the compounds were not convulsants. After determining neurological fitness of the animals,[9] they were challenged with increasing doses of the compounds until subjective evidence (changing or difficulty in breathing patterns or unsteady gait) of toxicity was noted. Subsequent analysis[8] of these data demonstrated no significant difference between the compounds but did reveal significant difference between the eventual ED_{50} range (21–122 mg/kg) and the TD_{50} range (202–402 mg/kg).

Further increases in doses finally resulted in death of the test animals. Death, which usually occurred within 5 min of injection, was preceded by tonic convulsive seizures associated with respiratory problems and ultimate collapse. Significant differences were noted between the lethal range (1.02–2.83 g/kg) and the toxicity range, although no significant difference between the compounds at either the ED_{50}, TD_{50}, or LD_{50} was identified.

At the ED_{50} dose, the test animals did not demonstrate salivation, lacrimation, or excessive urination nor were any tremors, twitches, or convulsions noted prior to pentylenetetrazole. Subtle nonquantifiable subjective behavioral changes were noted after dosage with the test compounds which were not observed in the control animals. The methoxy derivatives appeared to enhance alertness, whereas the halogen derivatives rendered the animals more passive.

The seizures induced in control animals were characterized by initial loss of righting followed by rigid tonic extension of the hind legs lasting for 2–3 min. More than one seizure occurred during the 30-min observation period. Three of the control animals died of respiratory failure within 5 min of pentylenetetrazole challenge. The vehicle alone provided no seizure protection.

Duration of Anticonvulsant Action (Table V). The unsubstituted compound did not provide satisfactory protection against pentylenetetrazole-induced convulsions. Maximum activity resulted in only two of five animals being protected. That compound showed the weakest *in vitro* carbonic anhydrase activity ($I_{50} = 7.1 \times 10^{-8}$) and is the only one subject to metabolic para hydroxylation.

Although there was no difference between the time of onset of activity with halogen or methoxy substitution, the methoxy derivatives, particularly the 2,4,5 isomer, persisted for a longer duration. The greater contribution to lipid solubility by a single halogen atom *vs.* three methoxy groups would afford the halogen derivative's more rapid passive transport and subsequent removal from the area of activity. In addition, the electron-rich trimethoxy aromatic rings would more readily form charge-transfer complexes retarding removal.

At the time of peak activity, the animals were sacrificed and their brains assayed for test compound. Presence of unsubstituted compound could not be determined after two separate attempts. The low order of carbonic anhydrase inhibition which is the basis of the analytical procedure demonstrated by this compound would indicate that the failure of identification was due to a lack of test sensitivity.

The test animals were maintained for 90 days of observation without the appearance of toxic effects.

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References

(1) T. Mann and D. Kellin, *Nature (London)*, **146**, 164 (1940).
(2) T. H. Maren, *Physiol. Rev.*, **47**, 595 (1967).
(3) J. R. Vaughan, Jr., J. A. Eichler, and G. W. Anderson, *J. Org. Chem.*, **21**, 700 (1956).
(4) R. W. Young, K. H. Wood, J. A. Eichler, J. R. Vaughan, Jr., and G. W. Anderson, *J. Amer. Chem. Soc.*, **78**, 4649 (1956).
(5) T. H. Chan and L. T. L. Wong, *J. Org. Chem.*, **34**, 2766 (1969).
(6) A. Albert and E. P. Sargeant in "Ionization Constants of Acids and Bases," Methuen Co., London, 1962.
(7) T. H. Maren, *J. Pharmacol. Exp. Ther.*, **130**, 26 (1960).
(8) F. S. Weil, *Biometrics*, **8**, 249 (1952).
(9) E. A. Swinyard, W. C. Brown, and L. S. Goodman, *J. Pharmacol. Exp. Ther.*, **106**, 319 (1952).
(10) Merck Index, 8th ed, Merck & Co., Rahway, N.J., 1968, p 672.

Potential Inhibitors of L-Asparagine Biosynthesis. 2. Chemistry and Biological Activity of β-Hydroxyaspartic Acid and Its Derivatives[1]

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Several derivatives of *erythro*-β-hydroxy-DL-aspartic acid (1) were prepared as potential inhibitors of L-asparagine synthetase (ASase) from rat Novikoff hepatoma. Benzylation of 1 gave the dibenzyl ester **2** which upon coupling with carbobenzoxyglycine afforded the blocked dipeptide **3**. Deblocking of **3** gave glycyl-*erythro*-β-hydroxyl-DL-aspartic acid (4) which could not be diazotized. The dimethyl ester of 1 was coupled with carbobenzoxyglycine to give the blocked dipeptide **7a** which was deblocked to give dimethyl glycyl-*erythro*-β-hydroxy-DL-aspartate hydrochloride (8). Diazotization of 8 gave impure diazo compound **9** which on reaction with HCl gave the chloro compound **10**. The methods of isolation, assay, and inhibition of ASase are described. At 10 m*M* concentrations **10**, 1, and its D and L enantiomers inhibit ASase by 45, 47, 36 and 66%, respectively.

