N-Lignoceroyl-sphingomyelin (IXc) crystallized from butyl acetate; m.p. 213–216° (with strong sintering at 180–190°); infrared spectrum: 3.01, 3.42, 3.50, 6.10, 6.42, 6.78, 8.12, 9.41, 10.33, 10.81, 12.01 μ.

Anal. Calcd. for $C_{47}H_{91}N_2O_7P$: C, 67.75; H, 11.7; N, 3.36; P, 3.7. Found: C, 67.73; H, 11.6; N, 3.49; P, 3.7.

REHOVOTH, ISRAEL

[CONTRIBUTION FROM PARKE, DAVIS AND COMPANY'S MULTIPLE FELLOWSHIP IN MEDICINAL CHEMISTRY, MELLON INSTITUTE]

Diazoacetic Esters of Hydroxyamino Acids[1]

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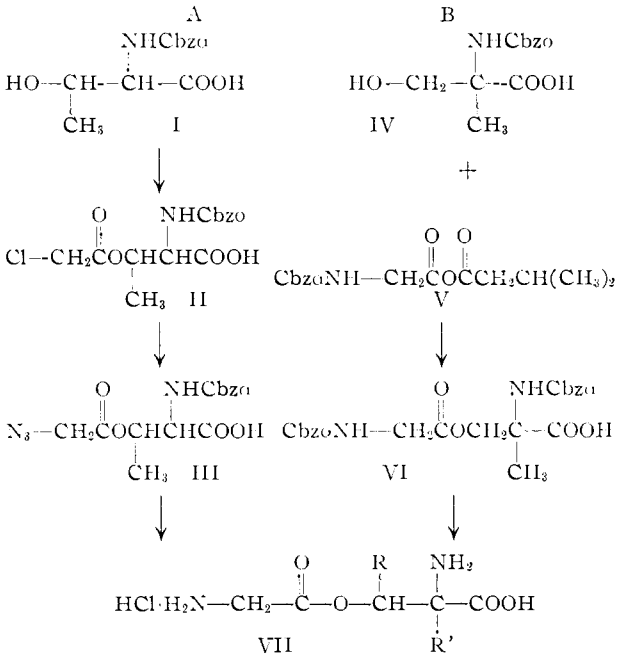
The preparation of the diazoacetic esters of DL-threonine, 2-methyl-DL-serine, 6-hydroxy-DL-norleucine and 4-hydroxy-L-proline are described.

As part of a program on the preparation of compounds for antitumor test, we have studied the effect of replacing the serine part of the azaserine molecule with various hydroxyamino acids. The present paper discusses the diazoacetic esters of DL-threonine, of 2-methyl-DL-serine, of 6-hydroxy-DL-norleucine and of 4-hydroxy-L-proline. The hydroxyamino acids were selected primarily because of their structural relationship to serine. In addition, dextrorotatory 2-methylserine has been reported to be a component of the antibiotic Amicetin,[3] and 6-hydroxy-norleucine has been shown to be similar to an anemia-producing factor of deaminized casein.[4]



The diazoacetic esters were obtained *via* the corresponding glycyl esters VII. The glycyl esters were prepared by *O*-acylation of the appropriate *N*-protected hydroxyamino acid with a reagent having a potential amino group, by procedures similar to those described previously for the synthesis of azaserine,[5] and outlined below for the syntheses of the glycyl esters of threonine (route A) and 2-methylserine (route B).

The *N*-*p*-nitrocarbobenzoxy derivative[6] of 4-hydroxy-L-proline was used for the preparation of the corresponding glycyl ester VII *via* route A in order to obtain crystalline intermediates. The glycyl ester of 6-hydroxynorleucine was prepared according to scheme B.

The glycyl esters of the hydroxyamino acids, as their hydrochloride salts, were diazotized in water at 5°, in the presence of excess nitrite ion at a *p*H of 4.5 to 5.5. The crude reaction mixtures were purified by passage through carbon columns.[7] The yields of the diazoacetic esters were quite low with the exception of the diazoacetic ester of 2-methylserine, which was obtained in 42–65% yields from the pure glycyl ester.

