Potential Inhibitors of L-Asparagine Biosynthesis. 5.[1a,b] Electrophilic Amide Analogues of (S)-2,3-Diaminopropionic Acid

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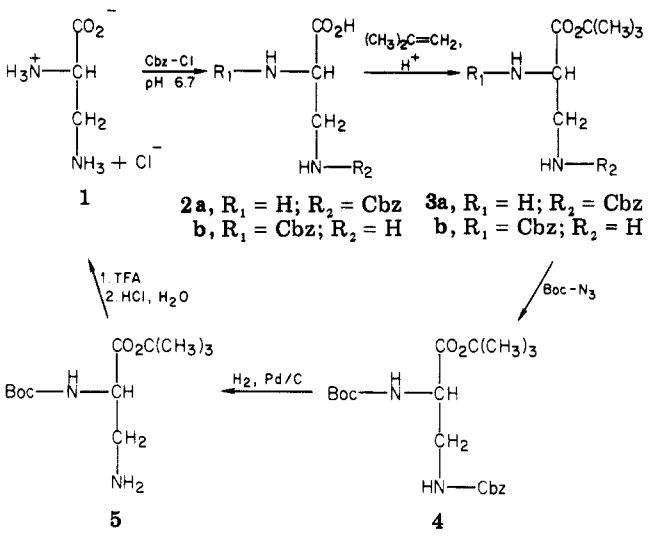
Three electrophilic amide analogues of (*S*)-2,3-diaminopropionic acid (**1**, DAP) have been prepared as potential inhibitors of L-asparagine synthetase (ASase, from Novikoff hepatoma, EC 6.3.5.4). DAP was selectively blocked by the carbobenzoxy (Cbz) group to give 3-*N*-Cbz-DAP (**2a**). Esterification of **2a** with isobutylene afforded *tert*-butyl 3-*N*-carbobenzoxy-(*S*)-2,3-diaminopropionate (**3a**), which was then blocked at the 2 position with the *tert*-butoxycarbonyl (Boc) group to give *tert*-butyl 2-[(*S*)-(*tert*-butoxycarbonyl)amino]-3-[(carbobenzoxy)amino]propionate (**4**). Selective cleavage of the Cbz group by H_2/Pd gave the key intermediate *tert*-butyl 2-*N*-(*tert*-butoxycarbonyl)-(*S*)-2,3-diaminopropionate (**5**), which was acylated, via the *N*-hydroxysuccinimide esters, with bromoacetic acid, dichloroacetic acid, and fumaric acid monoethyl ester to give *tert*-butyl 2-[(*S*)-(*tert*-butoxycarbonyl)-amino]-3-(2-bromoacetamido)propionate (**6a**), *tert*-butyl 2-[(*S*)-(*tert*-butoxycarbonyl)amino]-3-(2,2-dichloroacetamido)propionate (**6b**), and *tert*-butyl 2-[(*S*)-(*tert*-butoxycarbonyl)amino]-3-[(*E*)-3-(ethoxycarbonyl)acrylamido]-propionate (**6c**), respectively. Deblocking of **6a-c** gave the corresponding amino acids (*S*)-2-amino-3-(2-bromoacetamido)propionic acid hydrobromide (**7a**), (*S*)-2-amino-3-(2,2-dichloroacetamido)propionic acid (**7b**), and ethyl *N*-[(*S*)-2-amino-2-carboxyethyl]fumarate (**7c**). By a slightly different procedure, **5** was converted in two steps to (*S*)-2-amino-3-acetamidopropionic acid hydrobromide (**7d**). The inhibition of ASase by **7a-c** at 1 mM was 93, 19, and 37%, respectively, while **7d** was without inhibition at 2 mM. Compounds **7a-c** failed to increase the life span of mice infected with B16 melanoma.

Selected mammalian malignancies are susceptible to treatment with L-asparaginase (ASNase, EC 3.5.1.1), presumably due to the depletion of their cellular L-asparagine.[2] However, clinical resistance to therapy with ASNase has been shown to be due to an increased synthesis of L-asparagine via the enzyme L-asparagine synthetase (ASase, EC 6.3.5.4).[3-6] Horowitz et al.[7] have shown that many animal tumors which respond to ASNase treatment have very low levels of ASase and when these animals are subjected to subcurative doses of ASNase there is an increase in ASase activity as well as the development of resistance to ASNase.[8] There is thus a striking correlation between resistance to ASNase and possession of ASase activity, on the one hand, and sensitivity to ASNase and absence of ASase, on the other hand. Milman and Cooney[9] studied the distribution of ASase in the principal organs of several mammalian species and found very low levels of ASase activity in these normal tissues except, in certain species (not man), for the pancreas.[10] Thus, it appears that a biochemical difference exists between certain malignancies and normal tissues which could possibly be exploited therapeutically.

Scheme I[a]



[a] Cbz = $C_6H_5CH_2OOC$-; Boc = $(CH_3)_3COOC$-

Inasmuch as the data suggests that ASase activity is of central significance in relation to the effectiveness of ASNase therapy, we have been trying to find inhibitors of ASase. Such compounds could be beneficial from two standpoints. First they could be used against ASNase resistant lines, and secondly, they could be used in combination with ASNase sensitive tumors in an attempt to prevent the development of the resistant cells. Mammalian ASase synthesizes L-asparagine from L-aspartic acid, utilizing L-glutamine as the primary source of nitrogen.[11-13] In addition, it has been observed that ASase undergoes product inhibition by L-asparagine.[3,11-14] It was therefore believed that analogues of L-asparagine, containing functional groups which could covalently bind to the enzyme

