Introduction

Structural analogues of L-glutamine exhibit inhibitory properties towards enzymes utilizing this amino acid as a substrate, including glutaminase (EC 3.5.1.2), γ-glutamyl transpeptidase (EC 2.3.2.2) and several amidotransferases catalyzing the transfer of an amino group from the γ-amide function of L-glutamine to different acceptor molecules (Massiere and Badet-Denisot, 1998). One of these enzymes is glucosamine-6-phosphate synthase (GlcN-6-P synthase, EC 2.6.1.16) which catalyzes the first committed step in a pathway leading to the formation of UDP-N-acetylgalactosamine, providing N-acetyl-α-glucosamine for the formation of bacterial peptidoglycan and fungal chitin. For that reason, inhibitors of the enzyme are potential antimicrobial agents. Inhibitory properties of some glutamine analogues with respect to GlcN-6-P synthase and other amidotransferases have been reviewed (Pinkus, 1977).

In the present paper we describe the synthesis and enzyme inhibitory properties of four Nγ-alkyl analogues of L-glutamine, namely Nγ-ethyl- (1a), Nγ-methyl- (1b), Nγ,Nγ-diethyl- (1c) and Nγ,Nγ-dimethyl-L-glutamine (1d).

According to literature data (Tsushida and Takeo, 1984, 1985a; Chu et al., 1997) both Nγ-ethyl-L-glutamine (1a) (better known as theanine) and Nγ-methyl-L-glutamine (1b) have been found in green tea leaves. The research on rats with induced hypertension revealed that this two compounds cause significant reduction in blood pressure (Yokogoshi and Kobayashi, 1998; Yokogoshi et al., 1995). Recent studies have shown that theanine and Nγ-methyl-L-glutamine zinc(II) complexes exhibit insulinomimetic activity relative to isolated in vitro rodent adipocytes treated with epinephrine and also induce the release of free fatty acids (FFA) from fat cells (Matsumoto et al., 2005). Any particular biological properties of Nγ,Nγ-dialkyl derivatives of L-glutamine have not been reported so far.

Results and Discussion

Syntheses of Nγ-alkyl derivatives of L-glutamine have been reported by several authors (Tanaka, 1962; Hashizume, 1951; Lichtenstein, 1942; Saka-to et al., 1950; Craig et al., 1988; Furuyama et al., 1964; Gu et al., 2004; Tsushida and Takeo, 1985b). Theanine (Nγ-ethyll-L-glutamine) has been most often obtained by aminolysis of N-carbobenzyloxy-L-glutamic acid (Hashizume, 1951; Sakato et al., 1950) or L-glutamic acid γ-methyl ester (Kawagishi and Sugiyama, 1992; Tanaka, 1962; Lichtenstein, 1942; Furuyama et al., 1964; Barzily et al., 1956). Alternatively, N-phthaloyl-DL-glutamic anhydride was treated with a suitable amine to give Nγ-phthaloyl-Nγ-di- or -monoethyl-DL-glutamine, and subsequent removal of the phthaloyl residue with hydrazine led to the ultimate product,
$N$-alkyl-DL-glutamine (Friedman and Chatterji, 1959; Gu et al., 2004; Craig et al., 1988). The more general method involved conversion of L-glutamic acid into S-5-oxo-2-pyrrolidinecarboxylic acid (L-pyroglutamic acid), which was then opened upon the treatment with ethylamine or methylamine to give the corresponding product (Matsumoto et al., 2005; Lichtenstein, 1942; Hashizume, 1951; Tsushida and Takeo, 1985b). Biosynthesis of theanine (1a) from L-glutamic acid and ethylamine catalyzed by Pseudomonas nitroreducens was also reported (Abelian et al., 1993).

