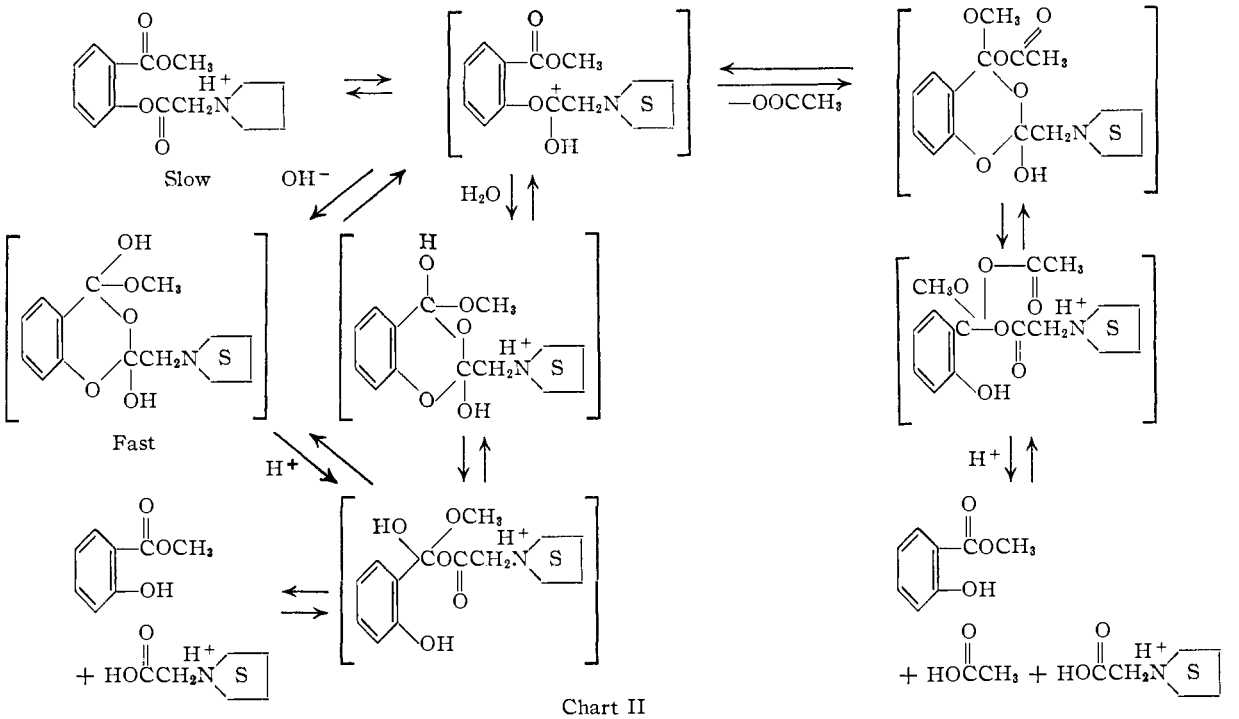


Chart II

tivation of the pyrrolidylacetyl carbonyl oxygen by the proton of the protonated nitrogen (either by adjacency[11] or direct transfer) (Chart I).

If the *o*-methyl carboxylate grouping is not involved in the mechanism, then general base catalysis may be expected in the hydrolysis of a dialkylaminoacetylphenate.

If the *o*-methyl carboxylate grouping is involved in the mechanism, an intramolecular condensation[4,12] may be postulated prior to hydrolysis. Of the two possible carbonyl activations, the most probable is in the pyrrolidylacetyl grouping as in II.

Acknowledgment.—Grateful appreciation is given to Dr. Fred Kagan and R. D. Birkenmeyer for the synthesis of the ester, to Miss Susan Theal for some of the titrations, and to Miss Kathryn Stimson for excellent technical assistance.

KALAMAZOO, MICH.

catalyzed hydrolysis by acetate ion. It also is consistent with their postulate that general base catalysis is of importance only for esters containing an alcohol that is a reasonably strong acid (pK_a <11), *i.e.*, methyl salicylate.

(11) The activation of the pyrrolidylacetyl carbonyl may also be by adjacency, *i.e.* as in preceding formula A.

(12) The possibility of a cyclic mechanism for salicylate ester hydrolysis cannot be ignored since this facilitates the explanation of the non-general base (*i.e.*, non-acetate) catalyzed "spontaneous hydrolysis" peculiar to acyl salicylates. (See Ref. (4) and references therein.)

