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SYNTHESIS AND RESOLUTION OF DHCGA, A NEW CONFORMATIONALLY RIGID 3,4-DEHYDROGLUTAMIC ACID ANALOGUE

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Abstract : (±) -1-amino-2-cyclohexene-1,3-dicarboxylic acid (DHCGA) , an unsaturated cyclic analogue of glutamic acid, was prepared from 3-carboxy-4-cyclohexenone by a Bucherer-Bergs reaction. Resolution was performed through coupling with L-leucine and separation of the resulting diastereoisomeric dipeptides. Spectral data, including optical rotation and circular dichroism of both enantiomers of DHCGA are reported.

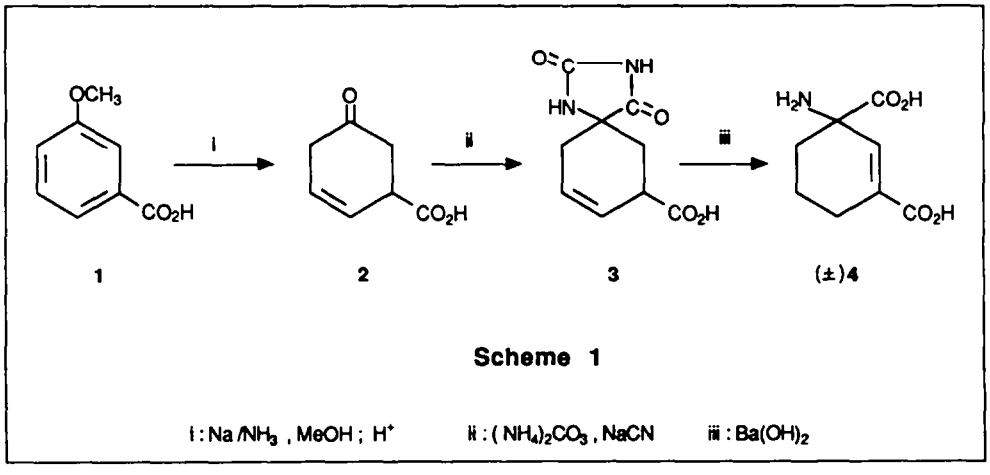
A remarkable pioneering investigation of the interactions of the active site of sheep brain glutamine synthetase with its substrate was achieved by Meister and coworkers[1-4] through the use of conformationally rigid cyclic glutamic acid analogues. In this opportunity, several cyclic substitutes of glutamic acid were synthesized and their stereochemistry thoroughly investigated[2-5]. More recently, considerable interest has been shown by neuropharmacologists in various rigid analogues of glutamic acid, for the elucidation of the receptors involved in its activity on the mammalian central nervous system[6]. In this respect, syntheses of several optically pure cyclic glutamic acid analogues have been described or reinvestigated[7-16].

Short peptides containing cyclic analogues of glutamic acid[15] have been synthesized in our laboratory[17], in order to be used as conformational probes of the active site of the vitamin K-dependent carboxylase. On the other hand, the synthesis of a derivative of β,γ-dehydro-DL-glutamic acid has been recently described and this compound was used in a mechanistic study of the vitamin K-dependent carboxylation of glutamic acid-containing substrates[18]. However, this study was hampered by the relative lability of this compound which rapidly isomerizes to an α,β-unsaturated derivative. It was attractive to combine both structural features by preparing a conformationally rigid 2,3-dehydrocycloglutamic acid (DHCGA) **4**, which in addition would be resistant to isomerization.

Synthesis of (±)-DHCGA

In our previous work about the synthesis of saturated cycloglutamic acids[15,17], we started from 3-carboxycyclohexanone which was prepared by a Birch reduction of 3-methoxybenzoic acid. At the final stage, it was found that the saturated cyclic amino acid was occasionally contaminated by an unsaturated homologue, having the characteristics of the now desired compound, and indicating that the Birch reduction was incomplete. A reexamination of the conditions of the reduction led to a predominant partial hydrogenation[19,20] which afforded, after mild acidic treatment, mainly 3-carboxy-4-cyclohexenone **2** (Scheme 1). However, as the attempts to purify completely this compound led to extensive losses, the crude ketoacid **2** (containing small amounts of the saturated compound) was directly converted to the corresponding spirohydantoin **3** under the usual Bucherer-Bergs conditions (NaCN and ammonium carbonate in aqueous ethanol). The crude hydantoin was then hydrolyzed by heating with aqueous barium hydroxide, and after elimination of barium ions, free crystallized (±)-DHCGA **4** was separated from small amounts of its saturated analogues by ion exchange chromatography.

The migration of the ring double bond into a conjugated 2,3-position during hydrolysis of the hydantoin was indicated by ^{13}C and ^{1}H-NMR data. A ^{13}C-resonance at 128.1 ppm was clearly ascribable to a sp^2 quaternary carbon atom bearing a -COOH group, while an uncoupled singlet at 7.0 ppm in the ^{1}H- NMR spectrum, integrating as one hydrogen atom, could only result from the occurrence of a double bond in the 2,3-position. Moreover, a strong absorption at 215 nm (ε_M = 18,000) in the ultraviolet spectrum of **4** (Fig.1) confirmed the presence of a double bond conjugated with one of the carboxylic groups.



