TABLE I

λ, mμ	Recrystd product from hydrogenolysis, [α], deg	L-Histidine, [α], deg
589	−13.5	+12.7
578	−14.0	+13.5
546	−16.5	+16.2
436	−32.5	+31.1

In another experiment a mixture of 4.36 g of L-2-chloro-3-[4(5)-imidazolyl]propionc acid, 3.0 ml of H_2O, 2.20 ml of 85% hydrazine hydrate, and 50 ml of MeOH was refluxed and the rate of liberation of Cl^- was determined (see Figure 1). Assuming that 1 mole of hydrazine was utilized in converting the α-chloro acid to the anion, the concentration of free hydrazine present at the start of this reaction was one-tenth that of the mixture with the standard excess of hydrazine.

An attempt was made to determine the rate of racemization of α-chloro acid under the refluxing MeOH conditions. L-2-Chloro-3-[4(5)-imidazolyl]propionic acid (4.36 g), 8.8 ml of hydrazine hydrate, and 40 ml of MeOH were refluxed 1 hr (30% completion based on liberated Cl^-). An aliquot (1% of mixture) was evaporated to dryness. The residue was chromatographed on a column of 50 g of silica gel using 4:1 MeOH–concentrated NH_4OH. The chlorine-containing fraction was partially evaporated and then flushed with MeOH. The partly crystalline residue, after washing with MeOH, gave 0.16 g (36%) of L-2-chloro acid, mp 192° dec, of about 96% optical purity. The mother liquor fraction on chromatography on 40 g of silica gel using 4:1 MeOH–H_2O gave 0.10 g (23%) of 2-chloro acid, mp 192° dec, of 35% optical purity. This material gave a single spot on tlc on a silica gel plate using 4:1 MeOH–H_2O. The average optical purity of the recovered α-chloro acid was 72%.

Diazotization of L-Histidine Methyl Ester.—To a mixture of L-histidine methyl ester dihydrochloride (2.57 g, 0.01 mole) and 14.3 ml of concentrated HCl at −3 to +3° was added over 6 min a solution of 1.92 g of $NaNO_2$ in 3.75 ml of water. The mixture was kept at about −5° for 45 min and then allowed to warm to room temperature over 1.5 hr. Following filtration to remove NaCl the solution was evaporated to dryness. After flushing twice with H_2O and twice with *t*-BuOH the residue was dissolved in 10 ml of H_2O and the solution was neutralized to pH 4.2. The product (0.84 g, 48%) was identical with the L-2-chloro-3-[4(5)-imidazolyl]propionic acid prepared from L-histidine. In a blank experiment in which the $NaNO_2$ was omitted, starting ester was recovered in 90.7% yield.

Hydrogenolysis of D-2-Hydrazino-3-[4(5)-imidazolyl]propionic Acid Hydrochloride.—Raney Ni catalyst (0.5 teaspoon), neutralized by storage under EtOAc, was added to a solution of D-2-hydrazino-3-[4(5)-imidazolyl]propionic acid hydrochloride (2.0 g) in 40 ml of MeOH and 50 ml of H_2O. The mixture was hydrogenated at 2.8 kg/cm² and 50° for 5 hr. Two-thirds of the theoretical quantity of H_2 was consumed. Following removal of the catalyst, the filtrate was evaporated to dryness. MeOH (20 ml) was added and the solution was evaporated to dryness. After repeating this step several times a crystalline product was isolated (0.55 g, mp 263–266°). A second fraction (0.25 g, softened at 207°, mp 240°) was isolated on evaporation of the mother liquors. Recrystallization of the first fraction from water gave D-histidine, mp 272–280°. Amino acid analysis (Spinco) showed 96.8% hisitidine. *Anal.* ($C_6H_9N_3O_2$) C, H, N. The ir spectrum was identical with that of L-histidine. The specific rotations (2% in 6 *N* HCl) compared to L-histidine are listed in Table I.