[CONTRIBUTION FROM THE CLAYTON FOUNDATION FOR RESEARCH, THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS]

Synthesis of 2-Cyclohexene-1-glycine and 1-Cyclohexene-1-alanine, Inhibitory Amino Acid Analogs

BY JEROME EDELSON, PRABHAT R. PAL,[1] CHARLES G. SKINNER AND WILLIAM SHIVE

RECEIVED MARCH 11, 1957

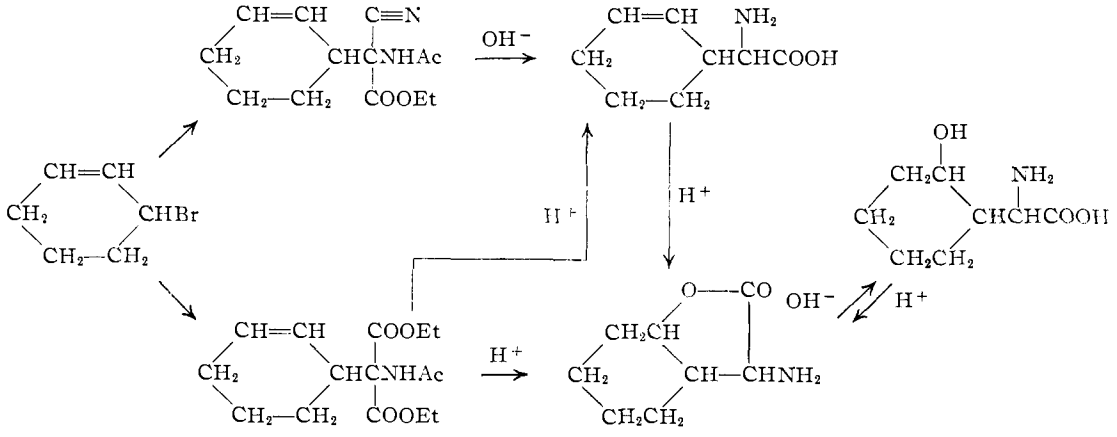
2-Cyclohexene-1-glycine and 1-cyclohexene-1-alanine were synthesized through the condensation of acetamidocyanoacetic ester and the appropriate halide followed by alkaline hydrolysis. In contrast tc the corresponding cyclohexane derivatives which were ineffective, 2-cyclohexene-1-glycine was found to be an antagonist of isoleucine for *Escherichia coli* 9723, and 1-cyclohexene-1-alanine was an antagonist of phenylalanine for *Leuconostoc dextranicum* 8086. The specificities of these amino acid antagonists have been correlated with the steric relationship of the cyclohexene group relative to the corresponding group of the natural amino acids.

Cyclopentaneglycine specifically inhibits the utilization of isoleucine[2] but 2-cyclopentene-1-glycine appears to be an antagonist of both isoleucine and valine in *Escherichia coli*.[3] The specificity of the cyclopentane analog as an isoleucine antagonist has been attributed to the slightly nonplanar structure of the cyclopentane ring. In *Leuconostoc dextranicum* 8086, cyclopentanealanine inhibits the utilization of leucine but not phenylalanine; this is in contrast to 1-cyclopentene-1-alanine which is an antagonist of phenylalanine but

(1) Rosalie B. Hite post-doctoral fellow, 1955–1956.

(2) W. M. Harding and W. Shive, *J. Biol. Chem.*, **206**, 401 (1954).

(3) R. L. Dennis, W. J. Plant, C. G. Skinner, G. L. Sutherland and W. Shive, THIS JOURNAL, **77**, 2362 (1955).



not of leucine.[4] The specificity of these amino acid analogs appears to result from whether or not the side chain emanates in the same plane as the ring.

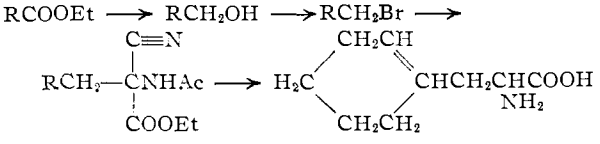
In an effort to compare the effect on biological activity of the incorporation of a double bond into a cyclohexane ring, the synthesis of 2-cyclohexene-1-glycine and 1-cyclohexene-1-alanine and a study of their biological activities in comparison with the corresponding cyclohexane derivatives were undertaken.

The synthesis of 2-cyclohexene-1-glycine was carried out as indicated in the accompanying equations.