(1) (a) For paper 4, see S. Brynes, G. J. Burckart, and M. Mokotoff, *J. Med. Chem.*, **21**, 45 (1978). (b) A portion of this work served as a basis for an award to L. W. Logue, Eastern-Regional Lunsford-Richardson Pharmacy Award, 1972. (c) Undergraduate participant.
(2) J. C. Wriston and T. O. Yellin, *Adv. Enzymol. Relat. Areas Mol. Biol.*, **39**, 185 (1973).
(3) C. M. Haskell and G. P. Canellos, *Biochem. Pharmacol.*, 18, 2578 (1969); *Cancer Res.*, **30**, 1081 (1970).
(4) M. D. Prager and N. Bachynsky, *Biochem. Biophys. Res. Commun.*, **31**, 43 (1968); *Arch. Biochem. Biophys.*, **127**, 645 (1968).
(5) S. Hongo, T. Matsumoto, and T. Sato, *Biochim. Biophys. Acta.*, **522**, 258 (1978).
(6) H. N. Jayaram, D. A. Cooney, H. A. Milman, E. R. Homan, and R. J. Rosenbluth, *Biochim. Pharmacol.*, **25**, 1571 (1976); H. A. Milman, D. A. Cooney, and J. M. Ward, *Toxicol. Appl. Phamacol.*, **50**, 573 (1979).
(7) B. Horowitz, B. Madras, A. Meister, L. Old, E. Boyse, and E. Stockert, *Science*, **160**, 533 (1968).
(8) H. N. Jayaram, D. A. Cooney, H. A. Milman, H. Greenberg, A. Goldin, and J. A. R. Mead, *Proc. Am. Assoc. Cancer Res.*, **14**, 109 (1973).
(9) H. A. Milman and D. A. Cooney, *Biochem. J.*, **142**, 27 (1974).
(10) H. A. Milman and D. A. Cooney, *Biochem. J.*, 181, 51 (1979); H. A. Milman, D. A. Cooney, and D. M. Young, *Am. J. Physiol.*, **236**, E746 (1979).
(11) M. K. Patternson, Jr., and G. R. Orr, *J. Biol. Chem.*, **243**, 376 (1968).
(12) B. Horowitz and A. Meister, *J. Biol. Chem.*, **247**, 6708 (1972).
(13) H. A. Milman, D. A. Cooney, and C. Y. Huang, *J. Biol. Chem.*, **255**, 1862 (1980).
(14) T. C. Chou and R. E. Handschumacher, *Biochem. Pharmacol.*, **21**, 39 (1972).

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protein, might be useful as inhibitors of ASase. We envisaged that selected 3-acylated derivatives of (*S*)-2,3-diaminopropionic acid (DAP, 1), which could be thought of as "reversed amide" analogues of L-asparagine, would act just as described above.

Biologically active derivatives of DAP acylated or similarly substituted in the 3 position are not unknown in the literature. Medicinal plants have yielded 3-*N*-oxalyl-DAP[15] (a neurotoxin), mimosine[16] (antitumor activity), and quisqualic acid[17] (anthelmintic). A fourth compound, with activity of interest to us, is seen in the recent report on 3-[(phosphonoacetyl)amido]-L-alanine (PA_2LA)[18] as an inhibitor of ASase from L5178Y/AR mouse tumors. This present study reports the synthesis and biological evaluation of three new "reversed-amide" analogues of L-asparagine, namely, **7a–c**. The choice of functional groups **a–c** was based on their use as irreversible inhibitors[19] in other enzyme systems.

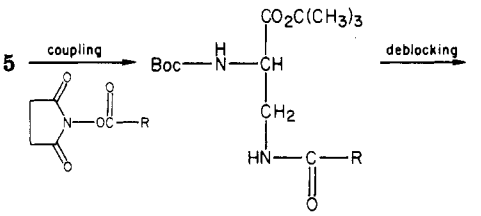
Chemistry. The common intermediate required for the synthesis of compounds **7a–d** was the properly blocked diamino acid **5**. Initially we considered the use of a copper complex[15,20] of 1 as the most direct route to **7a–c**. However, we chose the longer method shown in Scheme I because we believed that the high chemical reactivity of the functional groups (**7a–c**) attached to the 3-amino group would not be compatible with conditions needed to liberate the free amino acids from the copper complex.

The starting compound DAP (**1**) was prepared from L-aspartic acid by the method of Kitagawa et al.[21] Following the procedure of Kjaer and Larsen,[20] selective carbobenzoxylation of 1 at controlled pH gave crystalline, single spot (TLC) material which we initially believed to be pure 3-*N*-carbobenzoxy 2-(*S*)-2,3-diaminopropionic acid (3-*N*-Cbz-DAP, **2a**). Using the general method of Roeske[22] the latter compound was converted with acid-catalyzed isobutylene to the *tert*-butyl ester, which appeared on TLC as two spots, the upper R_f component being identified as **3a** and the lower component as **3b**. Obviously, the original compound **2a** was contaminated with the undesired isomer **2b**. We subsequently found that during the preparation of **2a**, even under proper pH control, some of the 2-*N*-Cbz compound **2b** invariably forms. However, recrystallization of the initially isolated product from water gives almost exclusively **2a** in the first crop, and subsequent crops are contaminated with **2b**. We were able to show this by direct comparison in the infrared with an authentic sample of **2a** prepared by an unambiguous route.[23a] Proof of structure **3b**, besides analysis, was made by selective cleavage of the *tert*-butyl ester to give 2-*N*-Cbz-DAP (**2b**) and direct comparison in the infrared with an authentic sample of **2b** prepared by an unambiguous route[24] (see Figure 1, supplementary material; see paragraph at the end of paper concerning supplementary material). In fact, we routinely determined the purity, and thus suitability for continued synthesis, of **2a** by whether or not its infrared spectrum was superimposable with the authentic sample. Subsequent crystalline crops of **2a** invariably were nonsuperimposable on either the infrared spectrum of **2a** or **2b** because of contamination with the latter compound. Furthermore, the above results were confirmed by using the color test of Larsen and Kjaer[25] which can distinguish between 2-substituted and 3-substituted DAPs.

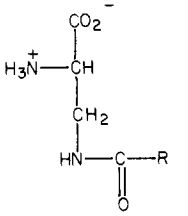
When pure **2a** was used, the procedure of Roeske[22] afforded pure *tert*-butyl ester **3a**, which upon reaction with *tert*-butoxycarbonyl azide,[26] by the method of Schroder and Klieger,[27] gave a 92% yield of the triply blocked amino acid **4**. Catalytic hydrogenation of **4** cleaved the Cbz blocking group, affording the key intermediate **5** in an overall yield, for the three steps from **2a**, of 59% (Scheme I). In order to check that the four steps from **1** to **5** proceeded without racemization, and thus the optical rotation observed for **5** is that of the L enantiomer (*S* configuration), we treated **5** with trifluoroacetic acid (TFA) to cleave the blocking groups and then with dilute HCl to obtain DAP·HCl (1). The optical rotation of the starting compound 1 (+24.8°) compared to 1 obtained from **5** (+23.8°) indicates that for the four steps there is little racemization.