In the present communication we describe the unequivocal and universal procedure of synthesis of the $N$-alkyl and $N,N$-dialkyl derivatives of L-glutamine 1a–d (Fig. 1). L-Glutamic acid, as a starting substrate, was first converted into N-carbobenzyloxy-L-glutamic acid $\alpha$-tert-butyl ester (2) in four steps, using previously described procedures (Boissonnas et al., 1955; Taschner et al., 1961). The $\gamma$-carboxyl function was converted into the methyl ester, while the amino and the $\alpha$-carboxyl group were protected by carboxbenzyl- and tert-butyl ester functions, respectively. Such strategy allowed a subsequent selective removal of $\gamma$-methyl ester by alkaline hydrolysis, to afford an appropriately protected substrate for $\gamma$-amide bond formation. Three different alternative methods of this reaction were tested: acid chlorides, mixed anhydrides, or $N,N'$-dicyclohexylcarbodiimide (DCCI)/N-hydroxysuccinimide (NSuOH). The first approach led, in our case, to the formation of N-carbobenzyloxy-L-pyroglutamic acid. Application of the mixed anhydrides method afforded a mixture of products, with urethane arising from an amine and chloroformate as the major component. Finally, the best results were obtained when 2 was treated with a suitable amine (ethylamine, methylamine, diethylamine, and dimethylamine) in the presence of a coupling compound – DCCI and N-hydroxysuccinimide. The reaction was continued at room temperature, usually for 3 days, and its progress was followed by TLC. Since some of the alkylamines are volatile at room temperature, an additional amount of this substrate was added when 2 was still present in the reaction mixture. The tert-butyl group of compounds 3a–d was selectively removed with trifluoroacetic acid (TFA) and the $\alpha$-amino function was deprotected by catalytic dehydrogenation. Compounds 1a–d were purified by ion-exchange chromatography and finally crystallized from different solvent systems.

![Fig. 1. Scheme of synthesis of the $N$-alkyl- and $N,N$-dialkyl-L-glutamine derivatives 1a–d.](imageURL)
The proposed general procedure of synthesis of Nγ-alkyl-glutamine derivatives is versatile. Removal of the protecting groups affords isobutene, toluene and carbon dioxide that may be easily removed from the post-reaction mixtures. The cumulative yields (≈60%) seem satisfactorily and are much better than those achieved by the previous methods, where values even lower than 10% were reported (Craig et al., 1988).

The synthesized compounds were tested as potential inhibitors of three glutamine-utilizing enzymes, namely GlcN-6-P synthase from Candida albicans, bacterial glutaminase, and mammalian γ-glutamyl transpeptidase. The obtained data on enzyme inhibition are summarized in Table I.

Compounds 1a–d appeared to be moderately strong inhibitors of all enzymes, as the determined IC50 and Ki values were in the millimolar range. The inhibition was in each case competitive with respect to L-glutamine. This type of inhibition is exemplified by the Lineweaver-Burk plot obtained for the GlcN-6-P synthase inhibition by 1c, shown in Fig. 2. Interestingly enough, the N,N-dialkyl derivatives 1c and 1d appeared to be stronger inhibitors of GlcN-6-P synthase activity than their N-monoalkyl counterparts 1a and 1b, while in the case of γ-glutamyl transpeptidase and glutaminase an opposite situation was noted. It is therefore worth mentioning that ammonia released from L-glutamine at the GlcN-6-P synthase active site is transferred to the acceptor binding site through an intramolecular tunnel (Teplyakov et al., 2001). It is therefore likely, that Nγ-substituted derivatives of L-glutamine are more effective inhibitors of the enzyme, as the N-alkyl and especially N,N-dialkyl substituents are supposed to block the entry to the tunnel, when the Nγ-substituted derivatives of L-glutamine bind to the active site instead of the natural substrate. The two other enzymes do not contain any intramolecular tunnel and their lower sensitivity to inhibition by N,N-dialkyl compounds may be rationalized in terms of restricted access of bulkier inhibitors to the glutamine binding sites.