3-Bromocyclohexene was condensed with either acetamidomalonic ester or acetamidocyanoacetic ester to form the corresponding cyclohexene derivatives. Alkaline hydrolysis of the substituted cyanoacetic ester afforded, after acidification, the desired 2-cyclohexene-1-glycine. Acid hydrolysis of the crude substituted malonic ester gave a considerable amount of the lactonized product of 2-cyclohexene-1-glycine which was converted to the 2-hydroxycyclohexaneglycine in alkaline solution. Although some cyclohexeneglycine was formed, it was difficult to separate it from the other products. 2-Cyclohexene-1-glycine from the cyanoacetic ester preparation readily was converted by treatment at room temperature with concentrated acid to the lactonized product.[5] The lactone is assigned the gamma rather than the delta structure by analogy with the reported ease of γ-lactone formation observed with γ,δ-unsaturated-α-amino acids,[6] and on the basis of its infrared absorption spectrum. The lactonized product isolated in this synthesis has an absorption band at about 5.67 μ which is in agreement with a γ- rather than a δ-lactone structure.[7] The lactone obtained from either acid hydrolysis of the malonic ester derivative or acid treatment of the unsaturated amino acid gave identical R_f values on paper chromatograms in several different solvents. Since the lactone of *cis*-2-hydroxycyclohexaneacetic acid forms more readily and is more stable than the corresponding *trans*-lactone,[8] the lactone formed from 2-cyclohexene-1-glycine is more probably the *cis* form.

Some further evidence concerning the steric configuration of 2-cyclohexene-1-glycine is afforded by the fact that, as subsequently shown, the compound is a competitive antagonist of isoleucine. It appears likely that the configuration on the β-carbon is analogous to that of isoleucine with the 5- and 6-methylene carbons of the cyclohexene ring corresponding to the ethyl group of isoleucine. Such an assumption would allow the complete assignment of configuration to the various derivatives.

1-Cyclohexene-1-alanine was prepared by the indicated sequence of reactions, in which R represents a 1-cyclohexenyl group.



Ethyl 1-cyclohexene-1-carboxylate was reduced with lithium aluminum hydride to 1-cyclohexene-1-carbinol which was converted by treatment with phosphorus tribromide in pyridine at $-40°$ to the corresponding bromide. The halide was condensed with ethyl acetamidocyanoacetate, and the condensation product was then subjected to alkaline hydrolysis to obtain upon acidification the desired amino acid.

In microbiological studies, 2-cyclohexene-1-glycine was found to inhibit the growth of *Escherichia coli*, and the growth inhibition is reversed in a competitive manner by isoleucine as indicated in Table I. In separate assays, 2-cyclohexene-1-glycine was found to be about half as effective in inhibiting the utilization of isoleucine as cyclopentaneglycine. On the other hand, cyclohexaneglycine[9] was ineffective as an inhibitor even at relatively high concentrations.

The introduction of a double bond into the 2-position of cyclopentaneglycine has been reported to result in an inhibitor which is reversed competitively by a mixture of isoleucine and valine but not by either alone. Thus, the specificity of cyclo-

(4) P. R. Pal, C. G. Skinner, R. L. Dennis and W. Shive, THIS JOURNAL, **78**, 5116 (1956).

(5) These data are in agreement with the observed behavior of crotylglycine as reported by H. L. Goering, S. J. Cristol and K. Dittmar, *ibid.*, **70**, 3310 (1948), in which they state that hydrolysis of the corresponding malonic ester derivative in acid gave poor yields "of the amino acid presumably due to lactonization."

(6) J. Fillman and N. Albertson, *ibid.*, **70**, 171 (1948).

(7) R. S. Rasmussen and R. R. Brattain, *ibid.*, **71**, 1073 (1949).

(8) M. S. Newman and C. A. VanderWerf, *ibid.*, **67**, 233 (1945).

(9) J. D. Fissekis, J. M. Ravel, C. G. Skinner and W. Shive, unpublished data.

pentaneglycine as an isoleucine antagonist appears to be the result of slightly puckered structure of the cyclopentane ring which resembles structurally the non-planar *sec*-butyl group of isoleucine but not the planar isopropyl group of valine; and, the introduction of a double bond in the ring results in a planar ring permitting the analog to antagonize both isoleucine and valine. It would be anticipated that the structure of the 2-cyclohexenyl group is such that carbons 1, 2, 3 and 4 would be planar while carbons 5 and 6 could either be both on the same side of the plane containing carbons 1, 2, 3 and 4; or, one on one side and one on the other. The latter structure would greatly resemble the cyclopentane structure with only the two carbons 5 and 6 out of planar configuration to a greater extent than the slightly puckered cyclopentane ring. On the other hand, the former structure would more closely resemble the boat form of cyclohexaneglycine. Since cyclohexaneglycine is ineffective as an inhibitor of isoleucine in this biological system, it would appear that the more probable structure of the cyclohexene ring in the biologically active form of 2-cyclohexane-1-glycine is that with carbons 5 and 6 on opposite sides of the plane of carbons 1, 2, 3 and 4. It is interesting to speculate that a microbiological method could be applied to the determination of the relative concentrations of the two possible steric configurations of such compounds.

