it slowly became dark brown in color. The reaction mixture was cooled, extracted with 150 ml. of ether, and the ether phase finally reduced in volume to yield a precipitate which was filtered, recrystallized from absolute alcohol and dried over calcium chloride in a vacuum desiccator to yield 14.7 g. of product, m.p. 75–76°.

Anal. Calcd. for $C_{12}H_{10}NO_2Cl$: C, 61.15; H, 4.28; N, 5.94. Found: C, 61.14; H, 4.50; N, 6.14.

Ethyl *cis*-2-Acetamido-2-cyano-6-phthalimido-4-hexenoate.—A sample of 6.5 g. of ethyl acetamidocyanoacetate was added to a solution of sodium ethoxide prepared from 1.4 g. of sodium treated with 100 ml. of magnesium-dried ethanol. The reaction mixture was warmed during the addition to effect complete solution of the cyanoacetate, and 10 g. of N-(*cis*-4-chloro-2-butenyl)-phthalimide then was added in small increments. After the latter addition was completed, the resulting mixture was heated under reflux for about 2 hr. After cooling, the sodium chloride was removed by filtration and the filtrate was reduced to dryness *in vacuo*. The residue was extracted three times with 50 ml. portions of carbon tetrachloride, and the combined organic extract was finally washed with water. The solvent now was removed *in vacuo* to yield 4.5 g. of residue which was crystallized from ethanol–water to yield 3.5 g. of product, m.p. 97–98°.

Anal. Calcd. for $C_{19}H_{19}N_3O_5$: N, 11.38. Found: N, 11.21.

***cis*-2,6-Diamino-4-hexenoic Acid (*cis*-4,5-Dehydrolysine).**—A mixture of 3 g. of *cis*-2-acetamido-2-cyano-6-phthalimido-4-hexenoate and 50 ml. of concentrated hydrochloric acid was heated under reflux for about 24 hr. The reaction mixture was cooled, the phthalic acid which precipitated was removed by filtration and the filtrate was reduced to dryness *in vacuo*. After the repeated addition and removal of small volumes of ethanol *in vacuo*, the dried residue was crystallized from ethanol–ethyl acetate to yield about 200 mg. of hygroscopic product. The hygroscopic nature of the sample was such that a melting point could not readily be determined. The material isolated gave a positive test with ninhydrin and the R_f values of this product in butanol:acetic acid:water (3:1:1) and 65% pyridine were 0.11 and 0.33, respectively. The sample was carefully weighed under anhydrous conditions using a weighing "pig" for an elemental analysis.

Anal. Calcd. for $C_6H_{12}N_2O_2 \cdot 2HCl$: N, 12.90. Found: N, 12.93.

N-(*trans*-4-Bromo-2-butenyl)-phthalimide.—A 40 g. sample of *trans*-1,4-dibromo-2-butene was heated to its melting point in an oil-bath under anhydrous conditions and, after it had completely melted, 8 g. of potassium phthalimide was added in small increments with frequent shaking. Upon completion of the addition (about 2 hr.), the reaction mixture was heated at about 100–105° for an additional 2 hr. The resulting dark brown reaction mixture was extracted with ether, and the ether extract was reduced to dryness with a water aspirator. The residue was dissolved in absolute ethanol, treated with Darco G-60, filtered and an equal volume of Skelly B then was added to the clear filtrate. While standing in the cold overnight, 10 g. of product separated, m.p. 95–96°.

Anal. Calcd. for $C_{12}H_{10}NO_2Br$: C, 51.45; H, 3.67; N, 5.00. Found: C, 51.77; H, 3.58; N, 5.04.

Ethyl *trans*-2-Acetamido-2-carboethoxy-6-phthalimido-4-hexenoate.—A sample of 8 g. of ethyl acetamidomalonate was added to a solution of sodium ethoxide prepared from 1 g. of sodium treated with 100 ml. of magnesium-dried ethanol, and, after the malonate derivative had dissolved, 10 g. of N-(*trans*-4-bromo-2-butenyl)-phthalimide was added in small increments with frequent shaking. The reaction mixture then was heated under reflux for about 4 hr. The sodium bromide which precipitated during the reaction was removed by filtration, and the filtrate was reduced to dryness *in vacuo*. The residue was dissolved in a small amount of ethanol; after which an equal volume of Skelly B was added, and the resulting solution was placed in the refrigerator for about one week. There was recovered about 7 g. of product, m.p. 126–127°.

Anal. Calcd. for $C_{21}H_{24}N_2O_7$: N, 6.72. Found: N, 6.81.

***trans*-2,6-Diamino-4-hexenoic Acid (*trans*-4,5-Dehydrolysine).**—A mixture of 7 g. of ethyl *trans*-2-acetamido-2-carboethoxy-6-phthalimido-4-hexenoate and 50 ml. of concentrated hydrochloric acid was heated under reflux for about 18 hr. The phthalic acid which formed during the hydrolysis was removed by filtration, and the filtrate was reduced to dryness *in vacuo*. The excess hydrochloric acid was removed by repeated addition and evaporation of small quantities of ethanol *in vacuo*. The resulting residue was crystallized from ethanol–ethyl acetate to yield 700 mg. of product, m.p. 180–185°. The R_f values of this compound in butanol:acetic acid:water (3:1:1) and 65% pyridine were 0.11 and 0.33, respectively. The ninhydrin spray reagent produced a yellow spot which slowly turned purple on standing.

