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Production of ring-substituted D-phenylglycines by microbial or enzymatic hydrolysis/deracemisation of the corresponding DL-hydantoins

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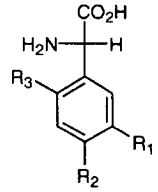
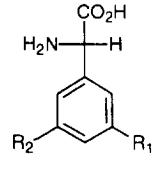
Abstract: A series of 17 ring-mono and -disubstituted D-phenylglycine derivatives was prepared in high enantiomeric purity by enzymatic hydrolysis and deracemisation of the corresponding DL-hydantoins, using D-hydantoinase activities of microorganisms or purified enzymes, followed by diazotation of the resulting *N*-carbamyl D-amino acids. No significant L-hydantoinase activity was found to produce the corresponding L-enantiomers.

Several DL-substituted phenylglycine derivatives **1–3** have been recently described as potent and selective effectors (agonists or antagonists) of the glutamate receptors of the central nervous system (CNS)[1,2]. Some of them are available as enantiomerically pure R- or S-enantiomers[1,3,4]; others are only available as racemic compounds. The enantiomers found to be active at metabotropic glutamate receptors[5–12] have been assigned the S-configuration, while some of the assigned R-forms are antagonists at ionotropic (NMDA) glutamate receptors[1].



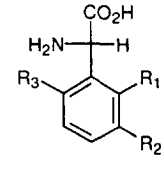
(±)-**1**

R_1= R_3= H; R_2= CO_2H
R_1= OH; R_2=R_3= H
R_1= OH; R_2=R_3= Cl
R_1= OH; R_2= CO_2H; R_3= H
R_1= Cl; R_2= OH; R_3= H
R_1= Cl; R_2= CO_2H; R_3= H
R_1= CO_2H; R_2= OH; R_3= H
R_1=R_2= CO_2H; R_3= H
R_1= OH; R_2= H; R_3= F
R_1= CO_2H; R_2= H; R_3= OH
R_1= H; R_2= CO_2H; R_3= I



(±)-**2**

R_1=R_2= OH
R_1= OH; R_2= CO_2H



(±)-**3**

R_1=R_3= Cl; R_2= OH

Enzymic processes for the preparation of enantiomerically pure D- or L-amino acids, and especially phenylglycine analogs, through the enantioselective hydrolytic ring opening of DL-hydantoins, have been frequently reported[13]. The enzymically produced *N*-carbamyl amino acids are then transformed chemically[13–15] or enzymically[13,15–17] to free amino acids with complete retention of configuration. In most cases, and especially in phenylhydantoin derivative hydrolyses, the spontaneous racemisation of the substrate under slightly alkaline conditions allows a quantitative conversion (deracemisation) to an enantiomerically pure D- or L-amino acid. D-Hydantoinases have been reported to be present in many microorganisms and extracts of mammalian and plant cells[13], and are currently used for the

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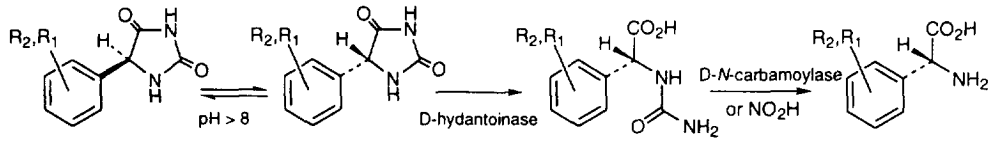

Scheme 1.

Table 1. Relative hydantoinase activities[a] with regard to ring-mono and -disubstituted DL-5-phenylhydantoins

	R_1	R_2	*Agrobacterium tumefaciens*	*Pseudomonas* sp.	*Pseudomonas fluorescens*	D-HYD-1	D-HYD-2
4	H	H	100[b]	100[c]	100[d]	100[e]	100[f]
5	2-Cl	H	71	4	61	8	4
6	3-Cl	H	102	82	89	38	44
7	4-Cl	H	66	64	103	36	12
8	2-Me	H	47	4	65	6	2
9	3-Me	H	61	64	105	46	33
10	4-Me	H	57	45	105	15	13
11	2-OMe	H	42	4	58	5	2
12	3-OMe	H	68	55	64	70	33
13	4-OMe	H	49	43	72	35	5
14	2-OH	H	6	7	42	2	2
15	3-OH	H	57	65	112	17	18
16	4-OH	H	43	21	55	7	2
17	3-OMe	5-OMe	59	9	70	8	2
18	3-OH[g]	5-OH	--	--	--	6	5
19	2-CO_2Me	H	17	3	61	1	1
20	4-CO_2Me	H	42	4	94	2	2

