Chemoenzymatic Synthesis of (4*S*)- and (4*R*)-4-Methyl-2-oxoglutaric Acids, Precursors of Glutamic Acid Analogues

Virgil Helaine[a] and Jean Bolte*[a]

Keywords: Amino acids / Enzyme catalysis / Glutamate / Oxoglutarate / Aminotransferase / Aminotransferase

Alkylation of dimethyl 2,2-dimethoxyglutarate followed by enzymatic resolution afforded (4*S*)- and (4*R*)-4-methyl-2-oxoglutaric acid in an enantiomerically pure form. The activity of glutamic oxalacetic transaminase towards these compounds has been measured. Their enzymatic transamination provides an efficient synthesis of (4*S*)- and (4*R*)-4-methyl-L-glutamic acids which are very useful for characterisation of glutamate receptors in the central nervous system.

Introduction

As one of the excitatory amino acids, glutamic acid plays a critical role in the central nervous system,[1] and numerous analogues have been synthesised for topological investigation of its various receptors.[2] Among these analogues, 4-methylglutamic acids are of particular interest since it has been shown that the (2*S*,4*R*) isomer **1** is a potent and selective agonist of the kainate receptor,[3] while the (2*S*,4*S*) isomer **2** is an agonist of one of the metabotropic receptors.[4]

Various chemical syntheses of 4-methylglutamic acid have been published, starting from pyroglutamic acid[5] or from another derivative of glutamic acid.[6] Two enzymatic ways are also possible starting from 4−methyl-2-oxoglutaric acid (MKG). One of them is by reductive amination catalysed by glutamate dehydrogenase.[7] We developed the other way which is based on the transamination catalysed by *G*lutamic *O*xalacetic *T*ransaminase (GOT).[8] These syntheses were carried out starting from racemic 4−methyl-2-oxoglutaric acid, and led to diastereomeric mixtures. By a careful control of the reaction′s progress, taking advantage of the enantioselectivity of GOT, it was possible to isolate diastereomerically pure samples. Now, a previous resolution of MKG would improve the process.

The synthesis of analogues of α-oxoglutaric acid has been little studied inspite of the fact that it is an important metabolite often associated with glutamic acid and an intermediate in the Kreb′s cycle. None of these analogues has been synthesised in an optically pure form. However, compounds like MKG could be useful as probes for mechanistic studies of aminotransferases or α-oxoglutarate dehydrogenase. They can also act as L-glutamic acid analogue precursors in vivo, by transamination with glutamic acid itself (or aspartic acid) catalysed by GOT.

In this paper, we describe the chemoenzymatic synthesis of both enantiomers of MKG, their behaviour towards GOT and their enzymatic conversion into 4-methyl-L-glutamic acids.

[a] Université Blaise Pascal, Département de Chimie, Laboratoire de Synthèse, Electrosynthèse et Etude de Systèmes d'Intérêt Biologique, associé au CNRS (UMR 6504), F-63177 Aubière Cedex, France
Fax: (internat.) +33-4/73407717
E-mail: jbolte@chimtp.univ-bpclermont.fr

