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

Synthesis of 2-Cyclopentene-1-glycine, an Inhibitory Amino Acid Analog

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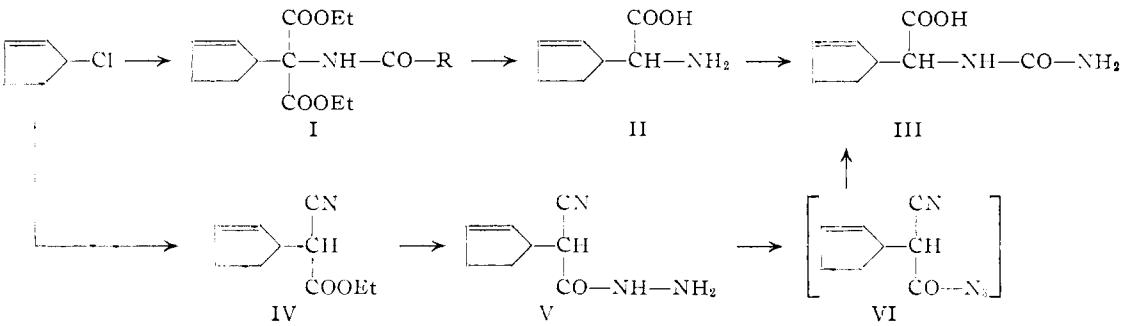
The preparation of 2-cyclopentene-1-glycine has been effected by condensation of 3-chlorocyclopentene with either acetamido- or formamidomalonic ester followed by the usual acid hydrolysis and decarboxylation. Attempts to prepare the amino acid from the condensation product of cyanoacetic ester and 3-chlorocyclopentene by the Curtius method of amino acid synthesis resulted in the formation of 2-(2-cyclopentyl)-hydantoic acid. 2-Cyclopentene-1-glycine is a potent growth inhibitor for *Escherichia coli*, and a mixture of valine and isoleucine but neither alone prevents the inhibition in a competitive manner.

Cyclopentaneglycine has been reported to inhibit competitively the utilization of isoleucine in *Escherichia coli*.[2] The cyclopentyl group apparently is structurally more similar to the *sec*-butyl group than to either the isopropyl or isobutyl group since this amino acid analog prevents the utilization of isoleucine rather than valine or leucine.

In order to compare the effect of a cyclopentene ring with that of a cyclopentane ring, the preparation of 2-cyclopentene-1-glycine was undertaken in this investigation. Condensation of 3-chlorocyclopentene, from cyclopentadiene and hydrogen chloride, with either acetamido- or formamidomalonic ester resulted in the formation of the substituted derivative I which on hydrolysis gave the desired product II.

In subsequent experiments, acetamidomalonic ester was condensed with the halide to obtain the intermediate, α-acetamido-2-cyclopentene-1-malonic ester, in crystalline form. Hydrolysis of this intermediate with dilute instead of concentrated hydrochloric acid resulted in the formation of no more than a trace of the contaminating material giving a purple coloration with ninhydrin reagent. Thus, the cyclopenteneglycine could be purified by fractional crystallization without an intermediate step involving chromatography on Polycel.

During the course of the above synthetic work, other possible synthetic routes were studied in order to avoid the tedious separation by chromatography of the mixture obtained in the initial experiments with formamidomalonic ester. One of these methods involved the Curtius reaction for



In initial experiments, in which formamidomalonic ester was condensed with 3-chlorocyclopentene, the intermediate was not isolated but hydrolyzed directly with concentrated hydrochloric acid. Such a procedure resulted in the formation of a mixture of two components, only one of which was inhibitory to the growth of *E. coli*. Paper chromatograms of the mixture using 95% methanol as the solvent for ascending development gave upon treatment with ninhydrin reagent a yellow spot, R_f 0.59, and a purple spot, R_f 0.65. Bioautographs with *E. coli* showed an inhibition zone corresponding to the position of the yellow spot developed with the ninhydrin reagent. Separation of the two components was effected by chromatographing the mixture on Polycel in 1-butanol saturated with water.