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Syntheses and Biological Activities of Some Cycloalkenealanines

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The alicyclic amino acids, DL-3-cyclopentene-1-alanine, DL-2-cyclopentene-1-alanine, DL-2-cyclohexene-1-alanine, and DL-1-cycloheptene-1-alanine were synthesized, and their effects upon the growth of several microorganisms were determined. DL-3-Cyclopentene-1-alanine inhibits the growth of three *Escherichia coli* strains and the lactic acid bacteria, *Lactobacillus plantarum* 8014; the growth inhibition of *E. coli* 10856 is reversed in a competitivelike manner by leucine only in the presence of a small supplement of methionine in a manner suggesting that the analog inhibits utilization of leucine and the biosynthesis of methionine. DL-2-Cyclohexene-1-alanine and DL-2-cyclopentene-1-alanine inhibit the growth of *Leuconostoc dextranicum* 8086, and the toxicities of both analogs were reversed in a competitivelike manner over a 10–20-fold range by increasing concentrations of leucine. DL-1-Cycloheptene-1-alanine prevented the growth of *Leuconostoc dextranicum* 8086 only at very high levels; no reversal of toxicity could be demonstrated with phenylalanine.

Several alicylic amino acids have been reported to have rather specific antimetabolite activity, and the structural relationships between the natural metabolites and the active analogs have demonstrated the importance of steric factors in the biological activity of these compounds. For example, 1-cyclopentene-1-alanine and 1-cyclohexene-1-alanine have been found to be competitive antagonists of phenylalanine in contrast to the corresponding saturated derivatives, cyclopentanealanine and cyclohexanealanine, the first of which has slight activity only as a leucine antagonist and the second of which has no demonstrable antimetabolite activity in the microorganisms studied. The emanation of the first carbon of the side chain in the plane of the 1-carbon and adjacent carbons of the alicyclic ring apparently is important for phenylalanine antagonism.[2] Other unsaturated alicyclic amino acid analogs which have been found to be active include 2-cyclopentene-1-glycine, 3-cyclopentene-1-glycine, 2-cyclohexene-1-glycine, and 3-cyclohexene-1-glycine, all of which are antagonists of isoleucine;[3,4] however, 2-cyclopentene-1-glycine also inhibits assimilation of

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valine, and 3-cyclopentene-1-glycine in addition to its isoleucine antagonism appears to prevent the biosynthesis of methionine. Since the corresponding cyclohexane derivatives of these cyclohexene analogs do not show detectable antimetabolite activity, it has been proposed that the biologically active conformation of the cyclohexene derivatives is not a boat conformation attainable also by the saturated analog but is one in which the two carbon atoms opposite the carbons with the double bond are on opposite sides of the plane of the four carbons associated with the double bond.[5] Similarly, 3-cyclohexene-1-alanine has been found to be an antagonist of leucine, while the corresponding saturated analog has no detectable antimetabolite activity.

In the present investigation, it was of interest to synthesize 3-cyclopentene-1-alanine in order to compare its activity with that of 3-cyclohexene-1-alanine. Also, 2-cyclopentene-1-alanine and 2-cyclohexene-1-alanine were prepared in order to determine the effect of the double bond in the 2 position in potential leucine antagonists. These analogs were synthesized and found to have activity as leucine antagonists, and one, 3-cyclopentene-1-alanine, was found to have an additional activity which appears to be associated with inhibition of the biosynthesis of methionine. In addition to these compounds, 1-cycloheptene-1-alanine was prepared in order to compare its biological activity with the corresponding cyclopentene and cyclohexene derivatives. Expansion of the cycloalkene ring from five to six members does not greatly alter activity, but expansion to a cycloheptene ring results in an analog, 1-cycloheptene-1-alanine, with considerable reduced toxicity and specificity in microbiological assay systems.

Experimental Section

General Procedures.—All melting points were taken on a Thomas–Hoover melting point apparatus in capillary tubes immersed in a stirred bath and are uncorrected. Pmr spectra were obtained using a Varian Associates A-60 spectrometer. The absorptions listed are given in τ units; the term "J" denotes a coupling constant with the value given in cycles per second (cps). The solvent and internal standard used for each spectrum were as follows: D_2O, NaOD, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt. Tlc was performed using Eastman "Chromagram" sheets, type K301R, Distillation Products Industries. These sheets were not activated prior to use; the solvent system used in each case is indicated. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