Scheme 1

i : Na /NH_3 , MeOH ; H^+ ii : $(NH_4)_2CO_3$, NaCN iii : $Ba(OH)_2$

Resolution of (±)-DHCGA

The resolution of DHCGA was effected through the separation of diastereoisomeric dipeptide derivatives . L-Leucine containing dipeptides have been frequently used for this purpose ,

essentially in analytical conditions[21-23]. When t-butyloxycarbonyl-L-leucine-hydroxysuccinimide ester (Boc-Leu-OSu) was reacted for several days with (±)-4 in aqueous dimethylformamide (DMF) - dioxane (DHCGA exhibited a poor solubility in most organic solvents), the coupling yield was low (about 15%), even in the presence of excess Boc-Leu-OSu and tertiary amine, because of the known decreased reactivity of the hindered amino group of α,α-dialkyl amino acids[24-26] and the resulting low reaction rate, compared to other Boc-Leu-OSu consuming reactions. The diastereoisomeric Boc-dipeptides, after acidic deprotection, were easily separated by ion exchange chromatography. Acidic hydrolysis, followed by simple chromatographic separation from leucine, allowed the recovery of pure DHCGA enantiomers (Scheme 2). When coupling was effected in dimethyl sulfoxide (DMSO) (3-6 days), in the presence of excess activated ester and tertiary amine, the yield was nearly quantitative, but extensive racemization of leucine (8-20%) was observed after dipeptide hydrolysis, associated evidently with a low optical purity of recovered DHCGA enantiomers*. Such features may proceed from a slow racemization of the activated ester (even if protected as a carbamate) through oxazolone formation[27], during long time coupling experiments in the presence of excess tertiary amine[28,29], resulting in a cross-contamination of DHCGA enantiomers. The occurrence of such a racemization mechanism was confirmed by the high amounts of D-leucine obtained (27%-43% in 1-3 days) when Boc-Leu-OSu was treated in similar conditions, but in the absence of any coupling partner. On the other hand, analogous observations have been recently reported[26,30], upon activation of peptides containing a

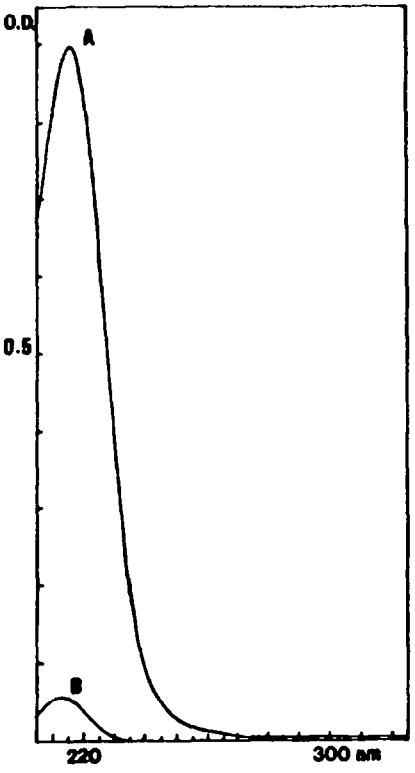


Figure 1: UV absorption of (±)-DHCGA (A) and (±)-*cis* -1-aminocyclohexane-1,3-dicarboxylic acid (B) (1 mg in 100 ml 0.1 N HCl)

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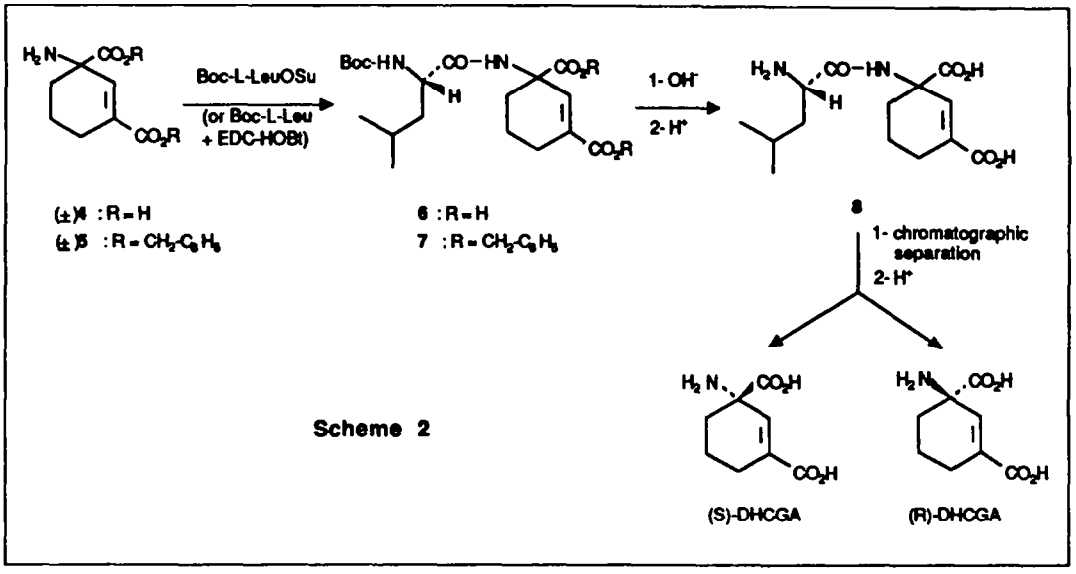
* As DHCGA enantiomers are assumed to be unable to racemize, any failure in performing a total resolution may arise: i) from a poor chromatographic separation of the dipeptide diastereoisomers: as a matter of fact, the large peak resolution generally obtained by ion exchange chromatography allowed an easy separation of the dipeptides; analysis by HPLC (Fig.2) indicated a cross contamination lower than 0.5%. ii) from a partial racemization of the leucine residue in any step *before* separation of the dipeptide diastereoisomers.



