Department of Biochemical Sciences[1], Charles University Prague, Faculty of Pharmacy, Hradec Králové, and Institute of Experimental Botany[2], Academy of Sciences of the Czech Republic, Prague, Czech Republic

Stereoisomers of 4-fluoroglutamic acid: influence on *Escherichia coli* glutamate decarboxylase

J. Dršata[1], M. Netopilová[1] and V. Tolman[2]

The pyridoxal-5′-phosphate dependent enzyme glutamate decarboxylase (GAD, EC 4.1.1.15) catalyses decarboxylation of L-glutamic acid to 4-aminobutyric acid (GABA, see e.g. [1]). Because of the key role of GAD in the synthesis of GABA, many inhibitors of this enzyme, acting by various mechanisms, have been prepared and studied [2].

The fluorinated derivatives of glutamate play an important role because substitution of a fluorine atom for hydrogen influences the chemical reactivity of amino acids without introducing a significant steric change [3]. Such a compounds is 4-fluoroglutamate, which was synthesized in 1961 as a mixture of four stereoisomers [4]. Unkeless and Goldman [3] noticed that only one half of DL-4-fluoroglutamate was decarboxylated by GAD from *Escherichia coli* to produce 2-fluoro-4-aminobutyrate (2-fluoro-GABA). The same authors [5] prepared in 1971 *erythro*- and *threo*-diastereoisomers of 4-fluoro-L-glutamate and characterised them as substrates of GAD with 3–4 times faster decarboxylation of the *threo*-diastereoisomer. They supposed [3] a little effect of the D-isomers on GAD, since in their experiments the mixture of both *erythro*- and *threo*-forms of D-4-fluoroglutamate in 5×10^{-2} M concentration inhibited *E. coli* GAD by 12% only, when the substrate was at 5×10^{-4} M concentration in the incubation mixture.

We took advantage of having all four stereoisomers of 4-fluoroglutamate, prepared by one of us [6] in order to study the influence of each of them on GAD from *E. coli* separately. The influence of the individual stereoisomers (at the final concentration 2×10^{-2} M) on GAD from *E. coli* was compared with the control reaction. The results (Table) show that the *erythro*-isomer of 4-fluoro-D-glutamate is a more potent inhibitor of GAD than other isomers. Because of limited solubility in the buffer used for keeping optimal pH of *E. coli* GAD, IC_{50} was estimated only for D-*erythro*-isomer. In the case of racemic 4-fluoroglutamate, IC_{50} was close to 2×10^{-2} M concentration, when this concentration caused 48% inhibition.

When we take into account the statement [3] that the mixture of both forms of D-4-fluoroglutamate in 5×10^{-2} M concentration decreased the GAD activity in the presence of 5×10^{-4} M DL-glutanate by 12% only, and supposing a competition of the fluoroderivatives with the natural amino acid [3], our results show a stronger effect on L-glutamate decarboxylation. Also both alternative substrates of GAD, i.e. the *erythro*- and *threo*- diastereoisomers of L-4-fluoroglutamate, may be considered as (competitive) inhibitors of the natural substrate — L-glutamate. In this view, their effect seems to be of the same order. In agreement with this presumption, we investigated the possibility of reactivation of the enzyme from its mixture with racemic fluoroglutamate by means of dialysis against the buffer. The activity of GAD preparation was completely restored in this experiment.

The considerably high stability of GAD preparation from *E. coli* made it possible to follow the effect of the individual stereoisomers in equipotent concentrations (1–5 $\times 10^{-2}$ M) on GAD for a longer time. Sodium azide (0.05%) was used to protect the enzyme preparation against microbial contamination in this experiment. Sodium azide itself was without any influence on the catalytic activity of GAD (unpublished results). The time profile of GAD activity under the influence of 4-fluoroglutamate showed that in the presence of L-glutamate and fluorinated derivatives at a concentration of the same order (10^{-2} M) it takes several days before the diastereoisomers of fluorinated L-glutamate as alternative substrates are distinguished from the diastereoisomers of fluorinated D-glutamate as real inhibitors (Fig.).