Bacterial Assays.—Stock cultures of *Leuconostoc dextranicum* 8086 were maintained by conventional bacteriological techniques. For inoculation of the assay medium, transfers were made from the stock culture into 10 ml of a yeast–peptone medium[6] and grown for approximately 18 hr. A 1.0-ml aliquot was transferred to 5 ml of fresh medium and incubated for 6–8 hr. This culture was centrifuged, and the cells were washed once with 10 ml of sterile physiological saline (0.8% NaCl). The cells were resuspended in 10 ml of saline and diluted 1:100 in sterile saline. For the inoculation, one drop of this diluted solution was added to each assay tube. For the assay a previously described medium[7] was modified as follows: for leucine antagonism, addition of DL-valine, 100 mg, and DL-isoleucine, 20 mg; for phenylalanine antagonism, deletion of DL-phenylalanine and DL-tyrosine and addition of DL-tryptophan, 6 mg, and DL-aspartic acid, 6 mg. The double-strength basal medium was prepared, heated at 100° for 15 min, and stored at room temperature under toluene. The purine–pyrimidine supplement was prepared by dissolving the components in a few milliliters of hot H_2O (glass distilled) containing a few drops of concentrated HCl before dilution to the specified volume. This supplement was stored at room temperature. Separate stock solutions of biotin, folinic acid, and *p*-aminobenzoic acid in H_2O were prepared at concentrations of 1.0 mg/ml and stored at −5°. The glucose and the supplements of vitamins, purines, and pyrimidines were added to an aliquot of the basal medium just before use. The pH of the complete medium was adjusted to 6.8 with NaOH.

Growth assays using *Escherichia coli* strains, ATCC 10856, 9723, and W were carried out using an inorganic salts–glucose medium described by Anderson.[8] The assays with *Lactobacillus plantarum* 8014 were carried out in a previously described medium,[9] except that leucine was present at 6 μg/ml. The general experimental detail has been reported elsewhere.[10]

The assay medium was autoclaved in capped tubes at 1.05 kg/cm² for 5 min, cooled, inoculated, and incubated for the indicated periods of time. The amount of growth was determined turbidimetrically in terms of galvanometer reading so adjusted that distilled H_2O read 0 and an opaque object read 100. The instrument has been described previously.[11]

3-Bromomethylcyclopentene.—3-Cyclopentene-1,1-dicarboxylic acid was converted to 3-cyclopentene-1-carboxylic acid,[12] which in turn was converted to 3-cyclopentene-1-methanol[13] by previously described procedures. A flask containing 28 g of 3-cyclopentene-1-methanol and 3 ml of pyridine was cooled to −15°, and 16 ml of PBr_3 was added dropwise over a 30-min period. When the addition was complete, the cold bath was removed, and the reaction mixture was stirred at room temperature for 2 hr; then 30 ml of petroleum ether (bp 30–60°) and 50 g of ice were added. The organic layer was separated, washed twice with 25-ml portions of H_2O, and then dried (Na_2SO_4). The petroleum ether was evaporated at reduced pressure, and the residue was distilled to yield 18 g of material, bp 59–61° (22 mm), $n^{25}D$ 1.4986. This material was unstable; if sealed under N_2 and stored at −18°, it could be kept for 3–4 days without undue decomposition. *Anal.* (C_6H_9Br) Br.

Ethyl α-Acetamido-α-cyano-3-cyclopentene-1-propionate.—A solution of 1.3 g of Na in 50 ml of EtOH was evaporated to dryness *in vacuo*, and the residue was suspended in 100 ml of DMSO. A solution of 9 g of ethyl acetamidocyanoacetate in 50 ml of DMSO was added to the suspension with vigorous stirring. When complete solution had occurred, 9 g of 3-bromomethylcyclopentene was added dropwise over a period of 30 min. The reaction mixture was stirred overnight at room temperature and then concentrated *in vacuo* to 25 ml. The residue was dissolved in 100 ml of H_2O, and this mixture was extracted twice with 100-ml portions of Et_2O. The Et_2O extracts were combined and evaporated to dryness. The residue was recrystallized from EtOH–H_2O, dried, and then recrystallized from toluene to give 8.7 g of material, mp 104–105°. *Anal.* ($C_{13}H_{18}N_2O_3$) C, H, N.

Ethyl α-Acetamido-α-cyano-1-cycloheptene-1-propionate was prepared from 11 g of 1-bromomethylcycloheptene[14–16] by the method described above. Recrystallization from Et_2O and then toluene yielded 10.2 g of white needles, mp 144–145°. *Anal.* ($C_{15}H_{22}N_2O_3$) C, H, N.