Anal. Calcd. for $C_6H_{12}N_2O_2 \cdot 2HCl$: C, 33.19; H, 6.50; N, 12.90. Found: C, 33.57; H, 6.59; N, 12.66.

Catalytic Hydrogenation of *cis* and *trans*-4,5-Dehydrolysine.—Both of the *cis* and *trans* isomers of 4,5-dehydro-DL-lysine were reduced in the same manner. A solution of 10 mg. of the appropriate isomer in 10 ml. of water was agitated in the presence of 50 mg. of palladium black under about 45 lb. of hydrogen pressure for 1 hr. The catalyst was removed and the appropriate dilutions were made from this 1 mg. per ml. solution for the microbiological assays using *Streptococcus faecalis*. The basal medium[8] was modified by omitting the DL-lysine, and the assays were supplemented with the components indicated and incubated at 30° for about 20 hr. The growth response curves obtained with the two hydrogenated samples were quantitatively identical with that found using an authentic sample of DL-lysine. The R_f values of both of the hydrogenated products were identical with that of lysine in several paper chromatographic systems.

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

A Conformation of Methionine Essential for its Biological Utilization

BY CHARLES G. SKINNER, JEROME EDELSON AND WILLIAM SHIVE

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The synthesis of the *cis* and *trans* forms of 2-amino-4-hexenoic acid (crotylglycine) and of 2-amino-3-methyl-4-pentenoic acid by condensation of the appropriate halide with ethyl acetamidocyanoacetate followed by hydrolysis of the condensation product is accompanied by an allylic rearrangement to give mixtures of crotylglycine and 2-amino-3-methyl-4-pentenoic acid. Biological properties previously ascribed to the *trans* form of 2-amino-4-hexenoic acid result from a small contamination of rearranged product, and purified samples are ineffective as an amino acid antagonist for *Escherichia coli*. In contrast, the *cis* form is a competitive antagonist of methionine for *E. coli*, so that the conformation of methionine on its site of utilization appears to be one in which the terminal group and the β-methylene group are in a *cis*-like configuration structurally resembling *cis*- rather than *trans*-crotylglycine.

Studies of various lysine analogs with restricted rotation have demonstrated that an essential conformation of lysine for binding at its site of utilization is such that the β and ϵ carbons are in a *trans*-

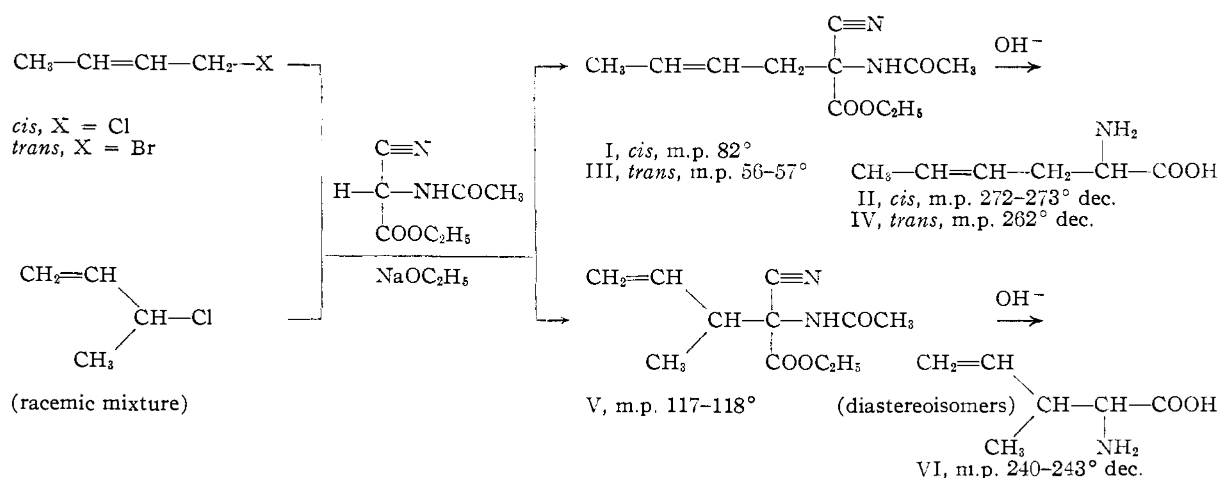

Fig. 1.—Reaction products of crotyl halides and 3-chloro-1-butene with ethyl acetamidocyanoacetate.

like configuration.[1–3] Since norleucine is an effective metabolic antagonist of methionine,[4] a study of the geometrical isomers of the 4,5-dehydro derivatives of norleucine should give some insight into the conformation of methionine necessary for its biological utilization.