Table: Influence of individual stereoisomers of 4-fluoroglutamate in 2×10^{-2} M concentration on GAD from *E. coli*

Stereoisomer of 4-fluoroglutamate		Relative GAD activity[a, b]
0 (control)		100%
D-*erythro*-	F H H NH₂ / HOOC COOH	28.6 ± 3.9%[c]
L-*erythro*-	F H H NH₂ / HOOC COOH	79.2 ± 1.5%
D-*erythro*-	F H H NH₂ / HOOC COOH	84.3 ± 0.5%
L-*erythro*-	F H H NH₂ / HOOC COOH	83.6 ± 2.2%

[a] Final concentration of L-glutamate 1.23×10^{-2} M
[b] Results are expressed in % of control activity ±S.D. as the mean of six samples
[c] $IC_{50} = 1.57 \times 10^{-2}$ M

It may be concluded that ale individual stereoisomers of 4-fluoroglutamate are more potent in their interference with catalysed decarboxylation of L-glutamate to GABA than it was assumed.

The specific role of GAD in *E. coli* is not clear. We consider the inhibitory effect of individual stereoisomers of 4-fluoroglutamate on *E. coli* GAD found in the present research as a starting point of our investigation of their influence on the enzyme from mammalian sources with the perspective that the compound might be useful as a tool in experimental epilepsy studies in laboratory animals [7].

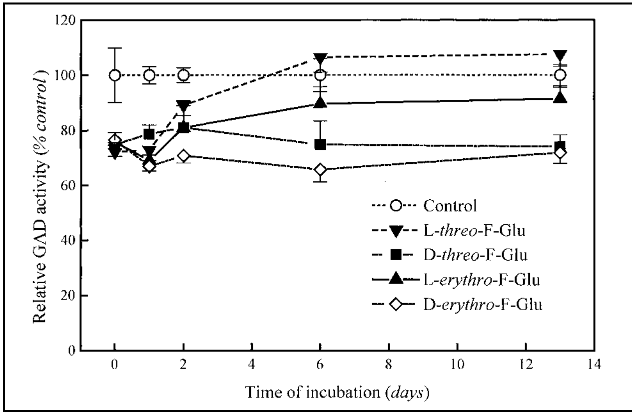

Fig.: Time profile of the activity of *E. coli* GAD under the influence of the individual stereoisomers of 4-fluoroglutamate. Relative GAD activity is expressed as % of control. Each point indicates the mean of six samples, vertical bars represent S.D.

Experimental

1. Stereoisomers of 4-fluoroglutamate

Individual stereoisomers were prepared by resolution of racemates through diastereomeric salts [6].

2. Enzyme preparation and chemicals

GAD from *Escherichia coli* (Sigma, 4.5 mg prot./ml suspension, 45 units/mg prot.) was used as the source of the enzyme.
The protein content was determined following the method of Lowry [8].
Chemicals used in GAD assay: L-1-^{14}C-Glutamate (Radiochemical Centre Amersham, specific activity 1.65 MBq · mmol^{-1}), pyridoxal-5′-phosphate (Koch-Light Labs.), 1,4-dithiothreitol (DTT, Merck), Bray's scintillation cocktail(Spolana, Neratovice). All chemicals used were at least of analytical grade.

3. Enzyme assay

The incubation mixture contained the enzyme, substrate L-glutamate-1-^{14}C, coenzyme pyridoxal-5′-phosphate, 1,4-dithiothreitol and the tested compound similarly as in [10] and was buffered to pH 5.0 (pH optimum for GAD from *E. coli*). The mixture was incubated at 37 °C for 30 min and the radioactivity of $^{14}CO_2$ liberated from the mixture and trapped by means of 0.1 ml of 30% KOH was measured in a dioxane scintillation cocktail using a 1219 Rackbeta scintillation counter LKB Wallac (Radioisotope Laboratory, Faculty of Pharmacy, Charles University, Hradec Králové).

Acknowledgement: This work was supported by the Charles University Grant 126/96.