The enzyme L-asparaginase has been shown to be an effective antitumor agent in both murine and human neoplasms,[2] the mechanism being the depletion of exogenous stores of L-asparagine.[2] It has also been shown that resistance to L-asparaginase therapy in certain murine tumors[3] and human neoplastic tissues[4] is related to the synthesis of endogenous L-asparagine *via* the enzyme L-asparagine synthetase (ASase). Mammalian ASase synthesizes L-asparagine from L-aspartic acid, utilizing L-glutamine as the primary source of nitrogen plus certain other cofactors.[5] We have thus embarked on a program aimed at the development of amino acid analogs related to aspartic acid and

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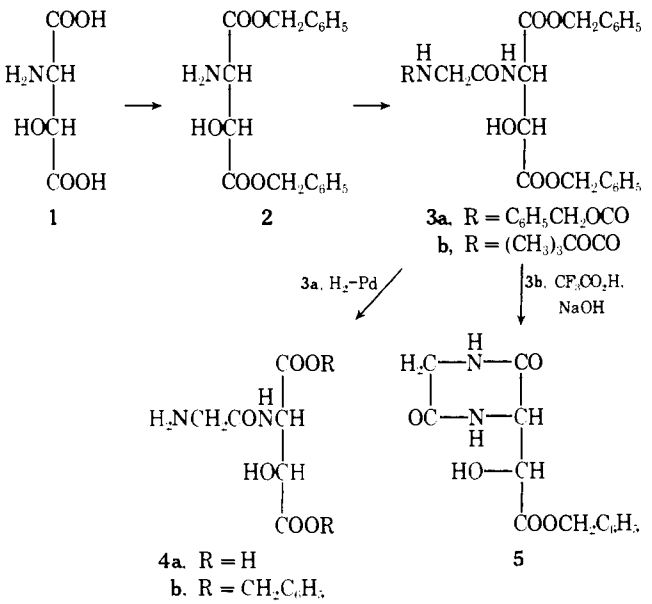
glutamine which could irreversibly inhibit ASase and perhaps be of use in those malignancies that are refractory to L-asparaginase therapy.

The present report describes a study of the synthesis and biological activity of β-hydroxyaspartic acid and some of its derivatives. The latter amino acid has been shown to be a competitive antimetabolite of aspartic acid[6] in several enzymic systems and, further, that it is the erythro form[7] (1) which is the biologically active diastereoisomer. Since L-aspartic acid is a substrate of ASase[5] it was thought that selected derivatives of 1 might inhibit this enzyme irreversibly, thus blocking the biosynthesis of L-asparagine. The presence of the hydroxyl group in 1 should enable one to use this "handle" to incorporate enzyme alkylating groups, thus leaving free all of the functional groups normally present in aspartic acid to interact with the enzyme in its usual fashion. In view of the use of the diazocarbonyl group in the design of irreversible enzyme inhibitors,[8] we attempted to synthesize the *O*-diazoacetyl derivative of 1. However, under the reaction conditions utilized, an unexpected β-elimination occurred, affording a fumarate derivative, the details of which have been published.[1] This elimination precluded our initial plan and thus we attempted the synthesis of the *N*-diazoacetyl derivative of 1. However, the latter compound could not be prepared, only its dimethyl *N*-chloroacetyl derivative **10**. The assumption was that although the nitrogen would be blocked, the hydroxyl, as an isostere,[9] could substitute for nitrogen thus allowing the amino acid to approach the active site of ASase. Furthermore, we wanted to selectively block certain of the functional groups of 1 in an attempt to elucidate which groups are necessary for binding to ASase.

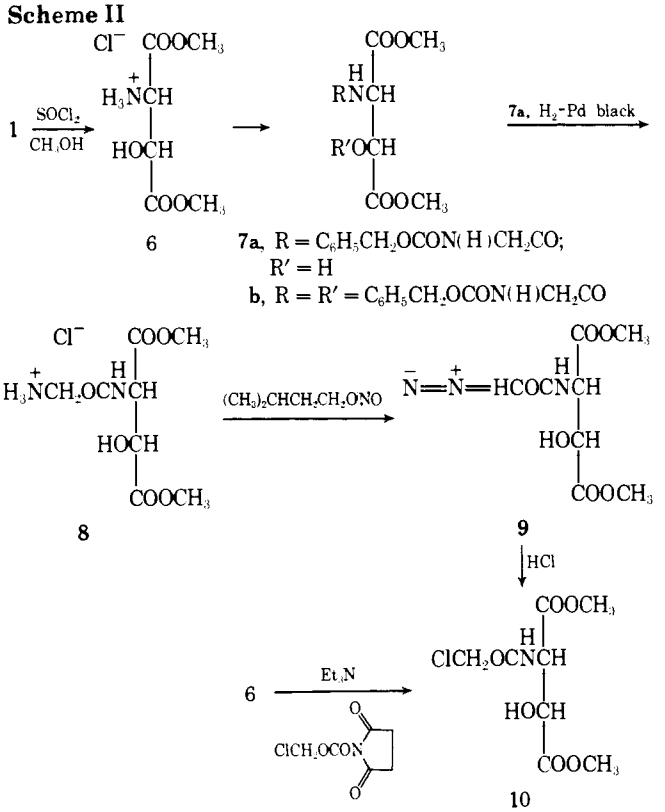
Chemistry. Esterification of DL-1 was accomplished with benzyl alcohol and *p*-toluenesulfonic acid, affording the dibenzyl ester **2**. The ester **2** was converted to the protected dipeptide **3a** using *N*-carbobenzoxyglycine and the mixed carbonic anhydride method of Anderson, *et al.*[10] The free dipeptide **4a** was readily obtained by catalytic hydrogenolysis of **3a** (Scheme I). Several attempts at diazotizing **4a**, by procedures successfully used in similar systems,[8,11] proved unfruitful.