TABLE I

REVERSAL OF INHIBITION OF 2-CYCLOHEXENE-1-GLYCINE BY ISOLEUCINE IN *Escherichia coli* 9723[a]

Compound, γ/5 ml.	DL-Isoleucine, γ/5 ml. assay 0	3	10	30
	Galvanometer readings[b]			
0	74	75	75	75
30	73	75		
100	74	73		
300	14	72	75	75
1000		14	60	69
3000			10	40
10000				5

[a] Incubated 18 hours at 37°. [b] A measure of culture turbidity; distilled water reads 0, an opaque object 100.

1-Cyclohexene-1-alanine inhibits the growth of *Leuconostoc dextranicum* 8086, and phenylalanine reverses the inhibition in a competitive manner as indicated in Table II. The inhibition index (the ratio of inhibitor to metabolite necessary for inhibition of growth) is approximately 50, and in separate assay 1-cyclohexene-1-alanine was found to be about equally effective as 1-cyclopentene-1-alanine in inhibiting growth of this organism. Since cyclopentanealanine is an antagonist of leucine rather than phenylalanine, it appears that the emanation of the alanine side chain in the same plane as the 1, 2 and 6 carbons of the ring is necessary for antagonism of phenylalanine. By contrast, cyclopentanealanine, a leucine antagonist, does not have the side chain emanating in the same plane as the ring and is structurally similar to isoleucine in this regard. Further, cyclohexanealanine does not exert any inhibitory effect on the growth of *L. dextranicum* even at relatively high concentrations.[9] The highly puckered cyclohexane ring is thus not sufficiently structurally similar to the isopropyl group of leucine to allow cyclohexanealanine to compete for the specific sites at which leucine interacts with enzymes.

TABLE II

REVERSAL OF INHIBITION OF 1-CYCLOHEXENE-1-ALANINE BY PHENYLALANINE IN *Leuconostoc dextranicum* 8086[a]

1-Cyclohexene-1-DL-alanine, γ/5 ml.	DL-Phenylanaine, γ/5 ml. 0	10	20	50	100
	Galvanometer readings[b]				
0	61	64	63	64	63
10	52				
20	50	62			
50	26	55	62		
100	17	46	56	62	
200	11	32	47	59	63
500	5	9	17	44	58
1000		3	7	23	42
2000			2	3	15
5000				1	2

[a] Incubated for 18 hours at 30°. [b] A measure of culture turbidity; distilled water reads 0, an opaque object, 100.

The incorporation of a double bond into two inactive compounds, cyclohexaneglycine and cyclohexanealanine, results in the formation of two very active amino acid inhibitors, 2-cyclohexene-1-glycine and 1-cyclohexene-1-alanine. These latter analogs inhibit the utilization of isoleucine and phenylalanine, respectively, and their biological activities apparently result from structural changes in the molecules so that they are no longer sterically hindered from combination with active sites. Even though the presence of a double bond in the cycloalkyl group may have some effect, the major factor in conferring enzyme specificity to the amino acid analogs appears to be the steric configuration of the molecules.

Experimental[10,11]

Biological Assays.—For assays with *Leuconostoc dextranicum* 8086 tyrosine and phenylalanine were omitted from a previously described amino acid medium,[12] and the medium was further modified by adding 0.1 γ of pantethine per 5-ml. assay tube, decreasing the concentration of leucine to 50 γ and tryptophan to 20 γ per 5-ml. assay tube, and by increasing the salts A concentration fourfold. Calcium pantothenate (3 mg. per 30 ml. of vitamin supplement), which was inadvertently omitted from the list of constituents of the basal medium previously described, was added.

For *Escherichia coli* 9723, a previously described inorganic salts–glucose medium[13] was employed. This assay technique recently has been reported in detail.[14]

In all assays, the inhibitors were dissolved in sterile water and added to the sterile assay tubes without being heated.