Scheme 2

C-terminal α,α–dialkyl amino acid such as α-amino isobutyric acid (Aib); this behaviour was tentatively attributed to a tautomeric hydrogen shift between the α-carbon atom of the (conventional) penultimate amino acid and the nitrogen of the adjacent oxazolone ring formed in dipeptides such as Z-X-Aib-OH upon activation of the C-terminal carboxylic group. Such a situation is possibly realized in our coupling reactions assuming that an activation of the α-carboxylic group of DHCGA may occur in the presence of excess activated ester. The use of an ester of the α,α-dialkyl amino acid will preclude such a side reaction[30].

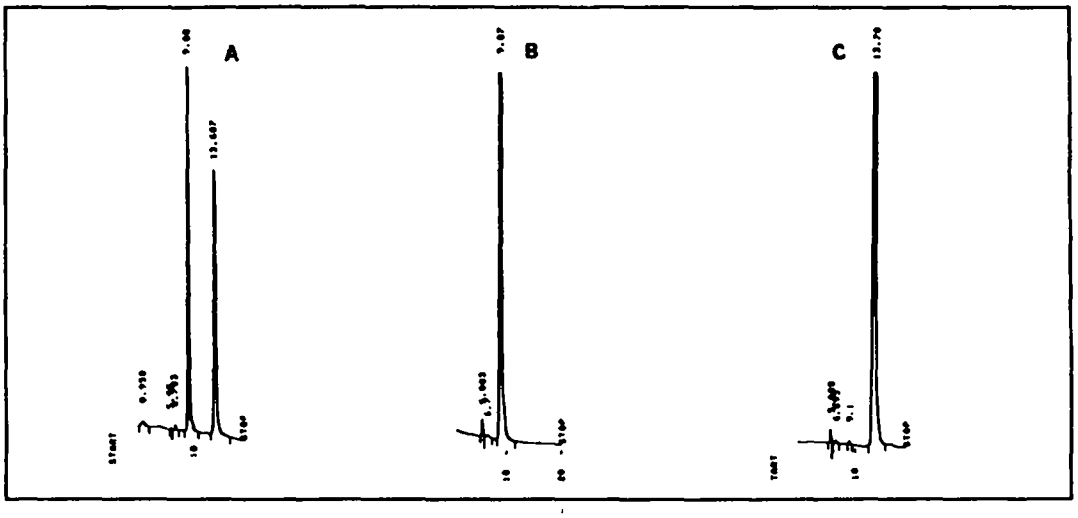


Figure 2: Reverse phase HPLC of diastereoisomeric Leu-DHCGA dipeptides: **A**, as a diastereoisomeric mixture, after coupling and deprotection; **B**, L-Leu-(S)-DHCGA (1st eluted from ion exchange column); **C**, L-Leu-(R)-DHCGA (2nd eluted). Nucleosil 5C18 column (250 x 4.6 mm); solvent: 0.05 M Na phosphate buffer, pH 3.0- MeOH (7:3), 0.5 ml.min^{-1}; detection at 210 nm

