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Cyclopentanealanine and 1-Cyclopentene-1-alanine, Inhibitory Analogs of Leucine and Phenylalanine

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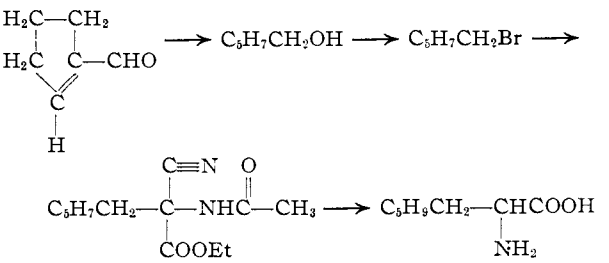
Cyclopentanealanine was prepared by the condensation of cyclopentylmethyl bromide with ethyl acetamidomalonate or ethyl acetamidocyanoacetate followed by hydrolysis and decarboxylation of the intermediate, and 1-cyclopentene-1-alanine was similarly prepared from 1-cyclopentene-1-methyl bromide and ethyl acetamidocyanoacetate. The introduction of a double bond in the cyclopentane ring at the point of attachment of the alanine side chain converts the amino acid analog from a leucine antagonist (cyclopentanealanine) to a phenylalanine antagonist (1-cyclopentene-1-alanine). Although the double bond itself may have some effect, the steric configuration appears to be of prime importance in conferring enzyme specificity to the analogs.

Cyclopentaneglycine inhibits specifically the utilization of isoleucine in many organisms,[3] and the toxicity of 2-cyclopentene-1-glycine is reversed in a competitive manner by a mixture of isoleucine and valine but not by either amino acid alone.[4] It was proposed that the cyclopentane group, which is not completely planar, is structurally more similar to the *sec*-butyl group of isoleucine than the planar isopropyl group of valine, and that the introduction of a double bond in the ring results in a more planar group which allows its glycine derivative also to compete with valine for essential enzymes.

In order to study further the structural features essential for selective combinations of amino acids with enzymes, the synthesis and biological testing of cyclopentanealanine and of 1-cyclopentene-1-alanine was undertaken. The former compound resembles leucine structurally, but since the ring is not completely planar it should not be more than a moderately active antagonist of leucine. The latter compound, however, has the first carbon of the side chain in the same plane as the cyclopentene ring. Thus, this compound would structurally resemble phenylalanine rather than leucine.

Cyclopentanealanine was prepared by alkylation of either ethyl acetamidomalonate or ethyl acetamidocyanoacetate in the usual manner with cyclopentylmethyl bromide using sodium ethylate. Both acid hydrolysis of the cyclopentyl derivative of acetamidomalonic ester and strong alkaline hydrolysis of the cyclopentyl derivative of ethyl acetamidocyanoacetate yielded cyclopentanealanine. The products from the two reactions had identical melting points and R_f values on paper chromatograms in various solvents. Mild alkaline hydrolysis of the acetamidocyanoacetate derivative produced the N-acetyl derivative of cyclopentanealanine which was subsequently hydrolyzed to the amino acid. Cyclopentanealanine was also converted to the corresponding hydantoic acid *via* potassium cyanate.

The synthesis of (1-cyclopentene-1-alanine) was effected through the series of reactions indicated. 1-Cyclopentene-1-carboxaldehyde was reduced with sodium borohydride to the corresponding unsaturated alcohol in 82% yield,[5] which was subsequently converted to 1-cyclopentene-1-methyl bromide with phosphorus tribromide in pyridine.



1-Cyclopentene-1-methyl bromide was condensed with ethyl acetamidocyanoacetate under typical conditions. The product was then hydrolyzed in dilute sodium hydroxide to yield 1-cyclopentene-1-alanine. This amino acid decolorized bromine water, and when developed by paper chromatography using the ascending technique (70 parts *sec*-butyl alcohol:10 parts formic acid:20 parts

TABLE I

REVERSAL OF INHIBITION OF CYCLOPENTANEALANINE BY LEUCINE

Cyclopentane-DL-alanine, γ per 5 ml.	Galvanometer readings[a] DL-Leucine, γ per 5 ml. 20	50	100	200	500
		Streptococcus lactis 8039[b]			
0		54	52	55	56
1000		39			
2000		4	49	59	59
5000			7	45	21
10000				2	3
		Leuconostoc dextranicum 8086[c]			
0	60	66	67	67	
500	31				
1000	14				
2000	10	56	55	61	
5000	5	39	50	50	
10000		3	21	21	
20000			6	9	

[a] A measure of culture turbidity; distilled water reads 0, an opaque object 100. [b] Incubated 25 hours at 30°. [c] Incubated 16 hours at 30°, medium contains 10 γ DL-phenylalanine per 5 ml.

