STEREOSPECIFIC SYNTHESIS OF ENANTIOMERS OF 4-HYDROXYGLUTAMIC ACID AND STUDY OF THEIR INHIBITING PROPERTIES WITH RESPECT TO GLUTAMINE SYNTHETASE

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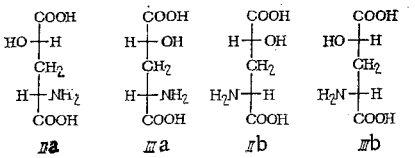
One of the promising methods for the selective blocking of the growth of cancer cells is suppression of their nucleic metabolism. Of great interest in this respect are inhibitors of the enzyme glutamine synthetase (GS) — one of the key enzymes of nitrogen metabolism. Included among compounds of this sort are substances that have high anticancer activity, which, however, have not found application in practical oncology because of their significant toxicity [11]. Inhibitors of nucleic metabolism are also of interest with respect to the search for substances that have antivirus activity.

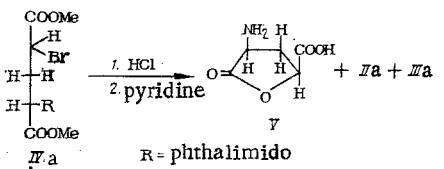
Among inhibitors of nucleic metabolism, C-derivatives of glutamic acid are known, including substances of natural origin (substances found in nature). Thus, 4-hydroxyglutamic acid (I) displays antimetabolite properties and antivirus activity [1] and has a growth-inhibiting effect [13]. In the opinion of several authors, the latter is due to suppression of the activity of GS. It has been shown that threo- and erythro-4-hydroxy-D,L-glutamic acids (II and III) are capable of inhibiting GS [5]; however, no systematic studies of the effect of the stereoconfigurations of these compounds on their inhibiting effect have been made. The present research is devoted to the development of a method for the stereospecific synthesis of enantiomers of I and to the study of their inhibiting effect with respect to GS.

Acid I was isolated for the first time from plants [12], after which it was obtained synthetically [7]. As a consequence of the presence in the I molecule of two asymmetric centers, it can exist in the form of four enantiomers: threo-4-hydroxy-L-glutamic acid (IIa), erythro-4-hydroxy-L-glutamic acid (IIIa), threo-4-hydroxy-D-glutamic acid (IIb), and erythro-4-hydroxy-D-glutamic acid (IIIb).



Enantiomers of acid I were earlier obtained by means of enzymatic synthesis [6] or by separation of diastereomeric racemates II and III by means of enzymes [8] or optically active bases. These methods are complex and involve many steps.

We have developed a new method for the stereospecific synthesis of enantiomers of acid I that is based on the use of glutamic acid derivatives that have genuine L or D configurations of the α-carbon atom. As the starting compound for the synthesis we used dimethyl esters of N-phthalyl-4-bromo-L- or D-glutamic acids (IVa, b), which were obtained by a method similar to that in [9] and were hydrolyzed with 20% HCl.



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Our initial attempt to carry out the separation of the optically active diastereoisomers under the conditions [8] used for this purpose in the case of acid I were unsuccessful. In this connection the separation of diastereoisomers IIa and IIIa was carried out in the following way. The hydrolysate obtained after refluxing bromo derivative IVa and HCl was separated from phthalic acid and evaporated *in vacuo*, and the residue was treated with pyridine in aqueous alcohol solution. The precipitate that separated upon cooling was removed by filtration. According to the results of thin-layer chromatography (TLC), this product was a mixture of threo-4-hydroxy-L-glutamic acid lactone (V) and acids IIa and IIIa. The IR spectrum contains absorption bands that confirm the existence of a five-membered lactone and I (λ_{max}, cm^{-1}): 1790 (five-membered lactone =O), 1720 (nonionized carboxy group (C=O), 1205 (C—O—C), and 1090 (O—H) [4]. When this mixture is treated with water, IIIa primarily passes into solution, whereas IIa and V remain in the precipitate in the form of a mixture. After two to three recrystallizations of the latter from water, the lactone ring undergoes opening, as a result of which pure acid IIa is formed (its constants are in agreement with those presented in [10]), the IR spectrum of which does not contain the absorption bands that are characteristic for five-membered lactones, but the spectrum does contain absorption bands that confirm its structure. The isolation of stereoisomers IIa and IIIa can be carried out somewhat differently. A mixture of the lactone V and acid IIa is treated in an aqueous medium with Ag_2CO_3 with heating, after which H_2S is passed through the mixture until the silver ions are precipitated completely in the form of Ag_2S, and acid IIa is precipitated by treatment of the resulting solution with alcohol. Erythro isomer IIIa is isolated from the mother liquor obtained after separation of the mixture of lactone V and acid IIa by adding it to alcohol with cooling. Recrystallization of this product from water gives acid IIIa, the constants of which are in agreement with those presented in [10].