(1) (a) In part from a thesis submitted by Robert L. Dennis to the Graduate School, The University of Texas, in partial fulfillment of the requirement for the degree of Master of Arts, August, 1951; (b) Eli Lilly and Co. Postdoctoral Fellow, 1949–1951.

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

amino acid synthesis, as indicated by the series of equations above. Condensation of cyanoacetic ester with 3-chlorocyclopentene proceeded as anticipated with only moderate yields of the product IV. The substituted cyanoacetic ester formed a crystalline hydrazide V which was then converted to the azide VI by interaction with nitrous acid. The crude product was then treated with aqueous barium hydroxide to form preferentially the 2-(2-cyclopentyl)-hydantoic acid, instead of the anticipated 2-cyclopentene-1-glycine. That the product was indeed the substituted hydantoic acid was further shown by its preparation from 2-cyclopentene-1-glycine by treatment with potassium cyanate in glacial acetic acid. The samples obtained by the two methods gave identical X-ray diffraction patterns.

The mechanism by which the hydantoic acid may be obtained from the cyano azide presumably involves the intermediate formation of a hydantoin, similar to the formation of hydantoins from disub-

stituted cyanoacetamides or malonamides treated with hypobromite.[3] Small yields of hydantoic acid have been obtained from malonamide,[4] but in general the disubstitution has been essential. The formation of a hydantoic acid in this manner is thus unusual for a monosubstituted derivative and may result from steric factors preventing hydrolysis in the usual manner to the amino acid.

In experiments to be reported separately, 2-cyclopentene-1-glycine is a potent inhibitor of growth of *Escherichia coli*. The inhibition is prevented in a competitive manner by a mixture of valine and isoleucine but not by either alone. Thus, the cyclopentene group is more closely related structurally to an isopropyl group than the cyclopentane ring since the cyclopentane analog affects only the utilization of isoleucine.

It is interesting to speculate that the structural differentiation of isoleucine and valine by enzymes result to some extent from the planar or nonplanar configuration of the group attached to the α-carbon atom. Thus, cyclopentaneglycine, containing the cyclopentane ring which has been reported to be slightly puckered rather than strictly planar,[5] would be expected to inhibit isoleucine utilization, while cyclopenteneglycine, containing the presumably planar cyclopentene group, might be expected to inhibit utilization of valine as well as isoleucine. On the other hand, the size of the group may be the determining factor so that the decrease in the distances of the carbon atoms containing the double bond in the cyclopentene derivative may account for its interesting variation in biological activity from the saturated derivative.

Experimental

3-Chlorocyclopentene.—Freshly distilled cyclopentadiene was treated with an equivalent amount of anhydrous hydrogen chloride with the reaction vessel submerged in an ice-salt-bath similar to the procedure of Noller and Adams.[6] A slight purple coloration occurs at the end of the reaction. The product after fractional distillation was used immediately for further synthesis since decomposition occurs rapidly.

2-Cyclopentene-1-glycine (II).—The typical malonic ester synthesis was employed using either formamido- or acetamidomalonic ester.

1. **Formamidomalonic Ester Method.**—The procedure of Galat[7] was employed for the preparation of ethyl formamidomalonate. The sodio derivative was prepared by dissolving 2.3 g. (0.1 mole) of sodium in 100 ml. of ethanol and adding 20 g. (0.1 mole) of ethyl formamidomalonate to the cooled alcoholic solution of sodium ethoxide. The reaction mixture was then cooled to 5°, and 10 g. (0.1 mole) of 3-chlorocyclopentene was added dropwise with stirring. The mixture was then allowed to warm to room temperature, and the reaction mixture was filtered to remove sodium chloride and evaporated under reduced pressure to remove the solvent. The residue, a yellow oil (4 g.), thus obtained was refluxed with 50 ml. of concentrated hydrochloric acid for four hours. The acid was removed under reduced pressure, and the sirupy residue was dissolved in water and treated with charcoal to remove colored impurities. The clear aqueous solution was neutralized (*p*H 7) with ammonium hydroxide, and evaporated under reduced pressure to a volume of 20 ml. Colorless granular material (0.8 g.) precipitated and was recrystallized from 60% isopropyl alcohol.