(1) Rosalie B. Hite Postdoctoral Fellow, 1955–1956.

(2) In part from a thesis submitted by Robert L. Dennis to the Graduate School, The University of Texas, in partial fulfillment of the requirements for the degree of Master of Arts, August, 1951.

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

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

(5) This may be compared to the procedure of E. Urion, *Compt. rend.*, **198**, 1518 (1934), wherein this aldehyde was reduced with a zinc–copper couple in 40% yield.

water) followed by treatment with the ninhydrin reagent, gave a yellow spot, R_f 0.84.

Cyclopentanealanine inhibits the growth of *Streptococcus lactis* 8039 and *Leuconostoc dextranicum* 8086 as indicated in Table I. Leucine reverses the toxicity for both organisms over a range of concentrations, but at high inhibitor concentrations (10 to 20 mg. per 5 ml.) the inhibition by cyclopentanealanine is not reversed by leucine. For *S. lactis*, high concentrations of leucine act synergistically with the inhibitor in further reducing growth. The inhibition indices are approximately 50 to 200 for *S. lactis* and *L. dextranicum*, respectively. The latter organism is comparatively more sensitive to cyclopentanealanine at low concentrations of leucine than would be anticipated from the results at higher concentrations of leucine. This may result from a synergistic action of the inhibitor with valine and isoleucine which are maintained at a constant concentration of 200 γ of the DL-forms per 5 ml. and which at higher concentrations are known to inhibit the utilization of leucine in this organism.[6] Cyclopentanealanine also inhibits the growth of *Lactobacillus arabinosus* 17–5 but does not inhibit the growth of *Escherichia coli* 9723 even at concentrations of 10 to 20 mg. per 5 ml. The toxicity of cyclopentanealanine for *L. dextranicum* is the same as that shown in Table I both in the absence of phenylalanine and in the presence of as much as 200 γ of DL-phenylalanine per 5 ml. Thus, cyclopentanealanine is primarily an antagonist of leucine under the testing conditions and does not inhibit the utilization of phenylalanine.

1-Cyclopentene-1-alanine inhibits the growth of *L. dextranicum* as indicated in Table II. Phenylalanine competitively reverses the toxicity over a fifty-fold range in concentration with an inhibition index of approximately 50. Increased leucine concentrations have no effect on the inhibition.

TABLE II

REVERSAL OF INHIBITION OF 1-CYCLOPENTENE-1-ALANINE BY PHENYLALANINE

Test organism, *Leuconostoc dextranicum* 8086, incubated at 30° for 17 hours.[a]

1-Cyclopentene-1-DL-alanine, γ per 5 ml.	DL-Phenylalanine, γ per 5 ml.					
	0	20	50	100	200	500
	Galvanometer readings					
0	58	60	62	62	61	58
200	44	58	58			
500	8	42	52	59		
1000	3	10	33	56	60	
2000		6	17	58	56	58
5000			9	13	41	58
10000				10	18	40

[a] DL-Leucine, 50 γ per 5 ml., added to medium.

The introduction of a double bond in the cyclopentane ring adjacent to the alanine side chain converts the amino acid analog from a leucine antagonist to a phenylalanine antagonist. As a result of this change in structure, the alanine side chain is attached to the cyclopentene ring in the same plane as the ring as is the case of phenylalanine, whereas the alanine side chain of cyclopentanealanine is attached in a non-planar configuration which resembles leucine. Although the double bond itself may possibly have some effect, the steric configuration appears to be of prime importance in conferring enzyme specificity to the analogs.

(6) L. T. H. Dien, J. M. Ravel and W. Shive, *Arch. Biochem. Biophys.*, **49**, 283 (1954).

Experimental[7,8]

Biological Assays.—For assays with *S. lactis* 8039 and *L. arabinosus* 17-5, a previously described amino acid medium[9] was modified by omitting the glutamic acid and leucine. L-Glutamine (200 γ per assay tube) was added aseptically to the autoclaved assays. For assays with *L. dextranicum* 8086, leucine and phenylalanine were omitted from the medium[9] and the medium was further modified by adding pantethine (0.1 γ per assay tube) and by increasing the salts A concentration fourfold. For all three of the above organisms, calcium pantothenate (3 mg. per 30 ml. of vitamin supplement), which was inadvertently omitted from the list of constituents of the basal medium described above, was added. For *E. coli*, a previously described inorganic salts–glucose medium[10] was employed. In all assays, the inhibitors were dissolved in sterile water and added to the sterile assay tubes without being heated.