Compounds IIb and IIIb are obtained by hydrolysis of bromo derivatives IVb with HCl and subsequent treatment of the hydrolysate with Ag_2CO_3 and H_2S. The yield of the mixture of IIb and IIIb is 74% of the theoretical value. Complete separation of the diastereoisomers does not occur in the case of a single recrystallization of the mixture from water. The best results were obtained when the process was carried out in analogy with that described for IIa and IIIa.

In order to ascertain the possible antimetabolite activity of the enantiomers of acid I we studied their inhibitor activity with respect to GS (E.C. 6.3.1.2). The activity of GS was judged from the formation of the phosphate (P) in the synthesis of the glutamine from the glutamate:

$$\text{L-glutamate} + \text{ATP} + NH_4^+ \xrightarrow[Mn^{2+}]{Mg^{2+}} \text{L-glutamine} + \text{ADP} + \text{P} + H_2O$$

The amount of the phosphate was determined spectrophotometrically in the form of a complex with ammonium molybdate by the method in [2]. It is apparent from the results obtained (see Table 1) that the stereoisomers of acid I, as for D-glutamic acid taken for comparison, have an inhibiting effect on the activity of GS, which depends substantially on the stereoconfiguration. Both diastereoisomers IIa and IIIa, as well as D-glutamic acid, have weak inhibitor activity. The stronger inhibitor in this series of compounds is acid IIb, which displays a pronounced inhibiting effect in a medium that contains both Mg^{2+} and Mn^{2+}. Acid II also has pronounced inhibitory activity, but the presence of IIb in the racemic mixture undoubtedly plays the principal role in this case. The data obtained constitute evidence that, in connection with the manifestation of a significant inhibiting effect by IIb and IIIb with respect to GS, these compounds are of interest for the study of their anticancer, antivirus, and other forms of biological activity in experiments performed on animals.

Thus, as a result of our research, we have developed a stereospecific method for the synthesis of enantiomers of I that excludes the use of toxic bases or enzymes that are difficult to obtain. We have established that the inhibiting effect of I with respect to GS depends markedly on its three-dimensional structure. We have discovered a highly active inhibitor of GS, viz., acid IIb, which is of interest for the study of its biological activity.

EXPERIMENTAL CHEMISTRY

The melting points were determined with a Hoffler apparatus. The IR spectra of mineral oil suspensions of the compounds were recorded with a UR-20 spectrometer (East Germany). Thin-layer chromatography (TLC) was carried out on Silufol plates in system a, viz., $CHCl_3$—17% NH_3—

MeOH (2:1:2) with development with a solution of ninhydrin. The angle of rotation of the plane of polarization was determined with an Al-EPL apparatus (USSR).

Threo- and Erythro-4-hydroxy-L-glutamic Acids (IIa and IIIa). A mxiture of 9 g of bromo derivative IVa and 150 ml of 20% HCl was refluxed for 6 h, after which it was cooled, the precipitated phthalic acid was separated by filtration, and the filtrate was evaporated to two thirds of its original volume at reduced pressure. An additional amount of phthalic acid was separated by filtration, the filtrate was again evaporated, water was added two to three times, and the mixture was evaporated again after each addition. The residue was dissolved in 90 ml of 70% alcohol, the solution was neutralized to pH 7.0 with pyridine, and the mixture was allowed to stand in a refrigerator for 3 days. The resulting semicrystalline precipitate was separated, and 9 ml of water was added to it. The precipitate that formed after the mixture was allowed to stand in a refrigerator for 1 day was separated and was found to consist of a mixture of acid IIa and lactone V. The mixture of IIa and V was recrystallized once and treated with 15 ml of water and 0.9 g of Ag_2CO_3, and the resulting mixture was heated on a water bath until CO_2 liberation ceased. Hydrogen sulfide was passed into the resulting solution until the silver ions had precipitated completely in the form of Ag_2S; the latter was separated, and the solution was poured with cooling to 0°C and stirring into 100 ml of alcohol. This resulted in the precipitation of acid IIa, which was separated after the mixture was allowed to stand in a refrigerator for 1 h. The IIa obtained in this way, after drying *in vacuo* over P_2O_5, was obtained as colorless crystals in the form of plates with mp 175-176°C and $[\alpha]_D^{20}$ − 14° (c 2, H_2O), in agreement with $[M]_D^{20}$ − 22.8° [literature data $[M]_D^{20}$ − 22.3° (c 1, H_2O) [10]] and R_f 0.22. Found, %: C 36.81; H 5.56; N 8.59. The following absorption bands were present in the IR spectrum (ν_{max}, cm^{-1}): 1570 (COO^-), 1645 (NH_3^+, deformation), 1720 (C=O in COOH), and 3150 (NH_3^+, stretching). The yield of optically pure acid IIa was 1 g or 26% of the theoretical value. Acid IIIa was isolated from the mother liquor after separation of the mixture of IIa and V by slow addition of it to ethanol with stirring and cooling to 0°C. The precipitate that formed after allowing the mixture to stand in a refrigerator for 2 days was removed by filtration and recrystallized twice from water. The product was obtained in the form of colorless crystals with mp 126-129°C (dec.), $[[M_D^{21}]$ + 61.9°C (c 1, 20% HCl) [literature values $[M_D^{26}]$ + 61.6°C (c 1, 20% HCl) [10]], and R_f 0.22. Found, %: C 34.80; H 5.85; N 8.20. $C_5H_9NO_5 \cdot 0.5H_2O$. Calculated, %: C 34.89; H 5.86; N 8.14. The yield of optically pure acid IIIa was 0.6 g or 15% of the theoretical value. A certain amount of acids IIa and IIIa were also isolated from the mother liquors by the described method for an overall yield of 45-50% of the theoretical value.