A paper chromatogram of this product developed with 95% methanol using the ascending technique showed on treatment with ninhydrin the presence of two spots, one having the typical purple color (R_f, 0.65) and the other being yellow (R_f, 0.59). Several recrystallizations from dilute isopropyl alcohol did not separate the mixture into its components.

The two components were separated by chromatography on type RCBF Polycel (Industrial Chemical Sales Division, West Virginia Pulp and Paper Co.) with 1-butanol saturated with water as the solvent. The mixture (10 mg.) was evaporated with water as the solvent. The mixture (10 mg.) was evaporated from an aqueous solution onto a small quantity of Polycel and added to the top of a 2 × 40 cm. column packed with dry Polycel. The packing was such that a rate of flow of about 5 ml. per hour of the developing solvent, 1-butanol saturated with water, was obtained. After 50 ml. of solvent had passed through the column, the substance giving a purple color with ninhydrin began to appear in solvent and fractions of 3 ml. were collected. Separation was accomplished, as indicated by paper chromatograms, with the first six fractions containing only the substance giving the purple color on treatment with ninhydrin, the next two fractions containing both components, and the subsequent fractions containing only the substance giving the yellow color with ninhydrin. The fractions containing only the latter component were evaporated to dryness under reduced pressure and recrystallized from a mixture of isopropyl alcohol and water. The crystals so obtained began decomposing at 230°.

Anal. Calcd. for $C_7H_{11}NO_2$: C, 59.56; H, 7.85; N, 9.92. Found: C, 58.94; H, 7.84; N, 10.01.

2. **Acetamidomalonic Ester Method.**—Ethyl acetamidomalonate was obtained from Winthrop-Stearns, Inc. (Special Chemical Division, 1450 Broadway, New York 18, N. Y.). To a cooled solution of 9.9 g. (0.43 mole) of sodium dissolved in 400 ml. of ethanol was added 100 g. (0.46 mole) of diethyl acetamidomalonate with stirring. After solution was effected the reaction mixture was cooled in an ice-bath, and 46.4 g. (0.45 mole) of 3-chlorocyclopentene was added over a period of about 30 minutes. After completion of addition, the reaction mixture was allowed to warm to room temperature and stirred an additional three hours. The sodium chloride was removed by filtration through a fluted filter paper, and the reaction mixture was then reduced in volume *in vacuo*. A total of 31.3 g. of unreacted diethyl acetamidomalonate was recovered in the initial precipitates obtained on reducing the volume. In the last stage of evaporation, a batch of crude crystals (45.6 g.), m.p. 45–49°, was obtained. Recrystallization was effected by dissolving the crystals in water and reducing the volume at room temperature to obtain 37.1 g. of diethyl α-acetamido-2-cyclopentene-1-malonate (I), m.p. 52–53°.

Anal. Calcd. for $C_{14}H_{21}O_5N$: N, 4.95. Found: N, 5.09.

A sample of 11.6 g. (0.04 mole) of diethyl α-acetamido-2-cyclopentene-1-malonate was added to 100 ml. of 10% hydrochloric acid, and the mixture was heated to reflux for 3 hours. The resulting straw colored solution was reduced to dryness by warming under reduced pressure. The excess hydrochloric acid was removed by repeated addition of ethanol and evaporation under reduced pressure. The residue was dissolved in about 25 ml. of water, decolorized with charcoal, and adjusted to *p*H 7 with concentrated ammonium hydroxide. After standing overnight the crystals were recovered and recrystallized from 60% isopropyl alcohol to yield 2.05 g. of 2-cyclopentene-1-glycine (II) which started colorizing at 227° and melted 252–255° dec.

