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Improved Chemical Synthesis of Optically Pure *N*-(9-Fluorenylmethoxycarbonyl)-γ,γ'-di-*tert*-Butyl-γ-Carboxyglutamic Acid

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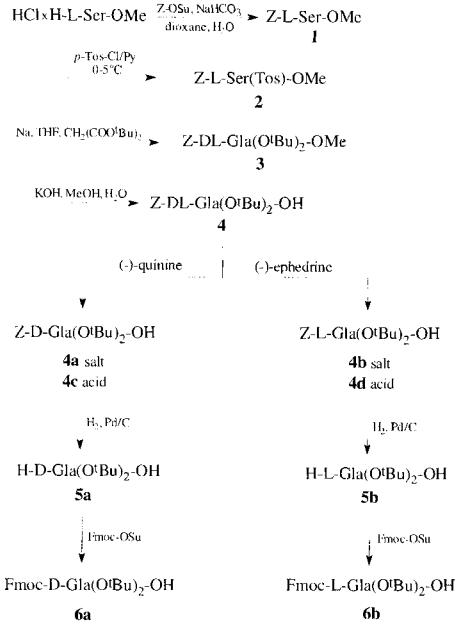
Because of the increasing interest in biochemistry and immunology as concerns γ-carboxyglutamic acid (Gla)-containing peptides, which are known to be responsible for the strong and specific coordination of calcium ions *via* relatively stable calcium complexes, synthetic organic chemistry has to provide this acid-labile amino tricarboxylic acid as the monomeric compound for both solid- and liquid-phase peptide synthesis. Although optically pure Gla can be produced by enzymatic carboxylation of L-glutamic acid, a number of synthetic approaches to protected Gla derivatives have been reported on the basis of the condensation of *N*-blocked *O*-tosyl-L-serine derivatives with different diesters of malonic acid [1–14]. All of these approaches have utilized sodium hydride as base suspended in abs. benzene, THF or DMF at low temperature to obtain the nucleophilic sodium salts of the respective malonate diesters. Unfortunately, the literature procedures in this respect are not unambiguous: some of the synthetic routes required a rather long reaction time (24 h), whereas others applied 1 h or only 10 min for this purpose. The conversion of L-serine derivatives into to the appropriate Gla derivatives was carried out in a time-consuming manner, the condensation being allowed to proceed to completion within 24–48 h (exceptionally 2 h). Furthermore, and presumably as a result of the use of excess malonate diesters, none of the syntheses could eliminate the tiresome purification of the products by silica gel column chromatography, and hence optimal yields could not be attained. Again, as the condensation is accompanied by extensive racemization at the α-carbon atom, much care is needed as regards separation of racemic *N*-blocked-γ,γ'-di-*tert*-butyl-DL-γ-carboxyglutamic acids. With consideration of all these points, the present work has focused on the large-quantity production of optically pure *N*-(9-fluorenylmethoxycarbonyl)-γ,γ'-di-*tert*-butyl-L-γ-carboxyglutamic acid, adaptable to solid- and liquid-phase peptide synthesis, with methodological improvement of the efficiency of the earlier syntheses, including the reaction conditions (time, temperature and work-up), the chemical purification, the optical separation of the condensation product, and the yield.

Formation of the sodium salt of di-*tert*-butyl malonate can be simplified by replacing sodium hydride by sodium, cp. scheme 1. When small pieces of sodium were added to the concentrated abs. THF solution of di-*tert*-butyl malonate during stirring in a N_2-atmosphere at room temperature, the sodium reacted quantitatively with the diester in 5 h, and the cessation of foaming indicated the completion of salt formation. *N*-benzyloxycarbonyl-*O*-tosyl-L-serine methyl-ester dissolved in abs. THF was added dropwise with stirring to the previously clear mixture at room temperature while the reaction was completed. *N*-benzyloxycarbonyl-γ,γ'-di-*tert*-butyl-DL-γ-carboxy-glutamic acid methyl ester (**3**) could be conveniently isolated as a crystalline substance in excellent yield (90%). Following hydrolytic cleavage of the racemic-methyl ester derivative (**3**) with methanolic KOH for 2 h at room temperature, the free acid (**4**) could be collected by acidification of the mixture with $NaHSO_4$ solution. Chemical resolution of the *N*-benzyloxycarbonyl-γ,γ'-di-*tert*-butyl-DL-γ-carboxyglutamic acid (**4**) was carried out by forming diastereomeric salts with chiral amines by a known procedure [7]. The separated free acids (**4c** and **4d**) were liberated from **4a** and **4b**, respectively, by acidic extraction. In order to remove the benzyloxycarbonyl protecting group, catalytic hydrogenation on Pd/charcoal was performed in aqueous acetic acid solution. The stereochemical purities of the products (**5a** and **5b**) were measured directly by conventional RP HPLC, using pre-column derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide according to Marfey [15], and each was confirmed to be more than 99% pure. Fmoc protected, optically pure *N*-(9-fluorenylmethoxycarbonyl)-γ,γ'-di-*tert*-butyl-γ-carboxyglutamic acid end-products (**6a** and **6b**) were prepared according to the standard literature method [16].