In this investigation, both the *cis* and *trans* forms of this dehydro derivative, crotylglycine, have been prepared, and the *cis* isomer but not the *trans* form has been found to be an effective competitive antagonist of methionine in *Escherichia coli* 9723. A preparation of the *trans* isomer[5] has been previously reported to be an amino acid antagonist,[6] but this biological activity can now be ascribed to a minor contaminant, 2-amino-3-methyl-4-pentenoic acid, which results from a rearrangement of the halide during the acetamidomalonic ester condensation step.

cis-Crotyl alcohol was prepared by catalytic hydrogenation of 2-butyne-1-ol using a previously reported procedure.[7] The resulting crotyl alcohol was fractionally distilled to give a sample of the *cis* isomer which on the basis of gas chromatographic analysis contained less than 2% of the *trans* isomer. The *cis*-crotyl alcohol was then converted by treatment with phosphorous trichloride to *cis*-crotyl chloride which was in turn condensed with the sodio derivative of ethyl acetamidocyanoacetate to give ethyl 2-acetamido-2-cyano-*cis*-4-hexenoate (I). The condensation product subsequently was hydrolyzed with barium hydroxide to yield the desired derivative, 2-amino-*cis*-4-hexenoic acid (*cis*-crotylglycine) (II). The reactions are summarized in Fig. 1.

A previously reported procedure[5] was utilized for the preparation of *trans*-crotylglycine except that *trans*-crotyl bromide was used instead of the corresponding chloride for condensation with ethyl acetamidocyanoacetate in alcoholic sodium ethoxide as indicated in Fig. 1. No evidence of any contamination of the *trans*-crotyl bromide by the *cis*-isomer was observed using gas chromatographic analysis. Alkaline hydrolysis of the cyanoacetic ester condensation product (III) gave a preparation of *trans*-crotylglycine (IV) which had antimicrobial activity similar to that previously reported.[6] However, subsequent recrystallizations of the preparation greatly diminished its activity such that the concentration necessary for inhibition of growth of *E. coli* increased from 10–20 γ/ml. to about 160 γ/ml. These results suggest that the initially observed antimicrobial activity of preparations of *trans*-crotylglycine was the result of a trace contaminant.

During the course of this study, the preparation of 2-amino-3-methyl-4-pentenoic acid (ω-dehydroisoleucine) as a possible isoleucine antagonist was undertaken as indicated in Fig. 1. Preliminary studies of the initial product obtained under refluxing conditions suggested that an allylic rearrangement of the unsaturated halide had occurred during the condensation reaction. Secondary allylic halides have been demonstrated to produce rearranged condensation products with nucleophilic reagents such as sodiomalonic ester.[8] Alkaline hydrolysis of the oily condensation product gave a mixture of amino acids consisting of approximately 90% 2-amino-4-hexenoic acid (crotyl glycine) (II or IV) and 10% 2-amino-3-methyl-4-pentenoic acid (VI).[9]

In an effort to obtain 2-amino-3-methyl-4-pentenoic acid (VI) free of crotylglycine, the conden-

(1) A. L. Davis, J. M. Ravel, C. G. Skinner and W. Shive, *Arch. Biochem.*, **76**, 139 (1958).

(2) A. L. Davis, C. G. Skinner and W. Shive, *Arch. Biochem. Biophys.*, **87**, 88 (1960).

(3) A. L. Davis, C. G. Skinner and W. Shive, *J. Am. Chem. Soc.*, **83**, 2279 (1961).

(4) W. M. Harding and W. Shive, *J. Biol. Chem.*, **174**, 743 (1948).

(5) H. L. Goering, S. J. Cristol and K. Dittmer, *J. Am. Chem. Soc.*, **70**, 3310 (1948).

(6) K. Dittmer, *Ann. N. Y. Acad. Sci.*, **52**, 1274 (1950).

(7) L. F. Hatch and S. S. Nesbitt, *J. Am. Chem. Soc.*, **72**, 727 (1950).

(8) R. E. Kepner, S. Winstein and W. G. Young, *ibid.*, **71**, 115 (1949).

(9) The composition of this mixture was determined by catalytic hydrogenation to form the corresponding saturated amino acids, followed by microbiological assays. The stimulation of growth of *Lactobacillus arabinosus* 17-5 under conditions such that the response to isoleucine and alloisoleucine are equivalent was used to determine the per cent. of VI originally present, and the inhibition of growth of *Escherichia coli* 9723 by the reduced material (in comparison with norleucine) was used to determine the per cent. of II and/or IV originally present. The composition was further established by preparing a mixture of subsequently isolated 2-amino-3-methyl-4-pentenoic acid (10%) and 2-amino-4-hexenoic acid (90%) and comparing the mixture with the isolated reaction products using X-ray powder diffraction techniques; the two powder diffraction patterns were identical.

sation of 3-chloro-1-butene with ethyl acetamidocyanoacetate was carried out at lower temperatures, and the condensation product (V) was carefully recrystallized to obtain a pure preparation. Alkaline hydrolysis of the intermediate produced the anticipated 2-amino-3-methyl-4-pentenoic acids (VI) which upon hydrogenation were found to promote the growth of *L. arabinosus* under the same conditions and at the same concentrations as does either isoleucine or alloisoleucine. Since the synthesis of 2-amino-3-methyl-4-pentenoic acid by the above procedure should result in the formation of two diastereoisomers corresponding to the dehydro derivatives of isoleucine and alloisoleucine, the composition of the preparation was determined by hydrogenation of the unsaturated amino acid(s) followed by an assay procedure using *Streptococcus faecalis* 8043 which requires isoleucine for growth but cannot effectively utilize alloisoleucine.[10] The hydrogenated reaction product was only 50% as effective as isoleucine for growth of this organism in contrast to being equally active with either isoleucine or alloisoleucine in promoting growth of *L. arabinosus*. Thus, it is evident that the preparation of 2-amino-3-methyl-4-pentenoic acid consisted of an equal mixture of the two diastereoisomeric forms, ω-dehydroisoleucine and ω-dehydroalloisoleucine. Attempts to separate these diastereoisomers by paper chromatographic techniques, by chromatography on a sulfonated-polystyrene resin and by fractional recrystallization of the acetyl derivatives were unsuccessful, even though the latter two techniques were satisfactory for the separation of mixtures of isoleucine and alloisoleucine.[11,12]