[a] Relative reaction rates (normalized to 100 for 5-phenylhydantoin **4** have been calculated from the amount of *N*-carbamyl amino acid formed from 50 mM substrate; incubations under nitrogen, at 50°C, during 30 min for microorganism suspensions, or 15 min for D-HYD-1 and D-HYD-2. [b] 0.0138 μmol. min^{-1}. mg dry weight. [c] 0.2 μmol. min^{-1}. mg dry weight. [d] 0.0152 μmol. min^{-1}. mg dry weight. [e] 815 μmol. min^{-1}. mg protein. [f] 771 μmol. min^{-1}. mg protein. [g] *N*-carbamyl amino acid determined by HPLC (see experimental).

production of D-amino acids (Scheme 1). Recently, stable enzymatic preparations (D-HYD-1 and D-HYD-2) have been made commercially available[18]. On the contrary, L-hydantoinases are more elusive and only a few applications to L-amino acid syntheses have been described[13].

In the present paper, we report the use of D-hydantoinase activity of various microorganisms or purified enzymic preparations for the synthesis of *N*-carbamyl derivatives of several ring-monosubstituted and ring-disubstituted D-phenylglycine analogs, easily converted to the corresponding D-amino acids, potentially useful as CNS-effectors.

The required racemic ring-substituted 5-phenylhydantoins (±)-**4**–**20** were synthesized according to the Bucherer–Bergs procedure[19], by condensation of the corresponding aldehydes with KCN and $(NH_4)_2CO_3$ in water–ethanol at 60°C, except for the 2-hydroxy- **14** and 3,5-dihydroxyphenylhydantoin **18** which were obtained by O-demethylation of the corresponding methoxyhydantoins. Preliminary hydrolyses were performed for screening by incubation of hydantoins, at pH 9.0 and 50°C, either with three selected bacterial strains, *Agrobacterium tumefaciens* CIP 67.1[16,20–22], *Pseudomonas fluorescens* CIP 69.13[23], and *Pseudomonas* sp. ATCC 43648, previously grown in the presence of 2-thiouracil as inducer[16], or with purified preparations of D-HYD-1 or D-HYD-2[18]. Results obtained by estimating the formation of *N*-carbamyl-amino acids by a colorimetric procedure[24] are reported in Table 1.

From these results it is clear that the highest activities are generally displayed by the enzymic preparations D-HYD-1 and D-HYD-2, with a very similar specificity pattern. These enzymes, like the *Pseudomonas* sp. culture, exhibited a decreased activity toward 2-substituted phenyl compounds

Table 2. Synthesis and characterization of ring-substituted D-phenylglycine derivatives prepared by using selected hydantoinase activities[a]
References cited within table: 3,4,7,14,15,25,26

	R_1, R_2	Hydantoinase activity	(b)	Reaction time (hours)	Yield (%)	$[\alpha]_D^{20}$ (c)	e.e (d) (%)
5	2-Cl	*A. tumefaciens*	NCA	43	70	− 125	-
			AA	48	65	− 102	>99
6	3-Cl	*A. tumefaciens*	NCA	21	76	− 118	>99
		Pseudomonas sp.	NCA	21	81	− 117	-
			AA	60	68	− 122	99
		D-HYD-2	NCA	24	83	− 118	96
9	3-Me	D-HYD-1	NCA	24	79	− 128	>99
		D-HYD-2	NCA	64	83	− 127	-
			AA	48	65	− 144	99
12	3-OMe	D-HYD-1	NCA	1	71	− 115	-
			AA	48	60	− 99	>99
15	3-OH	D-HYD-1	AA	(64) 24(e)	65	− 139 − 151(f)	97
17	3,5-$(OMe)_2$	D-HYD-1	NCA	40	63	− 102	97
18	3,5-$(OH)_2$	D-HYD-1	AA	(64) 24(e)	55	− 103 − 70(g)	98(h)