Results and Discussion

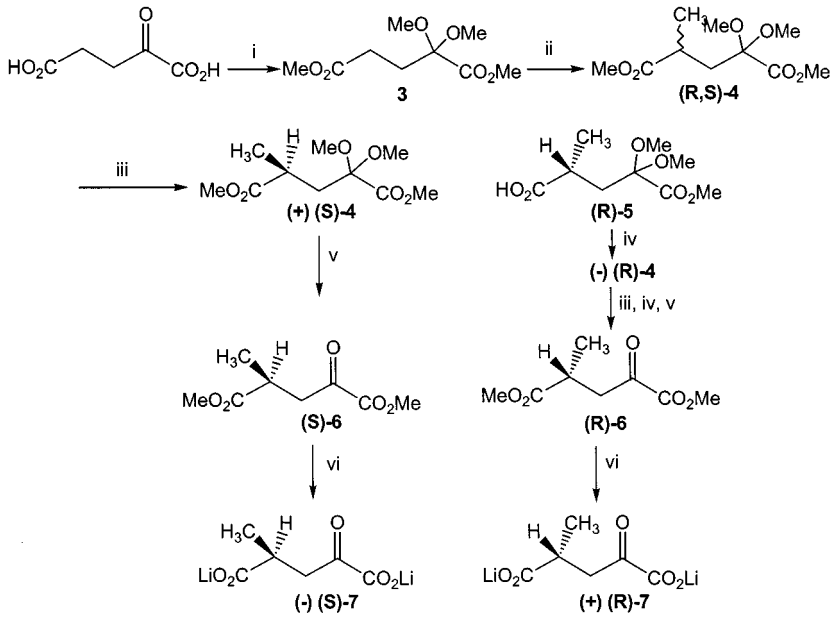
Previous syntheses of MKG, based on the method of Blaise and Gault,[9] are by condensation of methyl succinate onto diethyl oxalate, followed by hydrolysis and decarboxylation. The synthesis, however, leads to a mixture of 3- and 4-methyl-oxoglutaric acids and needs laborious washing and a distillation for purification. For small-scale synthesis, it is more convenient to start from commercial oxoglutaric acid as shown in Scheme 1.[10]

Oxoglutaric acid is easily protected as the dimethyl ester dimethyl acetal **3**. Then, the alkylation is achieved by action of LHDMS at −78°C in THF, followed by addition of methyl iodide. Dimethyl 2,2-dimethoxy-4-methylglutarate (**4**) was isolated with 73% yield. The ^{13}C-NMR spectrum of **4** was fully interpreted by experiments of semiselective polarisation transfer with selective proton decoupling (INEPT-DANTE). This allowed ^{13}C signals at $\delta = 176$ and 169 to be attributed to C^5 and C^1, respectively.

Racemic **4** was submitted to the action of hydrolases. The hydrolysis was monitored by maintaining the pH constant at 7 by NaOH addition which allows the calculation of the conversion rate. Enantiomeric excess of residual substrate and product were determined by ^{1}H NMR in the presence of europium salts. The enantioselectivities *E* of the enzymes were characterised by the ratio of the initial rates for each enantiomers, as proposed by Sih and co-workers[11] The results are reported in the Table.

Among the proteases tested, only the enzyme from *Aspergillus oryzae* was active, but without enantioselectivity. The Pig Liver esterase presents a small enantioselectivity. We tried to increase this specificity by addition of organic co-solvents.[12] The best results were reached with 25% of dimethyl sulfoxide in phosphate buffer. Various lipases were inactive, but *Pseudomonas mendocina* lipase showed an interesting enantioselectivity since the ester remaining after 50% of hydrolysis presented 75% of enantiomeric excess,

  1434−193X/99/1212−3403 $ 17.50+.50/0



Scheme 1. Reaction conditions: i) MeOH, $HC(OCH_3)_3$, H_2SO_4, (96%); ii) LHDMS, −78°C, MeI (73%); iii) *Pseudomonas mendocina* lipase, phosphate buffer, 4°C; iv) MeOH, $SOCl_2$; v) TFA; vi) LiOH (93%)