For the introduction of the functional groups a general procedure was used that was suitable for the preparation

Scheme II[a]



6a, R = CH_2Br
b, R = $CHCl_2$
c, R = CH=CHCO$_2$Et (trans)
d, R = CH_3



7a, R = CH_2Br
b, R = $CHCl_2$
c, R = CH=CHCO$_2$Et (trans)
d, R = CH_3

[a] Boc = $(CH_3)_3COOC$–. **7a,d**, HBr salts.

(15) S. L. N. Rao, P. R. Adiga, and P. S. Sarma, *Biochemistry*, **3**, 432 (1964); P. S. Cheema, G. Padmanaban, and P. S. Sarma, *J. Neurochem.*, **18**, 2137 (1971); J. W. Olney, C. H. Misra, and V. Rhee, *Nature (London)*, **264**, 659 (1976).
(16) W. D. DeWys and T. C. Hall, *Eur. J. Cancer*, **9**, 281 (1973).
(17) *Chem. Eng. News*, **56** (42), 62 (1978).
(18) H. N. Jayaram and D. A. Cooney, *Cancer Treat. Rep.*, **63**, 1095 (1979).
(19) P. S. Portoghese, V. G. *Telang*, A. E. Takemori, and G. Hayoshi, *J. Med. Chem.*, **14**, 144 (1971); A. Sweeny, Jr., R. H. Goldfarb, T. N. Salmon, G. Loewengart, R. Fox, and A. Sicignano, *ibid.*, **14**, 451 (1971).
(20) A. Kjaer and P. O. Larsen, *Acta. Chem. Scand.*, **13**, 1565 (1959).
(21) T. Kitagawa, T. Ozasa, and H. Taniyama, *Yakugaku Zasshi*, **89**, 285 (1969); this compound is now commercially available from several sources that supply amino acids.
(22) R. Roeske, *J. Org. Chem.*, **28**, 1252 (1963).
(23) (a) M. Zaoral, J. Kolc, and F. Sorm, *Collect. Czech. Chem. Commun.*, **35**, 1716 (1970); the authors thank Professor Sorm for sending us a sample of 3-*N*-Cbz-DAP (**2a**). (b) This optical rotation was not reported in ref 23a but recorded in our laboratory on Professor Sorm's sample.
(24) F. Brtnik and M. Zaoral, *Collect. Czech. Chem. Commun.*, **41**, 2969 (1976); the authors thank Professor Zaoral for sending us a sample of 2-*N*-Cbz-DAP (**2b**).
(25) P. O. Larsen and A. Kjaer, *Biochim. Biophys. Acta*, **38**, 148 (1960).
(26) *tert*-Butoxycarbonyl azide is no longer commercially available, but equally good results have been obtained using di-*tert*-butyl dicarbonate (see Experimental Section).
(27) E. Schroder and E. Klieger, *Ann. Chem.*, **673**, 208 (1964).

Table I. Inhibition of Asparagine Synthetase[a]

no.[b]	final concn, mM	% inhibn ± SD[c]
1	2	0
7a	1	93.0 ± 3.7
7b	1	18.7 ± 6.6
7c	1	37.4 ± 8.1
7d	2	0

[a] ASase partially purified from rat Novikoff hepatoma as described in paper 2 of this series (ref 31). [b] The compounds were preincubated with ASase and cofactors and then substrate aspartic acid was added. The formation of L-asparagine was determined as described earlier (ref 31). [c] Percent inhibition and true population standard deviation (as determined on a Hewlett-Packard 32E calculator) derived from an inhibition assay run in duplicate on 3 separate days (triplicate of duplicates) utilizing freshly prepared inhibitor solutions.

of **6a–c**. The method chosen was the activation of each corresponding acid by conversion to its *N*-hydroxysuccinimide ester[28] and then, without **6a-c**. of the active ester, coupling it with the amine **5**. Thus, bromoacetic acid was converted to its *N*-hydroxysuccinimide ester[29] and coupled with **5** to give **6a**. Deblocking of the protective groups of **6a** with anhydrous HBr in glacial acetic acid (HBr/AcOH) afforded **7a**, as its HBr salt, in a yield of 93% for the two steps. In a similar manner, dichloroacetic acid was converted to its active ester, which on reaction with **5** gave **6b**. With this compound we found that the blocking groups were best removed with liquid HF,[30] giving the desired **7b** as the free amino acid in an 86% yield for the two steps from **5**. The amine **5** on reaction with the active ester of fumaric acid monoethyl ester gave **6c**, which was selectively cleaved with TFA to the free amino acid **7c** in a yield of 44% for the two steps.

The prototype compound **7d**, which does not contain a reactive electrophilic function, was prepared by acylation of **5** with acetic anhydride and cleavage of the blocking groups with HBr/AcOH.

Biochemical Results. The ability of **7a–d** to inhibit the in vitro biosynthesis of L-asparagine is shown in Table I. The bromoacetamido compound **7a** shows significant inhibition (93% at 1 mM) of ASase from Novikoff hepatoma, whereas the two other electrophilic compounds **7b** and **7c** were considerably less active. On the other hand, the acetamido compound **7d**, which is devoid of an electrophilic group that could react with nucleophilic functions on the enzyme, was without any inhibitory activity. Compound **7d** was prepared and tested to see if this "reversed amide" could act like asparagine at the active site of ASase. Inasmuch as L-asparagine inhibits ASase by 63% at 2 mM,[31] it appears that acetamido compond **7d** does not mimic the former compound. Thus, the biological results suggest that the inhibitory activity of **7a–c** is not active-site specific.