**Material and Methods**

**General**

Homogeneity of the products and progress of the reactions were controlled by thin-layer chromatography (TLC). The following conditions were used for all derivatives: Merck silica gel 60 F254 plates (layer thickness, 0.1 mm); eluent mixtures: benzene/ethyl acetate/ethanol (60:20:5) (A), n-butanol/acetic acid/water (4:1:1) (B); the spots were visualized with iodine and ninhydrin reagent (for compounds containing free amine groups), heated at high temperature for about 1–2 min. Melting points are uncorrected.

**Table I. Inhibition of three glutamine-utilizing enzymes by Nγ-alkyl-glutamine derivatives.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>GlcN-6-P synthase</th>
<th>Glutaminase</th>
<th>γ-Glutamyl transpeptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 [mM]</td>
<td>Ki [mM]</td>
<td>IC50 [mM]</td>
</tr>
<tr>
<td>1a</td>
<td>14</td>
<td>0.78</td>
<td>24</td>
</tr>
<tr>
<td>1b</td>
<td>28</td>
<td>2.04</td>
<td>20</td>
</tr>
<tr>
<td>1c</td>
<td>9</td>
<td>0.49</td>
<td>39</td>
</tr>
<tr>
<td>1d</td>
<td>10.5</td>
<td>0.63</td>
<td>35</td>
</tr>
</tbody>
</table>

Fig. 2. Competitive inhibition of GlcN-6-P synthase by 1c with respect to L-glutamine. Inhibitor concentrations: (■) none; (●) 0.5 mM; (▲) 1.5 mM; (▲) 3.0 mM.
polarimeter. The IR spectra were recorded on a FT-IR Bruker IFS 66 spectrometer.

**L-Glutamic acid γ-methyl ester hydrochloride**

This compound was synthesized according to the method described by Boissonnas et al. (1955), in 55% yield. - M.p. 162–164° C (MeOH/ Et2O). - α_d +28.3° (c 2.5, 6 m HCl).

\[N^\alpha\text{-Carbobenzyloxy-L-glutamic acid a-tert-butyI ester (2)}\]

Compound 2 was synthesized according to the modified method described by Tschern et al. (1961). It was obtained in 55% yield (2.27 g). - R_f = 0.37 (B). - M.p. 79–82° C (CHCl3/hexane). - α_d +25° (c 1, MeOH). - 1H NMR (CDCl3): δ = 1.48 (s, 9H, C(CH3)3), 1.98 (m, 1H, CH3), 2.2 (m, 1H, CH2), 2.47 (m, 2H, CH2CO), 4.32 (m, 1H, CH2), 5.12 (s, 2H, CH2Ph), 5.45 (d, J = 7.8 Hz, 1H, NH), 7.37 (s, 5H, C6H5). - 13C NMR (CDCl3): δ = 177.9, 171.4, 156.7, 136.5, 128.8, 128.5, 128.4, 88.8, 67.4, 54.1, 29.7, 27.9, 27.6. - Literature data: oil, α_d –19.4° (c 2.2, MeOH) (Dolence et al., 1991).

\[N^\alpha\text{-Carbobenzyloxy-L-glutamine a-tert-butyI ester (3)}\]

Compound 2 (680 mg, 2.02 mmol) was dissolved in tetrahydrofuran (20 mL) at 0° C, with stirring, followed by addition of N,N-dicyclohexylcarbodiimide (417 mg, 2.02 mmol), N-hydroxysuccinimide (233 mg, 2.02 mmol), and an amine (4.04 mmol). The resulting mixture was stirred at room temperature for 3 d and then cooled to 0° C. The precipitate N,N-dicyclohexylurea was filtered off, and the solution was evaporated in vacuo. The residue was dissolved in ethyl acetate and washed with portions (10 mL) of water, 5% sodium hydrogen carbonate solution, water, 5% citric acid solution, and water, respectively. The organic phase was dried, and the solvent was removed under reduced pressure to give the crude product which was purified using column chromatography (silica gel Merck 70, 230 mesh, chloroform as an eluent).

