, H_2O)

*2-carbomethoxy-D-phenylalanine (**3e**)*. Prepared from **3c**. HPLC: tL = 2.74 min [minor, (L)-isomer], tD = 3.25 min [major, (D)-isomer], 94.5% ee; CE: t_m = 4.20 min MS calculated for $[M+H]^+$: $C_{11}H_{13}NO_5$ *m/z*: 240.08 found 240.15;

*3-carbomethoxy-D-phenylalanine (**4e**)*. Prepared from **4c** as brown powder mp 222–224°C; HPLC: tL = 3.40 min [minor, (L)-isomer], tD = 4.15 min [major, (D)-isomer], 91.9% ee; CE: t_m = 4.06 min; MS calculated for $[M+H]^+$: $C_{11}H_{13}NO_5$ m/z: 240.08 found 240.15; ^{1}H-NMR [D_2O] δ 2.81–2.89 (dd, J_1 = 5.90, J_2 = 8.46, 1H, H-C-H); 3.06–3.78 (dd, J_1 = 9.23 Hz, J_2 = 5.00 Hz, 1H, H-C-H); 3.72–3.76 (q, J = 4.44 Hz, 1H, C<u>H</u>-NH_2); 4.36 (s, 2H, O-C<u>H_2</u>-COOH); 6.72–6.80 (m, 3H, Ph-2-H, Ph-4-H, Ph-6-H); 7.19 (t, J = 7.82 Hz, 1H, Ph-5-H); $[\alpha]_D$ = + 20.1 (*c* = 0.001, H_2O);

*4-carbopropoxy-D-phenylalanine (**5e**)*. Prepared from **5c** as brown powder mp. 248–250°C, HPLC: tL = 2.10 min [minor, (L)-isomer], tD = 2.59 min [major, (D)-isomer], 99.36% ee; CE_{chiral}: t_m = 4.15 min [major, (D)-isomer t_m = 5.2 min [minor, (L)-isomer]; CE: t_m = 3.56 min MS calculated for $[M+H]^+$: $C_{13}H_{17}NO_5$ m/z: 268.11 found 268.16; ^{1}H-NMR [D_2O] δ 1.90 (qu, J = 6.86 Hz, 2H, -OOC-CH_2-C<u>H_2</u>-CH_2-O); 2.26 (t, J = 7.31 Hz, 2H, -OOC-C<u>H_2</u>-CH_2-CH_2-O); 2.87–2.95 (dd, J_1 = 6.67 Hz, J_2 = 7.95 Hz, 1H, H-C-H), 3.06–3.78 (dd, J_1 = 9.75 Hz, J_2 = 5.00 Hz, 1H, H-C-H); 3.78–3.83 (q, J = 4.36 Hz, 1H, C<u>H</u>-NH_2); 3.95 (t, J = 6.41 Hz, 2H, OOC-CH_2-CH_2-C<u>H_2</u>-O); 7.23 (d, J = 8.72 Hz, 2H, Ph-2-H, Ph-6-H), 7.11 (d, J = 8.46 Hz, 2H, Ph-3-H, Ph-5-H); $[\alpha]_D$ = + 16.2 (*c* = 0.001, H_2O)

Results

From Table I and Figures 3 and 4 it can be seen that the conversion yield of **1c** to the corresponding *N*-carbamoyl-D-amino acid after 96 h of biotransformation was only 23% with the *Va.*D-HYD enzyme and 18% with *r*D-HYD enzyme. Chiral HPLC analysis of 3-carboxy-D-phenylalanine prepared from substrate **1c** by the hydantoinase process showed the lowest enantiomeric purity among all the D-amino acids obtained (83% ee for *r*D-HYD and 76% ee for *Va.*D-HYD). The lowest bioconversion efficiency was observed for compound **3c** (22% conversion with *r*D-HYD, 17% conversion with *Va.*D-HYD). However, the resulting 2-carbomethoxy-D-phenylalanine showed a high optical purity (94.5% ee for *r*D-HYD and 98.7% ee for *Va.*D-HYD, respectively). The highest bioconversion efficiency of the examined substrates was shown for **2c** (86% conversion with *r*D-HYD, 97% conversion with *Va.*D-HYD) and **5c** (81% conversion with *r*D-HYD, 97% conversion with *Va.*D-HYD). Both D-hydantoinases

