

Latentiated Forms of the Transport-Inhibitory α -Amino Acid Adamantanine

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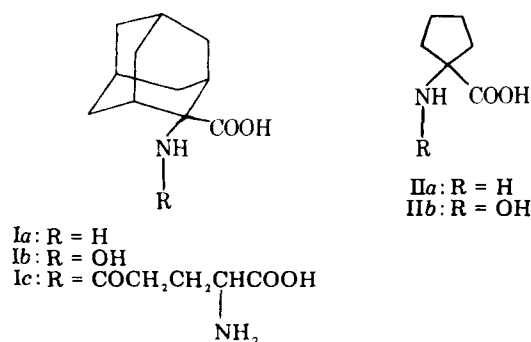
Abstract \square *N*-(γ -L-Glutamyl)adamantanine (Ic) and *N*-hydroxyadamantanine (Ib) were synthesized as latentiated forms of the transport-inhibitory α -amino acid adamantanine (Ia), and their biological properties were evaluated. Inhibition of the growth of P-388 tumor cells by Ib was comparable with that of the antitumor agent *N*-hydroxycycloleucine (IIb). In contrast, Ic was inactive in this system, presumably because it was not a substrate for γ -glutamyltranspeptidase. Nevertheless, the concept proposed here of using the enzyme, γ -glutamyltranspeptidase, to latentiate drugs *in vivo* by synthetic γ -glutamylolation of an amino or hydroxyl group on the drug molecule appears to be worthy of further exploration.

Keyphrases \square Adamantanine—synthesis of latentiated forms and biological evaluation \square Antitumor activity—latentiated forms of adamantanine, synthesis and biological evaluation \square Transport inhibitors—latentiated forms of α -amino acid adamantanine, synthesis

The tricyclo aliphatic α -amino acid adamantanine (2-aminoadamantane-2-carboxylic acid, Ia), a structural analog of the transport-inhibitory and antitumor amino acid cycloleucine (IIa), is highly effective in inhibiting the transport of L-methionine and L-leucine into Ehrlich ascites carcinoma cells *in vitro* (1). However, the bioavailability of Ia is compromised severely by its extreme insolubility. Accordingly, methods to convert Ia systematically to latentiated forms have been sought. The C-terminal and N-terminal glycine and leucine conjugates (dipeptides) of Ia were inactive, reflecting the inertness of these derivatives to cleavage by leucine aminopeptidase (2).

BACKGROUND

α -Amino acids and certain natural products that are conjugated on the amino group as γ -L-glutamyl derivatives can be considered as latentiated forms of the amino acid or natural product, since the γ -glutamyl group can be cleaved *in vivo* by γ -glutamyltranspeptidase, an enzyme that is abundant in transport-active organs such as the kidneys (3). Synthetic *N*-(γ -glutamyl)- α -amino acids were shown to be substrates for γ -glutamyltranspeptidase to varying degrees (4–6). *N*⁴-(1-Hydroxycyclopropyl)-L-glutamine, a compound found in the edible mushroom species *Coprinus atramentarius*, inhibits aldehyde dehydrogenase *in vivo* but not *in vitro* (7). *N*-(γ -L-Glutamyl)dopamine (8) and *N*-(γ -L-glutamyl)levodopa (9), both prodrugs of dopamine, have demonstrable renal vasodilator activities due to the release of the dopamine moiety *in vivo*.



N-Hydroxylated α -amino acids (α -hydroxyamino acids) also can be considered as precursors of the respective amino acids since the latter compounds can be generated on reduction of the hydroxyamino function *in vivo*. The antitumor agent 1-hydroxyaminocyclopentanecarboxylic acid (*N*-hydroxycycloleucine, IIb) is active against Ehrlich ascites carcinoma cells *in vivo* (10) and L-1210 leukemia (11); although no precise data are available to suggest the conversion of IIb to IIa, rat liver contains an enzyme that can reduce *N*-alkylhydroxyamines to the corresponding *N*-alkylamines (12).