(a) Incubation with 0.1 M hydantoins in 0.1-0.25 mM Tris-HCl buffer pH 9.0, under nitrogen, at 50°C. (b) NCA = *N*-carbamyl amino acid obtained after incubation during the time indicated and precipitated by acidification at pH 2-3[25]. AA = amino acid obtained par treatment[14,15] of NCA with NO_2H at 25°C during the time indicated, and purified by cation exchange chromatography. (c) unless indicated, $[\alpha]_D^{20}$ measured at c 1, 1N NH_4OH for N-carbamyl amino acids, or c 0.25, 1N HCl for amino acids. (d) unless indicated, e.e.% measured by GC on a ChirasilVal® capillary column, after derivatisation of amino acids to *N*-trifluoroacetyl-*O*-isopropyl esters[26]. (e) NCA not isolated and directly treated with NO_2H at 0°C. (f) c 0.13, 6N HCl; Lit.[7]: − 163. (g) c 0.1, H_2O; Lit.[4] for L-enantiomer: + 70. (h) measured by HPLC on a Crownpack CR(+) chiral column[3].

as compared with 3- or 4-substituted derivatives. On the contrary, *A. tumefaciens* or *P. fluorescens* cultures appear to be less specific and exhibit a more uniform but lower activity, independently of the ring substitution pattern. In all cases, the diazotation[14,15] of the purified *N*-carbamyl amino acid obtained afforded enantiomerically pure D-amino acids (96–99% e.e. by chiral GC).

Considering these preliminary results, the synthesis of several *N*-carbamyl D-phenylglycine derivatives and the corresponding amino acids therefrom was undertaken on a mmole scale, using selected hydantoinase activities. Qualitative and quantitative results are reported in Table 2, demonstrating the general feasibility of such a preparation of ring-substituted D-phenylglycine derivatives with good to excellent yields and high enantiomeric excesses. Due to some difficulties experienced in the purification of the *N*-carbamyl-D-(3-hydroxy)- and *N*-carbamyl-D-(3,5-dihydroxyphenyl)glycines, it was found more convenient and expeditious to prepare the corresponding amino acids directly by diazotation of the crude mixture resulting from the hydantoinase-mediated hydrolysis.

Of particular interest is the preparation, in moderate yield, but high enantiomeric excess, of D-(3,5-dihydroxyphenyl)glycine from the corresponding hydantoin, using D-HYD-1. This enzyme was recently reported[4] not to be able to hydrolyze this hydantoin compound.

Several attempts to use, in a symmetrical way, some L-hydantoinase activities which have been occasionally described[27–30] completely failed (data not shown), such activities being obviously too low with our substrates to be considered in preparative methods. Other hydantoinase linked activities, such as L-*N*-carbamyl amino acid amidohydrolases[31], or L-amino acid amide hydrolases[32] may offer an alternative answer, and are currently explored in our laboratory.

Conclusion

A number of D-phenylglycine derivatives mono- or disubstituted in the aromatic ring have been prepared in good yield and high enantiomeric purity by the deracemizing hydrolysis of the corresponding hydantoins, using either microbial cultures of current collection strains, or commercial purified D-hydantoinases from microbial origin. Such preparation methods may certainly be easily extended to a number of other di- or trisubstituted D-phenylglycine derivatives of outstanding interest in the studies of the glutamate receptors of the central nervous system. However, the corresponding L-phenylglycine derivatives cannot be obtained by a similar method, due to the present scarcity and low activity of L-hydantoinases.

Experimental

Materials and methods

Unless otherwise stated, all reagents were obtained from commercial suppliers and used without further purification. D-HYD-1 (120 U/mg, using 5-(4-hydroxy)phenyl hydantoin as substrate) and D-HYD-2 (200 U/mg, using 5-phenylhydantoin as substrate) were kindly donated by Dr Peter Rasor from Boehringer Mannheim Gmbh. Both enzymes[18] originate from thermophilic microorganisms and have been cloned and expressed in *Escherichia coli*. Reactions were monitored by TLC on Kieselgel $60F_{254}$ (Merck) and visualized by U.V. light (254 nm) or by spraying with ninhydrine or Erlich reagent (10% *p*-dimethyl-aminobenzaldehyde in 6 N HCl). Reverse phase HPLC was run on a Lichrospher 100 C_{18} (endcapped) column (4.5×125 mm, Merck) at a flow rate of 0.6 mL min^{-1}, with 0.1 M Na phosphate buffer pH 3.0–MeOH mixtures (95:5 to 70:30) as the solvent system. ^1H- and ^{13}C-NMR were respectively recorded at 250.13 and 62.9 MHz on a Brucker AM250 spectrometer, and chemical shifts (δ_H and δ_C) are given in ppm downfield from tetramethylsilane. Polarimetric analyses were carried out using a Perkin–Elmer 241C spectropolarimeter at the sodium D-line with a 1 dm pathlength cell; concentrations are given in g 100 mL^{-1}. UV–Visible spectra were recorded using a Uvikon 810 spectrophotometer (Kontron). Melting points were measured with a capillary melting point instrument (Büchi) and are uncorrected.