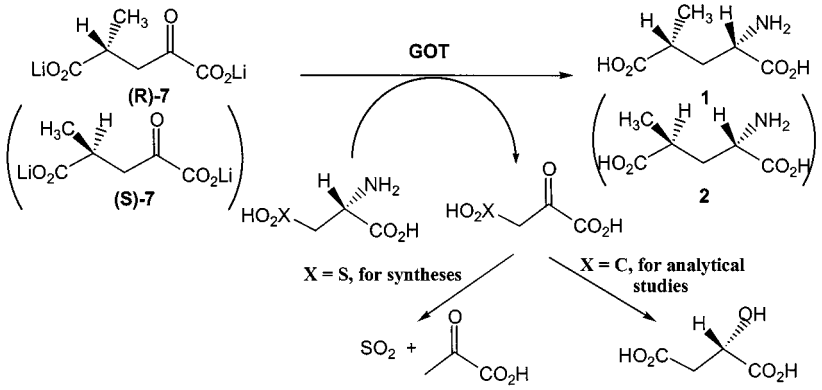
which corresponds to an enantioselectivity coefficient E = 15. Addition of organic solvents did not improve the resolution, so we looked for a possible temperature effect. The influence of the temperature on the enantioselectivity of enzymes has been pointed out for the first time in the case of a thermophilic alcohol dehydrogenase which is active on a large temperature range. In the last years, some temperature effects have been observed in lipase-catalysed reactions.[13] In our experiments, we observed that when the reaction was carried out at 4°C, the E value was increased to up to 40. At this temperature, the residual ester (+)-**4** was isolated in a 98% enantiomeric excess after 55% of conversion, with a 42% yield. The ^{13}C-NMR spectra of the hydrolysed product **5** showed that the C^1 signal at δ = 169 is unchanged whereas the C^5 signal is shifted to δ = 180.8, indicating that the hydrolysis has occurred, as expected, on the ester in C^5, near the asymmetric carbon atom. In order to improve its enantiomeric purity, **5** was esterified with $SOCl_2$ in methanol, to afford (−)-**4** which was submitted again to enzymatic hydrolysis. After 85% of conversion, **5** was isolated, esterified to give (−)-**4** in more than 98% *ee* and 44% overall yield. Hydrolysis of these diesters gave both enantiomers of MKG **7**.The usual protocol for hydrolysis of oxoglutaric diester, by heating in 1 N HCl led to the formation of unidentified by-products. To avoid these drastic conditions, we first hydrolysed the acetal in the presence of trifluoroacetic acid at room temperature to obtain (+)- and (−)-**6**, then the lithium salts of MKG (+)-**7** and (−)-**7** were obtained by hydrolysis under slightly basic conditions.

The absolute configuration of (−)-**4** and (+)-**4** as well as (+)- and (−)-**6** was attributed after the transamination of these compounds in the presence of GOT with cysteine sulfinic acid as amino-group donor according to Scheme 2. Indeed, the enzyme affords the amino acids of the natural L series, i.e. either (2*S*,4*R*)- or (2*S*,4*S*)-4-methylglutamic acids (which have already been described[7]) depending on the configuration of the MKG in reaction. The transamination was achieved as previously described[8] for the racemic MKG. (−)-MKG led with a 73% yield to the corresponding glutamate analogue, identified as (2*S*,4*S*)-4-methylglutamic acid, which allows the (*S*) configuration to be attributed to (+)-**4**. Of course, (−)-**4** of (*R*) configuration led to the (2*S*,4*R*) isomer with a 78% yield.

Table 1.

Enzymes	Solvent	Conversion rate (%)	Reaction time	*ee*[a] of residual ester (%)
Aspergillus oryzae protease	phosphate buffer	50	48 h	0
Porcine Liver Esterase	phosphate buffer	50	1 h	20
Porcine Liver Esterase	phosphate buffer/toluene	50	1 h	32
Porcine Liver Esterase	phosphate buffer/MeOH	50	1 h	52
Porcine Liver Esterase	phosphate buffer/DMSO	50	1 h	65
Pseudomonas cepacia lipase	phosphate buffer	no reaction	> 24 h	–
Pseudomonas mendocina lipase at 25°C	phosphate buffer	50	48 h	75 (E = 15)
Pseudomonas mendocina lipase at 4°C	phosphate buffer	55	3 d	98 (E > 40)

[a] The *ee* values were calculated by ^{1}H-NMR experiments with



Scheme 2. GOT-catalysed transamination of (4*S*)- and (4*R*)-4-methyl-2-oxoglutarates

The activity of GOT for these close analogues of α-oxoglutaric acid (KG) was determined in phosphate buffer at pH = 7.6 in the presence of aspartic acid (Scheme 2).(4*R*)-MKG is the best substrate. Its behaviour is very close to the natural substrate, with a K_m value of 0.16 mM (against 0.18 mM for KG) and a V_{max} value of 72% relative to KG. Apparently, GOT does not discriminate between its natural substrate and this analogue. (4*S*)-MKG is a poorer substrate with a K_m value of 9 mM and a V_{max} value of only 11% relative to KG. The enantioselectivity of the enzyme is mainly due to the best affinity of the enzymatic site for (4*R*)-MKG as indicated by the ratio of the K_m values (around 1:45), while the k_{cat} values are in a ratio of only 6:1.