Based on these considerations, several derivatives of Ia, the γ -glutamyl derivative *N*-(γ -glutamyl)adamantanine (Ic) and the *N*-hydroxylated analog *N*-hydroxyadamantanine (Ib), were synthesized as latentiated forms. These compounds were evaluated with respect to their ability to be released or converted to Ia by enzymatic action.

EXPERIMENTAL¹

Chemistry—*N*-(γ -L-Glutamyl)adamantanine (Ic) was synthesized by coupling *N*-phthaloyl-L-glutamic anhydride (III) with the *N,O*-bis(trimethylsilyl) derivative of adamantanine (IV), both prepared *in situ* (Scheme I). The procedure for the preparation of III was a modification (13) of the method of King and Kidd (14). Hydrazinolysis of the phthaloyl group of α -*N*-phthaloyl- γ -L-glutamyladamantanine (V) afforded Ic in a 74% yield. The fact that Ic was a γ -glutamyl peptide and not the isomeric α -peptide can be assessed by the method of synthesis, a procedure known to yield γ -glutamyl peptides (15), and was verified by chemical-ionization mass spectrometry².

N-Hydroxyadamantanine (Ib) was prepared *via* a three-step procedure (16–18) from adamantane-2-one oxime (VI) (Scheme II). Addition of hydrogen cyanide to VI gave 2-hydroxyamino-2-cyanoadamantane (VII), which was hydrolyzed successively to the carboxamide (VIII) with cold concentrated sulfuric acid and then to the desired Ib under more vigorous conditions.

Attempts to hydrolyze VII in one step to Ib at elevated temperatures led to considerable decomposition and reversion (19) to adamantane-2-one. Compound Ib was stable to acid but rapidly decomposed above pH 8, an alkaline instability typical for α -hydroxyamino acids (17). Compound Ib also gave a strong color test for hydroxylamine with triphenyltetrazolium chloride (20) and gave a quasimolecular ion (MH⁺, base peak) at the expected *m/e* 212 on chemical-ionization mass spectrometry.

α -N-Phthaloyl- γ -L-glutamyladamantanine (V)—To a solution of *N*-phthaloyl-L-glutamic acid (14) (19.25 g, 0.069 mole) in tetrahydrofuran (250 ml) was added dicyclohexylcarbodiimide (14.33 g, 0.070 mole), and the reaction mixture was stirred at room temperature for 2 hr. The mixture then was filtered. The filtrate was added to a solution of the trimethylsilyl derivative (IV), which was prepared *in situ* by heating Ia (13.56 g, 0.069 mole) and *N,O*-bis(trimethylsilyl)trifluoroacetamide (17.88 g, 0.069 mole) in 300 ml of acetonitrile under reflux for 2 hr. Heating under reflux was continued for 2 hr, and the solvent was evaporated to dryness.

The residue was suspended in 300 ml of water, and the solution was brought to pH 10 with 5% Na₂CO₃ solution. Ether (200 ml) was added

¹ Melting points were determined on a Mettler FP-2 hot-stage apparatus and are corrected. All solvent evaporations were carried out *in vacuo* on a mechanical rotating evaporator using a water aspirator. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn. IR spectra were determined on a Beckman IR-10 IR spectrophotometer, and the chemical-ionization mass spectra were provided by Dr. R. Foltz, Battelle Columbus Laboratories, Columbus, Ohio, using an AEI-MS-902 mass spectrometer equipped with an SRIC model CIS-2 combined chemical-ionization-electron-ionization ion source.

² R. Foltz, Battelle Columbus Laboratories, Columbus, Ohio, personal communication. The diagnostic value of chemical-ionization mass spectrometry for distinguishing γ -glutamyl dipeptides from the isomeric α -glutamyl dipeptides will be discussed in a separate report.

Table I—Formation of γ -Glutamylhydroxamate Catalyzed by γ -Glutamyltranspeptidase

γ -Glutamyl Donor	γ -Glutamylhydroxamate Formed, μ moles/mg of protein/10 min
Ic, 10 mM	<0.02
Glutathione, 10 mM	4.62
Glutathione + Ic, both at 10 mM	4.79

in three successive crops. Since the latter solids were yellow, they were dissolved in 50% ethanol and decolorized with charcoal. The decolorized solution was evaporated to dryness, and the solid residue was recrystallized from ethanol-acetone. This recrystallized product was combined with the 1.25 g of product isolated earlier and once again was recrystallized from ethanol-water to give a powder. This powder was dried under vacuum at 75° to give 2.78 g (63% yield), mp 220–224°; $[\alpha]_D^{25} +18.3^\circ$ (c, 1.0 mg in 1.0 N HCl, calculated as the monohydrate).