DL-3-Cyclopentene-1-alanine.—A stainless steel beaker containing 20 ml of 10% NaOH was heated on a steam cone to 95°, and then 2 g of ethyl α-acetamido-α-cyano-3-cyclopentene-1-propionate was added with stirring. The beaker was covered with a watch glass, and the heating was continued until no NH_3 could be detected (about 3 hr). The solution was cooled and acidified to pH 5 with concentrated HCl. After cooling over-

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night at 5°, the crystals were collected, recrystallized twice from H_2O, and then dried *in vacuo* (P_2O_5) to yield 1.3 g of material, mp 295–297° (dec from 225°). *Anal.* ($C_8H_{13}NO_3$) C, H, N. Ascending paper chromatograms, after treatment with ninhydrin, showed only one spot: R_f 0.71 (*n*-BuOH–AcOH–H_2O, 5:1:2), 0.56 (*i*-AmOH–pyridine–H_2O, 7:7:6), 0.74 (95% MeOH); pmr absorptions 4.26 (singlet, 2 protons), 6.78 (triplet, J = 7 cps, 1 proton), 7.15–8.65 (complex multiplet, 7 protons).

DL-1-Cycloheptene-1-alanine was prepared from 2 g of ethyl α-acetamino-α-cyano-1-cycloheptene-1-propionate by the method described above except the hydrolysis was carried out under reflux for 12 hr. Recrystallization (H_2O, then EtOH–H_2O) yielded 420 mg of fine white needles, mp 214–216° (dec above 200°). *Anal.* ($C_{10}H_{17}NO_2$) C, H, N.

Tlc of this material showed only one yellow spot after development with ninhydrin: R_f 0.67 (*n*-BuOH–AcOH–H_2O, 4:1:1), 0.76 (*t*-BuOH–2-butanone–H_2O–28% NH_4OH, 4:3:2:1), 0.85 (H_2O–MeOH, 1:1); pmr absorptions, 4.32 (triplet, J = 6 cps, 1 proton), 6.51–6.85 (multiplet, 1 proton), 7.30–8.13 (multiplet, 6 protons), 8.13–8.97 (multiplet, 6 protons).

5-(2-Cyclohexenylmethyl)hydantoin.—3-Hydroxycyclohexene was converted to 2-cyclohexenyl vinyl ether,[17] which in turn was converted to 2-cyclohexene-1-acetaldehyde[17] by previously described procedures. The conversion of 2-cyclohexene-1-acetaldehyde to 5-(2-cyclohexenylmethyl)hydantoin was accomplished by a procedure similar to the method described by Henze and Speer for synthesis of hydantoins from aldehydes.[18] 2-Cyclohexene-1-acetaldehyde (10 g) was dissolved in 200 ml of 50% EtOH in the presence of 13 g of KCN and 43 g of $(NH_4)_2CO_3$, and the mixture was heated to 60° for 4 hr. Crystals of the hydantoin appeared in the reaction mixture near the end of the reaction. The reaction mixture was evaporated *in vacuo* to about 125 ml and then placed overnight in the refrigerator. The crystals were then filtered, and after recrystallization from EtOH–H_2O, 6.2 g of white crystalline flakes were recovered, mp 241–242°. *Anal.* ($C_{10}H_{14}N_2O_2$) C, H, N.

5-(2-Cyclopentenylmethyl)hydantoin.—2-Cyclopentene-1-acetaldehyde[19] (9 g) was converted to the corresponding 5-substituted hydantoin[18] by the method described above for the cyclohexenyl homolog. There was recovered 6.5 g of white flakes, mp 204–205°. *Anal.* ($C_9H_{12}N_2O_2$) C, H, N.

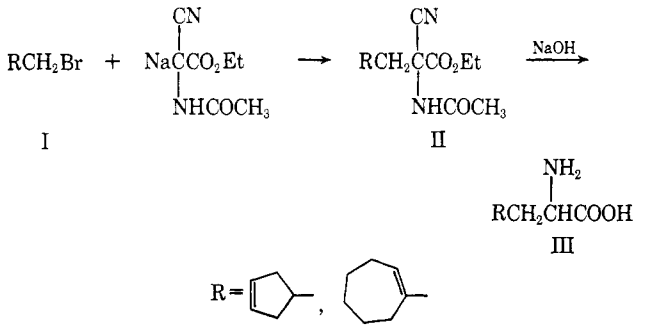
DL-2-Cyclohexene-1-alanine.—A mixture of 5 g of 5-(2-cyclohexenylmethyl)hydantoin, 40 g of $Ba(OH)_2 \cdot 8H_2O$, and 40 ml of H_2O was refluxed for 3 days. The insoluble material was removed, and the filtrate was neutralized with concentrated H_2SO_4. The precipitated $BaSO_4$ was filtered from the hot reaction mixture, and the cooled filtrate yielded a crystalline precipitate. After recrystallization from H_2O, there was recovered 2.2 g of white leaflets, mp 252–253° dec. *Anal.* ($C_9H_{15}NO_2$) C, H, N. Tlc of this material showed only one spot after development with ninhydrin: R_f 0.50 (*n*-BuOH–AcOH–H_2O, 4:1:1), 0.83 (*t*-BuOH–2-butanone–H_2O–28% NH_4OH, 4:3:2:1), 0.67 (MeOH); pmr absorptions, 4.26 (singlet, 2 protons), 6.66 (triplet, J = 7 cps, 1 proton), 7.67–8.20 (multiplet, 3 protons), 8.20–9.16 (multiplet, 6 protons).