General procedure for hydantoin synthesis (Bucherer–Bergs reaction)

The aldehyde (6 mmol) solubilized in ethanol (10 mL) was heated with a solution of $(NH_4)_2CO_3$ (30 mmol) and KCN (7.2 mmol) in water (10 mL) at 60°C during 2–12 h, using a water-cooled condenser. The reaction was monitored by TLC using CH_2Cl_2–MeOH (95:5) or CH_2Cl_2–MeOH–conc. NH_4OH (70:26:4) as developing solvent. The condenser was removed and the mixture was heated at 85°C during 2 h to eliminate excess $(NH_4)_2CO_3$. After cooling in an ice-bath, the resulting mixture was acidified to pH 2–3 with concentrated HCl, in an effective aspirating hood. Precipitated hydantoin was recovered by filtration, washed with water and dried *in vacuo*. Yield: 52–80%.

Synthesis of 5-(2′-hydroxy)- and 5-(3′,5′-dihydroxyphenyl)hydantoin

2-Methoxy or 3,5-dimethoxyphenylhydantoin obtained as previously described (1 mmol) and dissolved in CH_2Cl_2 (10 mL) were treated respectively with 3 or 4 equivalents of a 1M solution of BBr_3 in CH_2Cl_2[33]. After one night stirring at room temperature, excess BBr_3 was destroyed with water (5 mL) and ether (30 mL) was added. After filtration, the filtrate was extracted with 2 N NaOH, which was then acidified with HCl and extracted with EtOAc. Pure hydroxyphenylhydantoins were recovered after drying the EtOAc solution on $MgSO_4$ and evaporation *in vacuo*.

Microorganisms and cultures

Microbial strains were selected from the American Type Culture Collection (ATCC), Rockville, MD, USA, or the Pasteur Institute Collection (CIP), Paris, France. They were maintained on a Difco Nutrient agar solid medium, and grown in a liquid medium containing peptone 5 g, yeast extract 5 g, saccharose 10 g, KH_2PO_4 1 g, NaCl 0.5 g, $MgSO_4$ 0.1 g, $MnSO_4$, $1H_2O$ 0.1g, and 2-thiouracil

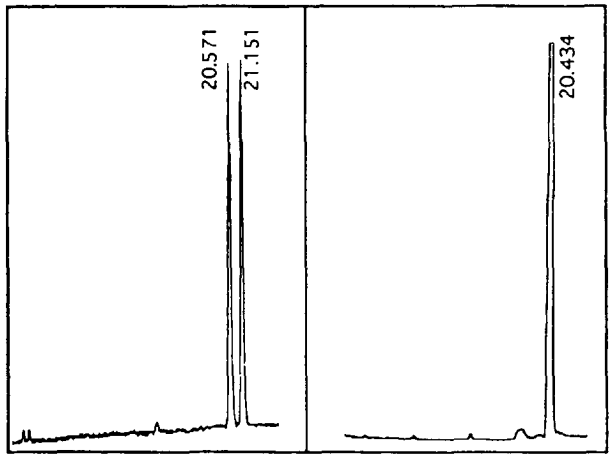

Figure 1. GC of *N*-TFA-*O*-*i*Pr ester of (±)-(*left*), or D-3-methoxy-phenylglycine from enzymic hydrolysis (*right*) on a ChirasilVal® column (see Experimental).

0.5 g per litre, adjusted to pH 7.0 with NaOH. Precultures and cultures were performed with rotatory shaking (220 rpm) at 30°C during 20 h and bacteria were harvested by centrifugation and washed twice in a 0.1 M Na phosphate buffer pH 7.0.