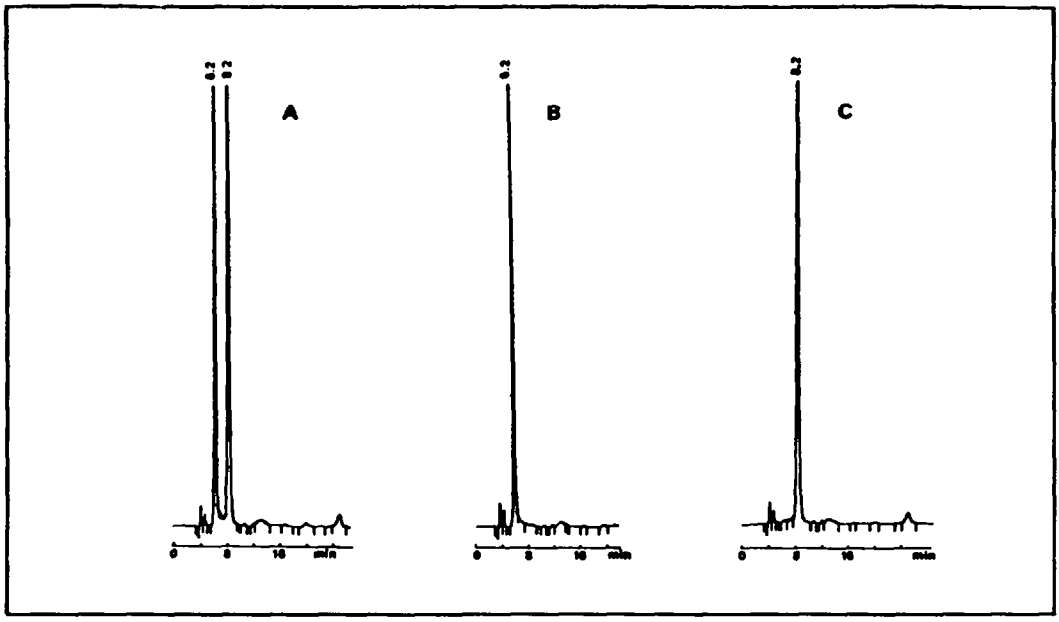


Figure 3: Reverse phase HPLC of DHCGA enantiomers after precolumn derivatization with N-acetyl-L-cysteine/ o-phthalaldehyde reagent[31]. A, (±)-DHCGA; B, (S)-DHCGA (from the 1st eluted peak of the ion exchange column); C, (R)-DHCGA (from the 2nd eluted peak). Nucleosil 5C18 column (250 x 4.6 mm); solvent: 0.1M Na phosphate buffer, pH 7.5-MeOH (8:2), 0.8 ml.min^{-1}; detection by fluorescence (excitation at 345 nm, detection at 445 nm).

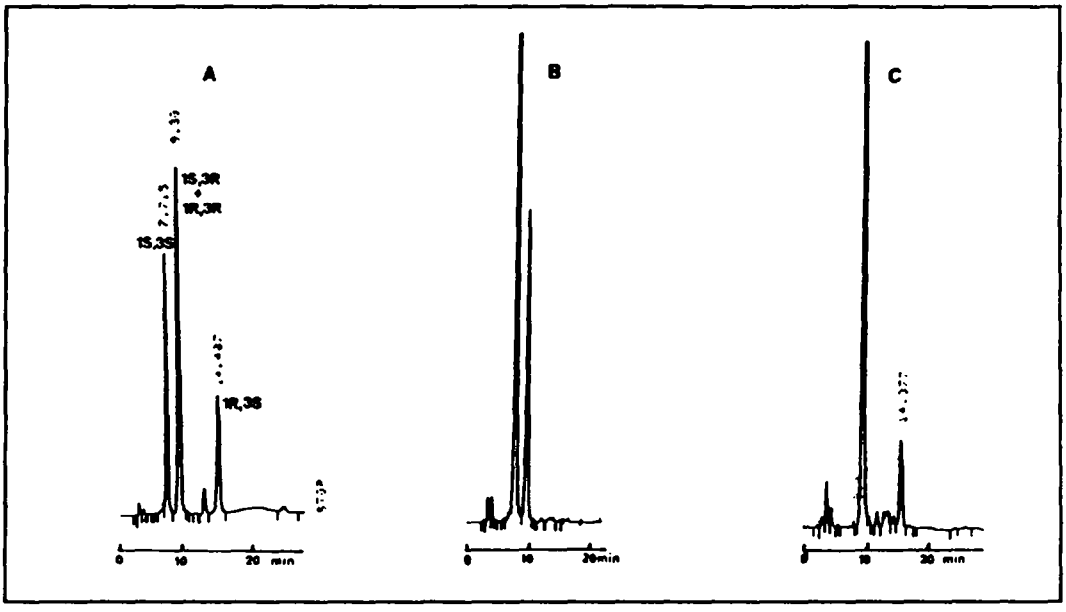
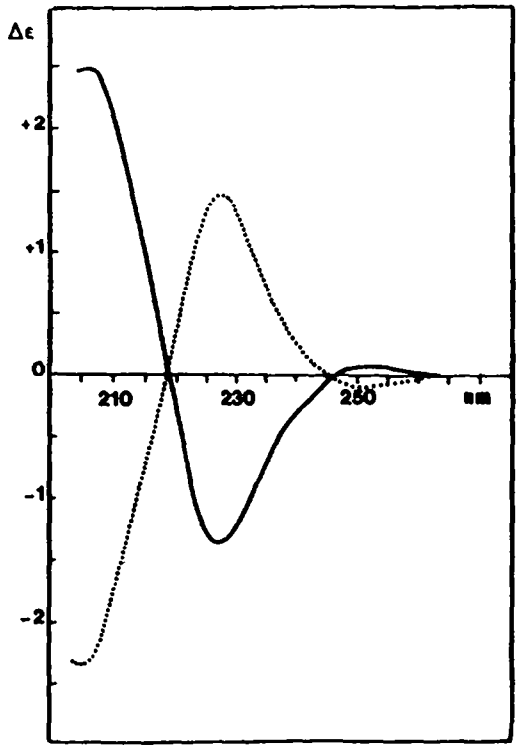


Figure 4: Determination of the absolute configuration of DHCGA enantiomers after catalytic hydrogenation. Comparison with (±)-*cis* and *trans* -1-aminocyclohexane-1,3-dicarboxylic acids (A) by reverse phase HPLC after precolumn derivatization with N-acetyl-L-cysteine/ o-phthalaldehyde reagent (see Fig.3). B, Hydrogenation product of (S)-DHCGA (from the 1st eluted peak of the ion exchange column); C, Hydrogenation product of (R)-DHCGA (from the 2nd eluted peak). Experimental conditions as in Fig.3.