Experimental Section

General: Melting points were determined with a Reichert hot-stage apparatus and are reported uncorrected. − IR spectra were recorded with a Perkin−Elmer 801 spectrophotometer. − Flash chromatography was carried out on 220−400 mesh silica gel. − Thin-layer chromatography was performed on Merck precoated silica gel 60 F_{254} plates and spots were visualised with vanillin [vanillin (1 g) is dissolved in MeOH (60 mL) and conc. H_2SO_4 (0.6 mL)]. − ^{1}H- (400 MHz), ^{13}C-NMR (100 MHz) experiments were performed with an AC 400 Bruker spectrometer. − Enzymes were from commercial origin: *Pseudomonas mendocina* lipase from Genencor, all others from Sigma.

Dimethyl 2,2-Dimethoxyglutarate (3): α-Oxoglutaric acid (60 g, 0.41 mol) was dissolved in 500 mL of methanol in which trimethyl orthoformate (160 mL, 1.4 mol, 3.5 equiv.) was introduced. Sulfuric acid (10 mL, 32 N) was added dropwise and the solution was refluxed during 12 h. The solution was concentrated in vacuo and ethyl acetate (200 mL) was added. The solution was washed with a saturated aqueous bicarbonate solution, dried with magnesium sulfate and concentrated to dryness to give 90.4 g (96%) of a colourless liquid. − ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.17, 2.26 (each m, each 2 H, 2 CH_2), 3.20 (s, 6 H, 2 CH_3 acetal), 3.60, 3.74 (each s, each 3 H, 2 CH_3 ester). − ^{13}C NMR (100 MHz, $CHCl_3$): δ = 28.1, 28.7 (C^3 and C^4), 49.9 (2 CH_3 acetal), 51.7, 52.6 (2 CH_3 ester), 101.5 (C^2), 169.0 (C^1), 172.8 (C^5).

(*R*,*S*)-Dimethyl 2,2-Dimethoxy-4-methylglutarate (4): Under argon was introduced 16.5 g (0.075 mol) of **3** into 200 mL of anhydrous THF. After cooling to −78°C, 1.2 equiv. of LHMDS (90 mL of a commercially available 1 M solution in THF) was added dropwise. After stirring for 1 h at −78°C, 14 mL of methyl iodide (0.225 mol, 3 equiv.) was added and the mixture was allowed to stand for another hour at this temperature before being raised to −30°C. After 1 h, water and ethyl acetate were introduced at room temperature and the solution transferred into a separatory funnel. The aqueous phase was neutralised with 1 N HCl and the organic phase was extracted, washed with a saturated aqueous sodium chloride solution, dried with magnesium sulfate and concentrated in vacuo to give 12.8 g (73% yield) of a colourless liquid after column chromatography on silica gel (R_f = 0.38, cyclohexane:ethyl acetate, 7:3). − ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.03 (d, 3 H, J = 8 Hz, CH_3), 1.74 (dd, 1 H, J = 4 Hz, J = 14 Hz, CH_2), 2.30 (dd, 1H, J = 8 Hz, J = 14 Hz, CH_2), 2.37 (m, 1 H, CH), 3.12 (s, 6 H, 2 CH_3 acetal), 3.53, 3.67 (each s, each 3 H, 2CH_3 ester). − ^{13}C NMR (100 MHz, $CDCl_3$): δ = 18.4 (4-CH_3), 34.5 (C^4), 37.0 (C^3), 49.7, 49.8 (2 CH_3 acetal), 51.5, 52.3 (2 CH_3 ester), 101.2 (C^2), 169.0 (C^1), 176.1 (C^5).