Scheme I



In order to obtain a diazoacetyl derivative of 1, it was felt that the carboxyls would have to be blocked as the corresponding esters. Initially **2** was coupled with *tert*-butoxycarbonylglycine by the mixed carbonic anhydride method,[10] affording the blocked dipeptide **3b**. Selective deblocking of the *tert*-butoxycarbonyl group with trifluoroacetic acid followed by neutralization did not give the desired amine **4b**, but instead the cyclized 2,5-piperazinedione derivative **5** (Scheme I). The structure of **5** was assigned on the basis of physical data (see Experimental Section). Cyclizations such as this are well known[12] in the literature but generally do not occur as rapidly and under such mild conditions (no heat) as we observed.

Scheme II



Similarly, 1 was converted to its dimethyl ester hydrochloride **6** by the method of Stammer, *et al.*[13] Coupling of the ester **6** with *N*-carbobenzoxyglycine was best accomplished *via* the *N*-hydroxysuccinimide method,[14] and the resulting protected dipeptide **7a** was hydrogenolyzed to the amine hydrochloride **8**. If the coupling of **6** with *N*-carbobenzoxyglycine was carried out by the mixed carbonic anhydride method[10] the results were variable, and, in addition to obtaining **7a**, a second component was formed and identified as the tetra-blocked amino acid **7b**. Diazotization of **8**, using isoamylnitrite,[15] readily gave the diazo ester **9** (Scheme II). Although the physical data [ir 4.72 μ; uv max 250 nm (ε 10,400)] substantiated the structure of **9** we were unable to confirm this by elemental analysis. It was felt that **9** was contaminated with small amounts of the chloro derivative **10**, since it was later observed that these two compounds had identical R_f values in our tlc system. However, treatment of **9** with anhydrous hydrogen chloride[16] gave compound **10**, which was identical with **10** prepared by coupling **6** with chloroacetic acid by the *N*-hydroxysuccinimide method.[14]

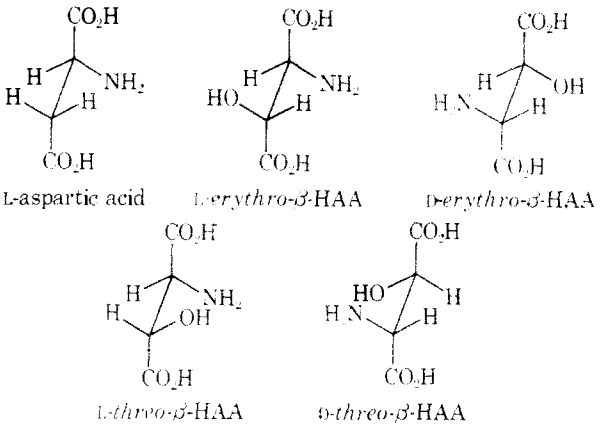
Biological Results. Rats infected with Novikoff hepatomas (asparaginase-resistant)[5d,‡] were utilized as the source of ASase. The enzyme as isolated[5a] had a specific activity of 0.11 μmol of asparagine synthesized per mg of protein in 30 min. L-Asparagine-^{14}C synthesized was isolated and the

‡The initial strain of Novikoff hepatoma was kindly supplied by Dr. Manford K. Patterson, Jr., The Samuel Roberts Noble Foundation, Inc., Ardmore, Okla. 73401.

Table I. Inhibition of Asparagine Synthetase[a]

Compound[b]	mM concn	% inhibition[c]
1	10	47[d]
threo-β-Hydroxy-DL-aspartic acid	10	0
erythro-β-Hydroxy-L-aspartic acid[e]	10	66[f]
erythro-β-Hydroxy-D-aspartic acid[e]	10	36[d]
4a	10	9
6	11	9
β-Methyl *erythro*-β-hydroxy-DL-aspartate[g]	11	16
10	10	45
erythro-β-Hydroxy-DL-asparagine[h]	10	14
L-Asparagine	2	63[d]

[a]ASase was purified from Novikoff hepatomas by a modification (see the Experimental Section) of previously reported procedures (ref 5a). [b]The compounds were preincubated with ASase, then substrate aspartic acid was added, and the formation of L-asparagine was determined as described in the Experimental Section. [c]Each assay was run in duplicate. [d]The mean result of two assays. [e]Prepared by the method of Okai, *et al.*[17] [f]The mean result of three assays. [g]Prepared by the method of Liwschitz and Singerman.[19] [h]Prepared by the method of Singerman and Liwschitz.[18]



[a]β-HAA = β-hydroxyaspartic acid.

radioactivity determined as described in the Experimental Section.

The ability of various β-hydroxyaspartic acid derivatives to inhibit the *in vitro* biosynthesis of L-asparagine is shown in Table I. None of the tested compounds had an inhibitory effect on ASase greater than that of its product L-asparagine, which at one-fifth the concentration inhibited ASase somewhat better than 1.