Anal.—Calc. for $C_{16}H_{24}N_2O_5 \cdot H_2O$: C, 56.13; H, 7.65; N, 8.18. Found: C, 56.01; H, 7.45; N, 7.87.

2-Hydroxyamino-2-cyanoadamantane (VII)—To a 500-ml glass pressure-reaction bottle were added adamantane-2-one oxime (VI) (21.25 g, 0.15 mole), monobasic potassium phosphate (120 g, 0.88 mole), 100 ml of water, 25 ml of ethanol, and 25.0 g (0.51 mole) of sodium cyanide. The bottle was sealed with a silicone rubber stopper fitted with a dial thermometer and placed in a shaking apparatus. The reaction mixture was heated gradually with shaking to 70° over 30 min, and the temperature was maintained at 70–78° for another 30 min.

The reaction mixture then was cooled, diluted with 2 liters of water, and continuously extracted with chloroform in a liquid-liquid extractor for 4 hr. The chloroform extract was treated with charcoal, and the solvent was evaporated to dryness. The yellow residue was dissolved in methylene chloride, and the solution was treated again with charcoal and filtered. The filtrate was concentrated to ~20 ml and diluted with 40 ml of hexane to give 5.70 g (19.8% yield) of pale-yellow crystals, mp 141–142°; IR (potassium bromide): 3455 (OH), 3270 (NH), and 2210 (CN) cm^{-1} .

Anal.—Calc. for $C_{11}H_{16}N_2O$: C, 68.72; H, 8.39; N, 14.57. Found: C, 68.70; H, 8.39; N, 14.52.

2-Hydroxyaminoadamantane-2-carboxamide (VIII)—Compound VII (2.00 g, 0.010 mole) was added slowly to 10 ml of 36 N H_2SO_4 with stirring, and the mixture was allowed to stand at room temperature for 90 min. The clear solution then was poured into 100 ml of ice-cold water, and the solution was brought carefully to pH 8 with 6 N NaOH. The solids which precipitated were collected, air dried, and then recrystallized from tetrahydrofuran to give 1.65 g (75% yield) of colorless plates, mp 177–178°; IR (potassium bromide): 3430 (OH), 3350, 3320, 3245, 3170 (NH_2 , NH), 1635 (C=O, amide I), and 1600 (NH deformation, amide II) cm^{-1} . An analytical sample was recrystallized from tetrahydrofuran and dried for 18 hr under vacuum, mp 177.5–178.5°.

Anal.—Calc. for $C_{11}H_{16}N_2O_2$: C, 62.83; H, 8.63; N, 13.32. Found: C, 62.94; H, 8.49; N, 13.34.

N-Hydroxyadamantane (Ib)—Compound VIII (0.300 g, 1.43 mmole) and 6 N HCl (3 ml) were added to each of three pressure-reaction tubes. The tubes were sealed and heated for 3 hr at 110°. The cooled reaction mixtures were filtered into a common receiver, and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in 20 ml of water and applied to a column of cation-exchange resin³ (80 g). The column was washed with water until the eluate was chloride free (300 ml) and then eluted with 2 N NH_4OH (400 ml).