The dibenzylester of (±)-DHCGA (p-toluenesulfonic acid salt) was thus prepared and coupled either with Boc-Leu-OSu (1.5 eq.) or with Boc-L-Leucine (Boc-Leu-OH) (1eq.) and N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC)- 1-hydroxybenzotriazole (HOBt) in DMF, in the presence of a stoechiometric amount of tertiary amine. The yields were respectively 54 and 81% with a negligible racemization of L-leucine (<1%). Optically pure DHCGA enantiomers (>98% ee, as estimated by reversed phase HPLC of their N-acetyl-L-cysteine/o-phthalaldehyde adducts[31], see Fig.3) were recovered in 45% overall yields (from racemic DHCGA) after alkaline saponification of the Boc-dipeptide dibenzylesters, mild acidic deprotection, chromatographic separation of the diastereoisomeric dipeptides and acidic hydrolysis (Scheme 2).



Their absolute configuration was established by catalytic hydrogenation (10% Pd on charcoal) to known enantiomers of *cis* + *trans* 1-amino-cyclohexane-1,3-dicarboxylic acids[15,17,31] (see Fig.4).

The circular dichroism (CD) curve of (S)-DHCGA in 0.1 N HCl (Fig.5) is characterized by a large negative Cotton effect at about 225 nm ($\Delta\varepsilon = 1.40$ litre mole^{-1}.cm^{-1}) The R-enantiomer displays a symmetrical positive Cotton effect at the same wavelength.

Figure 5: CD spectra of (S) (——) and (R)- DHCGA (.....)

In conclusion, the present work has established a simple synthetic method for the preparation of both optically pure DHCGA enantiomers, which are now available for advanced conformational studies of receptors or enzymes binding sites.

EXPERIMENTAL PROCEDURES

General: melting points were determined on a capillary tube Büchi apparatus and are uncorrected. A Bruker WM 250-FT NMR spectrometer was used for the collection of ^{1}H and ^{13}C-NMR data. A DEPT135 experiment[32] was systematically used for multiplicity assignment of ^{13}C-NMR resonances. Chemical shifts (δ ppm) relative to tetramethylsilane are reported after calculation with reference to residual protons of deuterated solvents. Multiplicities are reported as br (broad), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet); a * indicates that

assignments may be interchanged. UV spectra were recorded on an Uvikon 810 (Kontron) spectophotometer. CD spectra were recorded on a Jobin et Yvon Mark V Auto-dichrograph in a quartz cell with a path length of 0.1 cm; the concentrations of the solutions were 0.4 mg/ ml in 0.1 N HCl. Optical rotations were measured with a Perkin Elmer 241 polarimeter using a 1-dm tube. Microanalyses were performed at the Service Central de Microanalyses du CNRS, Gif-s-Yvette. Reverse phase HPLC was performed with an Altex Chromatem 380 pump, Rheodyne 7125 injector and Pye Unicam LC-UV detector (set at 210 nm) or Shimadzu RF-530 fluorescence HPLC monitor (set at 345 nm for excitation and 445 nm for emission); flow rates of 0.5-1 ml.min^{-1} were generally used with 250 x 4.6 mm internal diameter columns packed with Nucleosil 5C_{18}. GPC analyses were performed on a Varian 3700 gas chromatograph equipped with a polysiloxane-XE-60-S-valine-S-α-phenylethylamide fused silica capillary column (25 m x 0.25 mm, Chrompack), with helium as carrier gas (1.5 bar) and a flame ionization detector.

Boc-Leu-OH monohydrate was purchased from Bachem, Boc-Leu-OSu and HOBt (8% water) from Fluka, and EDC from Sigma. All other reagents were of the best commercially available grade.

3-Carboxy-4-cyclohexen-1-one **2** was prepared by Birch reduction[19,20] of m-methoxybenzoic acid in the presence of excess methanol. The acidification product (pH 1, room temperature) was extracted with $CHCl_3$ and used immediatly without purification; it usually contained less than 10% of the corresponding saturated homologue. A sample was repeatedly crystallized in a benzene-hexane mixture to give pure **2**, m.p. 104°C (lit.[20] 104°C)

^{13}C-NMR ($CDCl_3$): 207.36 and 177.60 (2xC=O), 127.25 and 124.07 (-CH=CH-), 42.24 (CH-COOH), 40.21 and 38.89 (2xCH_2)

(±)1-Amino-2-cyclohexene-1,3-dicarboxylic acid (DHCGA) **4**

To a solution of **2** (5 g, 35.7 mmol) in ethanol-water (1:1, 40 ml) was added by portions ammonium carbonate (15 g) then sodium cyanide (2 g) and the suspension was heated with magnetic stirring at 50-55°C for 5 hours; the temperature was then raised to 90°C for 1 hour to eliminate excess ammonium carbonate. After cooling and acidification with concentrated HCl, the crystalline hydantoin **3** was recovered by filtration and dried under vacuum (4g, 19 mmol, 53 %)

^{1}H-NMR (D_6-DMSO): 12.5 (br, 1H, COOH), 10.95 (s, 1H, NH), 8.57 (s, 1H, NH), 6.00 (m, 2H, CH=CH), 2.70-1.72 (m, 5H, 2xCH_2, CH)

^{13}C-NMR (D_6-DMSO): 178.12, 174.31 and 156.61 (3xC=O), 125.32 and 124.54 (-CH=CH-), 60.45 (tetrasubst.C), 38.48 (-CH-COOH), 33.30 and 31,99 (2xCH_2).