The ultraviolet absorption spectra of the four new diazoacetic esters show a single sharp maximum at 250 mμ and are comparable, on a molar basis, with the ultraviolet absorption spectrum of azaserine. The infrared absorption spectrum of each new diazoacetic ester is characterized, like the infrared spectrum of azaserine, by a strong band at 4.8 μ.

The new diazoacetic esters, and many of the intermediates, were submitted to Sloan-Kettering Institute for test against the Crocker sarcoma-180 tumor in mice, and to the Research Laboratories of Parke, Davis and Co. for various other biological tests. Preliminary reports[8] have indicated that these compounds have no appreciable activity in retarding the growth of tumors in mice at doses which were large in comparison with the minimum effective dose of azaserine.

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Experimental[9]

N-**Carbobenzoxy-DL-threonine.**—DL-Threonine (5.59 g., 0.05 mole) was partially dissolved in 40 ml. of normal sodium hydroxide in a beaker, and with stirring the *p*H was adjusted to 10.5 with more normal sodium hydroxide using a *p*H meter. The solution was cooled to 5–10° in an ice-bath and 9.5 g. (0.055 mole) of benzyl chloroformate was added in small portions. After each addition, the mixture was stirred until most of the chloroformate had reacted and the *p*H had dropped to 9.0–9.5. The *p*H was readjusted to 10.0–10.5 with 4 *N* sodium hydroxide and another portion of benzyl chloroformate was added. At the end of the addition, the mixture was stirred an additional 30 minutes at 10° and at a *p*H of 10 to hydrolyze any *O*,*N*-dicarbobenzoxy derivative. The mixture was extracted with 50 ml. of ethyl acetate and the extract was discarded. The aqueous portion was mixed with 100 ml. of ethyl acetate and the *p*H was adjusted to 1 with concentrated hydrochloric acid. The layers were separated and the aqueous portion was extracted again with ethyl acetate. The combined organic extracts were washed with water and dried over anhydrous sodium sulfate. The solution was evaporated *in vacuo* to an oil weighing 12 g. that crystallized upon standing a week. The solid was triturated with petroleum ether to yield 10.2 g., m.p. 69–75°. The solid was recrystallized from 25 ml. of benzene, 8.7 g., m.p. 81–83°. Samples were recrystallized repeatedly from benzene to a m.p. 82–83°, and dried over P_2O_5 at 0.1 mm.; however, satisfactory analyses were not obtained. The compound appeared to contain varying amounts of benzene.

O-**Chloroacetyl-***N*-**carbobenzoxy-DL-threonine.**—*N*-Carbobenzoxy-DL-threonine (1.25 g., 0.005 mole) was added to a cooled solution of 1.3 g. (0.0077 mole) of chloroacetic anhydride and 3.0 g. (0.025 mole) of *N*,*N*-dimethylaniline in 40 ml. of methylene chloride. The flask was stoppered loosely and cooled in an ice-bath for one hour with occasional swirling, and then allowed to stand at room temperature overnight, or refluxed gently for two hours. The dark reaction mixture was poured into water containing excess hydrochloric acid. The layers were separated and the organic layer was washed with dilute acid and then water. After drying over anhydrous sodium sulfate, the methylene chloride was evaporated *in vacuo*. The residue was dissolved in 6 ml. of benzene and crystallization was induced by adding small increments of petroleum ether during two days. The product separated as glistening needles. The yield was 0.83 g. (50%), m.p. 95–96°. An analytical sample which was recrystallized from a benzene–petroleum ether mixture melted at 96–97°.

Anal. Calcd. for $C_{14}H_{16}ClNO_6$: C, 51.00; H, 4.89; Cl, 10.75. Found: C, 51.38; H, 5.02; Cl, 11.04.