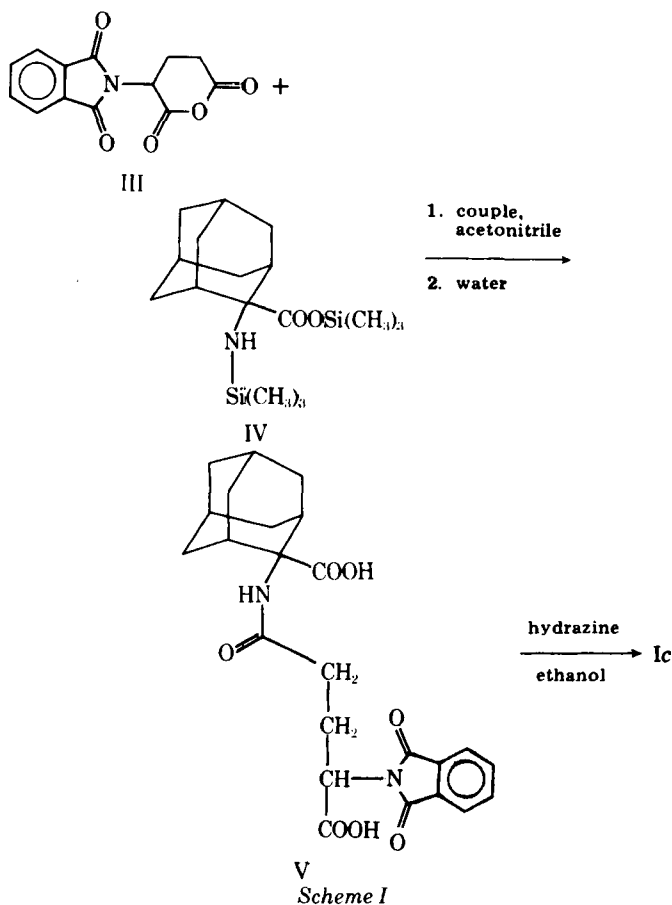
Evaporation of the eluate *in vacuo* gave 170 mg of crude product. This product was dissolved in 5 ml of ethanol, and the solution was decolorized with charcoal. By concentration to 2 ml, dilution with water (1 ml), and further evaporation of the solvent *in vacuo*, crystals precipitated. The crystals were collected and dried under vacuum at 65° for 18 hr to give 0.060 g (6.6% yield), mp 173.5–174.5°. The chemical-ionization mass spectrum of Ib exhibited peaks at m/e (relative intensity) 212 (MH^+ , 100), 196 (16), 166 (25), and 150 (60).

Anal.—Calc. for $C_{11}H_{17}NO_3$: C, 62.54; H, 8.11; N, 6.63. Found: C, 62.54; H, 8.15; N, 6.56.

In Vivo Metabolism Studies—Solutions of Ic dissolved in dilute aqueous hydrochloric acid (2.5 ml, pH 1) were administered orally at 40 and 400 mg/kg to male rats⁴ weighing ~250 g, and 24-hr urine samples were collected in stainless steel metabolism cages. The 24-hr urine sam-

³ Amberlite IRC-50 (H^+).

⁴ Sprague-Dawley strain.

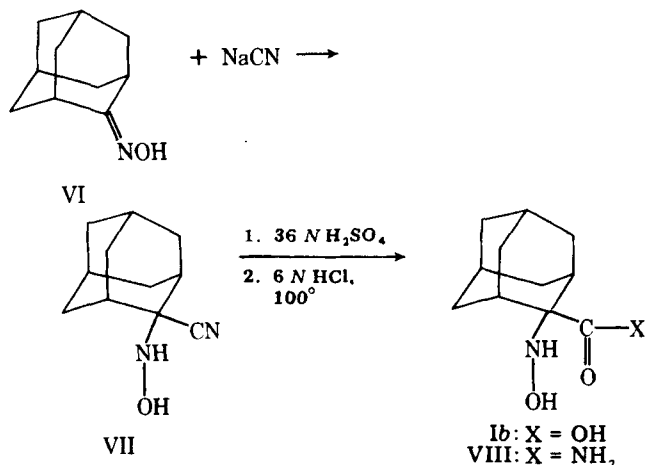


to the cloudy solution, and the three-phase system which contained solids was filtered. The collected solids were dissolved in fresh pH 10 aqueous sodium carbonate, and the solution was extracted with ether. The alkaline aqueous phases were combined, concentrated to remove the ether, and then acidified to pH 3 to precipitate V, which was collected and air dried to yield 23.4 g (74%), mp 135–137°.

Anal.—Calc. for $C_{24}H_{26}N_2O_7 \cdot H_2O$: C, 61.01; H, 5.97; N, 5.93. Found: C, 61.18; H, 5.80; N, 5.94.