This preparation of 2-amino-3-methyl-4-pentenoic acid inhibits the growth of *E. coli* in an inorganic salts–glucose medium at a concentration of approximately 0.6 γ/ml. The inhibitory activity of the analog is thus sufficiently potent to account for the antimicrobial activity of preparations of *trans*-crotylglycine which might contain small amounts of this product resulting from an allylic rearrangement during the alkylation step of the preparative procedure. In an effort to determine whether this were so, samples of *trans*-crotylglycine after successive recrystallizations were hydrogenated, and the amount of isoleucine present in the resulting mixture was determined using *S. faecalis*.[10] A comparison of the growth inhibiting properties of the corresponding unsaturated fraction using *E. coli* with that of the ability of the hydrogenated samples to replace isoleucine in growth of *S. faecalis* is presented in Table I. Since norleucine, which is the reduction product from the main component, crotylglycine, inhibits the response of *L. arabinosus* to alloisoleucine as indicated in Table II, it was not possible to determine the amount of the allo form present in the various recrystallized fractions. However, in view of the difficulty in separating these diastereoisomers of 2-amino-3-methyl-4-pentenoic acid through recrystallization, it is probable that the fractions contain about equimolar quantities of the two forms.

TABLE I

CONCENTRATION OF REARRANGED PRODUCT IN FRACTIONALLY RECRYSTALLIZED CROTYLGLYCINE

No. recrystalliz.	Amount required to inhibit growth, *E. coli*, γ/ml.	Isoleucine content in reduced sample, %
1	20	0.8
3	40	.4
4	80	.2
5	160	.1

TABLE II

EFFECT OF HIGH LEVELS OF NORLEUCINE ON GROWTH RESPONSE OF *Lactobacillus arabinosus* TO ISOLEUCINE AND ALLOISOLEUCINE

Concn. of isoleucine or alloisoleucine γ/ml.	Growth response in presence of DL-norleucine, mg./ml. — None: Isoleucine	None: Allo-isoleucine	1: Isoleucine	1: Allo-isoleucine	2: Isoleucine	2: Allo-isoleucine
	Galvanometer readings					
0	0	0	0	0	0	0
6	38	30	35	5	32	0
8	44	40	43	12	40	3
10	52	48	51	19	46	6
12	55	55	55	25	53	9

In order to account for the inhibitory effects of the recrystallized samples of *trans*-crotylglycine (Table I), in which the samples are effective at about one-half the concentration level as would be anticipated on the basis of their content of both diastereoisomers of 2-amino-3-methyl-4-pentenoic acid, the effect of crotylglycine upon the toxicity of 2-amino-3-methyl-4-pentenoic acid was determined. A supplement of 20 to 60 γ/ml. of *trans*-crotylglycine (which alone at these concentrations is non-toxic) caused a decrease of 2 to 4-fold in the amount of 2-amino-3-methyl-4-pentenoic acid necessary for inhibition of growth. Thus, it may be concluded that the toxic effects of *trans*-crotylglycine which have been observed are due to a trace contamination of the product formed from an allylic rearrangement during the condensation with the acetamidocyanoacetic ester. For *E. coli*, *trans*-crotylglycine does not appear to have any appreciable inhibitory activity on growth. In addition, the glycyl peptide of *trans*-crotylglycine did not have any inhibitory effects on the growth of *E. coli*.

A comparable investigation of the crystalline preparation of *cis*-crotylglycine after catalytic hydrogenation and a microbial assay for the presence of isoleucine using *S. faecalis* showed no evidence of any contamination of the anticipated norleucine with isoleucine. However, the crude reaction mixture, after alkaline hydrolysis and hydrogenation of the resulting amino acids, was found to contain about 0.4% isoleucine. Thus, an allylic rearrangement also appears to occur during the condensation of *cis*-crotyl chloride with acetamidocyanoacetate.

(10) A. Meister, *J. Biol. Chem.*, **196**, 813 (1952).

(11) A Beckman/Spinco Amino Acid Analyzer packed with sulfonated styrene-8% divinylbenzene co-polymer resin prepared for the Model 120 Amino Acid Analyzer was used for the column separation study. S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958); D. H. Spackman, W. H. Stein and S Moore, *ibid.*, **30**, 1190 (1958).

(12) Attempts to separate the acetyl derivatives were patterned after the method of J. P. Greenstein, S. M. Birnbaum and L. Levintow, "Biochemical Preparations," Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1953, p. 84.