General Procedure for the Resolution of Dimethyl (*R*,*S*)-2,2-Dimethoxy-4-methylglutarate (4): Substrate **4** (12.8 g, 54.7 mmol) was dissolved in phosphate buffer (650 mL, 0.1 M, pH = 7.0). *Pseudomonas mendocina* lipase (5 g) was then added and the pH was maintained by addition of 1 N NaOH. After addition of 30 mL of NaOH solution (55% conversion), the mixture was centrifuged and the supernatant was transferred into a separatory funnel. The aqueous phase was extracted with toluene and the organic phase was washed twice with a 10% solution of $NaHCO_3$, dried with $MgSO_4$ and concentrated in vacuo to afford 5.63 g (44%) of (*S*)-**4**; $[\alpha]_D^{25}$ = +31.9 (c = 2.1, $CHCl_3$), *ee* > 96% [^{1}H NMR with Eu(hfc)$_3$]. The combined aqueous extracts were brought to pH = 1−2 with 1 N HCl, saturated with NaCl and extracted three times with ethyl acetate. The combined organic phases were dried ($MgSO_4$) and concentrated in vacuo to afford (*R*)-**5**. (*R*)-**5** was esterified with $SOCl_2$ in refluxing MeOH in quantitative yield, and after the usual workup was hydrolysed again with the enzyme (c = 85%) in order to increase the enantiomeric purity. Thus, another esterification under the same conditions afforded a 42% overall yield (5.05 g) of (*R*)-**4**; $[\alpha]_D^{25}$ = −31.7 (c = 2.58, $CHCl_3$), *ee* > 96% [^{1}H NMR with Eu(hfc)$_3$].

Dimethyl (*R*)- and (*S*)-4-Methyl-2-oxoglutarate (6): To a solution of 5 g (21.3 mmol) of (*R*)-**4** or (*S*)-**4** in 25 mL of dichloromethane and 2.5 mL of water was added dropwise 25 mL of trifluoroacetic acid at room temperature. After 2 h of stirring, the solution was concentrated in vacuo to give a colourless oil.

Dimethyl (*R*)-4-Methyl-2-oxoglutarate: Liquid (3.8 g, 95%); $[\alpha]_D^{25}$ = +13.0 (c = 1.3, $CHCl_3$). − ^{1}H NMR (400 MHz, $CDCl_3$):

δ = 1.25 (d, 3 H, J = 7 Hz, CH_3), 2.88 (dd, 1 H, J = 5 Hz, J = 18 Hz, CH_2), 3.02 (m, 1 H, CH), 3.33 (dd, 1 H, J = 8 Hz, J = 18 Hz, CH_2), 3.7, 3.9 (each s, each 3 H, 2 CH_3 ester). − ^{13}C NMR (100 MHz,$CDCl_3$): δ = 17.0 (4-CH_3), 34.7 (C^4), 40.7 (C^3), 52.1, 53.1 (2 CH_3 ester), 161.0 (C^1), 175.5 (C^5), 192.1 (C^2).

Dimethyl (*S*)-4-Methyl-2-oxoglutarate: Liquid (3.74 g, 93%); $[\alpha]_D^{25}$ = −13.1 (c = 1.3, $CHCl_3$). Spectroscopic data are identical to those of dimethyl (*R*)-4-methyl-2-oxoglutarate.

Dilithium (*R*)- and (*S*)-4-Methyl-2-oxoglutarate (7): 3 g (16 mmol) of the obtained compounds [(*R*)- or (*S*)-**6**] were dissolved in 50 mL of water and 2 equiv. of lithium hydroxyde was added in portions, maintaining a pH below 10. When the pH was permanently alkaline, the solution was neutralised by addition of a weakly acidic resin (Amberlite IRC-50, H^+ form), filtered and then concentrated in vacuo to give a white solid.

Dilithium (*R*)-4-Methyl-2-oxoglutarate: Solid (2.88 g, 98%); $[\alpha]_D^{25}$ = +6.6 (*c* = 1, H_2O). − ^{1}H NMR (400 MHz, D_2O): δ = 1.20 (d, 3 H, J = 7 Hz, 4-CH_3), 2.78 (m, 1 H, CH), 2.87 (dd, 1H, J = 6 Hz, J = 18 Hz, CH_2), 3.15 (dd, 1 H, J = 7 Hz, J = 18 Hz, CH_2). − ^{13}C NMR (100 MHz, D_2O): δ = 18.3 (4-CH_3), 38.5 (C^4), 44.8 (C^3), 171.2 (C^1), 185.7 (C^5), 206.0 (C^2).