In summary, the use of sodium in solution instead of sodium hydride in suspension offers a very convenient synthetic route to protected Gla derivatives. The advantages of this method are the short reaction time at room temperature, the easy work-up, good yields and large-quantity production, furthermore



Scheme 1

excess reagent is unnecessary, and purification of the condensation product does not require column chromatography. The optical purities of the enantiomers can be determined directly without conversion of the Gla derivatives *via* hydrolysis and decarboxylation to the respective glutamic acid isomers.

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Experimental

All reagents and solvents were of reagent grade. L-Serine methyl ester hydrochloride was purchased from Sigma–Aldrich Kft. (Budapest, Hungary), 9-fluorenylmethylsuccinimidyl carbonate from Bachem (California, USA), and Marfey's reagent from Pierce Chemical Company (Rockford, IL, USA). For the preparation of *N*-benzyloxycarbonyl-L-serine methyl ester (**1**) [15], benzyl chloroformate was replaced by *N*-(benzyloxycarbonyloxy)-succinimide [18] prepared in our laboratory. *N*-benzyloxycarbonyl-*O*-tosyl-L-serine methyl ester (**2**) was prepared according to a literature method [19]. The quinine salt of *N*-benzyloxycarbonyl-γ,γ'-di-*tert*-butyl-D-γ-carboxyglutamic acid (**4a**) and the ephedrine salt of *N*-benzyloxycarbonyl-γ,γ'-di-*tert*-butyl-L-γ-carboxyglutamic acid (**4b**) were produced in the same manner as by Märki *et al.* [7]. Melting points were obtained with a PAMK VEB apparatus and are uncorrected. Optical rotations were measured on a Zeiss Polamat-A polarimeter at 20 °C. TLC was performed on silica gel precoated glass plates 60 F_{254} (Merck) and Chiralplate^R 811 055 (Macherey, Nagel, Dürren). Spots on TLC plates were visualized by means of *N,N,N',N'*-tetramethyl-4,4'-diaminodiphenylmethane (TMD) reagent after chlorination. Spectral data were acquired on a Bruker 400 MHz spectrometer (^{1}H NMR and ^{13}C NMR), or a Beckmann spectrometer (IR). Microanalyses were carried out with a CHN Analyser (Prague). HPLC measurements for optical purity control were performed on a M-600 low-pressure gradient pump equipped with an M-996 photoiode array detector and a Millenium 2010 Chromatography Manager data system (Waters Chromatography, Division of Millipore, Milford, MA, USA). The column used was Lichrospher RP18 C_{18} (15×4 mm, I.D.), with 5 μm particle size (Merck, Darmstadt, Germany). The chromatograph was operated isocratically at a flow rate of 0.8 cm^3/min with 0.01M potassium dihydrogenphosphate (pH 3). The mobile phase was 0.01M potassium dihydrogenphosphate (pH 3): methanol (1:1 v/v) or 0.01M potassium dihydrogenphosphate (pH 3): acetonitrile (6:4 v/v).