N-(γ -L-Glutamyl)adamantanine (Ic)—Compound V (6.00 g, 0.013 mole) was dephthaloylated by heating it with 95% hydrazine (0.440 g, 0.013 mole) in 100 ml of ethanol for 2 hr. After evaporation of the solvent to dryness, the residue was extracted four times with 50 ml of boiling water. The aqueous extracts were combined, heated to boiling, and then filtered to remove residual solids, and the filtrate was evaporated nearly to dryness. The residue was suspended in 50 ml of hot ethanol, and the insoluble solids (1.25 g) were collected by filtration.

Concentration of the filtrate gave a total of 2.70 g of additional solids



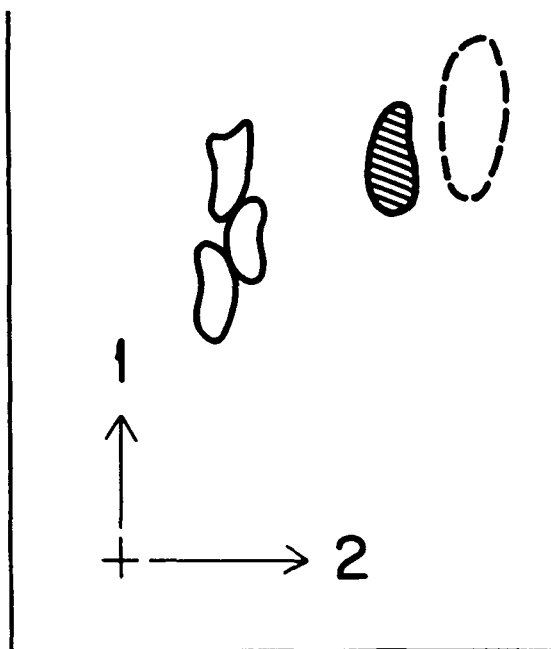


Figure 1—Composite two-dimensional chromatogram of the urine from a rat given 400 mg of γ -glutamyladamantanine (Ic)/kg and of a control urine sample to which Ic (shaded spot) (1 mg/ml) had been added. Solvent 1 was chloroform-methanol-17% ammonia (2:2:1); Solvent 2 was 1-butanol-acetic acid-water (4:1:1). The point of application is represented by +. The shaded spot was not seen in the urine of the rat treated with Ic, the chromatograms being otherwise similar.

ples from each rat prior to administration of the peptide served as control urine. The urine samples were spotted on 8 × 8-cm fluorescent silica gel plates and developed two dimensionally, first in chloroform-methanol-17% ammonia (2:2:1) and then in 1-butanol-acetic acid-water (4:1:1). The chromatograms were visualized with 0.3% ethanolic ninhydrin spray reagent. These chromatograms were compared to those of control urine and control urine to which the peptide was added. The results with the high dose are shown in Fig. 1.

In Vitro Enzyme and Growth Inhibition— γ -Glutamyltranspeptidase was isolated from hog kidney cortex (4), and the transfer of the γ -glutamyl group of Ic to hydroxylamine was determined spectrophotometrically (22). Glutathione, the natural substrate for this enzyme, was used as the standard. The results are recorded in Table I.

Growth inhibition of P-388 lymphoid leukemia cells in tissue culture was determined as described previously (21) using varying concentrations of Ib, Ic, and IIb (Table II). Compound Ic in hydroxypropylcellulose also was administered intraperitoneally to host BDF₁ mice bearing 10⁵ L-1210 lymphoid leukemia cells at doses of 400 mg/kg once daily for 9 days.

RESULTS AND DISCUSSION

Test doses of *N*-(γ -L-glutamyl)adamantanine (Ic) administered to rats at 40 and 400 mg/kg did not give rise to the excretion of the intact dipeptide (Fig. 1), in contrast to the metabolically inert dipeptides, leucyl- and glycyladamantanine, which were excreted intact in the urine (2). This result suggested that the compound was metabolized *in vivo* or else was poorly (or not at all) absorbed from the GI tract. Although Ic gives a strong ninhydrin chromophore (Fig. 1), the amino acid Ia is not chromogenic with ninhydrin or fluorescamine (23); hence, it was not possible to detect the free amino acid in the urine or plasma.