Dilithium (*S*)-4-Methyl-2-oxoglutarate: Solid (2.91 g, 99%); $[\alpha]_D^{25}$ = −6.6 (c = 1, H_2O). − Spectroscopic data are identical to those of dilithium (*R*)-4-methyl-2-oxoglutarate.

Enzymatic Studies: Activities of GOT as well as the Michaelis constants for (*R*)- and (*S*)-**7** were determined as described in ref.[14]

(4*R*)- and (4*S*)-L-Glutamic Acids 1 and 2: A solution (30 mL) containing (*R*)- or (*S*)-**7** (100 mg, 0.58 mmol), pyridoxal phosphate (0.5 mg, 15 μmol), and cysteine sulfinic acid was adjusted to pH = 7. Then GOT (300 units) was added and the mixture was stirred at room temperature. After 24 h, the solution was applied to a 16 × 2 cm column of Dowex 50X1(H^+). The resin was washed with 1 volume of water, then the amino acid was eluted with 0.1 N ammonia. The eluate was concentrated in vacuo, and the product purified on a 14 × 2 cm column of Dowex weakly basic (OH^-) eluted with 1 N formic acid to give after concentration **1** (72 mg, 78% yield) or **2** (68 mg, 73% yield, characterised by comparison with authentic samples.[8]

[1] G. Johnson, *Bioorg. Med. Chem. Lett*. **1993**, *3*, 9.
[2] F. C. Acher, F. J. Tellier, R. Azerad, I. N. Brabet, L. Fagui, P. R. J.- Pin, *J. Med. Chem*. **1997**, *40*, 3119−3129.
[3] Z.-Q. Gu, P. Hesson, J. C. Pelletier, M.-L. Maccecchini, *J. Med. Chem*. **1995**, *38*, 2518−2520.
[4] N. Todeschi, J. Gharbi-Benarous, F. Acher, V. Larue, J.-P. Pin, J. Bockaert, R. Azerad, J.-P. Girault, *Bioorg. Med. Chem*. **1997**, *5*, 335.
[5] E. Coudert, F. Acher, R. Azerad, *Synthesis* **1997**, 863; Z.-Q. Gu, D. P. Hesson, *Tetrahedron: Asymmetry* **1995**, *6*, 2101−2104.
[6] C. M. Moody, D. W. Young, *Tetrahedron Lett*. **1994**, *35*, 7277−7280.
[7] A. Righini-Tapie, R. Azerad, *J. Appl. Biochem*. **1984**, *6*, 361−366.
[8] F. Echalier, O. Constant, J. Bolte, *J. Org. Chem*. **1993**, *58*, 2747−2750.
[9] E. E. Blaise, H. Gault, *Bull. Soc. Chim. Fr*. **1994**, 458−464.
[10] P. Dowd, S.-C. Choi, F. Duah, C. Kaufman, *Tetrahedron* **1988**, *44*, 2137−2148.
[11] C.-S. Chen, Y. Fujimoto, G. Girdaukas, C. H. Sih, *J. Am. Chem. Soc*. **1982**, *104*, 7294−7299.
[12] P. A. Fitzpatrick, A. M. Klibanov, *J. Am. Chem. Soc*. **1991**, *113*, 3166; F. Terradas, M. Teston-Henry, P. A. Fitzpatrick, A. M. Klibanov, *J. Am. Chem. Soc*. **1993**, *115*, 390.
[13] V. T. Pham, R. S. Phillips, L. G. Ljungdahl, *J. Am. Chem. Soc*. **1989**, *111*, 1935; R. S. Phillips, *Trends Biotechnol*. **1996**, *14*, 13; T. Sakai, T. Kishimoto, Y. Tanaka, T. Ema, M. Utaka, *Tetrahedron Lett*. **1998**, *39*, 7881−7884.
[14] H. U. Bergmeyer, *Methods of Enzymatic Analysis*, 2nd ed. VCH, Weinheim, **1974**, p 144 and 462.

Received April 8, 1999
[O99198]