N-Benzyloxycarbonyl-γ,γ'-di-tert-butyl-DL-γ-carboxyglutamic acid methyl ester (**3**)

1.84 g (80 mmol) of small pieces of sodium was added to 17.9 ml (80 mmol) di-*tert*-butyl malonate dissolved in freshly distilled THF (10 ml) under a N_2-atmosphere with moderate stirring. After the reaction had been allowed to proceed to completion at room temperature (ca. 5 h), 30 g (75 mmol) **2** dissolved in abs. THF (250 ml) was added dropwise with vigorous stirring during 30 min. The precipitate was filtered off and the filtrate was concentrated *in vacuo* to dryness. The residue was dissolved in EtOAc (200 ml) and the organic phase was washed with water, dried over Na_2SO_4 and evaporated. The residual oil was triturated with *n*-hexane to give crystalline **3** (30 g, 90%), *m.p.* 56–58 °C, R_f = 0.72 (CH_2Cl_2:MeOH 9:1).

$C_{23}H_{33}O_8N$:	Calcd.:	C 61.18,	H 7.37,	N 3.10,
(451.5)	Found:	C 61.10,	H 7.30,	N 2.95.

N-Benzyloxycarbonyl-γ,γ'-di-tert-butyl-DL-γ-carboxyglutamic acid (**4**)

30 g (64.2 mmol) methyl ester **3** was hydrolysed by dissolving it in methanol (200 ml) and adding 6 g KOH dissolved in water (60 ml). The mixture was stirred at room temperature for 2 h. After acidification with 10% $NaHSO_4$, the solution was extracted with EtOAc at pH 2. The extract was washed with water, dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was crystallized from diethyl ether/*n*-hexane, and 22 g (76%) crystalline product was obtained, *m.p.* 62–63 °C, R_f = 0.52 (CH_2Cl_2:MeOH 9:1).

$C_{22}H_{31}O_8N$:	Calcd.:	C 60.39,	H 7.14,	N 3.20,
(437.5)	Found:	C 60.50,	H 7.06,	N 3.05.

Resolution of *N*-Benzyloxycarbonyl-γ,γ'-di-*tert*-butyl-DL-γ-carboxyglutamic acid (4) to the (–)-quinine salt of the D-isomer (4a) and the (–)-ephedrine salt of the L-isomer (4b)

Te suspension of 43.7 g (100 mmol) **4** and 48.6 g (150 mmol) (–)-quinine was dissolved in EtOAc (300 ml) at a slightly elevated temperature. The clear solution was allowed to stand in a refrigerator for some days. The crystalline product was collected by filtration and recrystallized from EtOAc to give 19.5 g (65%) **4a**, *m.p.* 141–143 °C, $[\alpha]_D = -86.8°$ (c = 1, MeOH) and $[\alpha]_D = -55.5°$ (c = 1, $CHCl_3$), respectively.

Removal of the excess (–)-quinine from the mother liquors by 10% $NaHSO_4$, the organic phase was dried over Na_2SO_4, concentrated *in vacuo* and 9.1 g (55 mmol) (–)-ephedrine

dissolved in EtOAc (100 ml) was added. After ad-ding *n*-hexane to the EtOAc solution, **4b** as (–)-ephedrine salt of the L-isomer was precipitated by cooling, which was re-crystallized from EtOAc/*n*-hexane solvent mixture. Yield 17 g (55%), *m.p.* 121–123 °C, $[\alpha]_D = -15.3°$ (c = 1, MeOH) and $[\alpha]_D = -7.9°$ (c = 1, $CHCl_3$), respectively.

Optical purities of **4a** and **4b** were measured as Marfey's derivatives of **5a** and **5b** in different mobile phase systems (see **5a** and **5b**).

γ,γ'-Di-tert-butyl-D-γ-carboxyglutamic acid (**5a**)

38.3 g (50.3 mmol) **4a** was suspended in EtOAc (250 ml) and (–)-quinine was removed by addition of 10% $NaHSO_4$ solution. The organic phase was dried over Na_2SO_4 and concentrated *in vacuo* to dryness. The residue (**4c**) was not investigated further, but catalytically hydrogenated over 10% Pd/charcoal in 10% aqueous acetic acid (300 ml). The catalyst was filtered off and the filtrate was concentrated *in vacuo* to dryness. The crude product was triturated with diethyl ether to give 13.3 g (87%) solid substance, *m.p.* 167–171 °C, R_f = 0.57 (acetonitrile: MeOH:H_2O 4:1:1, Chiralplate^R), $[\alpha]_D$ = –6.0° (c = 1, MeOH), optical purity: 99.4%. R_t-values for the Marfey's diastereomer of **5a** was 7 min and 25 min using 0.01M KH_2PO_4 (pH=3):MeOH (1:1 v/v) and 0.01M KH_2PO_4 (pH 3): acetonitrile (6:4 v/v) mobile phase systems, respectively.