Horowitz and Meister,[5c] working with ASase isolated from an asparaginase-resistant mouse leukemia (RADA1), have shown that *threo*-β-hydroxy-DL-aspartic acid at a 20 m*M* concentration had no inhibitory activity, while the erythro isomer at this same concentration only inhibited the ASase by 27%. At a 10 m*M* concentration, we also found that the threo isomer did not inhibit ASase from Novikoff hepatoma, but at this concentration we observed significant inhibition with **1**. Resolution[17] of **1** into its enantiomers has surprisingly shown that both D- and L-**1** can inhibit ASase. To prove that we were not getting racemization under the conditions used for the inhibition studies, we determined the optical rotations of both enantiomers in the same solution used for the incubation, except for the absence of ASase. There was no appreciable change observed in the rotations of D- or L-**1** after 45 min at 37°. In fact, the observed inhibition (47%) by DL-**1** is almost the average inhibition by the enantiomers D-**1** (36%) and L-**1** (66%).

Since D-aspartic acid cannot serve as a substrate for ASase, nor D-asparagine[5c] as an inhibitor of ASase, it is tempting to postulate that the reason D-**1** inhibits the enzyme is due to the β-hydroxy group acting as an isostere of nitrogen.[9] Neither of the *threo*-β-hydroxyaspartic acid enantiomers can assume a conformation analogous to L-aspartic acid (see below). However, this postulation does not fit the results observed with the dipeptide **4a**. If the β-hydroxyl of **4a** could substitute for nitrogen as depicted for D-erythro below, one might expect some inhibition by **4a**, unless the steric bulk of the amide group interferes. It is interesting to note that L-malic acid, a hydroxy isostere of L-aspartic acid, only inhibited ASase from RADA1 by 14% at 10 m*M*.[5c]

It has previously been shown[5] that mammalian ASase undergoes product inhibition by L-asparagine and further that it was noncompetitive with respect to L-aspartate but competitive with L-glutamine. We have prepared *erythro*-β-hydroxy-DL-asparagine by ammonolysis[18] of β-methyl *erythro*-β-hydroxy-DL-aspartate[19] but found that this analog of asparagine does not appreciably inhibit ASase.

Whereas the dimethyl eseter **6** was inactive as a reversible inhibitor of ASase, we observed some inhibitory activity with compound **10**. It was felt that this inhibition by **10** was probably not site specific and that the low order of activity did not warrant more extensive studies.

threo-β-Hydroxy-DL-aspartic acid and *erythro*-DL-**1** were screened for antitumor activity by the National Cancer Institute (NCI) in mice infected with L1210 lymphoid leukemia, but both compounds proved to be inactive.[§] The enantiomers D-**1** and L-**1** were also submitted to NCI but were not entered into the primary screen but instead tested[&] as potential inhibitors of the ASase from L5178Y-AR (Greenberg) lymphoma. Neither enantiomer inhibited the enzyme. It should be pointed out that both of the above tumor systems are L-asparaginase-resistant strains and therefore have elevated levels of ASase. It therefore appears as if **1** or its derivatives would be of no use in those malignancies that are refractory to L-asparaginase therapy.

Experimental Section

Melting points were taken on a Fisher-Johns apparatus and are not corrected. Infrared absorption spectra were recorded on either a Perkin-Elmer Infracord or a Beckman IR-8 spectrophotometer. Nmr spectra were recorded on a Varian A-60 or A-60D spectrometer in $CDCl_3$ with tetramethylsilane as internal standard. Thin-layer chromatography and preparative tlc (1.0 mm) were carried out with silica gel GF (Analtech, Inc.) and spots were located with either uv light or by spraying with 3% ceric sulfate in 3 *N* H_2SO_4 and then heating. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich. The petroleum ether used had a boiling point range of 30–60°. All concentrations were done under reduced pressure after first drying the organic layers with anhydrous Na_2SO_4. Mass spectra were determined on an LKB Model 9000 spectrometer at 70 eV. Radioactivity was determined on a Packard Model 3310 Tri-Carb scintillation spectrometer.

Dibenzyl *erythro*-β-Hydroxy-DL-aspartate (2). A mixture of *erythro*-DL-**1**[1] (1.00 g, 6.7 mmol), *p*-toluenesulfonic acid (1.30 g, 6.8 mmol), and benzyl alcohol (18.0 ml) in benzene (10 ml) was refluxed for 24 hr and the resultant water removed by way of a Dean-Stark trap. After cooling to 25°, the solution was poured into 150 ml of anhydrous ether. The resultant *p*-toluenesulfonate salt was dissolved in H_2O and made basic with a dilute NaOH solution, and the free amine extracted into $CHCl_3$. The organic layer was washed with H_2O, dried, and concentrated to a yellow oil. The oil

[§]Private communication from Dr. Harry B. Wood, Jr., Drug Development Branch, N.C.I., Bethesda, Md. 20014.

[&]Tested by Dr. David A. Cooney, Laboratory of Toxicology, N.C.I., Bethesda, Md. 20014.

was crystallized from benzene–petroleum ether to give 1.11 g (50%) of **2**: mp 83–84°; ir ($CHCl_3$) 5.75 μ (ester carbonyl); nmr δ 2.75 (s, 3 H, NH_2, OH, exchangeable with D_2O), 3.98 (d, J = 3 Hz, H_α), 4.58 (d, J = 3 Hz, H_β), 5.02 (s, 4 H, benzylic CH_2), 7.27 (s, 10 H, phenyl). *Anal.* ($C_{18}H_{19}NO_5$) C, H, N.