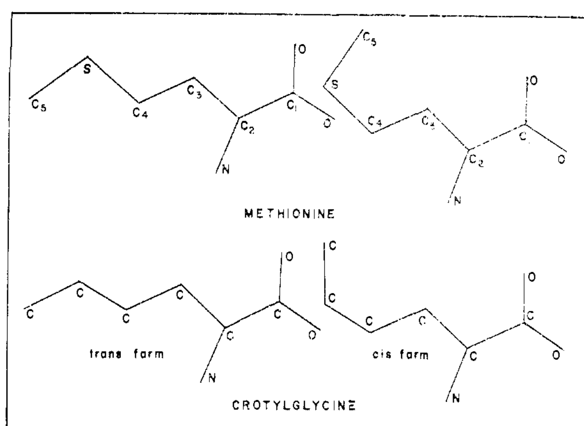

Fig. 2.—Scale models of DL-methionine (ref. 13) and *cis* and *trans*-DL-crotylglycine. The angles and bond lengths used to represent the crotylglycines were taken from "Tables of Interatomic Distances and Configuration in Molecules and Ions," Special Publication No. 11, The Chemical Society, Burlington House, London, W.1, 1958.

As indicated in Table III, *cis*-crotylglycine is inhibitory to the growth of *E. coli* at a level of about 10 γ per ml., and the growth inhibition is reversed competitively by methionine over more than a tenfold range of increasing concentrations with an inhibition index of approximately 100. In view of the lack of any appreciable toxicity of the *trans*-isomer, it appears that the configuration of methionine on its site of utilization is one in which the terminal methyl group and the β-methylene group are in a *cis*-like conformation resembling *cis*- rather than *trans*-crotylglycine.

TABLE III

REVERSAL OF *cis*-CROTYLGLYCINE TOXICITY IN *Escherichia coli* 9723 BY METHIONINE[a]

DL-*cis*-Crotylglycine, γ/ml.	Supplement: DL-Methionine, γ/ml. 0	0.2	0.5	1.0	2.0
	Galvanometer readings[b]				
0	75	75	74	71	75
2	75				
5	39				
10	2	73			
20		26	71		
50		9	14	67	
100				7	62
200					8

[a] Incubated for 17 hr. at 37°. [b] A measure of culture turbidity; distilled water reads 0, an opaque object, 100.

An examination of the crystalline structure of methionine is of some interest with regard to these conclusions. DL-Methionine crystallizes in two dimorphic forms of approximate equal stability, one of which has been termed the α-form, the other the β-form.[13] From X-ray crystal diffraction data, Mathieson concludes that, "in both α- and β-methionine, the atoms C_I, C_2, C_3, C_4 and S form an almost planar zigzag chain. For α-methionine, C_5 by the free rotation of the bond C_4–S lies out of this plane, whereas for β-methionine it is in the plane." A scale line drawing of the terminal zigzag planar chain of β-methionine[13] is presented in Fig. 2. This structure corresponds closely to *trans*-crotylglycine and would not be anticipated to be the biologically active conformer. The scale drawings of *cis* and *trans*-crotylglycine presented in Fig. 2 are averages derived from other *cis*- and *trans*-2-butene derivatives. A scale drawing of the conformer of methionine which most closely resembles the antagonistic *cis*-crotylglycine is shown also in Fig. 2, and it is derived from X-ray data on β-methionine[13] which actually possesses a structure intermediate between the two extremes pictured.

(13) A. McL. Mathieson, *Acta Cryst.*, **5**, 332 (1952).

The conformation of methionine essential for binding at its site of utilization may not possess a planar *cis*-like configuration for the terminal four atoms, but it appears that the position of the methyl group with respect to the plane containing C_3–C_4–S atoms would be more closely related to the *cis*-like rather than the *trans*-like configurations as indicated in Fig. 2. Norleucine would also be expected to be in a comparable conformation on the enzyme site in exerting its antagonistic effect on methionine utilization. It is not apparent from these data whether the biologically active conformation structurally assists binding at the active site, or the biologically inactive conformation is sterically hindered from binding.

The preparation of 2-amino-3-methyl-4-hexenoic acids herein reported is primarily an isoleucine antagonist for *L. arabinosus*, but it can also inhibit the utilization of valine. Further biological studies on these antagonisms are in progress.

Experimental[14]

Microbial Assays.—For the assays using *Escherichia coli* 9723 an inorganic salts-glucose medium[15] was employed, and this procedure previously has been reported in detail.[15] For the isoleucine assays with *Streptococcus faecalis* 8043, the media and assay procedure of Meister[10] was used. For *Lactobacillus arabinosus* 17-5, a previously described medium[17] was modified by omitting DL-isoleucine from the basal medium and by adding supplements as indicated in the tables; in addition, calcium pantothenate (100 γ/ml. of vitamin supplement) was added. *L. arabinosus* was incubated at 30°, and *E. coli* and *S. faecalis* were incubated at 37° for the time intervals indicated in the tables.

In all assays the analogs were dissolved in sterile water and added aseptically to the sterile assay tubes without being heated. The amount of growth was determined turbidimetrically in terms of galvanometer readings adjusted such that distilled water reads 0, and an opaque object 100.