For the past several years Arfin and co-workers[32] have been engaged in studying the regulation of ASase in Chinese hamster ovary (CHO) cells. In order to gain further insight into the mechanisms by which ASase is regulated and the relationship between the levels of enzyme activity and the charging of tRNA, Arfin et al.[32] have been attempting to isolate variants of CHO cells resistant to the action of analogues which affect asparagine metabolism. Thus, compounds **7a–c** were submitted to Dr. Arfin as potential inhibitors of ASase which might be toxic to the CHO cells, thereby allowing only the survival of variants of these cells which have altered asparagine metabolism. Preliminary results[33] of **7a–c** on the growth of TsH1 cells (a CHO cell line containing a temperature-sensitive leucyl-tRNA synthetase) are shown in Table II. Although the results shown in Table II are only qualitative, it has been found[34] that this procedure is fairly accurate as an initial screen for potentially toxic (to CHO cells) compounds.

Table II. Growth Inhibition of TsH1 Cells[a]

no.	concn, mg/mL (mM)	medium,[b] growth[c] –Asp, –Asn	–Asn	–Asp	complete
7a	1.0 (3.27)	–	–	–	–
	0.2 (0.65)	–	–	+	+
	0.04 (0.13)	+3	+2	+3	+3
	0.01 (0.03)	+3	+3	+3	+3
7b	1.0 (4.65)	–	–	+2	+2
	0.2 (0.93)	–	–	+3	+3
	0.04 (0.19)	+3	+3	+3	+3
	0.01 (0.04)	+3	+3	+3	+3
7c	1.0 (4.34)	+3	+3	+3	+3
	0.2 (0.87)	+3	+3	+3	+3
	0.04 (0.17)	+3	+3	+3	+3
	0.01 (0.04)	+3	+3	+3	+3

[a] TsH1 cells refers to a Chinese hamster ovary cell line containing a temperature-sensitive leucyl-tRNA synthetase (ref 35). [b] The cells were distributed into 24 well trays (30 000 cells/well) and allowed to attach overnight. The next day the medium was replaced with complete α ME medium (an enriched Eagle's minimal essential medium), lacking asparagine (–Asn) or aspartic acid (–Asp) or medium lacking both of these amino acids (–Asp, –Asn), each medium containing various concentrations of analogue. [c] Cells were stained with Giemsa 4 days after the addition of analogue and recorded as heavy growth (+3), medium growth (+2), light growth (+), or cells dead (–) (ref 36).

The results shown in Table II indicate that in the absence of asparagine both **7a** and **7b**, at a concentration of 0.2 mg/mL (0.65 and 0.93 mM, respectively) or greater, cause the death of TsH1 cells. Cell death due to **7a** is only slightly protected, and death due to **7b** is resonably protected by the addition of asparagine to the growth medium. This reversal of cell death by the presence of asparagine in the medium suggests that **7a** and **7b** interferes in the asparagine metabolism of TsH1 cells.

Compounds **7a–c** were submitted to the Drug Synthesis and Chemistry Branch of the National Cancer Institute (NCI) for in vivo screening in BDF_1 mice infected with B16 melanocarcinoma. The B16 tumor test system was chosen rather than P-388 lymphocytic leukemia, which is the routine screen for synthetic compounds, because the ASase

(28) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **85**, 3039 (1963).
(29) M. Wilchek and D. Givol, *Methods Enzymol.*, **46**, 153 (1977).
(30) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).
(31) M. Mokotoff, J. F. Bagaglio, and B. S. Parikh, *J. Med. Chem.*, 18, 354 (1975).
(32) S. M. Arfin, D. R. Simpson, C. S. Chiang, I. L. Andrulis, and G. W. Hatfield, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2367 (1977); I. L. Andrulis, G. W. Hatfield, and S. M. Arfin, *J. Biol. Chem.*, **254**, 10629 (1979); J. S. Gantt, C. S. Chiang, G. W. Hatfield, and S. M. Arfin, *J. Biol. Chem.*, **255**, 4808 (1980).
(33) The authors thank J. Stephen Gantt, Deparment of Biological Chemistry, California College of Medicine, University of California, Irvine, for the preliminary studies of **7a–c** on the TsH1 cells.
(34) Personal Communication from Dr. Stuart M. Arfin, Department of Biological Chemistry, California College of Medicine, University of California, Irvine.

activity of the former tumor system is greater than that of the latter.[35] Since compounds **7a–c** were designed as ASase inhibitors, it was felt that they would have a better chance of inhibiting tumor growth in a system which was ASNase resistant[35] and thus more dependent on its own synthesis of asparagine (higher ASase activity). The protocol presently in effect at NCI requires a minimal increase in survival of treated animals over controls resulting in a T/C ≥ 125% before a compound is considered active.[36] All three compounds tested failed to meet this criterion and were considered inactive.

Experimental Section

Melting points were determined on a Fisher-Johns apparatus and are uncorrected. The infrared spectral data were obtained with a Perkin-Elmer Model 267 grating infrared spectrophotometer as KBr disks. Optical rotations were determined with a Perkin-Elmer 241 or Rudolph 70 polarimeter. Radioactivity was determined with a Packard Model 3310 Tri-Carb scintillation spectrometer. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and Spang Microanalytical Laboratory, Ann Arbor, MI. L-Aspartic-^{14}C acid was obtained from Schwarz-Mann and purified as previously described.[31] Thin-layer chromatography was carried out with silica gel G-F (on glass, Analtech, Inc., or on aluminum sheets, EM Reagents) or cellulose GF (Quantum Industries), and spots were located with either UV light, charring (Al plates), or by treatment of the plate with HCl fumes, followed by heating and subsequent spraying with ninhydrin. All evaporations were performed in vacuo on a rotary evaporator. Organic solutions that had been previously extracted with aqueous solution were dried over anhydrous Na_2SO_4 prior to evaporation.

The apparatus for conducting the liquid HF reactions was constructed and used as previously described.[1a] More recently, a more efficient and useful apparatus was constructed from Teflon TFE valves and tubing purchased through Cole-Parmer Instrument Co. These valves and fittings did not leak HF gas.

(*S*)-2,3-Diaminopropionic Acid Hydrochloride (1). Method A. This compound was prepared by the method of Kitagawa et al.[21] in a 62% yield, $[\alpha]^{25}_D$ +24.8° (*c* 1.1, 1 N HCl) [reported[21] $[\alpha]^{12}_D$ +19° (*c* 1.0, H_2O); reported from Calbiochem-Behring Corp. $[\alpha]^{22}_D$ +24.9° (*c* 1.99, 1 N HCl).