The spirohydantoin **3** (2 g, 9.5 mmol) was suspended in water (100 ml) saturated with barium oxide (8 g) in a tightly closed bottle. The temperature was raised to 140°C with magnetic stirring for 45 min. After cooling, the pH of the suspension was adjusted to 4.0 with 4N H_2SO_4 and the barium sulfate precipitate eliminated by filtration and washed with hot water (4 x 150 ml). The filtrate and washings were brought to pH 9.0 with 1N NaOH and deposited on a Dowex 1X4 (200-400 mesh, acetate) column (50 x 5 cm). After washing with water (0.5 litre), DHCGA was eluted with 0.25 M AcOH (4 litres, 200 ml.h^{-1}) and the ninhydrin positive fractions evaporated to dryness under vacuum; the elution of the contaminating saturated homologues of DHCGA is delayed in such conditions. Pure (±)-DHCGA **4** (1g, 5.15 mmol., 54%) was obtained after crystallization in hot water and drying under high vacuum. M.p.(dec.) 293°C. λ max (in 0.1N HCl): 215 nm (ε_M=18,000). Found: C, 49.19; H, 6.09; N, 6.91. Calculated for $C_8H_{11}NO_4$, 0.5 H_2O: C, 49.48; H, 6.23; N, 7.21.

^{1}H-NMR (D_2O, CF_3COOD salt): 7.0 (s, 1H, -CH=), 2.80 (m, 1H, CH_2), 2.53-1.78 (m, 5H, 2xCH_2+CH)

^{13}C-NMR (D_2O-CF_3COOH, 8:2): 176.22 and 172.62 (2xCOOH), 143.51 (-CH=), 128.12 (=C-COOH), 61.65 (tetrasubst.C), 33.15, 29.93 and 24.52 (3xCH_2)

Synthesis of L-Leu-DHCGA

Method a : to (±)-**4** (388 mg, 2 mmol) suspended in a mixture of water (2 ml), dioxane (4 ml) and DMF (4 ml) were added triethylamine (0.84 ml, 6 mmol) and Boc-Leu-OSu (660 mg, 2 mmol). After one day stirring at room temperature, Boc-Leu-OSu (660 mg, 2 mmol.) and triethylamine (0.28 ml, 2 mmol) were again added. After two days, the solvents were removed in vacuo and the residue was dissolved in water (50 ml) added with a saturated $NaHCO_3$ solution (5 ml). After washing with EtOAc (2 x 50 ml), the solution was acidified cautiously to pH 3.0 with diluted HCl, saturated with NaCl, and extracted again with EtOAc (3 x 50 ml). The combined organic phases were dried over Na_2SO_4, evaporated and the residue purified by fast filtration on a Silicagel column (2 x 20 cm)

using iPrOH- 20% aqueous ammonia (9:3, v/v) as solvent to yield Boc-Leu-DHCGA as a colorless oil (121 mg, 15%)

Method b : to (±)-**4** (776 mg, 4 mmol) suspended in DMSO (20 ml) was added triethylamine (2.8 ml, 20 mmol), then Boc-Leu-OSu (2.63 g, 8 mmol). After 3 days stirring, the clear solution was diluted with water (100 ml) and a saturated $NaHCO_3$ solution (10 ml), then treated as described in method a. Yield: 1.50 g (92%) of a colorless oil which can be crystallized in MeOH-water. M.p. above 300°C. Found: C, 56.02, H, 7.86, N, 6.94. Calculated for $C_{19}H_{30}N_2O_7$, 0.5 H_2O: C, 56.01, H, 7.67, N, 6.87.

^{1}H-NMR (D_6-DMSO): 12.24 (s, 2H, 2xCOOH), 7.92 and 7.68 (2s, 1H, NH), 6.83 (br.s, 1H, =CH), 6.76 and 6.68 (2d, J= 9 Hz, 1H, NH), 3.93 (m, 1H, N-CH-CO), 2.49 (m, 2H, CH_2), 2.18 (m, 3H, CH_2 +CH), 1.60 (m, 2H, CH_2), 1.36 (s, 9H, $(CH_3)_3C$), 1.45-1.20 (m, 2H, CH_2), 0.83 (m, 6H, $(CH_3)_2C$).

^{13}C-NMR (CD_3OD): 176.6 and 175.4 (2xCOOH), 169.9 (CO-NH), 157.6 (N-COO-), 140.3 and 140.2 (-CH=), 128.0 (=C-COOH), 80.5 and 80.4 (Me_3-C), 58.14 (tetrasubst.C), 54.65 and 53.97 (N-CH-CO), 41.76 (CH_2), 33.96 and 33.69 (CH_2), 28.68 ($(CH_3)_3C$), 27.1 and 26.96 (CH_2); 25.86 and 25.73 (CH), 23.33 (CH_2), 22.15 and 21.95 ($(CH_3)_2C$)

Method c:

(±)-DHCGA-dibenzyl ester, p-toluenesulfonate **5**: (±)-**4** (194 mg, 1 mmol), p-toluenesulfonic acid monohydrate (202 mg, 1.06 mmol.), p-toluene sulfonylchloride (230 mg, 1.2 mmol.) and benzyl alcohol (1 ml) were heated at 90°C for 3 hours[33]. After cooling, ether was added and the resulting white precipitate was filtered, washed with an ether-hexane mixture and dried under vacuum to give the dibenzylester **5** as its p-toluenesulfonate salt (392 mg, 73%). M.p. 130°C. Found: C, 64.76, H, 5.87, N, 2.74, S, 5.98. Calculated for $C_{29}H_{31}NO_7S$: C, 64.79, H, 5.81, N, 2.60, S, 5.96.