Dibenzyl *N*-(Carbobenzoxyglycyl)-*erythro*-β-hydroxy-DL-aspartate (3a). An ice-cold solution of carbobenzoxyglycine[20] (0.121 g, 0.58 mmol) and triethylamine (0.059 g, 0.58 mmol) in 2.0 ml of dioxane was treated with isobutyl chloroformate (Eastman, 0.079 g, 0.58 mmol). After 10 min a solution of **2** (0.200 g, 0.61 mmol) in 2.0 ml of dioxane was added and stirred for 2.5 hr, while allowing it to come to room temperature. The mixture was poured into 25 ml of cold H_2O. After extracting with 50 ml of $CHCl_3$ the organic layer was washed twice with 10% HCl solution, twice with saturated $NaHCO_3$ solution, and once with H_2O and dried. The solvent was concentrated and the resulting oily product crystallized from benzene–petroleum ether, affording 0.178 g (59%) of **3a**, mp 129–130°. Recrystallization gave an analytical sample of **3a**: mp 129.5–130.5°; ir (KBr) 5.78 (ester and carbamate carbonyl) and 6.02 μ (amide carbonyl); nmr δ 3.87 (d, J = 6 Hz, CH_2NH, singlet after D_2O exchange), 4.55 (m, H_β), 4.98, 5.02, 5.08 (singlets, 6 H, benzylic CH_2), 5.22 (m, H_α), 7.28 (m, 15 H, aromatic). *Anal.* ($C_{28}H_{28}N_2O_8$) C, H, N.

Glycyl-*erythro*-β-hydroxy-DL-aspartic Acid (4a). The blocked dipeptide **3a** (0.178 g, 0.34 mmol) was dissolved in dioxane (5 ml) and 0.1 *N* HCl solution (3.4 ml) and then palladium black (Englehard, 0.125 g) was added. The mixture was hydrogenated at room temperature and atmospheric pressure and after 2 hr the theoretical amount of H_2 gas was consumed. Water (15 ml) was added and the catalyst removed by filtration. Concentration of the filtrate and crystallization from H_2O–ethanol gave, in two crops, 0.057 g (81%) of **4a**, mp 227–230° dec. Analysis showed **4a** to be the free dipeptide and not the HCl salt. *Anal.* ($C_6H_{10}N_2O_6$) C, H, N.

Dibenzyl *N*-(*tert*-Butoxycarbonylglycyl)-*erythro*-β-hydroxy-DL-aspartate (3b). A solution of **2** (0.20 g, 0.60 mmol) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (Aldrich, 0.25 g, 0.060 mmol) in acetonitrile (8 ml) was treated dropwise with a solution of *tert*-butoxycarbonylglycine (Sigma, 0.105 g, 0.60 mmol) in acetonitrile (5 ml). After 24 hr of stirring, the mixture was concentrated to an oily residue and treated with 10 ml of H_2O. The product was extracted into $CHCl_3$ and then washed successively with 10% HCl solution, saturated $NaHCO_3$ solution, and H_2O and dried. Concentration of the solvent afforded **3d** as yellow oil, 0.27 g (crude 93%), which migrated as a single spot on tlc (5% CH_3OH–$CHCl_3$) but resisted attempts at crystallization; nmr δ 1.42 (s, *tert*-butyl), 3.80 (m, 3 H, CH_2N, OH), 4.58 (d, J = 2.5 Hz, H_β), 5.00, 5.05 (singlets, 4 H, benzylic CH_2), 5.2 (m, H_α), 7.27, 7.28 (singlets, 10 H, aromatic). This material was suitable for the next step.

β-Benzyl Glycyl-*erythro*-β-hydroxy-DL-aspartate Diketopiperazine (5). The blocked dipeptide **3b** (0.27 g, 0.55 mmol) was dissolved in trifluoroacetic acid (4.0 ml) and stirred at 10–15° for 15 min. After concentrating, the resulting oil was treated with 2.5% NaOH solution (20 ml) and then extracted three times with $CHCl_3$ (20 ml each). The combined organic layers were washed with H_2O, dried, and concentrated to give a semisolid product, a portion of which no longer was soluble in $CHCl_3$. The $CHCl_3$ insoluble material, 0.021 g, was collected by filtration and the filtrate again concentrated, affording another 0.020 g of insoluble material. A tlc examination of the $CHCl_3$ soluble material indicated the presence of several other components, from which no other identifiable products were obtained. The combined solids were crystallized from H_2O–ethanol to give 0.032 g (21%) of **5**: mp 253–256° dec; ir (Nujol) 5.71 (ester carbonyl), 5.85, 5.97 μ (amide carbonyl); mass spectrum *m/e* (rel intensity) 278 (22, M^+), 260 (6), 171 (14), 143 (49) 114 (47), 91 (100). *Anal.* ($C_{13}H_{14}N_2O_5$) C, H, N.