Method B. Compound **5** (0.053 g, 0.02 mmol), prepared as described below, was dissolved in TFA (2.0 mL) and allowed to stir for 15 min. The excess reagent was removed by evaporation, and the residue was triturated with Et_2O, evaporated, and then dissolved in 3 N HCl (1.0 mL) by warming. The aqueous solution was concentrated to dryness, redissolved in 0.5 mL of H_2O, and MeOH was added until crystallization ensued, affording 0.021 g (75%) of **1**, identical in the IR with that prepared above: $[\alpha]^{25}_D$ +23.8° (*c* 1.0, 1 N HCl).

3-*N*-Carbobenzoxy-(*S*)-2,3-diaminopropionic Acid (2a). Following a procedure similar to that of Kjaer and Larsen,[20] compound **1** (5.64 g, 40.1 mmol) was dissovled in a 0.05 M phosphate buffer (pH 7.0, 100 mL, Fisher Scientific Co.) and cooled in ice, and the pH of the solution (which was 5.5) was adjusted to 6.7 with 10% NaOH solution. Carbobenzoxy chloride (8.6 mL, 60.2 mmol, Chemical Dynamics) was dissolved in toluene (8.0 mL) and added dropwise, with vigorous stirring, over 100 min, the pH being monitored (meter) and adjusted to between 6.5 and 7.0 by the addition of a 10% NaOH solution. While the mixture was stirring and cooling, the pH was maintained near 6.5 for another 4.5 h, and then the mixture was refrigerated overnight. The resulting precipitate was collected by filtration, and the solid was washed with H_2O and Et_2O. Crystallization from H_2O gave white crystals: yield 5.21 g (54.3%); mp 235–240 °C dec; $[\alpha]^{25}_D$ −11.9° (*c* 1.02, 1 N HCl), −4.1° (*c* 0.96, 0.1 M NaOH) [reported[20,23a] mp 227–229, 245 °C; $[\alpha]^{21}_D$ −18.7 (*c* 1.0, 1 N HCl),[20] −11.0° (*c* 1.01, 1 N HCl),[23b] −4° (*c* 1.1, 0.1 M NaOH),[20] −3.8° (*c* 0.6, 0.1 M NaOH)[23a]]. This sample of **2a** was identical in the IR with a sample supplied by Dr. Sorm.[23a] Concentration of the above aqueous mother liquor gave a second crop of crystals, 1.95 g, whose IR spectra indicated it was impure **2b** (see below).

Table III

	development with ninhydrin		development with cupric reagent followed by ninhydrin	
	color	intensity	color	intensity
2a	violet	strong	none	
2b	violet	weak	yellow-brown	medium

***tert*-Butyl 3-*N*-Carbobenzoxy-(*S*)-2,3-diaminopropionate (3a).** Compound **2a** (3.44 g, 14.5 mmol), pure according to IR, was dissolved in a solution of dioxane (60 mL, peroxide free) and concentrated H_2SO_4 (4.0 mL, 146 mequiv) contained in a 500-mL Parr bottle. This solution was cooled in ice and treated with (previously condensed) isobutylene (ca. 65 mL). This mixture was immediately placed on a Parr shaker-type hydrogenation apparatus[22] (Model 3911), stoppered, and allowed to shake overnight (initial pressure 24 psi) at room temperature. After 21 h (final pressure about 22 psi), the excess isobutylene was removed, in vacuo, directly on the Parr apparatus. The contents of the flask were cooled in ice and then poured into a H_2O–Et_2O mixture (80:200 mL) containing NaOH (6.0 g, 150 mequiv). The layers were separated and the aqueous layer was washed with two 50-mL portions of Et_2O. The combined Et_2O solution was washed with 5% NaOH, H_2O, and saturated salt solution and dried. The solution was evaporated to give **3a** as a pale yellow oil (crude 3.57 g, 84%), which according to TLC (7% MeOH/$CHCl_3$) was ca. 98% pure and was suitable for the preparation of **4**. The analytical sample was obtained by converting the amine **3a** to its maleate salt and crystallization from EtOH–Et_2O: mp 163–164 °C; $[\alpha]^{28}_D$ +12.6° (*c* 1.03, EtOH). Anal. ($C_{19}H_{26}N_2O_8$) C, H, N.

***tert*-Butyl 2-*N*-Carbobenzoxy-(*S*)-2,3-diaminopropionate (3b).** Beginning experiments on introducing the *tert*-butyl ester group, as described above, utilized material which was thought to be pure **2a** but actually was contaminated with **2b**. Utilizing the method above afforded an oil whose TLC pattern indicated two components. The mixture (0.49 g) was separated on a column of silica gel (Brinkmann 0.05–0.2 mm, 22 g, column dimensions 1.5 × 33 cm) packed as a slurry in 30% Et_2O/$CHCl_3$. Development of the column with 30% Et_2O/$CHCl_3$, 15% Et_2O/$CHCl_3$, $CHCl_3$, 2% MeOH/$CHCl_3$, and finally 4% MeOH/$CHCl_3$ eluted the compounds, affording 0.22 g of **3a** as the upper R_f component and 0.12 g of **3b** as the lower R_f component. Conversion of amine **3b** to its maleate salt and crystallization from acetone–Et_2O gave the analytical sample, mp 135–137 °C; $[\alpha]^{28}_D$ −19.0° (*c* 0.95, EtOH). Anal. ($C_{19}H_{26}N_2O_8$) C, H, N.