^{1}H-NMR (D_6-Me_2CO): 8.75 (br.s, 3H, NH_3), 7.65 (d, 2H, ArH), 7.35 (m, 10H, ArH), 7.10 (d, 2H, ArH), 7.01 (s, 1H, -CH=), 5.21 and 5.15 (2s, 4H, 2xCH_2Ar), 3.14-2.79 (m, 4H, 2xCH_2), 2.63-2.17 (m, 2H, CH_2), 2.30 (s, 3H, Ar-CH_3)

Coupling with L-leucine : A solution of **5** (349 mg, 0.65 mmol.), Boc-Leu-OSu (234 mg, 0.715 mmol.) and N-methylmorpholine (79 µl, 0.715 mmol) in DMF (3 ml) was stirred for 2 days at room temperature; Boc-Leu-OSu (86 mg, 0.26 mmol) was again added and after 4 days, the solvents were removed under vacuum . The residue was dissolved in EtOAc (20 ml), washed with water (2 x 15 ml), dried over Na_2SO_4 and purified by Silicagel chromatography (2 x 16 cm column) with hexane-EtOAc (7:3) to afford 204 mg (54%) of diastereoisomeric Boc-Leu-DHCGA-dibenzylesters **7** as an amorphous solid

^{1}H-NMR ($CDCl_3$): 7.34 and 7.31 (2s, 10H, ArH), 7.06 (br.s, 1H, -CH=), 6.49 and 6.38 (2 br.s, 1H, NH), 5.16 (m, 4H, 2xCH_2), 4.71 and 4.59 (2 br.d, J=7 and 8 Hz, 1H, NH), 3.95 (m, 1H, N-CH-CO-), 2.70 and 2.66 (2s, 2H, CH_2), 2.48 (m, 1H, CH_2), 2.27 (m, 2H, CH_2), 1.94 (m, 1H, CH_2*), 1.68-1.25 (m, 3H, CH_2 + CH*), 1.38 and 1.35 (2s, 9H, $(CH_3)_3C$), 0.85 (m, 6H, $(CH_3)_2C$).

Method d : To an ice-cooled solution of **5** (581 mg, 1.08 mmol), Boc-Leu-OH, 1 H_2O (270 mg, 1.08 mmol) and HOBt (193 mg, 1.41 mmol) in DMF (5 ml) were added triethylamine (166 µl, 1.19 mmol) and EDC (249 mg, 1.30 mmol). After stirring 1 hour at 0°C, then 24 hours at room temperature, the solvent was removed under vacuum and the residue dissolved in EtOAc (40 ml) was washed with water at pH 3.0, then pH 8.0 and processed as in method c to give the diastereoisomeric protected peptides **7** (507 mg, 81%). M.p.105°C.

To **7** (507 mg, 0.878 mmol) dissolved in acetonitrile-DMSO (6 ml, 5:1) was added 1N NaOH (2.2 ml). The cloudy suspension clarifies in a few hours and, after 3 days stirring at room temperature, the solution was evaporated in vacuum; the residue was dissolved in water (50 ml) added with a saturated $NaHCO_3$ solution (2 ml). After washing with EtOAc (50 ml) the aqueous phase was cautiously acidified to pH 3.0 with solid $KHSO_4$, then extracted again with EtOAc (3 x 50 ml). The combined organic phases were dried over Na_2SO_4 and evaporated to give the diastereoisomeric mixture of Boc-Leu-DHCGA **6** as an oil (314 mg, 87%) which crystallized in MeOH-water at 4°C. M.p. above 300°C.

Boc-Leu-DHCGA **6** (536 mg, 1.31 mmol) was dissolved in a 1N solution of dry HCl in AcOH (12 ml) and kept at room temperature for 30 min. The solvents were evaporated, and the residue repeatedly treated with toluene and evaporated in vacuum to give quantitatively the diastereoisomeric mixture of deprotected dipeptides **8** as a white powder (435 mg).

A solution of crude **8** (430 mg) in water (300 ml) was adjusted to pH 8.0, then deposited on a Dowex AG 1X4 (200-400 mesh, acetate) column (5 x 50 cm). After washing with water (500 ml), the dipeptides were eluted at 200 ml.h^{-1} in 10 ml fractions with 0.1 M AcOH (5 litres) and localized by the ninhydrin reaction. L-Leu-(S)-DHCGA emerged in fractions 250-310, L-Leu-(R)-DHCGA in

fractions 330-420. Both peptides were recovered by evaporation under vacuum of the corresponding pooled fractions.