DL-2-Amino-*trans*-3-hexenoic Acid (*trans*-Crotylglycine).—This compound was prepared by a previously reported procedure[6] except that *trans*-crotyl bromide was used instead of the chloride, and the final amino acid derivative, formed from the hydrolysis of the intermediate condensation product with ethyl acetamidocyanoacetate, was fractionally recrystallized five times. At each successive stage of crystallization, the product was assayed for its inhibitory activity for *E. coli*,

(14) All melting points are uncorrected. The authors are indebted to Dr. J. M. Ravel and Mrs. Jean Humphreys for assistance with the microbial assays and to Charles Hedgcoth and Allen Lane for some of the elemental analyses. The remainder of the elemental analyses were carried out by Alfred Bernhardt, Germany. All of the paper chromatographic data were obtained using the ascending technique in the solvents indicated, and the chromatograms were developed with ninhydrin reagent. For the vapor phase chromatograms, a Cenco Vapor Phase Analyzer packed with di-2-ethylhexyl phthalate was used at 160° and at a flow rate pressure of 10 lb./sq. in.

(15) E. H. Anderson, *Proc. Natl. Acad. Sci., U. S.*, **32**, 120 (1946).

(16) F. W. Dunn, J. M. Ravel and W. Shive, *J. Biol. Chem.*, **219**, 809 (1956).

(17) J. M. Ravel, L. Woods, B. Felsing and W. Shive, *ibid.*, **206**, 391 (1954).

and a hydrogenated sample was concurrently assayed for the percentage of isoleucine present using *S. faecalis*. The latter data are representative of the concentration of dehydroisoleucine present as a contaminant with the isomeric crotylglycine.

The concentration of alloisoleucine could not be determined by the *L. arabinosus* assay previously indicated since the high concentration of *nor*-leucine produced from the hydrogenation of the crotylglycine present in the original mixture inhibited the response of this organism to alloisoleucine. The five-times recrystallized sample was about 99.8% pure as determined by microbial assays, m.p. 262° (reported[5] m.p. 260°), and gave the anticipated elemental analyses for C, H and N.

Ethyl 2-Acetamido-2-cyano-*cis*-4-hexenoate.—To a solution of sodium ethoxide prepared from 5.1 g. of sodium treated with 200 ml. of ethanol, 38 g. of ethyl acetamidocyanoacetate was added, followed by 22 g. of *cis*-crotyl chloride.[18] The reaction mixture was stirred in an ice-bath for about 24 hr., after which 100 ml. of anhydrous ether was added and the mixture was heated under reflux for about four days. The inorganic salts were removed by filtration, and the filtrate was warmed *in vacuo* to remove the ether. Upon addition of water to the resulting residue, an oil separated which solidified after standing at room temperature for several hours. There was recovered 33.5 g. of product. A sample was recrystallized from aqueous acetone for elemental analysis, m.p. 82°.

Anal. Calcd. for $C_{11}H_{16}N_2O_3$: N, 12.49. Found: N, 12.30.

DL-2-Amino-*cis*-3-hexenoic Acid (*cis*-Crotylglycine).—A mixture of 29.5 g. of ethyl 2-acetamido-2-cyano-*cis*-4-hexenoate and 50 g. of barium hydroxide octahydrate was heated under reflux in a stainless steel vessel for 14 days. After cooling, a stream of carbon dioxide was passed through the reaction mixture to precipitate most of the barium ions as barium carbonate. The resulting mixture was filtered, and sulfuric acid was added to complete the precipitation of the barium ions as barium sulfate. After filtering, the filtrate was finally adjusted to *p*H 6 with 10% sodium hydroxide and reduced *in vacuo* to about one-half the original volume. Upon cooling in the refrigerator overnight, there was recovered 1.2 g. of product which, after recrystallization several times from water, had a m.p. of 272–273° dec.

Anal. Calcd. for $C_6H_{11}NO_2$: C, 55.79; H, 8.59; N, 10.85. Found: C, 56.12; H, 8.66; N, 10.84.

N-Chloroacetyl-DL-2-amino-*trans*-3-hexenoic Acid.—Following a previously reported technique,[19] 200 mg. of *trans*-crotylglycine suspended in 20 ml. of ethyl acetate containing 175 mg. of chloroacetyl chloride was heated to reflux for about 1.5 hr. The resulting mixture was filtered, and 32 mg. of unreacted *trans*-crotylglycine was recovered. The filtrate was evaporated *in vacuo* to a pale yellow oil, which, upon crystallization from ethyl acetate:*n*-hexane yielded 245 mg. of long needles, m.p. 104–106°.

Anal. Calcd. for $C_8H_{12}NO_3Cl$: C, 46.74; H, 6.33. Found: C, 46.93; H, 6.37.

N-Glycyl-DL-2-amino-*trans*-3-hexenoic Acid.—A sample of 140 mg. of the chloroacetyl derivative of *trans*-crotylglycine was dissolved in 3 ml. of 30% ethyl alcohol, and the resulting solution was added dropwise, with stirring, to 10 ml. of concentrated ammonium hydroxide at 0–5°. The reaction mixture was then sealed and left at room temperature for about 24 hr. The resulting solution was evaporated to dryness *in vacuo*, and the solid residue was recrystallized from water–ethanol to yield 81 mg. of product, m.p. 231–232°.

Anal. Calcd. for $C_8H_{14}N_2O_3$: C, 51.60; H, 7.58; N, 15.05. Found: C, 51.23; H, 7.74; N, 15.10.