2-*N*-Carbobenzoxy-(*S*)-2,3-diaminopropionic Acid (2b). A sample of **3b** maleate (0.030 g, 0.073 mmol) was dissolved in H_2O and basified with 1 N NaOH, and the liberated amine was extracted into CH_2Cl_2. The organic layer was washed with a saturated salt solution and evaporated to a residue. The residue was dissolved in 50% TFA–CH_2Cl_2 (2.0 mL) and stirred in ice for 30 min and at room temperature for 30 min. Evaporation of the excess reagent and trituration with anhydrous Et_2O gave a solid product, which was crystallized from H_2O. In two crops, 0.011 g (60%) of **2b** was obtained: mp 233–234 °C; $[\alpha]^{24}_D$ −6.0° (*c* 0.25, 1 N NaOH) [reported[24] mp 240–241 °C; $[\alpha]^{22}_D$ −7.4° (*c* 0.4, 1 N NaOH)]. This sample of **2b** was identical in the IR with a sample supplied by Dr. Zaoral.[24]

Color Test of 2 and 3-Cbz-DAP. Using the procedure of Larsen and Kjaer,[25] ca. 10 μg each of **2a** and **2b** was spotted on individual filter papers and either treated directly with the ninhydrin solution or treated first with the cupric reagent and then with ninhydrin. The color results are given in Table III.

***tert*-Butyl 2-[(*S*)-(*tert*-Butoxycarbonyl)amino]-3-[(carbobenzoxy)amino]propionate (4). Method A.** The ester **3a** (3.57 g, 12.1 mmol) was dissolved in pyridine (17 mL, previously dried over KOH) and treated with *tert*-butoxycarbonyl azide[26] (3.0 mL, 21.2 mmol, Aldrich). After remaining at room temperature for 3 days, the excess solvent was evaporated in high

(35) D. A. Cooney, V. D. King, R. G. Cable, B. Taylor, Jr., and I. Wodinsky, *Cancer Res.*, 26, 3238 (1976).

(36) Instruction 14, "Screening Data Summary Interpretation and Outline of Current Screen", Drug Evaluation Branch, National Cancer Institute, Bethesda, MD, p 17.

vacuo. The remaining oil was dissolved in EtOAc and washed successively with 10% citric acid, 5% Na_2CO_3, H_2O, and saturated salt solution. Evaporation of the solvent gave compound **4** as a pale yellow gum, 4.37 g (crude 92%), which was one component on TLC (6% MeOH/$CHCl_3$). This was not purified further but used directly in the next step.

Method B. When *tert*-butoxycarbonyl azide was no longer commercially available, the following method was used. Compound **3a** (4.52 g, 15.4 mmol) was dissolved in purified dioxane (50 mL) and treated with a solution of di-*tert*-butyl dicarbonate (Fluka, 3.36 g, 15.4 mmol) in dioxane (25 mL). The solution remained overnight and then the solvent was removed by evaporation. The residue was dissolved in EtOAc and worked up as in Method A, affording 5.92 g (crude 98%) of **4**, suitable for the next step.

***tert*-Butyl 2-*N*-(*tert*-Butoxycarbonyl)-(*S*)-2,3-diaminopropionate (5). Method A.** Compound **4** (4.37 g, 11.1 mmol) was dissolved in absolute EtOH (60 mL) and hydrogenated at atmospheric pressure with either Pd black or 10% Pd/C as catalyst (0.6 g). When H_2 was no longer consumed, the reaction was stopped and the catalyst was removed by filtration. After evaporation of the solvent, the resulting oil was dissolved in hot *n*-heptane, decolorized (with the aid of Norit-A and Celite), and allowed to crystallize (refrigeration), affording 2.15 g of **5** as pale yellow crystals, mp 61–63 °C. Concentration of the mother liquor gave an oil, which was purified on preparative silica gel plates (Analtech GF, 1000 μm). The desired product was obtained from the plates and crystallized from *n*-heptane, yielding another 0.08 g of **5**, mp 58–60 °C: total yield 77%. The analytical sample was obtained by recrystallization from *n*-heptane to give off-white crystals, mp 63–64 °C; $[\alpha]^{28}_D$ −23.9° (*c* 1.05, EtOH). Anal. ($C_{12}H_{24}N_2O_4$) C, H, N.

Method B. If on occasion the hydrogenolysis of **4** in EtOH did not proceed, as if the catalyst was inactive, then an alternative procedure was used. Compound **4** was hydrogenated in glacial AcOH with 10% Pd/C as catalyst. When H_2 was no longer consumed, the catalyst was removed by filtration and the AcOH evaporated. The oily residue was triturated with toluene and then evaporated to remove traces of AcOH. Upon refrigeration in the presence of *n*-heptane, the oil solidified and the solid, **5** acetate salt, was collected by filtration. The solid was powdered and washed several times with *n*-heptane, which removed a higher R_f (TLC, 4% MeOH/$CHCl_3$ + 1% NH_4OH) contaminant. The resulting **5** acetate salt was dissolved in H_2O and basified with 20% NaOH, and the free amine was extracted into EtOAc. Evaporation of the solvent left an oil, which was crystallized from *n*-heptane to give crystalline **5**, mp 62–64 °C, identical with that prepared above.

***tert*-Butyl 2-[(*S*)-(*tert*-Butoxycarbonyl)amino]-3-(2-bromoacetamido)propionate (6a).** *N*-Hydroxysuccinimide (0.39 g, 3.39 mmol) and bromoacetic acid were dissolved in dioxane (20 mL), and then dicyclohexylcarbodiimide (DCC) in dioxane (10 mL) was added. After 30 min the dicyclohexylurea (DCU) was removed by filtration, and to the clear filtrate was added the amine **5** (0.80 g, 3.08 mmol) dissolved in dioxane (10 mL). TLC evaluation (4% MeOH/$CHCl_3$) after 70 min indicated the absence of starting material and thus the mixture was concentrated to dryness. The resulting product was dissolved in EtOAc, filtered free of a small amount of DCU, and then washed successively with 10% citric acid, 5% Na_2CO_3, and saturated salt solution. Evaporation of the solvent afforded a yellow oil, crude 1.29 g (theory 1.17 g), which was virtually one spot on TLC. This was not purified further but used directly in the next step.