L-Leu-(S)-DHCGA : 210 mg, 49%. M.p. (dec.) above 300 °C. $[\alpha]_D^{21}$ +47.6° (c 3.0, 4N HCl). Diastereoisomeric excess by reverse phase HPLC >98%, see Fig.2. Found: C, 50.12, H, 7.51, N, 8.11. Calculated for $C_{14}H_{22}N_2O_5,2H_2O$: C, 50.29, H, 7.84, N, 8.38.

^{1}H-NMR (D_2O): 7.04 (s, 1H, -CH=), 3.95 (dd, J1= 8.5Hz, J2= 7Hz, 1H, N-CH-CO), 2.92-2.70 (m, 2H, CH_2), 2.42 (br.s, 2H, CH_2), 2.0-1.4 (m, 5H, 2xCH_2+CH), 0.88 and 0.89 (2d, J= 7Hz and 6.5Hz, 6H, 2xCH_3).

^{13}C-NMR (D_2O): 181.16, 173.56 and 171.88 (3xC=O), 142.24 (-CH=), 129.77 (=C-COOH), 61.39 (tetrasubst.C), 54.34 (N-CH-CO), 42.29 (CH_2), 32.26, 30.47 and 24.76 (3xCH_2), 26.43 (C-Me_2), 24.23 and 23.41 (2xCH_3).

L-Leu-(R)-DHCGA : 207 mg, 48%. M.p. (dec.) about 300 °C. $[\alpha]_D^{21}$ -20.3° (c 3.0, 4N HCl). Diastereoisomeric excess by reverse phase HPLC >98%, see Fig.2. Found: C, 50.36, H, 7.44, N, 8.22. Calculated for $C_{14}H_{22}N_2O_5,2H_2O$: C, 50.29, H, 7.84, N, 8.38.

^{1}H-NMR (D_2O): 7.05 (s, 1H, -CH=), 3.98 (t, J= 7.5Hz, 1H, N-CH-CO), 2.58 (br.s, 2H, CH_2), 2.50-1.85 (m, 4H, 2xCH_2),1.70-1.55 (m, 3H, CH_2+CH), 0.93 (d, J= 6Hz, 6H, 2xCH_3).

^{13}C-NMR(D_2O): 181.12, 173.56 and 172.17 (3xCO), 142.91 (-CH=), 129.06 (=C-COOH), 61.30 (tetrasubst.C), 54.21 (N-CH-CO), 42.39 (CH_2), 35.37, 27.92 and 25.09 (3xCH_2), 26.47 (C-Me_2), 23.94 and 23.55 (2xCH_3).

(S)(+)- and (R)(–)-DCHGA:

(S)(+)-DHCGA : L-Leu-(S)-DHCGA (200 mg, 0.60 mmol) was refluxed in 6N HCl (100 ml) during 18 hours. After evaporation to dryness in vacuum, the residue was repeatedly dissolved in water (50 ml) and evaporated. The solution of the residual white solid in water (100 ml) was adjusted to pH 8.0 with 0.5N NaOH then deposited on a small column (1.2 x 20 cm) of Dowex 1X4 (acetate). After washing with water (100 ml), which eluted leucine, the column was eluted with 0.25 M AcOH (50 ml.h^{-1}) in 10 ml fractions. A small amount (6 mg) of residual peptide was eluted as soon as the pH of the eluent became acidic (fractions 5-7), while pure (S)-DHCGA (97 mg, 83%) was eluted in fractions 13-18. M.p.(dec.) 281°C. $[\alpha]_D^{21}$ +29.7° (c 1.2, 4N HCl); enantiomeric excess determined by reverse phase HPLC (after derivatization with the N-acetyl-L-cysteine/o-phthalaldehyde reagent, see Fig.3): >98%. ^{1}H and ^{13}C-NMR data identical to the racemic compound.

(R)(–)-DHCGA : L-Leu-(R)-DHCGA (200 mg, 0.60 mmol) was similarly hydrolyzed to give (R)-DHCGA (94 mg, 80%). M.p. (dec.) 284°C. $[\alpha]_D^{21}$ -30.0° (c 1.2, 4N HCl); enantiomeric excess determined by reverse phase HPLC (after derivatization with the N-acetyl-L-cysteine/ o-phthalaldehyde reagent, see Fig.3): >98%. ^{1}H and ^{13}C-NMR data identical to the racemic compound.

Checking for racemization during the coupling with L-leucine : a sample of Boc-Leu-DHCGA (2 mg) was heated in 4N HCl (1 ml) at 110°C in a sealed tube during 4 hours. The solution was evaporated to dryness and the residue, derivatized to N-trifluoroacetyl O-isopropyl ester as previously described[34], was analyzed by chiral GPC (120°C) for leucine enantiomers, using excess samples in order to be able to detect less than 0.5% contaminating enantiomer.

Determination of the absolute configuration of DHCGA enantiomers :

(+) or (–)-DHCGA (5 mg) in AcOH (3 ml) was hydrogenated at room temperature under atmospheric pressure in the presence of 10% Pd on charcoal (5 mg) during 16 hours. After filtration of the catalyst and evaporation of the solvent, an aliquot was derivatized with the N-acetyl-L-cysteine/ o-phthalaldehyde reagent and analyzed by reverse phase HPLC[31] (see Fig.4), together with authentic *cis* and *trans* 1-aminocyclohexane-1,3-dicarboxylic acid enantiomers previously described[15] .

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