Dimethyl *N*-(Carbobenzoxyglycyl)-*erythro*-β-hydroxy-DL-aspartate (7a) and Dimethyl *N,O*-(Dicarbobenzoxyglycyl)-*erythro*-β-hydroxy-DL-aspartate (7b). (a) A solution of carbobenzoxyglycine[20] (0.915 g, 4.38 mmol) and *N*-hydroxysuccinimide (Sigma, 0.500 g, 4.35 mmol) in dioxane (30 ml) was treated at room temperature with a solution of dicyclohexylcarbodiimide (0.906 g, 4.40 mmol) in dioxane (15 ml). After stirring for 30 min, the dicyclohexylurea was removed by filtration and the filtrate treated with a mixture of **6**[13] (0.92 g, 4.31 mmol) and triethylamine (0.44 g, 4.35 mmol) in dioxane (30 ml). After 3.5 hr, the mixture was filtered, the filtrate concentrated to dryness, and the residue dissolved in CH_2Cl_2 and successively washed with 5% HCl solution, 10% Na_2CO_3 solution, and saturated NaCl solution and then dried. Evaporation of the solvent afforded a crude oil which was again dissolved in CH_2Cl_2 and filtered free of insoluble dicyclohexylurea. Evaporation of the solvent gave **7a** as a gum (1.58 g, crude 95%), which could not be induced to crystallize but which appeared as a homogeneous product on tlc (10% CH_3OH–$CHCl_3$); ir ($CHCl_3$) 5.74 (ester and carbamate carbonyls), 5.93 μ (amide carbonyl); nmr δ 3.68, 3.78 (singlets, 6 H, OCH_3), 3.95 (d, J = 6 Hz, CH_2NH), 4.50 (m, H_β), 5.10 (s, benzylic CH_2), 5.18 (m, H_α), 5.78 (m, 1 H, NH), 7.28 (s, 5 H, aromatic).

(b) Carbobenzoxyglycine[20] (0.482 g, 2.31 mmol) was dissolved in THF (6 ml), cooled in ice, and then treated successively with triethylamine (0.47 g, 4.65 mmol) and dropwise with a solution of isobutyl chloroformate (Eastman, 0.32 g, 2.34 mmol) in THF (14 ml). After 15 min, compound **6**[13] (0.492 g, 2.30 mmol) was added and the mixture stirred at room temperature for 4 hr. The mixture was concentrated to dryness and the residue suspended in H_2O (10 ml) and then extracted with $CHCl_3$ (25 ml). The organic layer was successively washed with H_2O, 2.5% HCl solution, saturated $NaHCO_3$ solution, and H_2O and then dried. Evaporation of the solvent gave a yellow oil (crude 0.84 g), which appeared on tlc (4% CH_3OH–$CHCl_3$) as a two-component mixture. A portion (0.15 g) of this oil was purified on two preparative tlc plates (5% MeOH–$CHCl_3$). The lower R_f band (0.11 g) corresponded to **7a** while the upper R_f band (0.025 g) was identified as **7b**. Two recrystallizations of **7b** from ethyl acetate–petroleum ether gave the analytical sample, mp 134–135°. *Anal.* ($C_{26}H_{29}N_3O_{11}$) C, H, N.

Dimethyl Glycyl-*erythro*-β-hydroxy-DL-aspartate Hydrochloride (8). A mixture of **7a** (0.28 g, 0.76 mmol), palladium black (Englehard, 0.20 g) in CH_3OH (7.5 ml), and 0.09 *N* HCl (9.0 ml, 0.81 mmol) was hydrogenated at 25° and 1 atm. After 30 min the theoretical amount of H_2 was consumed and the catalyst was removed by filtration and washed with H_2O. The combined filtrates were concentrated to dryness and the residue crystallized from CH_3OH–ether giving 0.12 g (58%) of **8**, mp 95–98°. A portion of **8** was recrystallized twice to give the analytical sample: mp 109–113°; ir (KBr) 5.78 (ester carbonyl), 5.88 μ (amide carbonyl); nmr (D_2O) δ 3.78, 3.83 (singlets, 6 H, OCH_3), 3.93 (s, $COCH_2$), 5.21 (d, J = 3 Hz, H_α), H_β obscured by DOH peak. *Anal.* ($C_8H_{15}N_2O_6Cl$) C, H, N.

Dimethyl *N*-(Diazoacetyl)-*erythro*-β-hydroxy-DL-aspartate (9). A suspension of **8** (0.052 g, 0.19 mmol) in $CHCl_3$ (5 ml) was treated with a 0.10-ml solution of triethylamine (0.022 g, 0.22 mmol) in $CHCl_3$ (0.30 ml of Et_3N, 0.70 ml of $CHCl_3$) and stirred for 30 min. To this suspension was then added a 0.10-ml solution of isoamyl nitrite (Aldrich, 0.028 g, 0.24 mmol) in $CHCl_3$ (0.32 ml of isoamyl nitrite, 0.68 ml of $CHCl_3$), followed by a 0.10-ml solution of acetic acid (3.6 mg, 0.060 mmol) in $CHCl_3$ (0.36 ml of acetic acid, 0.64 ml of $CHCl_3$), and then the mixture was refluxed for 3 hr. The insolubles were removed by filtration and the filtrate was concentrated to dryness. A tlc (10% MeOH–$CHCl_3$) of the resulting yellow oil showed a strong quenching spot under uv plus several other minor spots. Preparative tlc (10% MeOH–$CHCl_3$) of the oil afforded 12–18 mg of **9**, which appeared to be homogeneous (tlc): ir ($CHCl_3$) 4.72 (diazo), 5.73 (ester carbonyl), 5.96 (shoulder, amide carbonyl of impurity), 6.09 μ (amide carbonyl conjugated with diazo).