(*S*)-2-Amino-3-(2-bromoacetamido)propionic Acid Hydrobromide (7a). A solution of 10% HBr in glacial AcOH was prepared by bubbling HBr gas through a 10% solution of resorcinol in TFA directly into the AcOH and measuring the weight gain. Compound **6a** (1.29 g, crude) was dissolved in 10% HBr/AcOH (20 mL) and allowed to stir at room temperature for 40 min. The excess reagent was evaporated, and the residue was twice triturated with anhydrous Et_2O and evaporated. Trituration again with anhydrous Et_2O and decantation of the solvent left a semisolid residue, which was twice crystallized from AcOH–Et_2O to afford 0.88 g (93% for the two steps) of **7a** (crystals were always collected under a dry atmosphere in a Labconco glove box), mp 142–146 °C, which appeared pure on TLC (*n*-BuOH-AcOH-H_2O, 3:1:2, silica gel and on cellulose). Repeated crystallizations and drying of the sample under high vacuum at 40 °C over P_2O_5 (to remove AcOH) gave the analytical sample as white crystals: mp 142–145 °C; $[\alpha]^{24}_D$ −10.5° (*c* 1.11, 1 N HCl). Anal ($C_5H_{10}N_2O_3Br_2$) C, H, N.

***tert*-Butyl 2-[(*S*)-(*tert*-Butoxycarbonyl)amino]-3-(2,2-dichloroacetamido)propionate (6b).** The identical procedure described above for **6a** was used with the following quantities: *N*-hydroxysuccinimide (0.48 g, 4.17 mmol), dichloroacetic acid (0.54 g, 4.20 mmol), DCC (0.95 g, 4.61 mmol), amine **5** (1.00 g, 3.85 mmol), and dioxane (60 mL). Workup as above gave **6b** as a gum, one component on TLC (4% MeOH/$CHCl_3$), weighing 1.59 g crude (1.43 g, theory). This was not purified further but used directly in the next step.

(*S*)-2-Amino-3-(2,2-dichloroacetamido)propionic Acid (7b). Compound **6b** (1.59 g, crude) was placed in the reaction vessel of the HF apparatus along with anisole (2.0 mL). Hydrogen fluoride was condensed in the reservoir flask which contained CoF_3 (1 g, as drying agent). The HF in the reservoir flask was allowed to distil into the reaction flask, and thus **6b** was stirred in liquid HF (ca. 8 mL) for 30 min at dry ice/acetone temperature. After evaporation of the HF, the residue was treated with 6% AcOH/H_2O (75 mL) and the anisole was removed by extraction into EtOAc. The acidic aqueous layer was lyophilized to a white powder, which was redissolved in H_2O (20 mL) and the pH was adjusted to 6.0 (pH meter) by the addition of dilute $KHCO_3$. The neutralized solution was lyophilized and the white powder crystallized from AcOH–Et_2O, yielding in two crops 0.71 g (86% for the two steps) of **7b**, mp 188–191 °C, which appeared pure on TLC (*n*-BuOH–AcOH–H_2O, 3:1:2, silica gel and on cellulose). The analytical sample was obtained by recrystallization and, after drying at 100 °C in vacuo over P_2O_5, had mp 186–187 °C; $[\alpha]^{24}_D$ −2.8° (*c* 0.99, H_2O). Anal. ($C_5H_8N_2O_3Cl_2$) C, H, N.

***tert*-Butyl 2-[(*S*)-(*tert*-Butoxycarbonyl)amino]-3-[(*E*)-3-(ethoxycarbonyl)acrylamido]propionate (6c).** The identical procedure described above for **6a** was used with the following quantities: *N*-hydroxysuccinimide (0.24 g, 2.09 mmol), fumaric acid monoethyl ester (0.30 g, 2.09 mmol), DCC (0.43 g, 2.09 mmol), amine **5** (0.50 g, 1.93 mmol), and dioxane (30 mL). Workup as above gave **6c** as a gum, ca. 98% pure according to TLC (5% MeOH/$CHCl_3$), weighing 1.37 g crude (theory 1.19 g). This was not purified further but used directly in the next step.

Ethyl *N*-(*S*)-(2-Amino-2-carboxyethyl)fumaramate (7c). Compound **6c** (1.37 g, crude) was dissoved in TFA (5.0 mL) and then stirred for 25 min at room temperature. The TFA was evaporated, and the resulting residue was twice triturated with anhydrous Et_2O and evaporated. Trituration again with anhydrous Et_2O and decantation of the solvent gave a solid which was dried in vacuo over P_2O_5 and NaOH. Crystallization from AcOH containing a few drops of H_2O gave nearly white crystalline **7c**, 0.20 g (44% for the two steps), mp 232–234 °C dec, one component on TLC (*n*-BuOH-AcOH-H_2O, 3:1:2, silica gel); $[\alpha]^{24}_D$ −30.7° (*c* 1.07, 1 N HCl). Anal. ($C_9H_{14}N_2O_5$) C, H, N.

***tert*-Butyl 2-[(*S*)-(*tert*-Butoxycarbonyl)amino]-3-acetamidopropionate (6d).** Compound **5** (0.15 g, 0.57 mmol) was dissolved in dry pyridine (3.0 mL), and then acetic anhydride (0.80 mL) was added. After 2 h the excess reagents were removed by evaporation, the residue was treated with H_2O (1.0 mL), and 15 min later $CHCl_3$ (30 mL) was added. The layers were separated, and the organic layer was washed successively with 10% citric acid, 5% Na_2CO_3, H_2O, and saturated salt solution. Evaporation of the solvent gave **6d** (crude 0.17 g) as a colorless gum, one component according to TLC (7% MeOH/$CHCl_3$). This was not purified further but used directly in the next step.

(*S*)-2-Amino-3-acetamidopropionic Acid Hydrobromide (7d). Compound **6d** (0.17 g, crude) was dissolved in 10% HBr in glacial acetic acid (8.0 mL, prepared as described for **7a**) and stirred for 35 min at room temperature. Workup with anhydrous Et_2O as described for **7a** gave a solid, which was crystallized from 2-propanol–Et_2O to give off-white **7d** (0.067 g, 52% for the two steps), mp 172–174 °C. Recrystallization gave the analytical sample, mp 176–177 °C; $[\alpha]^{25}_D$ −41.9° (*c* 1.0, H_2O). Anal. ($C_5H_{11}N_2O_3Br$) H, N; C: calcd, 26.45; found, 26.98.