O-**Azidoacetyl-***N*-**carbobenzoxy-DL-threonine.**—A mixture of 3.3 g. (0.01 mole) of *O*-chloroacetyl-*N*-carbobenzoxy-DL-threonine in 50 ml. of dioxane and 1.3 g. (0.02 mole) of sodium azide dissolved in 10 ml. of water was heated under gentle reflux for four hours. During the reflux period, another 5 ml. of water was added to maintain a homogeneous solution. The reaction mixture was concentrated *in vacuo* to a small volume. The yellow sirup was redissolved in 75 ml. of water and filtered from a trace of insolubles. The aqueous solution was acidified with 5 ml. of concentrated hydrochloric acid and the precipitated oil was extracted into four 50-ml. portions of ethyl acetate. After drying over anhydrous sodium sulfate, the solvent was evaporated *in vacuo*. The pale yellow oil (*ca.* 4 g.) was dissolved in 10 ml. of hot benzene and 7 ml. of petroleum ether was added. Upon cooling overnight at 0°, a product weighing 2.3 g. (68.5% yield) was collected, m.p. 77–81°. An analytical sample was prepared by three recrystallizations from benzene–petroleum ether; colorless needles, m.p. 83–84°.

Anal. Calcd. for $C_{14}H_{16}N_4O_6$: C, 50.00; H, 4.79; N, 16.66. Found: C, 50.38; H, 4.59; N, 16.77.

O-**Glycyl-DL-threonine Monohydrochloride.**—*O*-Azidoacetyl-*N*-carbobenzoxy-DL-threonine (1.7 g., 0.005 mole) was dissolved in 30 ml. of ethanol and 5 ml. of *N* hydrochloric acid in 30 ml. of water was added. Hydrogenation of this mixture was carried out in the usual manner in the presence of 0.5 g. of 10% Pd–C catalyst. The catalyst was collected on a Filter-cel mat and washed with water. The filtrate was concentrated to a very small volume and 50 ml. of absolute ethanol was added. Upon standing and scratching, the gum crystallized and 0.9 g. (89% yield) of a colorless solid was obtained that melted with decomposition at 135–140°. The crude product was purified by crystallization from water–ethanol. The hydrochloride melted at 162–163° dec.

Anal. Calcd. for $C_6H_{13}ClNO_4$: C, 33.89; H, 6.16; N, 13.18. Found: C, 33.73; H, 6.00; N, 13.35.

O-**Diazoacetyl-DL-threonine.**—*O*-Glycyl-DL-threonine monohydrochloride (1.59 g., 7.5 meq.) was dissolved in 30 ml. of water and cooled in an ice-bath. A solution of 1.04 g. (15 meq.) of sodium nitrite in 10 ml. of water was added in one portion. The solution turned yellow, and a moderate evolution of gas was observed. After 20 minutes, the mixture was frozen and lyophilized. The ultraviolet absorption spectrum of the yellow hygroscopic powder had a single maximum at 250 mμ, $E^{1\%}_{1cm}$ 177. This crude powder was dissolved in 40 ml. of water and was passed over a column prepared from 20 g. of Darco G-60 and 20 g. of Celite 503. The column was washed with 250 ml. of water and then with 2% aqueous acetone. The fraction of effluent (130–210 ml.) from the latter solvent mixture showing a strong positive ninhydrin test was collected. The yellow solution was frozen and lyophilized. The slightly hygroscopic pale yellow amorphous powder weighed 240 mg. (17% yield), m.p. 137–143° dec., λ 250 mμ, $E^{1\%}_{1cm}$ 930. The theoretical $E^{1\%}_{1cm}$ based on azaserine[7] is 1050.

N-*p*-**Nitrobenzyloxycarbonyl-4-hydroxy-L-proline** was prepared according to the procedure of Carpenter and Gish.[6] After a recrystallization from amyl acetate, the product melted at 133–135°, $[\alpha]^{21.5}D$ −37° (*c* 1, *N* sodium hydroxide).

O-**Chloroacetyl-***N*-*p*-**nitrobenzyloxycarbonyl-4-hydroxy-L-proline.**—*N*-*p*-Nitrobenzyloxycarbonyl-4-hydroxy-L-proline (3.1 g., 0.01 mole) was added to an ice-cold solution of 3.4 g. (0.02 mole) of chloroacetic anhydride and 6.0 g. (0.05 mole) of *N*,*N*-dimethylaniline in 60 ml. of methylene chloride. The solution was kept in an ice-bath for 1.5 hours with occasional swirling. The mixture was heated under reflux another hour and then poured into water containing excess hydrochloric acid. The layers were separated and the organic layer was washed with dilute hydrochloric acid and water. The solution was dried over anhydrous magnesium sulfate and the solvent was evaporated *in vacuo*. The residue was triturated in a small volume of dry ether for crystallization. The colorless solid weighed 3.2 g. (84%), m.p. 120–122°. An analytical sample was recrystallized from ethyl acetate–petroleum ether, m.p. 121–122°, $[\alpha]^{21.5}D$ −44° (*c* 1, ethanol).