High Temperature Condensation of 3-Chloro-1-butene and Ethyl Acetamidocyanoacetate.—To a solution of 13.3 g. of sodium treated with 500 ml. of ethanol was added 82 g. of ethyl acetamidocyanoacetate, dropwise and subsequently 55 g. of 3-chloro-1-butene. The resulting reaction mixture was heated to reflux for two days during which time the alcoholic solution slowly changed from alkaline to acidic. After cooling, the salts which had precipitated were removed, and the solvent was reduced to dryness *in vacuo*. Repeated attempts to crystallize the resulting residual oil failed to yield a solid product, and it finally was hydrolyzed directly by placing it in a steel beaker in the presence of 200 ml. of 20% sodium hydroxide and heating over a steam cone for three days. The cooled reaction mixture then was adjusted to *p*H 6 with concentrated hydrochloric acid, and, after cooling in the refrigerator, there was recovered crystalline material. This crude product was recrystallized from water to yield 13 g. of material, m.p. 259° dec.

Anal. Calcd. for $C_6H_{11}NO_2$: C, 55.80; H, 8.59; N, 10.85. Found: C, 56.23; H, 8.52; N, 10.84.

A mixture of 100 mg. of the above material, 100 ml. of water and 50 mg. of palladium black was shaken under 3 atmospheres of hydrogen pressure for 1 hr. in a Parr Apparatus. Microbiological analysis of this hydrogenated product with *L. arabinosus* as the test organism indicated it to be about 10% isoleucine (or alloisoleucine) on a weight basis. The reaction mixture was reduced to dryness *in vacuo*, and the resulting solid residue was fractionally recrystallized from water to yield, as the major product, DL-norleucine which subsequently was identified by comparing it to an authentic sample by infrared techniques.

Ethyl 2-Acetamido-2-cyano-3-methyl-4-pentenoate.—A sample of 138 g. of ethyl acetamidocyanoacetate was added to a solution of 18.4 g. of sodium dissolved in about 600 ml. of ethanol. After solution was completed, 84 g. of 3-chloro-1-butene were added dropwise while the reaction mixture was cooled in an ice-bath, and the resulting mixture was stirred at room temperature for 8 days. The inorganic salts which had precipitated were removed, and the filtrate was reduced to dryness *in vacuo* to yield a viscous oil. The residue then was extracted with chloroform and the chloroform phase was washed with water, after which the organic layer was dried over calcium sulfate. After decantation from the drying agent, sufficient ether was added to cause a slight turbidity, and the resulting solvent mixture was placed in a deep-freeze chest overnight. There were recovered 17 g. of solid material, m.p. 115°. A sample was recrystallized from ethyl acetate–*n*-hexane for elemental analysis, m.p. 117–118°.

Anal. Calcd. for $C_{11}H_{16}N_2O_3$: C, 58.91; H, 7.19; N, 12.49. Found: C, 59.19; H, 6.98; N, 12.47.

DL-2-Amino-3-methyl-4-pentenoic Acid.—A mixture of 15 g. of ethyl 2-acetamido-2-cyano-3-methyl-4-pentenoate was treated with 75 ml. of a suspension containing 70 g. of barium hydroxide octahydrate and heated to reflux for 3 days in a steel vessel. The insoluble material was removed, and the filtrate was then treated with carbon dioxide to remove the residual barium ions. The mixture was again filtered, and the filtrate was adjusted to *p*H 5 with dilute sulfuric acid to precipitate the final traces of barium sulfate. The latter filtrate was reduced in volume *in vacuo* and ethyl alcohol was added to induce crystallization. There were recovered 4.1 g. of material, m.p. 240–243° dec.

Anal. Calcd. for $C_6H_{11}NO_2$: C, 55.79; H, 8.59; N, 10.85. Found: C, 56.03; H, 8.91; N, 10.71.

R_f values in butyl alcohol:acetic acid:water (4:1:1) and 65% pyridine are 0.61 and 0.88, respectively, as determined by the ascending technique using ninhydrin reagent to develop the color.

Catalytic Hydrogenation of DL-2-Amino-3-methyl-4-pentenoic Acid.—A solution of 100 mg. of 2-amino-3-methyl-4-pentenoic acid in 100 ml. of water was treated with 50 mg. of palladium catalyst and placed under 3 atmospheres of hydrogen pressure for about 1 hr. The catalyst was removed by

Compound studied	Relative isoleucine activity, %	
	Lactobacillus arabinosus	*Streptococcus faecalis*
DL-Isoleucine	100	100
DL-Alloisoleucine	100	1
DL-Norleucine	0	0
DL-2-Amino-3-methyl-4-pentenoic acid	0	0
Hydrogenated product from 2-amino-3-methyl-4-pentenoic acid	108	56

(18) This compound was prepared by the method of L. F. Hatch and S. S. Nesbitt, *J. Am. Chem. Soc.*, **72**, 727 (1950), and was about 98% pure as evidenced by a vapor phase chromatographic analysis.

(19) E. Ronwin, *J. Org. Chem.*, **18**, 1546 (1953).

filtration, and the filtrate was assayed for isoleucine content using *L. arabinosus* which responds to both isoleucine and alloisoleucine and *S. faecalis* under conditions such that only isoleucine and not alloisoleucine will promote growth.[10] That the 2-amino-3-methyl-4-pentenoic acid herein reported is approximately 50% DL-dehydroisoleucine and 50% DL-allodehydroisoleucine is apparent from the biological data.

