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Enzymatic Resolution of Z- γ , γ' -di-*tert*-butyl-D,L-Carboxyglutamic Acid Methyl Ester

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Abstract: A rapid and simple method for the resolution of Z- γ , γ' -di-*tert*-butyl-D,L-carboxyglutamic acid methyl ester is described. The new procedure is based on the enzymatic enantioselective saponification of the methyl ester by the endoprotease papain. Using a simple HPLC protocol the enantiomeric excess of Z-di-*tert*-butyl-L-Gla-OH has been shown to be higher than 99.5%. The procedures described in this communication allow the resolution, analysis, and downstream process of multi-gram amounts of the Gla derivative in 48 h.

The amino acid γ -carboxyglutamate (Gla) is a vitamin K-dependent post-translational modification of glutamate. This amino acid is present in different families of proteins and peptides such as proteins of the coagulation cascade, osteocalcins and conantokins.¹⁻⁵ The conantokins are the only antagonists of the *N*-methyl-D-aspartate (NMDA) receptor of peptidic nature known to date.^{6, 7} Calcium binding to the gamma-carboxyglutamate residues is believed to influence the activity of these proteins and peptides through the induction of conformational changes.^{2, 3, 5}

The solid-phase synthesis of Gla containing peptides requires the preparation of appropriate carboxyglutamic acid protected derivatives. Fmoc- γ , γ' -di-*tert*-butyl-L-Gla-OH has been used in solid-phase peptide synthesis under the Fmoc/*tert*-butyl protocol.^{2, 5, 7} Most of the syntheses of Gla derivatives are based on the condensation of Z-*O*-tosyl-Ser-OMe with dialkyl-malonate.^{5, 8-10} After this the Z group must be removed and the Fmoc introduced. The bottle neck in all the syntheses described so far is the resolution of the racemic mixture resulting from the condensation reaction. In what has resulted to be the most commonly used method, the intermediate Z- γ , γ' -di-*tert*-butyl-D,L-Gla-OMe is saponified and the enantiomers resolved by the selective precipitation/crystallization using quinine, ephedrine, and/or tyrosine hydrazine salts of Z-D,L-Gla-OH protected on the side chain carboxylates.^{5, 8-10} These procedures use large amounts of reagents and solvents in successive precipitation and re-crystallization steps. This makes the method expensive and time consuming. The enzymatic resolution of stereoisomeric mixtures is a powerful alternative in many cases.¹¹ However, there exists the general believe that enzymatic methods do not work with Gla derivatives.^{8, 9} The only example involving an enzyme in the resolution of a partially protected Gla derivative uses three steps after the condensation reaction.¹² Here we present an efficient enzymatic method for the direct enantiomeric resolution of Z- γ , γ' -di-*tert*-butyl-D,L-Gla-OMe. The enantioselective saponification by papain under the conditions described below yields Z- γ , γ' -di-*tert*-butyl-L-Gla-OH in one enzymatic reaction and one simple extraction. The reaction was carried out either in acetonitrile/buffer or in dimethylformamide/buffer mixtures. In a typical experiment, Z- γ , γ' -di-*tert*-butyl-D,L-Gla-OMe (30 g, 69 mmol) was suspended in a mixture of dimethylformamide/buffer¹³ 60/40 v/v (200 ml). To this solution, papain (100 mg) and dithiothreitol (200 mg)

dissolved in buffer (1 ml) were added. The reaction was carried out at room temperature (25°C) and monitored by HPLC (Fig. 1a,b). After 20 h the reaction was stopped by adding acetic acid (50 ml). The mixture was then evaporated under vacuum and the residue dissolved in ethyl acetate, washed with citric acid (3x100 ml) and then extracted with Na₂CO₃/NaHCO₃ 0.1 M pH 10 buffer (7x50 ml) (Fig. 1c). The aqueous phase was then acidified with citric acid, extracted with ethyl acetate (3x100 ml), dried over anhydrous Na₂SO₄ and the solvent evaporated to dryness. A white solid was obtained (13 g, 90% yield with respect to the L-isomer) and identified as the L-isomer.¹⁴

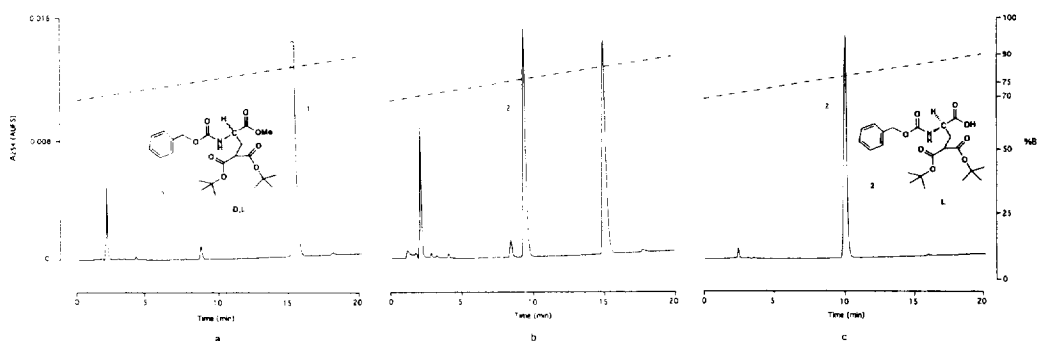


Fig. 1 HPLC profiles of the enzymatic saponification of *L*- γ -DL-*tert*-butyl-DL-Glu-OMe. Reaction progress: (a) initial, (b) after 20 h. Separation: (c) aqueous Na₂CO₃/NaHCO₃ layer. Reaction and extraction conditions in the main text. Chromatographic conditions: Column: Lichrosphere RP-18, 5 μ m, 0.46 x 25 cm; solvent A: aqueous 0.10% TFA; solvent B: H₂O/CH₃CN (1:4) 0.08% TFA. Gradient elution from 70 to 90% B in 20 min. Flow: 1 ml/min. Detection: 215 nm.

A simple method was used to evaluate the enantiomeric purity of the product. A diastereomeric mixture was obtained by derivatization with H-L-Tyr-OMe which was separated and analyzed by reversed-phase HPLC.¹⁵ The enantiomeric excess was found to be higher than 99.5%. Both the enzymatic resolution and the enantiomeric excess analysis are fast, clean, simple and inexpensive methods. The whole process including reaction, extraction and analysis takes no more than 48 hours with minimal waste of solvents and reagents.

Acknowledgments

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- Mellvaine phosphate/citrate buffer 0.1 M pH 6.17 containing 1.5 mM of EDTA.
- [α]_D²⁰ (c=1, MeOH) -10.9 \pm 0.2 (lit -10.9 \pm , refs. 5, 8). ¹H-NMR (CDCl₃, 250 MHz) 1.43, 1.41 (18H, 2s, 2 t-Bu), 2.00-2.50 (2H, broad, β -CH₂), 3.33 (1H, dd, J₁=6.4 Hz, J₂=7.5 Hz, γ -CH), 4.42 (1H, m, α -CH), 5.09 (2H, s, CH₂ Bzl), 5.42 (1H, d, J=8.8 Hz, NH), 7.32 (5H, s, ArH).
- The method is based on the approach of Görög and co-workers (Görög, S.; Herényi, B.; Löw, M. *J. Chromatogr.* **1986**, *353*, 417). Here, commercially available L-tyrosine methyl ester and reversed-phase HPLC are used instead of O-(4-Nitrobenzyl)-L-tyrosine methyl ester and normal silica HPLC.

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