DL-2-Cyclopentene-1-alanine.—5-(2-Cyclopentenylmethyl)hydantoin (3 g) was converted to the corresponding alanine derivative by the method described above. There was obtained 1.7 g of white prisms, mp 295–297° (dec from 250°). *Anal.* ($C_8H_{13}NO_2$) C, H, N. Tlc of this material showed only one spot after development with ninhydrin: R_f 0.66 (*n*-BuOH–AcOH–H_2O, 4:1:1), 0.72 (*t*-BuOH–2-butanone–H_2O–28% NH_4OH, 4:3:2:1), 0.84 (MeOH–H_2O, 1:1); pmr absorptions, 4.17 (singlet, 2 protons), 6.71 (triplet, J = 7.5 cps, 1 proton), 7.00–7.46 (multiplet, 1 proton), 7.46–8.86 (multiplet, 6 protons).

Results and Discussion

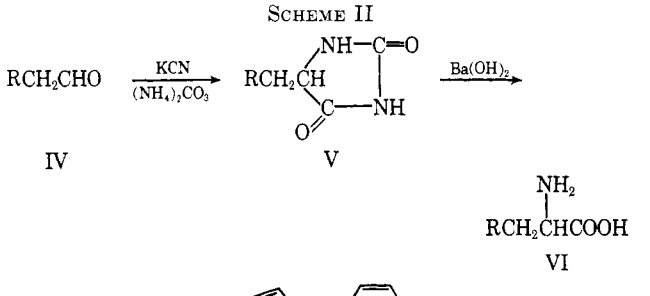
For the synthesis of 3-cyclopentene-1-alanine and 1-cycloheptene-1-alanine, the appropriate bromomethylcycloalkene (I) was condensed with the sodio derivative of ethyl acetamidocyanoacetate to form the corresponding ethyl α-acetamido-α-cyanocycloalkenepropionate (II), which upon alkaline hydrolysis, gave the desired cycloalkene-1-alanine (III) as indicated in Scheme I.

SCHEME I



The preparation of 2-cyclopentene-1-alanine and 2-cyclohexene-1-alanine was accomplished by converting the appropriate 2-cycloalkene-1-acetaldehyde (IV) to the corresponding 5-(2-cycloalkenylmethyl)hydantoin (V) followed by alkaline hydrolysis of the hydantoin to form the 2-cycloalkene-1-alanine (VI) as indicated in Scheme II.

SCHEME II



In microbiological studies, 3-cyclopentene-1-alanine was found to prevent visible growth of *Escherichia coli* 9723, 10856, and W and *Lactobacillus plantarum* 8014 at concentrations of 6, 6, 30, and 300 μg/ml, respectively, after an 18-hr incubation period. Competitive reversal of the growth inhibitions by 3-cyclopentene-1-alanine was not obtained with any single amino acid in any of the several organisms studied. Detailed studies of the effect of the amino acid analog upon *E. coli* 10856 revealed that leucine alone reverses the inhibition at low levels of the inhibitor but has no effect at higher concentrations, as shown in Table I. However,

TABLE I

REVERSAL OF THE INHIBITION OF DL-3-CYCLOPENTENE-1-ALANINE IN *E. coli* 10856

	Galvanometer readings[a]						
	DL-Leucine, μg/ml						
Inhibitor	0	0.03	3.0	0	0.01	0.03	0.1
concn,	DL-Methionine, μg/ml						
μg/ml	0	0	0	3	3	3	3
0	66	60	62	59	59	59	59
1	67			61			
3	7	64	63	61	59	60	
10		63	64	3	61	61	60
30		0	3	0	1	65	63
100			0		0	20	27
300							0

[a] A measure of culture turbidity; distilled water reads 0, an opaque object 100.