Anal. Calcd. for $C_{15}H_{15}ClN_2O_8$: C, 46.58; H, 3.91; Cl, 9.17. Found: C, 46.64; H, 3.75; Cl, 9.35.

O-**Azidoacetyl-***N*-*p*-**nitrobenzyloxycarbonyl-4-hydroxy-L-proline.**—A solution of 1.0 g. (0.015 mole) of sodium azide in 10 ml. of water was added to a solution of 1.9 g. (0.005 mole) of *O*-chloroacetyl-*N*-*p*-nitrobenzyloxycarbonyl-4-hydroxy-L-proline in 30 ml. of dioxane and the mixture was heated under reflux for three hours. The reaction mixture was evaporated *in vacuo* and the residue was dissolved in 60 ml. of water and clarified by filtering. The filtrate was acidified with 5 ml. of concentrated hydrochloric acid and the precipitated oil was extracted into three 40-ml. portions of ethyl acetate. The combined extracts were washed with water, dried over anhydrous magnesium sulfate and the solvent was evaporated *in vacuo*. A small volume of dry ether was added to the residue which crystallized readily with gentle warming and scratching. The solid weighed 1.65 g. (86%) and melted at 104–108°. The azidoacetic ester melted at 105–106° after a recrystallization from ethyl acetate–petroleum ether, $[\alpha]^{21.5}D$ 32° (*c* 1, ethanol).

Anal. Calcd. for $C_{15}H_{15}N_5O_8$: C, 45.80; H, 3.85; N, 17.81. Found: C, 45.80; H, 3.96; N, 17.60.

O-**Glycyl-4-hydroxy-L-proline Monohydrochloride.**—*O*-Azidoacetyl-*N*-*p*-nitrobenzyloxycarbonyl-4-hydroxy-L-proline (7.8 g., 0.02 mole) was dissolved in 75 ml. of absolute methanol and 0.02 mole of concentrated hydrochloric acid and 0.5 g. of 10% Pd–C catalyst were added. The mixture was hydrogenated one hour, fresh catalyst was added and shaking was continued another hour. The

(9) Microanalyses were performed by Dr. C. Tiedke, Laboratory of Microchemistry, Teaneck, N. J.; Mr. C. W. Beazley, Micro-Tech Laboratory, Skokie, Ill., and Mr. G. Stragand, University of Pittsburgh.

catalyst was collected on a Filter-cel mat and the filtrate was evaporated *in vacuo*. The residue was dissolved in 30 ml. of water and cooled in an ice-bath. The *p*H was adjusted carefully to 6.2 with 4 *N* sodium hydroxide. Toluidine (1.4 g.) was removed by filtration. The filtrate was extracted quickly with five portions of ether, and the *p*H of the aqueous solution was readjusted to 3.5 with concentrated hydrochloric acid and decolorized with charcoal. Solutions of this glycyl ester hydrochloride were diazotized directly as described below.

O-Diazoacetyl-4-hydroxy-L-proline.—The aqueous solution of *O*-glycyl-4-hydroxy-L-proline hydrochloride as prepared above was cooled in an ice–water-bath and a solution of 2.7 g. (0.04 mole) of sodium nitrite in 7 ml. of water was added in one portion. The pH rose immediately to 4.3 and then more slowly to 4.9. After 30 minutes the cloudy yellow solution was filtered, frozen and lyophilized. The yellow powder was chromatographed on a column prepared from 25 g. of Darco G-60 and 25 g. of Celite-503 by the technique described above. The yellow eluate was collected, frozen and lyophilized to yield 315 mg. (10% based on the azidoacetic ester) of hygroscopic yellow solid, m.p. 103–107°. The diazoacetic ester had a single sharp maximum in the ultraviolet, λ H_2O max. 250 mμ, $E^{1\%}_{1cm}$ 1000.