Anal. Calcd. for $C_7H_{11}NO_2$: C, 59.56; H, 7.85; N, 9.92. Found: C, 59.00; H, 7.79; N, 10.09.

The filtrate was reduced in volume to yield another batch of crystals (1.1 g.).

Ethyl α-Cyano-2-Cyclopentene-1-acetate (IV).—To a solution of 23 g. (1 mole) of sodium in 650 ml. of ethanol was added 113.1 g. (1 mole) of ethyl cyanoacetate. To the cooled reaction mixture was added 102 g. (1 mole) of 3-chlorocyclopentene over a period of about one hour. The sodium chloride precipitated in such small crystals that filtration through a fluted filter paper was necessary. The solvent was removed under reduced pressure, and the product was distilled *in vacuo*. There was obtained about 40 g. of forerun and 55–60 g. of product (30% *ca.* yield), b.p. 86–

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(4) H. Weidel and E. Roithner, *Monatsh.*, **17**, 172 (1896).

(5) J. E. Kilpatrick, K. S. Pitzer and R. Spitzer, THIS JOURNAL, **69**, 2483 (1947).

(6) C. R. Noller and R. Adams, *ibid.*, **48**, 2444 (1926).

(7) A. Galat, *ibid.*, **69**, 965 (1947).

88° (0.5 mm.), $n^{25}D$ 1.4637, d^{25}_4 1.0485; Σ *MR* 47.11, *MR* calcd. 47.14.

Anal. Calcd. for $C_{10}H_{13}NO_2$: C, 67.02; H, 7.31. Found: C, 66.99; H, 7.03.

α-Cyano-2-cyclopentene-1-acethydrazide (V).—A solution of 17.9 g. (0.1 mole) of ethyl α-cyano-2-cyclopentene-1-acetate and 3.3 g. (0.1 mole) of 95% hydrazine in 55 g. of ethanol was heated to reflux for one hour. On removal of the solvent there was obtained 10.5 g. of solid (64% yield), m.p. 90–91°.

Anal. Calcd. for $C_8H_{11}N_3O$: C, 58.16; H, 6.71; N, 25.44. Found: C, 58.10; H, 6.42; N, 25.60.

Curtius Reaction with α-Cyano-2-cyclopentene-1-acethydrazide.—A reaction mixture containing 5 g. (0.03 mole) of α-cyano-2-cyclopentene-1-acethydrazide, 20 ml. of water, 2.5 ml. of concentrated hydrochloric acid and 100 ml. of ether was cooled to 0° in an ice–salt-bath. To this cold mixture 2.1 g. (0.03 mole) of sodium nitrite in 20 ml. of water was added slowly while the reaction mixture was kept below 5°. After completion of addition, the ether phase was recovered and dried with sodium sulfate overnight.

After addition of 50 ml. of ethanol, the ether was removed by evaporation, and the alcoholic solution heated to reflux for two hours. The alcohol was then removed to yield a brown sirupy residue which was heated to reflux for two hours with a saturated aqueous solution of barium hydroxide containing 0.03 mole of base. The reaction mixture was filtered, and the excess barium ions were removed by careful precipitation with sulfuric acid. The clear filtrate was evaporated to a small volume and yielded 1.5 g. of solid material[8] which formed long white needles, recrystallized from water, m.p. 197–200° dec.

Anal. Calcd. for $C_8H_{12}N_2O_3$: C, 52.16; H, 6.57; N, 15.21. Found: C, 52.33; H, 6.24; N, 15.43.

A comparison of the X-ray diffraction pattern of this compound with a sample of 2-(2-cyclopentyl)-hydantoic acid (III), prepared from the amino acid using the potassium cyanate–glacial acetic acid procedure, proved to be identical.[9] Further, this compound was degraded, by heating with alkali under pressure, to a ninhydrin-active material which was identical with cyclopentene glycine as indicated by paper chromatography.