γ,γ'-Di-tert-butyl-L-γ-carboxyglutamic acid (**5b**)

The above procedure was applied for **5b**, starting from 28.7 g (46.4 mmol) **4b**. Yield 13.1 g (89%), *m.p.* 169–172 °C, R_f = 0.62 (acetonitrile: MeOH:H_2O 4:1:1, Chiralplate^R), $[\alpha]_D$ = +6.1° (c = 1, MeOH), optical purity: 99.9%. R_t-values for the Marfey's diastereomer of **5b** was 5 min and 16 min using 0.01M KH_2PO_4 (pH 3): MeOH (1:1v/v) and 0.01M KH_2PO_4 (pH 3): acetonitrile (6:4 v/v) mobile phase systems, respectively.

N-(9-Fluorenylmethoxycarbonyl)-γ,γ'-di-tert-butyl-D-γ-carboxyglutamic acid (**6a**)

13 g (42.8 mmol) **5a** and 13.7 g (42.8 mmol) 9-fluorenylmethylsuccinimidyl carbonate were added to 7.1 g (85.6 mmol) $NaHCO_3$ suspended in aqueous dioxane (300 ml, 1:1 v/v). The mixture was stirred at room temperature for ca. 5 h (TLC control), then concentrated *in vacuo* and acidified with 10% $NaHSO_4$ solution to pH 2. The oily precipitate was extracted with EtOAc (300 ml), and the extract was washed with water, dried over Na_2SO_4 and evaporated *in vacuo* to dryness. The residue was crystallized from EtOAc/*n*-hexane to give 18.85 g (82%) product, *m.p.* 118–121 °C, R_f = 0.25 (CH_2Cl_2:MeOH 9:1), $[\alpha]_D$ = +7.4° (c = 1, MeOH).

$C_{29}H_{35}O_8N$:	Calcd.:	C 66.27,	H 6.71,	N 2.67,
(525.6)	Found:	C 65.82,	H 6.50,	N 2.43.

N-(9-Fluorenylmethoxycarbonyl)-γ,γ'-di-tert-butyl-L-γ-carboxyglutamicacid (**6b**)

The same procedure for **6b** was performed as for **6a**. Yield 16.4 g (78%), *m.p.* 74–76 °C (as **6a** and **6b** are enantiomers, the different melting points are unexpected!), R_f = 0.25 (CH_2Cl_2:MeOH 9:1), $[\alpha]_D$ = –7.6° (c = 1, MeOH).

$C_{29}H_{35}O_8N$:	Calcd.:	C 66.27,	H 6.71,	N 2.67.
(525.6)	Found:	C 65.49,	H 6.90,	N 2.94.

^{1}H NMR ($CDCl_3$) δ 1.46, 1.47 (2s, 18H, 2^{t}Bu), 2.20–2.50 (m, 2H, βCH_2), 3.85 (t, 1*H*, γCH, J_1=1.6 Hz, J_2=1.8 Hz), 4.22 (t, 1H, αCH, $J_{1,2}$=1.8 Hz), 4.33 (b, 1*H*, NH), 4.44 (b, 2H, CH_2), 5.54 (d, 1*H*, CH, J=3.4 Hz), 7.30–7.80 (b, 8H, aromatic CH). – IR (KBr) ν_{max}: 3400, 1680, 1620, 1330, 1220, 1105, 700 cm^{-1}.

Derivatization of 5a and 5b

5a or **5b** (5 μmol) was dissolved in water (100 μl), and 1M $NaHCO_3$ solution (40 μl) and 1% solution of Marfey's reagent in acetone (200 μl) was added. The solution was incubated at 40 °C for 60 min, cooled, and 2M HCl solution (20 μl) was added. After the cessation of foaming, the sample was ready for analysis.

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