\[N^\alpha\text{-Ethyl-N^\alpha\text{-carbobenzyloxy-L-glutamine a-tert-butyI ester (3a)}}\] Yield 64%. - R_f = 0.32 (B). - α_d – 13.5° (c 1.04, MeOH). - 1H NMR (CDCl3): δ = 1.13 (t, 3H, CH3), 1.45 (s, 9H, C(CH3)3), 1.93 (m, 2H, CH2), 2.21 (m, 2H, CH2), 2.27 (m, 2H, CH2N), 3.27 (m, 2H, CH2Ph), 4.27 (m, 1H, CH2), 5.11 (m, 2H, CH2Ph), 5.54 (d, J = 7.3 Hz, 1H, NH), 6.0 (m, 1H, NH), 7.35 (s, 5H, C6H5). - 13C NMR (CDCl3): δ = 172.3, 171.5, 156.9, 136.7, 129.0, 128.7, 128.6, 82.99, 67.5, 54.5, 34.97, 33.15, 29.7, 28.4, 15.22.

\[N^\alpha\text{-Methyl-N^\alpha\text{-carbobenzyloxy-L-glutamine a-tert-butyI ester (3b)}}\] Yield 61%. - R_f = 0.27 (B). - α_d –22.6° (c 0.93, MeOH). - 1H NMR (CDCl3): δ = 1.44 (s, 9H, C(CH3)3), 1.96 (m, 1H, CH2), 2.1 (m, 1H, CH2CO), 2.78 (d, 3H, NCH3), 4.21 (m, 1H, CH2), 5.1 (s, 2H, CH2Ph), 5.68 (d, J = 7.3 Hz, 1H, NH), 6.03 (m, 1H, NH), 7.35 (s, 5H, C6H5). - 13C NMR (CDCl3): δ = 173.3, 171.5, 156.9, 136.7, 129.0, 128.7, 128.6, 83.09, 67.56, 54.47, 32.95, 28.46, 27.0.

\[N^\alpha\text{-Diethyl-N^\alpha\text{-carbobenzyloxy-L-glutamine a-tert-butyI ester (3c)}}\] Yield 70%. - R_f = 0.52 (B). - α_d –16.8° (c 1.9, MeOH). - 1H NMR (CDCl3): δ = 1.15 (t, 6H, CH3), 1.47 (s, 9H, C(CH3)3), 2.03 (m, 1H, CH2), 2.20 (m, 1H, CH2), 2.32–2.46 (m, 2H, CH2CO), 3.27 (m, 2H, CH2N), 3.36 (m, 2H, CH2N), 4.23 (m, 1H, CH2), 5.09 (m, 2H, CH2Ph), 5.7 (d, 1H, NH), 7.35 (s, 5H, C6H5). - 13C NMR (CDCl3): δ = 172.3, 171.5, 156.9, 136.7, 129.0, 128.7, 128.6, 82.99, 67.5, 54.5, 34.97, 33.8, 29.7, 28.4, 15.2.

\[N^\alpha\text{-Dimethyl-N^\alpha\text{-carbobenzyloxy-L-glutamine a-tert-butyI ester (3d)}}\] Yield 61%. - R_f = 0.38 (B). - α_d –17.4° (c 0.98, MeOH). - 1H NMR (CDCl3): δ = 1.47 (s, 9H, C(CH3)3), 2.01 (m, 1H, CH2), 2.21 (m, 1H, CH2), 2.32–2.46 (m, 2H, CH2CO), 2.94 (s, 3H, NCH3), 2.96 (s, 3H, NCH3), 4.26 (m, 1H, CH2), 5.1 (m, 2H, CH2Ph), 5.68 (d, J = 7.4 Hz, 1H, NH), 7.37 (s, 5H, C6H5). - 13C NMR (CDCl3): δ = 173.5, 171.9, 157, 138.6, 129.0, 128.7, 128.6, 83.09, 67.56, 54.47, 34.5, 33.8, 28.46, 27.1.