Preparation of ASase, ASase Assay, and Inhibition. Rats innoculated with Novikoff hepatomas were utilized as the source of ASase. Excised tumors were kindly supplied by Dr. Manford

K. Patterson, Jr. (The Samuel Roberts Noble Foundation, Inc., Ardmore, OK 73401), and ASase was isolated as previously described.[31] The enzyme fraction used in the inhibition studies had a specific activity of 0.51 μmol of asparagine synthesized per milligram of protein in 30 min. L-Aspartic-^{14}C acid was incubated with L-glutamine, ASase, and other needed cofactors, and the L-asparagine-^{14}C synthesized was isolated as previously described.[31] The inhibitors were preincubated with ASase and necessary cofactors and then substrate aspartic acid was added, as outlined in paper 4 of this series.[1a]

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Supplementary Material Available: Comparison infrared spectra of **2a** and **2b** (Figure 1) (1 page). Ordering information is given on any current masthead page.

Methotrexate Analogues. 13. Chemical and Pharmacological Studies on Amide, Hydrazide, and Hydroxamic Acid Derivatives of the Glutamate Side Chain

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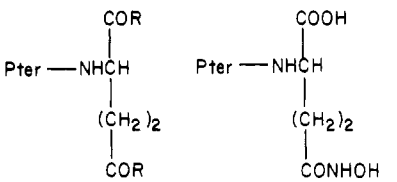
Carbodiimide-mediated condensation of 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA) with several alkyl, aralkyl, and aryl amines, in the presence or absence of *N*-hydroxysuccinimide, was employed in order to prepare new lipid-soluble bis(amide) derivatives of methotrexate (MTX) as potential prodrugs. MTX dianilide was likewise prepared, in comparable yield, from APA and L-glutamic acid dianilide via the mixed carboxylic–carbonic anhydride method. Dihydrazide and bis(*N*-methylhydrazide) derivatives of MTX were formed readily from MTX diethyl ester. However, reaction with hydroxylamine led to MTX γ-monohydroxamic acid as the sole isolated product. The bis adduct appears to form, but is unstable during workup. The identity of the product was confirmed by independent mixed anhydride synthesis from APA and the γ-monohydroxamate of L-glutamic acid. Treatment of MTX dimethyl ester with *N,N*-dimethylhydrazine unexpectedly yielded MTX γ-monomethyl ester. MTX dianilide was active against L1210 leukemia in mice, with a +155% increase in life span at a dose of 160 mg/kg given ip in 10% Tween 80 on a q3d × 3 schedule. The bis(*p*-chlorobenzylamide), bis(*p*-methoxybenzylamide), and dihydrazide were also active against L1210 leukemia in vivo, but to a lesser extent than the dianilide. The γ-monohydroxamic acid derivative showed activity (+111% ILS at 40 mg/kg) similar to that of MTX and was found to bind to a partially purified dihydrofolate reductase preparation from L1210 cells with an ID_{50} of 0.005 μM as compared to 0.007 μM for MTX. In vivo experiments in mice indicated that the pharmacokinetic properties of this compound and of MTX are similar but failed to demonstrate any advantage over MTX in terms of selective uptake into tumor (sc implanted P388 leukemia) or improved penetration of the central nervous system. The activities of the dianilide, bis(benzylamide), and dihydrazide derivatives in vivo are of interest in view of their low toxicity relative to MTX against cells in culture, which suggests that these derivatives are probably acting as prodrugs in the intact animal.

Several previous reports from this laboratory have dealt with the chemical synthesis and biological evaluation of prodrug derivatives of methotrexate (4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid, MTX). Classes of compounds which have been studied include diesters,[1a–e] bis(amides),[2,3] α- and γ-glutamyl conjugates,[4] and more recently a series of monoesters.[5a,b] In this paper we describe several additional examples of the amide type, whose structures (1–11) and methods of synthesis are shown in



1-11 12

Pter = 4-amino-4-deoxy-N^{10}-methylpteroyl

Table I. The monohydroxamic acid **12**, a heretofore unknown MTX analogue differing only in the replacement of the γ-COOH group by γ-CONHOH, was also prepared. Compound **12** was a good inhibitor of dihydrofolate reductase, was moderately toxic to human and mouse leukemic cells in culture, and showed in vivo antitumor activity comparable to that of MTX against L1210 leukemia in mice. Interest in this compound stemmed from the fact that, while the γ-CONHOH group is structurally very

(1) (a) A. Rosowsky, *J. Med. Chem.*, **16**, 1190 (1973); (b) G. A. Curt, J. S. Tobias, R. A. Kramer, A. Rosowsky, L. M. Parker, and M. H. N. Tattersall, *Biochem. Pharmacol.*, **25**, 1943 (1976); (c) A. Rosowsky and C.-S. Yu, in "Chemistry and Biology of Pteridines", R. L. Kisliuk and G. M. Brown, Eds., Elsevier/North Holland, New York, 1979, pp 377–381; (d) G. P. Beardsley, A. Rosowsky, R. P. McCaffrey, and H. T. Abelson, *Biochem. Pharmacol.*, **28**, 3069 (1979); (e) A. Rosowsky, H. Lazarus, G. C. Yuan, W. R. Beltz, L. Mangini, H. T. Abelson, E. J. Modest, and E. Frei III, *ibid.*, **29**, 648 (1980); (f) G. P. Beardsley and A. Rosowsky, *AACR Proc.*, **21**, 264 (1980).

(2) A. Rosowsky, W. D. Ensminger, H. Lazarus, and C.-S. Yu, *J. Med. Chem.*, **20**, 925 (1977).

(3) For related work on bis(amides) of MTX, see the following papers: (a) J. R. Piper and J. A. Montgomery, in "Chemistry and Biology of Pteridines", R. L. Kisliuk and G. M. Brown, Eds., Elsevier/North Holland, New York, 1979, pp 261–265; (b) F. M. Sirotnak, P. L. Chello, J. R. Piper, J. A. Montgomery, and J. I. DeGraw, *ibid.*, pp 597–602.

(4) A. Rosowsky and C.-S. Yu, *J. Med. Chem.*, **21**, 170 (1978).

(5) (a) A. Rosowsky, G. P. Beardsley, W. D. Ensminger, H. Lazarus, and C.-S. Yu, *J. Med. Chem.*, **21**, 380 (1978); (b) H. T. Abelson, G. P. Beardsley, W. D. Ensminger, E. J. Modest, and A. Rosowsky, *AACR Proc.*, **21**, 265 (1980).

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