showed very high enantioselectivity towards **5c** with enantiomeric excesses of the corresponding 4-carboxypropoxy-D-phenylalanine 99.4% ee (*V.a.*D-HYD) and 98.5% ee (*r*D-HYD). Additionally, the ee of the 4-carboxypropoxy-D-phenylalanine obtained from *r*D-HYD was calculated using chiral CE and compared with the HPLC result (98.5% ee for HPLC and 97.3% ee for chiral CE respectively). The conversion yield of **4c** to the corresponding *N*-carbamoyl-D-phenylalanine derivative was 67% with *r*D-HYD as catalyst and 81% with *V.a.*D-HYD. *V.a.*D-HYD also showed very high enantioselectivity towards **4c**. The ee of 3-carbomethoxy-D-phenylalanine obtained from **4c** by using *V.a.*D-HYD was 99.4% ee, whereas using *r*D-HYD it was only 91.9% ee.

Discussion

A broad range of hydantoin derivatives which can be converted non- or stereoselectively to *N*-carbamoyl-amino acids has been reported in the literature. However, the substrate specificity, activity and stereoselectivity of hydantoinases vary with the type of substrate and the source of the enzyme (Syldatk et al. 1999; Sharma & Vohra 1999). For instance, the D-hydantoinase obtained from adzuki bean (*Vigna angularis*) prefers PH as the substrate rather, than 5-(4-hydroxyphenyl)hydantoin (*p*HPH). It was shown, that specific activity towards PH was 5.3–7-fold higher of that towards *p*HPH (Fan & Lee 2001). The hydantoinases were also shown to be substrate-dependent in terms of enantioselectivity. The hydantoinase from *Arthrobacter aurescens* DSM 3745 was reported to be highly L-selective for indolylmethyl-hydantoin, non-selective for methylthioethylhydantoin (with preference for the D-enantiomer) and D-selective for methylhydantoin (May et al. 1998). Recent analyses of substrate docking and re-engineering of the D-hydantoinase binding pocket using mutagenesis showed that the activity, efficiency and substrate specificity of D-hydantoinase depends on the size and spatial orientation of the substrate. In that study it was shown that modification of the enzyme's hydrophobic binding pocket allows better access of substrates to the catalytic centre increasing enzyme activity (Lee et al. 2009; Cai et al. 2009). However, the efficiency of hydantoin-derivative hydrolysis catalysed by D-hydantoinase may also be reduced due to the low racemisation rate of the substrate. The velocity of chemical racemisation of monosubstituted hydantoins bearing an aromatic substituent in 5-position may vary according to the electronic nature of the substituent (Syldatk et al. 1992; Schnell et al. 2003). In addition, it was shown that the activity of D-hydantoinase is also affected by product inhibition (Kikugawa et al. 1994; Soong et al. 1999). To our knowledge, the D-hydantoinases examined during this study were able to hydrolyse all new substrates into corresponding *N*-carbamoyl amino acids D-selectively with significant enantiomeric purity. However, the efficiency and enantioselectivity differed depending on the enzyme and on substrate. Generally, *V.a.*D-HYD revealed higher hydrolysis efficiency than *r*D-HYD, which is in accordance with manufacturer specifications. Surprisingly, very high enantioselectivity and efficiency of **5c** hydrolysis by using *r*D-HYD and *V.a.*D-HYD was observed. This substrate has a very long side chain in the 4 position of the phenyl ring. Similar results were obtained for **2c**, the substrate with a carboxylic substituent in the 4 position of the phenyl-ring. On the other hand, very low hydrolysis efficiency and the lowest enantioselectivity among all substrates *r*D-HYD and *V.a.*D-HYD were exhibited towards **1c**. This substrate possess the same carboxylic substituent as **2c** but at the 3 position of the phenyl ring. Surprisingly, another substrate with a larger substituent in the 3 position, **4c**, was hydrolysed by the D-hydantoinases examined with significant conversion yield and high enantioselectivity. Substrate **3c**, which has a similar substituent was converted with very low efficiency. However, chiral analysis of the phenylalanine derivative obtained from **3c** showed a high ee of the D-enantiomer.

Conclusions

*r*D-HYD and *V.a.*D-HYD enantioselectivity and hydrolysis efficiency towards new (*R,S*) 5-benzylhydantoin derivatives is substrate-dependent. The enzymes have a preference for substrates with substituents in the 4 position of the phenyl ring regardless of their size. However, the exact reasons for differences in the hydrolysis efficiency and enantioselectivity of the substrates examined remain to be elucidated.

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