Diethyl α-Acetamido-α-cyclopentylmethylmalonate.—A solution of sodium ethoxide was prepared by dissolving 1.7 g. of sodium in 100 ml. of magnesium-dried ethyl alcohol. To this clear solution was added 16.0 g. of diethyl α-acetamidomalonate, and the mixture was stirred with slight warming until solution was complete. Then, 12.0 g. of cyclopentylmethyl bromide dissolved in 25 ml. of benzene was added slowly, and the reaction mixture heated to reflux for 20 hours. The precipitated salts were removed, the solution was treated with Darco G-60, neutralized to *p*H 7 with alcoholic hydrogen chloride and finally the excess solvent was removed under reduced pressure. The solid thus obtained was taken up in one liter of boiling water and allowed to crystallize slowly. Further crops of crystals were obtained by reducing the volume of solvent slowly with an air jet over a two-day period. There was thus obtained 6.6 g. (30% of theory) of lustrous flakes of crystals, m.p. 55°.

Anal. Calcd. for $C_{16}H_{25}NO_5$: C, 60.18; H, 8.42; N, 4.68. Found: C, 60.21; H, 8.27; N, 5.01.

Ethyl α-Acetamido-α-(cyclopentylmethyl)-cyanoacetate.—To a solution of 1.7 g. of sodium in 100 ml. of ethanol was added 7.36 g. of ethyl α-acetamidocyanoacetate. The turbid mixture was stirred, and 12.0 g. of cyclopentylmethyl bromide was added over a period of 30 minutes. The reaction mixture was then heated under reflux for 24 hours; then treated with Darco G-60, and filtered through a Celite mat. The brownish solution was then evaporated to dryness under reduced pressure. The residue was treated with chloroform, and water was added to effect solution of the remaining solid material. The chloroform phase was dried over calcium sulfate. After evaporation of some of the chloroform and dilution with ether, there was recovered 2.4 g. of product which was recrystallized from alcohol–water, m.p. 125°.

Anal. Calcd. for $C_{13}H_{20}N_2O_3$: C, 61.88; H, 7.99. Found: C, 61.73; H, 7.81.

The filtrate did not yield any additional crystalline material; however, the brown oil obtained on evaporation was hydrolyzed in 20% sodium hydroxide to recover 330 mg. of cyclopentanealanine.

N-Acetylcyclopentane-DL-alanine.—A suspension of 1 g. of diethyl α-acetamido-α-cyclopentylmethylmalonate in 5 ml. of 6 *N* sodium hydroxide was heated under reflux for one hour, during which time the organic material slowly dis-

(7) All melting points were determined on a Fisher–Johns melting point block.

(8) 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. Russell Claybrook for the chemical analyses.

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

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

solved. The clear yellow solution was then acidified with concentrated hydrochloric acid and heated to reflux for 20 minutes, filtered while hot, and allowed to cool in a refrigerator. There was recovered a total of 320 mg. (46%) of product, which was recrystallized from water alcohol to give micro-needles, m.p. 165–167°.

Anal. Calcd. for $C_{10}H_{17}NO_3$: C, 60.28; H, 8.60; N, 7.03. Found: C, 60.28; H, 7.96; N, 7.30.

Cyclopentane-DL-alanine (A).—A mixture of 3.6 g. of diethyl α-acetamido-α-(cyclopentylmethyl)-malonate and 75 ml. of 5 *N* hydrochloric acid was heated under reflux for three hours during which time complete solution of the organic phase was effected. The reaction mixture was taken to dryness under reduced pressure, and the residue was then twice dissolved in ethanol and taken to dryness to remove the residual excess hydrochloric acid. Finally, the solid residue was taken up in warm water, filtered and adjusted to *p*H 7 with concentrated ammonium hydroxide. There was recovered 1.33 g. (68%) of product, which darkened at 220° and melted with decomposition at 245°.

Anal. Calcd. for $C_8H_{15}NO_2$: C, 61.12; H, 9.62; N, 8.91. Found: C, 61.23; H, 9.65; N, 9.13.

A paper chromatogram of this amino acid treated with ninhydrin showed only one purple spot, R_f 0.64 in 95% methyl alcohol.

(B).—A sample of 1.8 g. of ethyl α-acetamido-α-(cyclopentylmethyl)-cyanoacetate was heated under reflux for 15 hours in the presence of 25 ml. of 3 *N* sodium hydroxide. The organic material slowly dissolved, and the ammonia was liberated. The reaction mixture was then cooled, filtered and carefully treated with concentrated hydrochloric acid to *p*H 9. The precipitated silicate was removed and the solution finally adjusted to *p*H 8. The reaction mixture cooled overnight in a refrigerator gave a precipitate which was filtered, washed with cold water, and dried under vacuum over P_2O_5. There was recovered 1.1 g. (98%) of product which was identical in R_f value to the material previously isolated and which darkens at 220° and melts with decomposition at 242–244°.