Measurement of enzymatic activities

Hydantoinase activity of bacterial cultures or purified enzymes toward the various phenylhydantoins tested was determined by estimating the amount of *N*-carbamyl amino acids formed in the following conditions: hydantoins (50 µmol) in 0.1 M Tris-HCl buffer pH 9.0 (1 mL) were incubated under nitrogen at 50°C with a bacterial amount corresponding to about 2 mL of liquid growth medium (2–7 mg dry weight), or a diluted solution of D-HYD-1 (0.06 U) or D-HYD-2 (2.5 U). After 30 min for bacteria, or 15 min for purified enzymes, the reaction was stopped by adding 12% trichloroacetic acid (100 µL) and the *N*-carbamyl amino acid formed was estimated colorimetrically: a 10% dimethylaminobenzaldehyde in 6 N HCl (100 µL) was added to a sample, the volume was completed to 2 mL with water and the suspension was centrifuged (bacteria) or filtered (enzyme). Absorbance at 438 nm of the supernatant or filtrate was measured and compared with a calibration curve of *N*-carbamyl-phenylglycine.

Measurement of enantiomeric purity

Chiral GC was performed on a ChirasilVal® capillary column (0.32 mm×50 m, Pierce Chem Co.) with flame ionisation detection, after derivatization of amino acids to *N*-trifluoroacetyl-*O*-isopropyl esters[26], at an oven temperature of 150°C (carrier gas: helium, 1 bar). A typical separation is illustrated in Figure 1. Chiral HPLC was run at room temperature on a Crownpack CR(+) column (4×150 mm, J.T. Baker), at a flow rate of 1 mL min^{-1}, with a solution of 5.15 g of perchloric acid (70%, Merck) in 1 L of water (pH 1.5) as eluent[3]. Detection was performed at 280 nm (Figure 2).

General procedures for preparative hydrolyses

a) Hydantoin (1 mmol) in 20 mL of a bacterial suspension (*A. tumefaciens*: 236 mg dry weight; *Pseudomonas* sp.: 220 mg dry weight) in 0.1 M Tris-HCl buffer pH 9.0 was incubated with rotatory shaking at 50°C under nitrogen and the reaction was monitored by reverse phase HPLC. After completion, bacteria were eliminated by centrifugation and the supernatant was concentrated *in vacuo* to about 5 mL. *N*-Carbamyl amino acids were precipitated by acidification with concentrated HCl. The resulting precipitate was filtered, washed with water and dried *in vacuo*.

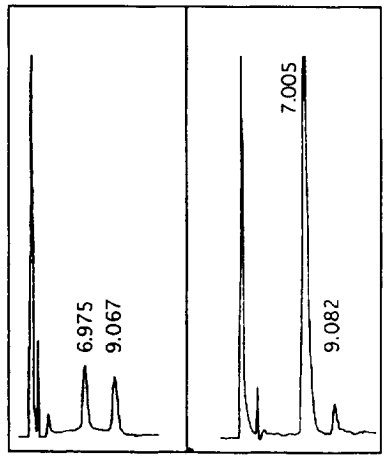

Figure 2. HPLC of (±)-(*left*), or D-3,5-di-hydroxyphenylglycine from enzymic hydrolysis (*right*) on a Crownpack CR(+) column (see Experimental).

b) Hydantoin (1 mmol) was dissolved in 10 mL of a 0.25 M Tris-HCl buffer pH 9.0 containing 1 mM $MnCl_2$, added with a solution of D-HYD-1 (40 µL, 267 U) or D-HYD-2 (200 µL, 2670 U), and incubated with rotatory shaking at 50°C under nitrogen. The reaction was monitored by reverse phase HPLC. After completion, the solution was filtered on Celite and the filtrate concentrated *in vacuo* to about 5 mL. *N*-Carbamyl amino acids were isolated as described above.

General procedure for the preparation of amino acids

N-Carbamyl amino acids (0.5 mmol) and $NaNO_2$ (0.5 mmol) were dissolved in 3.5 N HCl (20 mL) at room temperature. The diazotation reaction was monitored by HPLC or TLC (using the Erlich reagent and a ninhydrin reagent). After completion of the reaction, the reaction mixture was diluted with deionized water (100 mL) and deposited on a AG50X4 (H^+, 20–50 mesh) cation exchange column. The column was washed with water, water–THF (1:1), water, and the pure amino acids were eluted with pyridine–water (1:9) and recovered by evaporation *in vacuo*. Yields: see Table 2.

D-*(2-Chlorophenyl)glycine*

M.p. 160–161°C. ^{1}H-NMR (D_2O): 7.55 (m, 4H, ArH), 5.21(s, 1H, α-CH); ^{13}C-NMR (D_2O): 176.04 (COOH), 136.54 and 135.30 (quat. ArC), 133.92, 133.07, 130.86 (ArCH), 58.71 (α-CH). $[\alpha]_D^{20}$ see Table 2. Chiral GC (as *N*-TFA-*O*-*i*Pr ester), Retention times: 14.24 (R); 14.45 (S) min.