γ-Lactone of *cis*-2-Hydroxycyclohexane-1-glycine Hydrochloride.—Diethyl α-acetamido-2-cyclohexene-1-malonate was prepared by the typical malonic ester procedure. To a cooled solution of 5.05 g. of sodium in 200 ml. of absolute ethanol, 48 g. of diethyl acetamidomalonate was added with stirring. The reaction mixture was cooled in an ice-bath.

(10) All melting points were determined on a Fisher–Johns melting point block and are uncorrected.

(11) The authors are indebted to Dr. J. M. Ravel and her staff at the Biochemical Institute, The University of Texas, Austin, Texas, for the biological testing data, and to Mr. J. R. Claybrook and F. D. Talbert for the chemical analyses.

(12) J. M. Ravel, L. Woods, B. Felsing and W. Shive, *J. Biol. Chem.*, **206**, 391 (1954).

(13) E. H. Anderson, *Proc. Natl. Acad. Sci.*, **32**, 120 (1946).

(14) F. W. Dunn, J. M. Ravel and W. Shive, *J. Biol. Chem.*, **219**, 810 (1956).

and 37.7 g. of 3-bromocyclohexene[15] was added over a period of 30 minutes. The reaction mixture was then allowed to come to room temperature and stirred an additional hour. After the sodium bromide was removed by filtration, the solution was treated with Darco G-60 and filtered through a Celite mat. The filtrate was finally evaporated *in vacuo* to yield a colorless oil. All attempts to crystallize this intermediate failed to yield a solid product.

A mixture of 10 g. of the above oil and 100 ml. of 8 *N* hydrochloric acid was heated on a steam-bath overnight. The resulting solution was evaporated *in vacuo* to yield a yellow powder. Excess hydrochloric acid was removed by the repeated addition of ethanol and evaporation under reduced pressure. To 1.3 g. of the yellow powder, enough absolute ethanol was added to effect solution. Diethyl ether was then added until the solution became faintly turbid, and the sample was refrigerated overnight to yield a crystalline material. The crystals were removed and recrystallized from ethanol–ether. There was recovered 200 mg. of product, m.p. 246°.

Anal. Calcd. for $C_8H_{14}NO_2Cl$; C, 50.13; H, 7.36. Found: C, 50.27; H, 7.34.

A paper chromatogram by the ascending technique, after treatment with ninhydrin, showed only one yellow spot; R_f 0.66, in 95% methanol; R_f 0.63, in butanol:acetic acid:water (4:1:1); R_f 0.82, in 65% pyridine.

The infrared spectrum of this compound produced the following major absorption peaks (wave lengths in μ; intensity in units of percentage transmission using a potassium bromide pellet containing approximately 0.5% concentration of the compound): 2.95 (35), 3.45 (29), 3.55 (30), 5.67 (11), 6.70 (52), 8.32 (27), 10.47 (31). The area of absorption attributed to the carbonyl of a five-membered lactone ring is 5.65 μ.[7]

This lactone after being dissolved in 10% sodium hydroxide solution and heated on a steam-cone for three hours produced a new compound which was identified by paper chromatography in the above three developing solvent systems as being 2-hydroxycyclohexane-1-glycine.

***cis*-2-Hydroxycyclohexane-1-glycine.**—Ten ml. of 10% sodium hydroxide was added to a 500-mg. sample of the above lactone. The reaction mixture was heated on a steam-cone for four hours, and then neutralized with concentrated hydrochloric acid. The solid that separated was recrystallized from alcohol–water to yield 130 mg. of product, which starts decomposing about 280°, m.p. above 300°.

Anal. Calcd. for $C_8H_{15}NO_3$: C, 55.47; H, 8.73; N, 8.07. Found: C, 55.78; H, 8.43; N, 8.35.

A paper chromatogram by the ascending technique, after treatment with ninhydrin, showed one brownish spot; R_f 0.46, in 95% methanol; R_f 0.53, in butanol–acetic–water (4:1:1); R_f 0.72, in 65% pyridine. A sample of this compound was dissolved in concentrated hydrochloric acid and allowed to stand at room temperature for several days. Paper chromatograms of the resulting solution showed a single ninhydrin spot in the above solvents which was identical with the γ-lactone derivative described above.