References

1 Martin, D. L.; Rimvall, K.: J. Neurochem. **60**, 395 (1993)
2 Tunnicliff, G.: Int. J. Biochem. **22**, 1235 (1990)
3 Unkeless, J. C.; Goldman, P.: Mol. Pharmacol. **6**, 46 (1970)
4 Hudlický, M.: Coll. Czech. Chem. Commun. **26**, 1414 (1961)
5 Unkeless, J. C.; Goldman, P.: Mol. Pharmacol **7**, 293 (1971)
6 Tolman, V.; Šimek, P.: 30th Conference "Advances in organic, bioorganic and pharmaceutical chemistry", 6–8 November 1995, Liblice, Czech Rep., Abstracts p. 71.
7 Netopilová, M.; Dršata, J.; Ulrichová, J.: Pharmazie **51**, 589 (1996)
8 Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J.: J. Biol. Chem. **193**, 265 (1951)

Received March 3, 1999
Accepted April 27, 1999

Prof. Dr. Jaroslav Dršata
Charles University Prague
Faculty of Pharmacy
50005 Hradec Králové
Czeck Republic

Department of Pharmacognosy and Botany[1], Pharmaceutical Faculty, Comenius University, Bratislava, Slovak Republic and Institute of Organic Chemistry and Biochemistry[2], Academy of Sciences of the Czech Republic, Prague, Czech Republic

Triterpenoid saponins from *Cynara cardunculus* L.

P. Mučaji[1]; D. Grančai[1], M. Nagy[1], M. Buděšínský[2] and K. Ubik[2]

Cynara cardunculus L. (Asteraceae) is a plant occasionaly cultivated for the leaves which are blanched and eaten as a vegetable (cardoon). In folk medicine it is often used for the treatment of hepatic insufficiency [1]. Previous chemical investigation of involucral bracts of this plant established the occurence of sterols and triterpenoids [2], cynarine and coumarins [3–4] and flavonoids [5–6]. From the roots of *Cynara cardunculus* L. seven ursane-type saponins (cynarasaponins A–G) and three oleanane-type saponins (cynarasaponins H–J) have been isolated [7]. Now, we describe the isolation and identification of the triterpenoid saponins cynarasaponin A (**1b**) and cynarasaponin H (**2b**) and its methyl esters (**1a, 2a**) from the involucral bracts of this plant.
NMR spectra of the sample A (**1a** + **2a**) show the mixture of two structurally similar compounds in a ratio of ca. 43 : 57. The character of spectra indicates the presence of three saccharide units and a triterpenoid as an aglycone. Heavy overlap of proton multiplets in the ^{1}H NMR spectrum makes its detailed analysis difficult. On the other hand, the ^{13}C NMR spectrum shows resolved peaks for the most of carbon atoms. The comparison of our ^{13}C NMR data (Table) with literature data [7] proves that two components correspond to the methyl esters of cynarasaponin A and cynarasaponin H (**1a** + **2a**) containing oleanolic and ursolic acid, respectively, as aglycone. MS (see Experimental) are in agreement with the proposed structures.
Also NMR spectra of the sample B (**1b** + **2b**) showed the mixture of two components in a similar ratio (ca. 45 : 55). The ^{1}H and ^{13}C NMR spectra are very similar to those of the above discussed sample A (see Table). The only significant difference – the absence of a methoxy group signal – allowed to suggest the structures of cynarasaponin A and cynarasaponin H (**1b** + **2b**) as components of the mixture. The change from $COOCH_3$ to COOH is accompanied by a downfield shift of corresponding carbonyl signal in the ^{13}C NMR spectrum (from $\delta \sim 170.5$ to ~ 176.5). The suggested structure was supported by MS results. Standard FAB-MS and linked scans showed that the aglycone is substituted by both one hexose and by a chain consisted of one pentose and glucuronic acid.
Additional evidence for structures **1a** + **2a** and **1b** + **2b** provided the MS and NMR spectra of the aglycone, prepared by hydrolysis of sample B with HCl (see Experimental). Its EI-MS is in a good agreement with known spectra of oleanolic and ursolic acid [8]. The ^{1}H NMR spectrum has confirmed a mixture of ursolic and oleanolic acid in the ratio 45 : 55 (for data – see Experimental) by comparison with ^{1}H NMR spectra of authentic samples.

Experimental

1. Equipment

MS were measured on a ZAB-EQ mass spectrometer (Micromass, Manchester, U.K.) using both electron ionization (EI) for aglycon and fast atom bombardment (FAB) ionization with a magic bullet matrix and Xe at 8 kV as a bombarding gas. Daughter ion linked scans at B/E = const. and parent