2-(2-Cyclopentyl)-hydantoic Acid (III).—To an ice-cold solution of 350 mg. (0.0025 mole) of 2-cyclopentene-1-glycine and 284 mg. (0.0035 mole) of potassium cyanate in 20 ml. of water, 210 mg. (0.0035 mole) of glacial acetic acid was added over a period of about 20 minutes. The resulting solution was warmed on a steam-bath for 45 minutes and then allowed to cool to room temperature. After neutralization to a congo red end-point, the solution was placed in a refrigerator overnight. A precipitate, 200 mg., was obtained and recrystallized from water to yield white needles, m.p. 197–198° dec.

Anal. Calcd. for $C_8H_{12}N_2O_3$: N, 15.21. Found: N, 15.20.

(8) The residue possessed a small amount of ninhydrin-active material which on paper chromatography corresponded to 2-cyclopentene-1-glycine; however, no crystalline material could be isolated.

(9) These data were furnished by Dr. S. H. Simonsen and Mr. J. L. Ogilvie of the Department of Chemistry.

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[CONTRIBUTION FROM THE RESEARCH DIVISION, ARMOUR & CO.]

The Degradation of Collagen. II. The Solubilization Process in the Acid *p*H Range

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The partial dissolution of intact collagen has been followed as a function of *p*H and the duration of extraction at 60°. At this temperature collagen does not undergo heat shrinkage. A certain portion of the collagen dissolves readily at each *p*H and the amount of this primary solubilization at a given *p*H approaches a limiting upper value. After the primary solubilization has become extensive at *p*H 2.0 the breakdown of the collagen structure proceeds at a significantly greater rate. Dye binding data indicate an opening up of the structure of the insoluble residues but no increase in the number of basic groups on the protein (<1 mmole/100 g.). Carboxyl groups are exposed during the degradations, but only up to the amount expected to be available based upon the analysis for glutamic and aspartic acids. The dissolution process also brings about a change in the effective *pK* of some of the titratable groups. While there is thus little evidence for extensive peptide bond hydrolysis in the residues, the total nitrogen content of the insoluble residues decreases with increasing degradation whereas the soluble extracts, lower in nitrogen than the original collagen when the fraction solubilized is small, gradually increase in total nitrogen. These data support the view that intact collagen may be composed of a series of related proteins of not quite identical composition. The initial phases of the collagen–soluble collagen (gelatin) transition appear to take place with a very limited amount of peptide bond hydrolysis.

The collagen–gelatin transition and the accompanying or subsequent degradation of the soluble material has been studied most extensively through the examination of the thermal degradation of soluble commercial gelatins and extrapolation back to the "parent gelatin" molecule.[1,2] However, since these studies involved the use of a pretreated starting "undegraded" gelatin preparation their results cannot be readily carried over to a discussion of the initial collagen–gelatin transformation. As Kanagy[3] has pointed out, the usual prolonged liming pretreatment will result in the formation of gelatin without the application of heat. Acid-precursor gelatins, on the other hand, seem to resemble collagen in several respects and are quite different from the alkali-precursor gelatins.[4] In order to help bridge this gap in our understanding of the collagen–gelatin conversion we have examined the dissolution of an essentially untreated purified bovine hide collagen in the acid *p*H range.

Experimental

The collagen containing corium of a fresh steer hide, mechanically split from the dermal and fleshy layers, was purified as described previously.[5] The repeated washings with organic acids used in this treatment essentially remove all of the low temperature acid-soluble collagenous protein, variously called "procollagen" or "extracted-skin collagen." The intact, low-temperature acid-insoluble collagen residue was used in all subsequent investigations.

Solubilization studies were carried out in a direct fashion. Weighed samples of collagen, in the form of very small fibrous cubes, were placed in contact with ten times their weight of water, or the equivalent volume of salt or buffer solution. The collagen was allowed to become thoroughly

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