Ethyl α-Acetamido-α-cyano-2-cyclohexene-1-acetate.—To a solution of 5.7 g. of sodium in 200 ml. of ethanol, 35.0 g. of ethyl acetamidocyanoacetate was added; then to the cooled reaction mixture, 47.1 g. of 3-bromocyclohexene was added over a period of 30 minutes. The sodium bromide was removed by filtration, and the filtrate was poured into ice-water to make a total volume of two liters. This mixture was allowed to remain four hours in the cold before the crystals were removed. Recrystallization from 50% acetone yielded 28 g. of product, m.p. 146–148°.

Anal. Calcd. for $C_{13}H_{18}N_2O_3$: C, 62.38; H, 7.26; N, 11.19. Found: C, 62.32; H, 7.00; N, 11.21.

2-Cyclohexene-1-glycine.—A 3.0-g. sample of ethyl α-acetamido-α-cyano-2-cyclohexene-1-acetate was heated to reflux for 24 hours in 25 ml. of 10% sodium hydroxide. The insoluble precipitate formed was removed by filtration, and the filtrate was neutralized with concentrated hydrochloric acid to *p*H of 6. The crude product that precipitated was recrystallized from water to yield 620 mg. (33%) of white flakes, which started decomposing at about 205°, m.p. 290–293° dec.

Anal. Calcd. for $C_8H_{13}NO_2$: C, 61.90; H, 8.44; N, 9.03. Found: C, 61.86; H, 8.10; N, 9.24.

A paper chromatogram, by the ascending technique, after treatment with ninhydrin, showed one greenish spot; R_f 0.64, in 95% methanol; R_f 0.66, in butanol–acetic–water (4:1:1); R_f 0.73, in 65% pyridine. A sample of this compound dissolved in water was found to decolorize bromine–water and reduced a permanganate solution.

When a sample of 2-cyclohexene-1-glycine was dissolved in concentrated hydrochloric acid and allowed to stand at room temperature for several days, the resulting solution was found to contain, on developing a paper chromatograph, two ninhydrin spots. These spots were identified in three different solvent systems as being unchanged 2-cyclohexene-1-glycine and the corresponding γ-lactone derivative described above.

The N-benzoyl derivative was prepared in the usual manner, m.p. 171–172°.

Anal. Calcd. for $C_{15}H_{17}NO_3$: N, 5.40. Found: N, 5.64.

Ethyl 1-Cyclohexenemethyl-acetamidocyanoacetate.—To a sample of 0.41 g. of sodium dissolved in 15 ml. of ethanol was added 3.0 g. of ethyl acetamidocyanoacetate, and the mixture was warmed to effect solution. Then, 3.0 g. of 1-bromomethyl-1-cyclohexene[16] was added and the mixture was stirred for one hour, following which it was heated to reflux for an additional 6 hours. The reaction mixture was then poured over ice-water, and an oil separated which eventually solidified to yield a crystalline mass (3.0 g.). After crystallization from 50% aqueous acetone there was recovered 2.1 g. of product, m.p. 123–124°.

Anal. Calcd. for $C_{14}H_{20}N_2O_3$: C, 63.61; H, 7.63. Found: C, 63.87; H, 7.46.

An aqueous solution of this compound decolorized both bromine water and neutral permanganate solution.

1-Cyclohexene-1-DL-alanine.—A solution of 1.8 g. of the cyanoacetamido derivative above was suspended in 20 ml. of 10% sodium hydroxide solution and heated to reflux for 20 hours. The cooled solution was acidified with concentrated hydrochloric acid to *p*H 6.5, and the precipitated amino acid was recovered by filtration. The filtrate was reduced in volume to yield a second crop of crystals. The total yield of crude material was 1.36 g. The product was dissolved in hot water and treated with Darco G-60. On cooling there was recovered 1.1 g. of product, which started decomposing at 215°; m.p. 227°.

Anal. Calcd. for $C_9H_{15}NO_2$: C, 63.88; H, 8.94; N, 8.28. Found: C, 64.18; H, 8.89; N, 8.31.

AUSTIN 12, TEXAS

(15) K. Ziegler, A. Spath, E. Schaaf, W. Schumann and E. Winkelmann, *Ann.*, **551**, 110 (1942).

(16) M. Mousseron and F. Winternitz, *Bull. soc. chim.*, 604 (1946). Our sample, b.p. 94° (27 mm.), $n^{25}D$ 1.5109, was prepared by the action of phosphorus tribromide on the corresponding carbinol, b.p. 75–77° (7 mm.), $n^{25}D$ 1.4890 (reported 1.4905, 1.4881, THIS JOURNAL, **75**, 939 (1953)), in turn prepared by the action of lithium aluminum hydride on ethyl 1-cyclohexene-1-carboxylate.