N-Acetyl Derivatives of 2-Amino-3-methyl-4-pentenoic Acid.[20]—A 5 g. sample of 2-amino-3-methyl-4-pentenoic acid indicated above was added to a mixture of 9 ml. of acetic anhydride in 57 ml. of glacial acetic acid and heated to its boiling point for about 12 minutes. The resulting solution was reduced in volume *in vacuo* to yield a yellow oil which was taken up in 7 ml. of water and cooled in the refrigerator. There was recovered 1.2 g. of product, m.p. 115°, which was fractionally recrystallized three times to give 600 mg. of material, m.p. 129–130°.

Anal. Calcd. for $C_8H_{13}NO_3$: N, 8.19. Found: N, 8.35.

Repeated attempts to obtain another crystalline derivative from the mother liquors resulting from the first batch of crystals above were unsuccessful. Alkaline hydrolysis of the final product, m.p. 129–130°, produced a derivative with comparable inhibitory activities to those of the original material. Further, after hydrogenation, the reduced material upon microbial analysis was indicated to be about an equimolar mixture of the two forms, isoleucine and alloisoleucine, indicating that no appreciable resolution of the diastereoisomers had been accomplished.

(20) This procedure was patterned after that of Greenstein, *et al.*,[12] for the separation of isoleucine and alloisoleucine.

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Studies on Polypeptides. XIX. Improved Synthetic Routes to Histidylphenylalanylarginyltryptophylglycine, a Key Intermediate in the Synthesis of ACTH Peptides[1-3]

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Improved procedures are described for the synthesis of the 4-L form of the pentapeptide histidylphenylalanylarginyltryptophylglycine which served as a key intermediate for the construction of the adrenocorticotropically active portion of the ACTH molecule.

In a previous communication[4] we have described the preparation of the 4-L form of the pentapeptide histidylphenylalanylarginyltryptophylglycine (I), a key intermediate in the synthesis of analogs of α-MSH[5] and of the adrenocorticotropically active portion of the ACTH molecule.[3] A different route to this same peptide was reported in a preliminary communication by Schwyzer and Li,[6] but experimental details pertaining to their synthesis are unavailable.

In our original preparation of I[4] we employed N,N′-dicyclohexylcarbodiimide (DCC)[7] to couple carbobenzoxyhistidylphenylalanylnitroarginine (II) with benzyl tryptophylglycinate (III) and subjected the ensuing crude benzyl ester (IV) to exhaustive hydrogenation. Evaluation of the stereochemical homogeneity of the hydrogenation product with leucine aminopeptidase (LAP) and trypsin pointed to marked racemization of the arginine moiety. The crude carbobenzoxyhistidylphenylalanylarginyltryptophylglycine benzyl ester IV was then subjected to fractional crystallization from ethanol and the most sparingly soluble material, obtained in a yield of 19%, proved to be the desired 4-L form of the protected pentapeptide benzyl ester. This material on hydrogenation afforded the 4-L form of the pentapeptide I which was completely digestible by LAP. Hydrogenation of the material from mother liquors gave a pentapeptide whose acid hydrolysate contained the expected amino acids in the correct molar ratios but which was only partially digestible by LAP and trypsin. This material must have contained a mixture of L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophylglycine and of L-histidyl-L-phenylalanyl-D-arginyl-L-tryptophylglycine. Since the possibility existed that the incomplete digestibility of the pentapeptide I from mother liquors could have been the result of an inhibition of the enzymes by impurities in the peptide, we have now employed a strictly chemical approach to confirm the results which were obtained by the use of enzymes. Samples of the mother liquor pentapeptide were hydrolyzed with acid, and arginine was isolated from the hydrolysate by the flavianate procedure.[8] The resulting arginine monohydrochloride exhibited an optical rotation of ±1° in water and thus was unquestionably of the DL-variety. A sample of L-arginine monohydrochloride ($[\alpha]_D$ +12.1°) that was subjected to the same isolation procedure exhibited an optical rotation of +11.2° under identical experimental conditions.

These results provided unequivocal support for the conclusions which were reached by the sole use of enzymatic techniques.

Since the 4-L pentapeptide I served as a key intermediate for further synthetic work and since our previously developed route afforded this compound in low yield, it became of importance to develop

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(2) Most of the peptides and peptide derivatives mentioned in this communication are of the L-configuration. In the interest of space conservation we have eliminated for each individual amino acid residue the customary L-designation where the configuration is clearly L.

(3) See *J. Am. Chem. Soc.*, **83**, 487 (1961), for paper XVIII in this series, erroneously numbered XIII.

(4) K. Hofmann, M. E. Woolner, G. Spühler and E. T. Schwartz, *J. Am. Chem. Soc.*, **80**, 1486 (1958).

(5) K. Hofmann, H. Yajima and E. T. Schwartz, *ibid.*, **82**, 3732 (1960).

(6) R. Schwyzer and C. H. Li, *Nature*, **182**, 1669 (1958).

(7) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

(8) G. J. Cox, *J. Biol. Chem.*, **78**, 475 (1928).