D-*(3-Chlorophenyl)glycine*

M.p. 179–180°C. ^{1}H-NMR (D_2O): 7.34 (m, 4H, ArH), 4.34 (s, 1H, α-CH); ^{13}C-NMR (D_2O, NaOD): 183.26 (COOH), 147.10 and 136.52 (quat. ArC), 133.11, 130.25, 129.64, 128.04 (ArCH), 62.94 (α-CH). $[\alpha]_D^{20}$ see Table 2. Chiral GC (as *N*-TFA-*O*-*i*Pr ester), Retention times: 15.96 (R); 16.36 (S) min.

D-*(3-Methylphenyl)glycine*

M.p. 205–206°C. ^{1}H-NMR (D_2O, NaOD): 7.24 (m, 4H, ArH), 4.32 (s, 1H, α-CH), 2.35 (s, 3H, CH_3); ^{13}C-NMR (D_2O, NaOD): 184.03 (COOH), 145.17 and 141.79 (quat. ArC), 131.66, 130.95, 130.34, 126.68 (ArCH), 63.36 (α-CH), 23.31 (CH_3). $[\alpha]_D^{20}$ see Table 2. Chiral GC (as *N*-TFA-*O*-*i*Pr ester), Retention times: 10.85 (R); 11.11 (S) min.

D-*(3-Methoxyphenyl)glycine*

M.p. 180–181°C. ^{1}H-NMR (D_2O, NaOD): 7.31, 6.95 (2m, 4H, ArH), 4.30 (s, 1H, α-CH), 3.80 (s, 3H, OCH_3),^{13}C-NMR (D_2O, NaOD): 183.70 (COOH), 161.85 and 146.86 (quat. ArC), 132.90, 122.55, 115.97, 115.24 (ArCH), 63.31 (α-CH), 58.24 (OCH_3). $[\alpha]_D^{20}$ see Table 2. Chiral GC (as *N*-TFA-*O*-*i*Pr ester), Retention times: 20.57 (R); 21.15 (S) min.

D-*(3-Hydroxyphenyl)glycine*

5-(3′-Hydroxyphenyl)hydantoin (192.5 mg, 1 mmol) in a 0.2 M Na borate buffer, pH 9.0 containing 1 mM $MnCl_2$ (10 mL), was hydrolyzed with D-HYD-1 (40 µL, 267 U) at 50°C under nitrogen. The reaction was monitored by reverse phase HPLC (0.1 M phosphate buffer pH 3.0–MeOH, 95:5). After 64 h, the reaction mixture was filtered on Celite and the crude *N*-carbamyl amino acid filtrate was evaporated to dryness. The resulting product was dissolved in 3.5 N HCl, and to the ice-cold solution was added $NaNO_2$ (0.65 mmol) dissolved in ice-cold water (10 mL). After 24 h at 0°C, the mixture was evaporated to dryness *in vacuo* and the resulting amino acid purified by cation exchange chromatography, as described above. Yield: 108 mg, 65%. M.p. 204–205°C. ^{1}H-NMR (D_2O, NaOD): 7.42, 7.01 (2m, 4H, ArH), 4.84 (s, 1H, α-CH); ^{13}C-NMR (D_2O, NaOD): 182.70 (COOH), 165.24 and 145.38 (quat. ArC), 133.04, 119.85, 118.83, 118.47 (ArCH), 63.10 (α-CH). $[\alpha]_D^{20}$ see Table 2. Chiral GC (as *N*-TFA-*O*-*i*Pr ester), Retention times: 11.25 (R); 11.61 (S) min.

D-*(3,5-Dihydroxyphenyl)glycine*

The compound was prepared by the same method as above using the crude hydrolysis product of the corresponding hydantoin (208.5 mg, 1 mmol) with D-HYD-1. Yield: 102 mg, 55%. M.p. 205–206°C. ^{1}H-NMR (D_2O, NaOD): 6.55 (m, 2H, ArH), 6.49 (m, 1H, ArH), 4.69 (s, 1H, α-CH); ^{13}C-NMR (D_2O, NaOD): 175.75 (COOH), 160.30 and 139.51 (quat. ArC), 109.99, 106.41 (ArCH), 61.00 (α-CH). $[\alpha]_D^{20}$ see Table 2. Chiral HPLC, Retention times: 6.98 (R); 9.08 (S) min.

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