**Synthesis of N^\alpha\text{-alkyl-L-glutamine (1)}**

Compound 3 (0.5 mmol) was treated with trifluoroacetic acid (2.1 mL) at room temperature. Progress of acidolysis was followed by TLC. After the acidolysis was completed, excess of trifluoroacetic acid was removed under reduced pressure. The residue was washed several times with diethyl ether. The crude product was purified by column chromatography on silica gel (Merck, 70–230 mesh) using chloroform as the eluent. The N^\alpha\text{-alkyl-N^\alpha\text{-carbobenzyloxy-L-glutamine derivatives were obtained as colourless oils, dissolved in ethanol and hydrogenated over 10% Pd/C for a few hours. The progress of the reaction was fol-}
lowed by TLC. The catalyst was filtered off and washed with a little amount of water/ethanol. The combined solution was evaporated in vacuo, and the residue was purified on Dowex 50X8–100[H+] resin with 10% NH3 as an eluent. The solvent was removed by evaporation and the residue dissolved in an appropriate solvent (see below) to obtain products 1a–d as white crystals.

\[ N^\gamma\text{-Ethyl-L-glutamine (1a): Yield 62\%}. \]

\[ R_t = 0.61 \text{ (A)}. \]

\[ [\alpha]_20^\text{D} +12^\circ \text{ (c 0.5, H}_2\text{O}). \]

\[ \text{M.p. 218–216}^\circ\text{C (hot H}_2\text{O/acetone).} \]

\[ ^1\text{H NMR (D}_2\text{O):} \delta = 0.95 \text{ (t,} J = 7.3 \text{ Hz, 3H, NCH}_2\text{CH}_3), 1.97 \text{ (m, 2H, CH}_2), 2.23 \text{ (m, 2H, CH}_2), 3.04 \text{ (q,} J = 7.3 \text{ Hz, 2H, NCH}_2\text{CH}_3), 3.61 \text{ (t,} J = 6.1 \text{ Hz, 1H, CH}^\text{β}). \]

\[ \text{– Literature data: m.p. 214–216}^\circ\text{C,} [\alpha]_20^\text{D} +6.6^\circ \text{ (c 1, H}_2\text{O).} \]

\[ \text{Kawagishi and Sugiyma, 1992; m.p. 215}^\circ\text{C (MeOH)} \text{ (Tanaka, 1962); m.p. 217}^\circ\text{C (H}_2\text{O/acetone).} \]

\[ \text{– M.p. 192–178}^\circ\text{C,} [\alpha]_20^\text{D} +7.9^\circ \text{ (Sakato, 1950; Sakato et al., 1950); m.p. 200}^\circ\text{C,} [\alpha]_20^\text{D} +6.25^\circ \text{ (Lichtenstein, 1942).} \]

\[ N^\gamma\text{-Methyl-L-glutamine (1b): Yield 59\%.} \]

\[ R_t = 0.18 \text{ (A)}. \]

\[ [\alpha]_20^\text{D} +6.3^\circ \text{ (c 1, H}_2\text{O).} \]

\[ \text{M.p. 200–202}^\circ\text{C (H}_2\text{O/acetone).} \]

\[ ^1\text{H NMR (D}_2\text{O):} \delta = 2.13 \text{ (m, 2H, CH}_2), 2.43 \text{ (m, 2H, CH}_2), 2.72 \text{ (s, 3H, NCH}_2\text{CH}_3), 3.69 \text{ (m, 1H, CH}^\text{β}). \]

\[ \text{– Literature data: m.p. 202–203}^\circ\text{C,} [\alpha]_20^\text{D} +7.14^\circ \text{ (c 10, H}_2\text{O) (Barzily et al., 1956); m.p. 192}^\circ\text{C (r),} [\alpha]_20^\text{D} +5.9^\circ \text{ (c 1, H}_2\text{O) (Tanaka, 1962); m.p. 202}^\circ\text{C (r) (Hashizume, 1951); m.p. 192}^\circ\text{C,} [\alpha]_20^\text{D} +6^\circ \text{ (Lichtenstein, 1942).} \]