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TABLE II

REVERSAL OF DL-2-CYCLOHEXENE-1-ALANINE TOXICITY IN *L. dextranicum* 8086 BY LEUCINE[a]

DL-2-Cyclohexene-1-alanine, μg/ml	Galvanometer readings[b] DL-Leucine, μg/ml			
	1	2	5	10
0	47	64	66	64
200	11	62	63	64
500	6	61	63	60
1000	5	40	59	61
2000		15	56	58
3000		7	51	56
4000			23	55

[a] Incubated for 17 hr at 30°. [b] A measure of culture turbidity; distilled water reads 0, an opaque object 100.

TABLE III

REVERSAL OF DL-2-CYCLOPENTENE-1-ALANINE TOXICITY IN *L. dextranicum* 8086 BY LEUCINE[a]

DL-2-Cyclopentene-1-alanine, μg/ml	Galvanometer readings[b] DL-Leucine, μg/ml				
	2	5	10	25	50
0	45	51	57	52	50
500	9	47	50	52	50
1000		35	45	45	44
2000		4	31	37	39
3000			18	31	34
4000			2	4	33

[a] Incubated for 17 hr at 30°. [b] A measure of culture turbidity; distilled water reads 0, an opaque object 100.

a minimal supplement of methionine, which alone has no extensive effect in reversing the inhibition, permits leucine to reverse the growth inhibition in a competitive manner over a limited range of concentrations. The small amount of methionine required to exert this effect is approximately the same at different concentrations of inhibitor. These results suggest that this analog not only inhibits the utilization of leucine but also inhibits in some manner the biosynthesis of methionine. This effect is comparable to that found in studies with 3-cyclopentene-1-glycine, the growth inhibitory effect of which is reversed competitively by isoleucine only in the presence of small amounts of methionine.[4]

2-Cyclohexene-1-alanine, though not as effective as 3-cyclohexene-1-alanine, inhibits the growth of *L. dextranicum* as indicated in Table II. The inhibition is prevented over a 10–20-fold range of increasing concentrations of leucine in a competitivelike manner. The inhibition index (ratio of concentration of analog to metabolite necessary for preventing detectable growth during the incubation period) is approximately 1000. The mutual antagonisms of leucine, isoleucine, and valine in this particular organism prevent a demonstration of a competitive relationship over a broader range of concentrations.

2-Cyclopentene-1-alanine was found to be somewhat more potent than 2-cyclohexene-1-alanine as a growth inhibitor of *L. dextranicum*. The growth inhibition is reversed over a 20-fold range of increasing concentrations of leucine in a competitivelike manner with an inhibition index of approximately 400 as indicated in Table III. In contrast to 3-cyclopentene-1-alanine, 2-cyclopentene-1-alanine did not inhibit the growth of the three strains of *E. coli*. Thus, the seemingly minor alteration of the structure by moving the double bond from the 3 to the 2 position causes complete loss of activity for *E. coli*. It appears likely that the distance between the carbon atoms of the double bond may have a specific steric effect, but an effect of the π electrons cannot be excluded. Even though 2-cyclohexene-1-alanine and 2-cyclopentene-1-alanine have some structural features similar to the corresponding 1-cycloalkenealanines, no reversal of toxicity of these compounds could be demonstrated with phenylalanine.

1-Cycloheptene-1-alanine was found to prevent the growth of *L. dextranicum* only at high concentrations of approximately 5 mg/ml, and no reversal of toxicity could be demonstrated with phenylalanine. Unlike 1-cyclopentene-1-alanine and 1-cyclohexene-1-alanine, both of which are phenylalanine antagonists, 1-cycloheptene-1-alanine, although toxic at high levels, appears to be a weak nonspecific inhibitor. Presumably, 1-cycloheptene-1-alanine, although differing from 1-cyclohexene-1-alanine by ring expansion of only one methylene group, cannot effectively compete with phenylalanine at the site of its utilization because of its increased ring size. Apparently, planarity of carbon atoms adjacent to and including the carbon atom to which the alanine moiety is attached is not a sufficient criterion for phenylalanine antagonism, but in addition there are necessary limits of the size and planarity of the remainder of the β substituent.

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