These results encouraged the evaluation of Ic for substrate activity with a partially purified preparation of γ -glutamyltranspeptidase from hog kidney cortex (22). However, Ic did not transfer its γ -glutamyl moiety to the test acceptor hydroxylamine (Table I). Under the same conditions, glutathione, the natural substrate for this enzyme, readily formed γ -glutamylhydroxamate, indicating that the enzyme preparation was active. Moreover, Ic did not inhibit the latter reaction when present at equimolar concentrations with glutathione.

As can be seen from Table II, Ic did not inhibit the growth of P-388 leukemia cells *in vitro*, nor did Ic increase the survival time of host BDF₁ mice bearing 10⁵ L-1210 leukemia cells, even at daily doses of 400 mg/kg

Table II—Inhibition of Growth of P-388 Lymphoid Leukemia Cells *In Vitro*

Compound	I ₅₀	
	$\mu\text{g/ml}$	mM
Ib	168	0.80
Ic	>1000	>3
IIb ^a	50	0.34
<i>N</i> -Hydroxyurea ^b	2.3	0.03

^a Prepared according to a combination of literature procedures (17, 18), mp 196–198° dec. (corrected) [lit. (26) mp 200–202° dec.]. ^b Schwartz/Mann Division, Becton-Dickinson and Co., Orangeburg, N.Y.

for 9 days (data not shown). However, *N*-hydroxyadamantanine (Ib) had activity comparable to *N*-hydroxycycloleucine (IIb) in the P-388 system (Table II), although these compounds were nearly 10 times less active in this system than *N*-hydroxyurea, which was the standard test compound.

The lack of substrate activity of Ic for γ -glutamyltranspeptidase probably is due to steric hindrance at the peptide bond. Various glycine and leucine dipeptides of adamantanine (both N-terminal and C-terminal) also are resistant to hydrolysis by leucine aminopeptidase (2). Meister (24) showed that γ -glutamyl derivatives of α -alkyl-substituted α -amino acids, e.g., γ -glutamyl- α -aminoisobutyrate, are not good substrates for γ -glutamyltranspeptidase. Since Ia also may be considered as α -alkyl substituted, the present example provided by Ic reinforces this observation.

Although the latentiation of Ia as peptide derivatives has not yet been achieved, perhaps due to the unique structure of this amino acid, the concept of γ -glutamyltranspeptidase-mediated amino acid release should be capable of extension appropriate to the latentiation of biologically active molecules other than amino acids, e.g., drugs. By utilizing this enzyme from the γ -glutamyl cycle (24), selected drugs may be transported *in vivo* as chemically conjugated synthetic γ -glutamyl derivatives with subsequent release at target organ sites. The successful use of this approach to the development of dopamine prodrugs was alluded to previously (8, 9). For the moiety attached to the γ -glutamyl end of the molecule, there is a low order of specificity. For example, whereas the natural substrate is glutathione itself, γ -L- or γ -D-glutamyl-*p*-nitroanilide (4) and *S*-substituted glutathione derivatives (25) are active substrates for the transpeptidase. Furthermore, the amide linkage is not mandatory since γ -esters of L-glutamic acid also serve as substrates (4). Since a large number of pharmacological agents possess free amino or hydroxyl groups, the applications of this concept appear to be unlimited. These possibilities are under study.

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Anti-Inflammatory Activity of Amine Cyanoboranes, Amine Carboxyboranes, and Related Compounds

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Abstract □ Amine cyanoboranes and amine carboxyboranes (boron analogs of α -amino acids) were shown to inhibit inflammation. The analogs effectively blocked general inflammation, induced arthritis, and the writhing reflex associated with inflammation pain, while the inflammation associated with pleurisy was marginally inhibited. The boron analogs were shown *in vitro* to inhibit the release of lysosomal enzymes from liver and polymorphonuclear neutrophils. Furthermore, prostaglandin synthesis was blocked by these agents at a low concentration, *i.e.*, 10^{-6} M. Liver oxidative phosphorylation processes also were uncoupled by these agents, but the migration of polymorphonuclear neutrophils was unaltered at 10^{-4} M. The elevation of cyclic adenosine monophosphate levels in polymorphonuclear neutrophils correlated positively with *in vivo* antiarthritic activity. Initial studies in rodents demonstrated that these boron analogs can be used at safe therapeutic doses.