Threo- and Erythro-4-hydroxy-D-glutamic Acids (IIb and IIIb). Acids IIb and IIIb were obtained by hydrolysis of dimethyl N-phthalyl-4-bromo-D-glutamic acid (IVb) by a method similar to that described above for IIa and IIIa. Acid IIb was obtained as colorless crystals with mp 172-173°C $[\alpha]_D^{20}$ +14° (c 3, H_2O), and R_f 0.22. Found, %: C 36.61; H 5.52; N 8.50. $C_5H_9NO_5$. Calculated, %: C 36.81; H 5.56; N 8.59. The yield of acid IIb was 23.5% of the theoretical value. Acid IIIb was obtained as colorless crystals with mp 164-166°C, $[\alpha]_D^{20}$ − 20° (c 2, H_2O), and R_f 0.22. Found, %: C 36.67; H 5.82; N 8.68. $C_5H_9NO_5$. Calculated, %: C 36.81; H 5.56; N 8.59. The yield of acid IIIb was 18% based on the theoretical value. The overall yield of pure diastereoisomers IIb and IIIb was ∼42% and can be increased to 46% by additional recrystallization of the intermediate fractions, which consist of a mixture of

TABLE 1. Inhibition of the Activity (in %) of GS by Stereoisomers of 4-Hydroxyglutamic Acid

Inhibitor	Mg^{2+}	Mn^{2+}
II	62; 48	75; 48
IIa	8; 13	...; 8
II a	63; 80	64; 76
III	8; 17	35; 43
IIIa	6; 13	...; 19
IIIb	20; 35	35; 54
D-Glutamic acid	8; 12	...; 15

Note: The first numbers are for an inhibitor concentration of 10^{-2} M, whereas the second numbers are for an inhibitor concentration of $2 \cdot 10^{-2}$ M.

IIb and IIIb, with subsequent washing of the precipitated IIb by heating to 40°C with water in a ratio of 2.5 ml per gram of the mixture.

EXPERIMENTAL BIOLOGY

The optical density was measured with an SF-16 spectrophotometer (USSR). The GS was isolated from *Chlorella pyrenoidosa* cells by the method described in [3]. The mixture for the experiments (final volume 1.6 ml) consisted of 0.1 M L-sodium monoglutamate, 0.05 M NH_4Cl, 0.005 M ATP, 0.1 M Tris-HCl [sic] and 0.015 M $MgSO_4$ with pH 8.0 or 0.1 M acetate buffer and 0.005 M $MnSO_4$ with pH 5.5. Weighed samples of the stereoisomers of I were dissolved in the mixture, and the pH was brought up to the necessary value. The enzyme preparation was introduced in the amount necessary for the formation of 0.05-0.8 mmole of phosphate in the sample. Incubation was carried out at 37°C for 15 min. The reaction was stopped by the addition of 4 ml of a 1% solution of $FeSO_4$ in 0.3 M H_2SO_4. Coloration developed after the addition of 0.4 ml of a 6.6% solution of $(NH_4)_6Mg_7O_{24}\cdot 4H_2O$ in 7.5 N H_2SO_4. The optical density was measured at 700 nm relative to a solution containing the same components as those for the experiment but without the enzyme. In addition, monitoring for the compound under investigation was carried out.

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