Anal. Calcd. for $C_7H_9N_3O_4$: N, 21.10. Found: N, 20.87.

***N*-Carbobenzoxy-6-hydroxy-DL-norleucine** was prepared from 6-hydroxy-DL-norleucine[10] in 76% yield by the procedure described above for the preparation of *N*-carbobenzoxy-DL-threonine. The colorless solid was recrystallized from an ethyl acetate–petroleum ether mixture, m.p. 112–113°.

***O*-(*N*-Carbobenzoxyglycyl)-*N*-carbobenzoxy-6-hydroxy-DL-norleucine.**—The procedure is essentially that of Nicolaides.[5] A solution of 4.8 g. (0.04 mole) of isovaleryl chloride in 10 ml. of methylene chloride was added dropwise to a stirred and cooled (0°) solution of 8.4 g. (0.04 mole) carbobenzoxyglycine and 4 g. (0.04 mole) of triethylamine in 60 ml. of methylene chloride. The mixture was stirred another two hours at 0° as a precipitate of triethylamine hydrochloride formed. A precooled (0°) solution of 11.2 g. (0.04 mole) of *N*-carbobenzoxy-6-hydroxynorleucine and 4.0 g. (0.04 mole) of triethylamine in 60 ml. of methylene chloride was added in one portion. The mixture was stirred for 7 hours at 0° and the temperature was allowed to rise slowly to 15–20° overnight. An equivalent of triethylamine (4 g.) was added and the mixture was extracted with two 40-ml. portions of water to remove isovaleric acid and unchanged *N*-carbobenzoxy-6-hydroxy-DL-norleucine as their triethylamine salts. The washed methylene chloride solution was evaporated *in vacuo*. The oil was redissolved in 40 ml. of ethyl acetate. The ethyl acetate solution, in turn, was extracted with three 35-ml. portions of water. The aqueous extracts were acidified with concentrated hydrochloric acid. The precipitated oil was re-extracted into ethyl acetate. After drying the organic extracts over anhydrous magnesium sulfate, the solvent was evaporated *in vacuo* to yield 12 g. (64%) of colorless oil. The crude bis-carbobenzoxy compound was not crystallized.

O-Glycyl-6-hydroxy-DL-norleucine Monohydrochloride.—The crude *O*-(*N*-carbobenzoxyglycyl)-*N*-carbobenzoxy-6-hydroxy-DL-norleucine from above (12 g., calcd. as 0.025 mole) was dissolved in 50 ml. of absolute ethanol and 25 ml. of *N*-hydrochloric acid was added. The mixture was hydrogenated in the presence of 0.5+ g. of 10% Pd–C catalyst for three hours. The bottle was vented several times, and at the end of one hour another 0.5 g. of fresh catalyst, wet down with water, was added. The catalyst was collected by filtering and the filtrate was concentrated *in vacuo* to *ca.* 15 ml. Absolute ethanol (100 ml.) was added and then dry ether to cloudiness. More ether was added in small portions to complete the crystallization. The crude product weighed 2.0 g. (33%) and melted over the range 187–195°. An analytical sample was recrystallized from water–ethanol, m.p. 200–202° dec.

Anal. Calcd. for $C_8H_{17}ClN_2O_4$: C, 39.91; H, 7.12; N, 11.64. Found: C, 39.57; H, 7.19; N, 11.19.

O-Diazoacetyl-6-hydroxy-DL-norleucine.—*O*-Glycyl-6-hydroxy-DL-norleucine hydrochloride (1.2 g., 0.005 mole) was dissolved in 25 ml. of water and cooled in an ice-bath. A solution of 0.75 g. (0.012 mole) of sodium nitrite in 3 ml. of water was added in one portion. After standing one hour, the yellow solution was frozen and lyophilized. The yellow solid was redissolved in 25 ml. of water and chromatographed over a mixture of 15 g. of Darco G-60 and 15 g. of Celite 503. The column was washed with water (300 ml.) and eluted with 4% aqueous acetone. The aqueous acetone effluent (150–370 ml. fraction) was frozen and dried by vacuum sublimation to yield 300 mg. of the diazoacetic ester, m.p. 214–218° dec. The yellow amorphous solid had a single sharp absorption maximum in the ultraviolet, λ H_2O max. 250 mμ, $E^{1\%}_{1cm}$ 835.