Dimethyl *N*-(Chloroacetyl)-*erythro*-β-hydroxy-DL-aspartate (10). (a) A solution of *N*-hydroxysuccinimide (0.051 g, 0.44 mmol) and chloroacetic acid (0.042 g, 0.44 mmol) in dioxane (5 ml) was treated with a solution of dicyclohexylcarbodiimide in dioxane (2 ml). After 45 min the precipitated dicyclohexylurea was removed by filtration and the filtrate added to a stirred mixture of **6** (0.10 g, 0.47 mmol), triethylamine (0.045 g, 0.45 mmol), and dioxane (3 ml). After 1 hr, the insolubles were removed by filtration and the filtrate was concentrated to dryness. The residue was dissolved in CH_2Cl_2 and again filtered free of dicyclohexylurea and concentrated to give 0.084 g of a gum. Crystallization of the gum from ether–petroleum ether afforded 0.047 g (39%) of **10**, mp 65–68°. Two recrystallizations gave the analytical sample: mp 68–70°; ir ($CHCl_3$) 5.73 (ester carbonyl), 5.95 μ (amide carbonyl); nmr δ 3.77, 3.85 (singlets, 6 H, OCH_3), 4.13 (s, $COCH_2$), 4.55 (d, J = 3 Hz, H_β), 5.18 (m, H_α). *Anal.* ($C_8H_{12}NO_6Cl$) C, H, N, Cl.

(b) A solution of **9** (0.012 g) in $CHCl_3$ (3 ml) was treated dropwise with a $CHCl_3$ solution saturated with dry HCl until the pH was distinctly acidic. The solvent was evaporated and the residue purified on one 20 × 20 cm tlc plate (250 μ thick, 10% MeOH–$CHCl_3$). The major band was isolated and gave 4 mg of **10**, identical in the ir with **10** prepared as above.

***erythro*-β-Hydroxy-L-aspartic Acid and *erythro*-β-Hydroxy-D-aspartic Acid.** Both of these were prepared by the method of Okai, *et al.*[17] The L enantiomer had $[\alpha]^{25}D$ +46.0° (*c* 1.0, H_2O)

[lit.[17] $[\alpha]^{20}D$ +47.0° (c 1.0, H_2O)]. The D enantiomer had $[\alpha]^{25}D$ −42.8° (c 1.0, H_2O) [lit.[17] $[\alpha]^{20}D$ −46.8° (c 1.0, H_2O)].

Preparation of ASase. The Novikoff hepatoma strain was maintained by transferring it weekly into two female Holtzman rats (180–200 g, Holtzman Co., Madison, Wis.) according to the general method of McCoy and Neuman.[21] When the enzyme was to be isolated, 14 Holtzman 1-yr-old retired breeder rats were infected intraperitoneally with the hepatoma suspension and the tumor masses harvested after 6 days. The resultant tissue (100 g wet wt) was then homogenized and ASase isolated as previously described.[5a] The medium B as reported[5a] was modified** so as to contain 100 m*M* Tris–HCl buffer, pH 8.0; EDTA, 2.1 g; glycerin, 10% by weight; dithiothreitol (Sigma), 5 m*M* in a total volume of 1 l. The enzyme fraction used in this work was obtained from the precipitate after the third $(NH_4)_2SO_4$ addition.[5a] This was dissolved in 9 ml of medium B and kept frozen. Initial experiments with this fraction required several thawings and refreezings whereupon the initial activity (0.16 μmol of asparagine synthesized per mg protein per 30 min, see below for details) decreased to about 25% of the original activity. This fraction was concentrated to about 3 ml by use of an Amicon Model 50 ultrafiltration cell using a UM-20E filter. The concentrated fraction was then purified by chromatography on a column of Sephadex G-100 (2.5 × 24 cm), equilibrated and eluted with medium B. Fractions were collected immediately after the void volume (determined with Pharmacia Blue Dextran) and those containing the highest specific activity were pooled (9 ml). The pooled solution was concentrated to 3 ml using the Amicon ultrafiltration unit described above. Protein on this fraction (9.4 mg/ml) was determined by an adaptation[22] of the method of Lowry. Crystalline bovine serum albumin was used to construct a standard curve. This repurified ASase (specific activity of 0.11 μmol of asparagine synthesized per mg protein per 30 min) was divided into 0.2-ml portions and kept frozen at −60°. The repurified enzyme lost little or no activity after 1 year at this temperature.

ASase Assay and Inhibition. Two stock solutions were prepared. Solution "A" contained, in each 0.5 ml, disodium ATP (Sigma, 8.0 μmol), $MgCl_2 \cdot 6H_2O$ (Fisher, 8.0 μmol), and Tris buffer (100 μmol) adjusted to pH 8.0. Solution "B" contained, in each 0.2 ml, L-glutamine (Sigma, 20.0 μmol), L-aspartic acid (Sigma, 2.0 μmol), and L-aspartic acid-^{14}C (Schwarz-Mann, further purified by the method of Patterson, *et al.*,[23] 1 × 10^6 dpm per μmol). The incubation mixture contained 20 λ of the enzyme, 0.5 ml of "A," 0.2 ml of "B," and 0.3 ml of H_2O. After incubation for 30 min at 37°, the reaction was terminated by the addition of 1.0 ml of 0.8 *M* $HClO_4$ and the mixture neutralized to a pH of *ca.* 6 with a 5 *N* KOH solution.[3b] After centrifugation, the asparagine-^{14}C was separated from aspartic acid-^{14}C as previously described,[23] and 1.5 ml of the eluate was counted in 15 ml of counting fluid.[3d] A control without enzyme was used.