\[ N^\gamma,N^\gamma\text{-Diethyl-L-glutamine (1c): Yield 56\%.} \]

\[ R_t = 0.38 \text{ (A)}. \]

\[ [\alpha]_20^\text{D} +1.0^\circ \text{ (c 0.9, H}_2\text{O).} \]

\[ \text{M.p. 168–170}^\circ\text{C (EtOH/acetone).} \]

\[ ^1\text{H NMR (D}_2\text{O):} \delta = 0.96 \text{ (t,} J = 7.1 \text{ Hz, 3H, CH}_3), 1.04 \text{ (t,} J = 7.5 \text{ Hz, 3H, CH}_3), 2.04 \text{ (m, 2H, CH}_2), 2.46 \text{ (m, 2H, CH}_2), 3.22 \text{ (q,} J = 7.3 \text{ Hz, 2H, CH}_2\text{N), 3.25 \text{ (q,} J = 7.3 \text{ Hz, 2H, CH}_2\text{N), 3.65 \text{ (t, 1H, CH}^\text{β}).} \]

\[ \text{– Literature data: m.p. 170–172}^\circ\text{C,} [\alpha]_20^\text{D} +1.16^\circ \text{ (c 1.25, EtOH) (Craig et al., 1988); rac m.p. 167–168}^\circ\text{C (Friedman and Chatterji, 1959).} \]

\[ N^\gamma,N^\gamma\text{-Dimethyl-L-glutamine (1d): Yield 68\%.} \]

\[ R_t = 0.20 \text{ (A).} \]

\[ [\alpha]_20^\text{D} +11.0^\circ \text{ (c 1, H}_2\text{O).} \]

\[ \text{M.p. 183–184}^\circ\text{C (EtOH/acetone).} \]

\[ ^1\text{H NMR (D}_2\text{O):} \delta = 2.09 \text{ (q,} J = 6.7 \text{ Hz, 2H, CH}_2), 2.54 \text{ (m,} J = 6.8 \text{ Hz, 2H, CH}_2), 2.87 \text{ (s, 3H, NCH}_3), 3.0 \text{ (s, 3H, NCH}_3), 3.72 \text{ (m, 1H, CH}^\text{β}). \]

\[ \text{– Literature data: m.p. 173–174}^\circ\text{C,} [\alpha]_20^\text{D} +11.3^\circ \text{ (c 2, H}_2\text{O) (Capaneris et al., 1978).} \]

\[ \text{Determination of enzyme inhibitory activity} \]

\[ \text{Candida albicans} \text{ GlcN-6-P synthase was purified to near homogeneity according to a previously published procedure (Sachadyn et al., 2000). The enzyme activity was determined by the modified Elson-Morgan method (Janiak et al., 2003).} \]

\[ \gamma\text{-Glutamyl transpeptidase from bovine kidney and} E.\text{ coli} \text{ glutaminase were purchased from Sigma. The activity of} \gamma\text{-glutamyl transpeptidase was determined using} \gamma\text{-glutamyl p-nitroanilide (GLUPA) as a substrate, under conditions described by Persijn et al. (1971), and glutaminase was assayed colourimetrically, according to the procedure of Gella and Pascual (1982). For determination of the IC}_{50} \text{ values, the concentration of L-glutamine in GlcN-6-P synthase and glutaminase assays was 10 mM, while the concentration of GLUPA in } \gamma\text{-glutamyl transpeptidase determination was 1 mM. In incubation mixtures for } K_i \text{ determination, glutamine/GLUPA concentrations were variable in the 0.2–2 mM range. All the measurements were done in triplicate. Inhibitory constants were determined from the secondary plots of } k_{\text{app}} \text{ versus inhibitor concentration, derived from the Lineweaver-Burk plots.} \]