N-Carbamylcyclopentane-DL-alanine.—A solution of 280 mg. of cyclopentanealanine and 200 mg. of potassium cyanate was dissolved with warming in 20 ml. of water. The solution was chilled and 0.15 ml. of glacial acetic acid added over a period of 15 minutes with efficient stirring. The reaction mixture was then warmed on a steam-cone for 30 minutes after which it was cooled, and acidified to a congo red end-point with concentrated hydrochloric acid. The precipitate was recovered, washed with cold water and dried under vacuum over P_2O_5 to yield 140 mg. (39%) of product (recrystallized from alcohol–water) melting at 210–211° dec.

Anal. Calcd. for $C_9H_{16}N_2O_3$: C, 53.98; H, 8.05; N, 13.99. Found: C, 53.81; H, 7.90; N, 13.81.

1-Cyclopentene-1-methanol.—A solution of 15 g. of 1-cyclopentene-1-carboxaldehyde[11] dissolved in 50 ml. of methanol was cooled to 10° and 1.9 g. of sodium borohydride, dissolved in 100 ml. of methanol, added dropwise to the well stirred solution over a period of about 30 minutes while maintaining the temperature at 10°. The reaction mixture was stirred for an additional 30 minutes at room temperature, and finally heated over a steam-cone for 15 minutes. The solution was reduced to about one-half volume by heating over steam under reduced pressure. The resulting sample was acidified to a congo red end-point with hydrochloric acid, and the aqueous phase was then extracted with ether. The ether phase was washed with potassium carbonate solution and finally dried over anhydrous potassium carbonate. After removal of the solvent, the residue was fractionated under reduced pressure to yield 12.5 g. (82%) of product, b.p. 75° (20 mm.), $n^{20}D$ 1.4760.[12]

1-Cyclopentene-1-methyl Bromide.—A mixture of 22.5 g. of 1-cyclopentene-1-methanol and 7 ml. of dry pyridine was placed in a flask fitted with a dropping funnel and the system protected from moisture with a calcium chloride drying tube. After cooling the solution to −40°, 12 ml. of phosphorus tribromide was added dropwise in about 30 minutes with shaking. Finally, after remaining at room temperature overnight, water was carefully added with external cooling. The aqueous phase was extracted with petroleum ether (40–60°), and the organic phase washed with sodium bicarbonate and then water. After drying over calcium sulfate, the excess solvent was removed and the residue distilled under reduced pressure. There was obtained 28 g. (76%) of product, b.p. 59–60° (15 mm.).[13]

Ethyl α-Acetamido-α-(1-cyclopentene-1-methyl)-cyanoacetate.—1-Cyclopentene-1-methyl bromide (28 g.) was condensed with 28.0 g. of ethyl acetamidocyanoacetate in the presence of one equivalent of sodium ethoxide dissolved in 120 ml. of dry ethanol. After removal of the solvent, there was recovered 37 g. (85%) of product, which after recrystallization from water melted at 107°. A water solution of this compound decolorized potassium permanganate and bromine water indicating the presence of a double bond.

Anal. Calcd. for $C_{13}H_{18}N_2O_3$: N, 11.20. Found: N, 11.28.

1-Cyclopentene-1-DL-alanine.—A solution of 12.0 g. of ethyl α acetamide-α-(1-cyclopentene-1-methyl)-cyanoacetate in 150 ml. of 10% sodium hydroxide was heated under gentle reflux for 20 hours. After cooling the reaction mixture and adjusting the solution with hydrochloric acid to *p*H 6, 7.3 g. of crude product was obtained. The filtrate was further acidified and reduced to dryness over a steam-cone and the residue was then extracted with ethanol. Finally, the solvent was removed, and the solid was taken up in water and treated with ammonium hydroxide to recover an additional 1.15 g. of the amino acid.

The combined precipitates were recrystallized from water to yield 6.0 g. (81%) of product, m.p. 245° dec.

Anal. Calcd. for $C_8H_{13}NO_2$: C, 61.91; H, 8.44; N, 9.03. Found: C, 61.91; H, 8.57; N, 9.08.

The N-benzoyl derivative of 1-cyclopentene-1-alanine was prepared in the usual manner, m.p. 140°.

Anal. Calcd. for $C_{15}H_{17}NO_3$: N, 5.40. Found: N, 5.33.

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(11) J. B. Brown, H. B. Henbest and E. R. H. Jones, *J. Chem. Soc.*, 3634 (1950).

(12) The recorded values are: b.p. 66° (11 mm.) and $n^{20}D$ 1.4770 by E. Urion, *Compt. rend.*, **198**, 1518 (1934).

(13) The recorded value is b.p. 56–59° (15 mm.); L. Piaux, *Compt. rend.*, **199**, 1127 (1934).