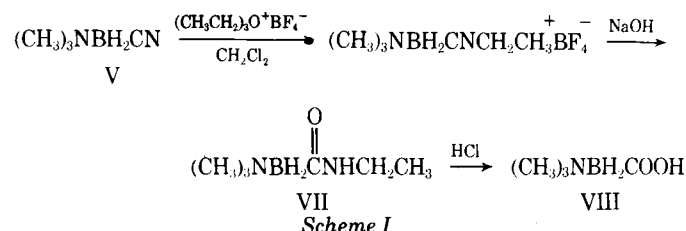
Keyphrases □ Anti-inflammatory activity—evaluation of amine cyanoboranes, amine carboxyboranes, and related compounds □ Amine cyanoboranes and amine carboxyboranes—evaluation for anti-inflammatory activity

The antineoplastic activity of some α -amino boron analogs was reported previously (1). While studying their metabolic effects on tumor cell metabolism, it was noted that these agents interfered with oxidative phosphorylation processes of mitochondria, inhibited lysosomal enzymatic hydrolytic activities, and elevated cyclic adenosine monophosphate levels. Since commercially available anti-inflammatory agents, *e.g.*, phenylbutazone, salicylates, and indomethacin, have similar effects on cellular metabolism, testing of the boron analogs for anti-inflammatory activity in rodents was undertaken and the data are reported here.

EXPERIMENTAL

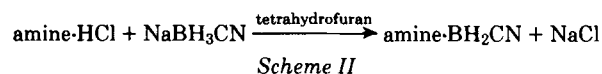
Chemistry—Consideration of the isoelectronic formalism between carbon and boron results in the prediction of boron analogs of dipolar α -amino acids, *e.g.*, glycine ammonia carboxyborane, alanine ammonia carboxymethylborane, and betaine triethylamine carboxyborane. Interest in these boron analogs lies mainly in their potential biological activity when compared with the enormous biological activity of the α -amino

acids. A highly significant step toward demonstrating the existence of this class of compounds was the synthesis of trimethylamine carboxyborane, the protonated boron analog of betaine (2) (Scheme I).



Trimethylamine carboxyborane, a white, crystalline solid whose X-ray crystal structure was determined, is stable in air and water.

Amine Cyanoboranes—One approach to the synthesis of boron analogs of the α -amino acids involves the conversion of an amine cyanoborane to a boroamino acid according to the procedure outlined in Scheme I. To provide adequate quantities of the precursor amine cyanoboranes, a general, convenient, high-yield synthesis (3, 4) of this class of compounds was developed (Scheme II).



All previous syntheses (5–9) of amine cyanoboranes were limited as to the yield and reaction scale. Several synthetic procedures were developed (5, 6) following initial reports from these laboratories (10, 11) on the preparation of cyanoborane oligomers, *i.e.*, $(\text{BH}_2\text{CN})_x$, by the addition of dry hydrogen chloride to a solution containing cyanohydroborate in ether. Thus, addition of amines to solutions of cyanoborane [which also can be prepared (8) by the reaction of cyanohydroborate and halogens] gives the amine cyanoborane. Typical yields have been ~25%, whereas yields up to 90% have been obtained with the amine hydrochloride procedure.

Following Scheme II, amine cyanoboranes were prepared where the amine was trimethylamine (V), dimethylamine (VI), pyridine (XV), 3-dimethylaminopropionitrile (XIX), or *N*-methylmorpholine (XIV). Bis(cyanoboranes) (XI and XX) were prepared (4) from ethylenediamine and tetramethylethylenediamine. However, attempts to prepare the parent ammonia cyanoborane (XVII) by this procedure were not successful. The only other report of the preparation of XVII (7) involved the reaction of trimethylamine iodoborane (IX) with sodium cyanide in liquid ammonia. Attempts to repeat this reaction in these laboratories resulted