The inhibition studies were performed in duplicate in the following manner. A solution of the inhibitor dissolved in Tris solution (0.3 ml, 1.0 μmol of Tris), 0.5 ml of "A," and 20 λ of enzyme was preincubated for 15 min at 37°. Solution "B" (0.2 ml) was added and the mixture incubated for another 30 min and worked up as described above. The per cent inhibition is then the ratio of asparagine-^{14}C synthesized in the presence of inhibitor to that in the absence of inhibitor.

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References

(1) M. Mokotoff and B. S. Parikh, *J. Org. Chem.*, **37**, 2786 (1972) (paper 1).
(2) R. H. Adamson and S. Fabro, *Cancer Chemother. Rep.*, **52**, 617 (1968); D. A. Cooney and R. E. Handschumacher, *Annu. Rev. Pharmacol.*, **10**, 421 (1970).
(3) (a) J. D. Broome and J. H. Schwartz, *Biochim. Biophys. Acta*, 138, 637 (1967); (b) M. K. Patterson, Jr., and G. R. Orr, *Biochem. Biophys. Res. Commun.*, **26**, 228 (1967); (c) M. D. Prager and N. Bachynsky, *ibid.*, 31, 43 (1968); (d) M. D. Prager and N. Bachynsky, *Arch. Biochem. Biophys.*, **127**, 645 (1968).
(4) C. M. Haskell and G. P. Canellos, *Biochem. Pharmacol.*, 18, 2578 (1969); C. M. Haskell and G. P. Canellos, *Cancer Res.*, **30**, 1081 (1970).
(5) (a) M. K. Patterson, Jr., and G. R. Orr, *J. Biol. Chem.*, **243**, 376, (1968); (b) M. Benuck, F. Stern, and A. Lajtha, *J. Neurochem.*, 17, 1133 (1970); (c) B. Horowitz and A. Meister, *J. Biol. Chem.*, **247**, 6708 (1972); (d) T. C. Chou and R. E. Handschumacher, *Biochem. Pharmacol.*, **21**, 39 (1972).
(6) W. Shive and C. G. Skinner in "Metabolic Inhibitors," Vol. I, Hochster and Quastel, Ed., Academic Press, New York, N.Y., 1963, p 21, and references cited therein.
(7) M. L. Kornguth and H. J. Sallach, *Arch. Biochem. Biophys.*, **91**, 39 (1960).
(8) H. DeWald and A. M. Moore, *J. Amer. Chem. Soc.*, **80**, 3941 (1958); E. D. Nicolaides R. D. Westland, and E. L. Wittle, *ibid.*, **76**, 2887 (1954); A. Singh, E. R. Thornton, and F. H. Westheimer, *J. Biol. Chem.*, **237**, 3006 (1962); R. E. Handschumacher, C. J. Bates, P. K. Chang, A. T. Andrews, and G. A. Fischer, *Science*, **161**, 62 (1968).
(9) T. C. Daniels and E. C. Jorgensen in "Textbook of Organic Medicinal and Pharmaceutical Chemistry," 6th ed, Wilson, Gisvold, and Doerge, Ed., J. B. Lippincott Co., Philadelphia, Pa., 1971, p 43.
(10) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **89**, 5012 (1967).
(11) (a) T. Curtius and E. Welde, *Chem. Ber.*, 43, 862 (1910); (b) H. A. DeWald, D. C. Behn, and A. M. Moore, *J. Amer. Chem. Soc.*, 81, 4364 (1959); (c) L. Baldini and G. Brambilla, *Cancer Res.*, **26**, 1754 (1966).
(12) M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience, New York, N.Y., 1966, p 47; D. E. Nitecki, B. Halpern, and J. W. Westley, *J. Org. Chem.*, **33**, 864 (1968); C. Shin, M. Masaki, and M. Ohta, *Bull. Chem. Soc. Jap.*, **43**, 3223 (1970).
(13) C. W. Jones, III, D. E. Leyden, and C. H. Stammer, *Can. J. Chem.*, **47**, 4363 (1969).
(14) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **85**, 3039 (1963); **86**, 1839 (1964).
(15) N. Takamura, T. Mizoguchi, K. Koga, and S. Yamada, *Tetrahedron Lett.*, 4495 (1971).
(16) H. A. DeWald, D. C. Behn, M. S. Morgan, A. G. Renfrew, and A. M. Moore, *J. Amer. Chem. Soc.*, 81, 4367 (1959); H. E. Scheffer and J. A. Moore, *J. Org. Chem.*, **28**, 129 (1963).
(17) H. Okai, N. Imamura, and N. Izumiya, *Bull. Chem. Soc. Jap.*, **40**, 2154 (1967).
(18) A. Singerman and Y. Liwschitz, *Tetrahedron Lett.*, 4733 (1968); H. Okai and N. Izumiya, *Bull. Chem. Soc. Jap.*, **42**, 3550 (1969).
(19) Y. Liwschitz and A. Singerman, *J. Chem. Soc. C*, 1696 (1967).
(20) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 2, Wiley, New York, N.Y., 1961, p 891.
(21) T. A. McCoy and R. E. Neuman, *J. Nat. Cancer Inst.*, 16, 1221 (1956).
(22) G. L. Miller, *Anal. Chem.*, 31, 964 (1959).
(23) M. K. Patterson, Jr., G. R. Orr, and T. A. McCoy, *Anal. Biochem.*, 6, 543 (1963).