***N*-Carbobenzoxy-2-methyl-DL-serine.**—*N*-Carbobenzoxy-2-methyl-DL-serine was prepared in 88% yield from 2-methyl-DL-serine[11] according to the procedure described above for other carbobenzoxy derivatives of amino acids. The colorless powder melted at 111–112.5° after several recrystallizations from ethyl acetate–petroleum ether.

Anal. Calcd. for $C_{12}H_{15}NO_5$: C, 56.91; H, 5.99; N, 5.53. Found: C, 56.98; H, 5.91; N, 5.34.

***O*-(*N*-Carbobenzoxyglycyl)-*N*-carbobenzoxy-2-methyl-DL-serine.**—A solution of the triethylamine salt of *N*-carbobenzoxy-2-methyl-DL-serine (30 g., 0.12 mole) in 180 ml. of methylene chloride was added to the mixed anhydride of carbobenzoxyglycine and isovaleric acid prepared from 14.4 g. of isovaleryl chloride, 25 g. of carbobenzoxyglycine and 12 g. of triethylamine in 180 ml. of methylene chloride as described above for the bis-carbobenzoxy derivative of the glycyl ester of 6-hydroxy-DL-norleucine. Work-up of the reaction mixture yielded 51 g. (94%) of oily bis-carbobenzoxy derivative.

O-Glycyl-2-methyl-DL-serine Hydrochloride.—Crude *O*-(*N* - carbobenzoxyglycyl) - *N* carbobenzoxy - 2 - methyl-DL-serine, (51 g., *ca.* 0.114 mole) was dissolved in 125 ml. of absolute ethanol and added to 120 ml. of *N* hydrochloric acid containing 3 g. of 10% Pd–C catalyst. The mixture was hydrogenated in the usual manner. The catalyst was collected by filtering, and the filtrate was evaporated *in vacuo*. Absolute ethanol was added in small portions to the sirup with warming until crystallization began. More alcohol was added portionwise to complete the precipitation of the hydrochloride. The amino acid weighed 8.9 g., m.p. 180–182° (40% based on *N*-carbobenzoxy-2-methylserine).

Anal. Calcd. for $C_6H_{13}ClN_2O_4 \cdot {}^1/_2H_2O$: C, 32.50; H, 6.37; N, 12.64. Found: C, 32.61; H, 6.48; N, 12.31.

O-Diazoacetyl-2-methyl-DL-serine.—***O*-Glycyl-2-methyl-DL-serine** monohydrochloride (1.1 g., 0.005 mole) was dissolved in 25 ml. of water and cooled to 5° in an ice–water-bath. A solution of 0.75 g. (0.012 mole) of sodium nitrite in 7 ml. of water was added in one portion. The *p*H of the reaction mixture rose from 3.8 to 5.1 and a yellow color developed very slowly. The mixture was cooled at 5° for an hour and then allowed to stand at room temperature for 30 minutes. The solution was passed through a column prepared from a mixture of 15 g. of Darco G-60 and 15 g. of Celite 503. The column was washed with 400 ml. of water and these washings were discarded. The column was then washed with 4% aqueous acetone. The second 100-ml. fraction (column holdup volume 100 ml.) was frozen and lyophilized. The yield of pale yellow powder ranged from 400 to 600 mg. (42–65%) m.p. 159–161° dec. The diazoacetic ester showed a single sharp absorption maximum in the ultraviolet λ H_2O max. 250 mμ, $E^{1\%}_{1cm}$ 1072. A sample was recrystallized for analysis by solution in several drops of water and adding warm absolute ethanol to cloudiness, m.p. 160–161° dec.

Anal. Calcd. for $C_6H_9N_3O_4$: N, 22.45. Found: N, 21.68.

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