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THE SYNTHESIS OF *l*(−)-LEUCYLGLYCYLGLYCINE[1]

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The preparation of relatively large quantities of a number of peptides (2, 3) required for certain enzyme studies during the war period was undertaken in the writers' laboratory. The preparation of *l*(−)-leucylglycylglycine is described in the present report, since there are no entirely adequate literature methods for the synthesis of this tripeptide. The only recorded synthesis is that of Abderhalden and Fodor (4) who prepared 2.5 g. of the crude peptide by amination of *d*-α-bromoisocapronylglycylglycine. The latter compound was synthesized by the reaction of *d*-α-bromoisocapronyl chloride and glycine anhydride. The preparation of less than 1 g. of carbobenzoxy-*l*(−)-leucylglycylglycine by the hydrazide procedure has been described by Bergmann *et al.* (5).

EXPERIMENTAL

2,5-Diketopiperazine (glycine anhydride). This method is a modification of that described by Sannié (6). A mixture containing 700 g. of technical glycine and 3500 ml. of technical ethylene glycol is heated for 50 minutes at 174–176° with continuous stirring in a 5-l. round-bottom flask. The mixture is cooled for 20 hrs. in the refrigerator, the suspension is centrifuged and the brown crystalline precipitate is washed on a Büchner funnel with absolute methanol (1500 ml. total volume) until the washings are nearly colorless. The brown-colored 2,5-diketopiperazine (about 320 g.) is dissolved in 2.2 l. of boiling water and the solution is cooled overnight in the refrigerator. The suspension is filtered and the light brown crystals are washed with absolute methanol (500 ml.) and dried in air. The product (about 250 g.) is dissolved in 2.5 l. of boiling water, the solution is decolorized with 20 g. of carbon (Nuchar XXX) and the suspension is filtered on a steam-heated Büchner funnel. If the filtrate is yellow, the decolorization procedure is repeated using 5 g. of carbon. The colorless filtrate is cooled overnight in the refrigerator, the suspension is filtered and the crystals are washed in turn with 100 ml. of ice-water, 80 ml. of 50% methanol and 80 ml. of absolute methanol. The yield is 210 g. (40%) of air-dried, pure white 2,5-diketopiperazine. An additional 22% of the product may be readily obtained from the filtrates but the approximately 14% remaining in the glycol residue is not conveniently recovered. The remaining 25% of the original glycine is converted to other products as indicated by the ammoniacal odor of the gases evolved and the black color of the reaction mixture.

Glycylglycine hydrochloride monohydrate. A mixture, prepared by adding 205 g. (1.8 moles) of diketopiperazine to 1100 ml. of hot C. P. concentrated HCl, is immediately heated to boiling, boiled for 90–100 seconds[2] and immediately cooled in an ice-water bath. After an

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[2] The first-order reaction rate constants for the hydrolysis in boiling concentrated HCl of diketopiperazine and glyclyglycine, were found to be about 0.05 and 0.0006, respectively, per second. The optimum single-heating time was calculated from these values to be 90 seconds. The yield is increased about 3% by reheating the filtrate as suggested by Fischer and Fourneau (7). The first heating should be limited to 30 seconds and the second (of the filtrate) to 90 seconds.

hour the crystal mass is filtered on acid-resistant paper. The crystals are washed with three 90-ml. portions of 95% ethanol and dried at 50°. The yield of glycylglycine hydrochloride monohydrate is 300 g. (89%). The product may be purified by recrystallization from water and 95% ethanol.

Anal. Calc'd for $C_4H_{11}ClN_2O_4$: N, 15.01; Eq. wt., 186.6.

Found: N, 14.84, 14.83; Eq. wt. by electrometric titration to *p*H 5.6, 185.2.

Glycylglycine ethyl ester hydrochloride.[3] The following procedure is based on the alternative method of Fischer and Fourneau (7). The preferred method (treatment of diketopiperazine with ethanol-HCl) of these workers was abandoned since only impure products difficult to purify were produced in several trials. A 3-l., three-necked, round-bottom flask is fitted with a reflux condenser, a motor stirrer, an outlet to a gas trap (8) and an inlet tube for dry HCl. One hundred sixty grams (0.86 mole) of glycylglycine hydrochloride monohydrate and 2400 ml. of absolute ethanol are placed in the flask, about 250 g. of dry hydrogen chloride from a tank or generator is introduced with stirring and cooling, and the mixture is boiled vigorously for just 10 minutes on the steam-bath. The steam-bath is immediately replaced by an ice-water bath and the mixture is stirred continuously for 2 hours. The suspension is filtered and the crystals are washed with 50 ml. of ice-cold absolute ethanol. The crude ester is recrystallized from 2400 ml. of absolute ethanol and the product is dried at 50°. An additional 140 g. (0.21 mole) of glycylglycine hydrochloride monohydrate is suspended in the ethanolic filtrate and the peptide is esterified and recrystallized as described. The total yield of recrystallized product is 250 g. (84%). The product contains less than 0.5% of free dipeptide as determined by titration with standard base using methyl red indicator. The melting point is 181–182°. Fischer and Fourneau (7) reported the melting point as 182°, with decomposition.

Glycylglycine ethyl ester (7). Eighty grams (0.41 mole) of glycylglycine ethyl ester hydrochloride is dissolved in a mixture of 56 ml. of water and 170 ml. of chloroform. The mixture is placed in a freezing bath and is cooled to −15°. A solution of 18 g. (0.45 mole) of NaOH in 44 ml. of water, cooled to −15°, is added slowly with constant stirring. One hundred grams of anhydrous K_2CO_3, cooled to −15°, is added and the mixture is thoroughly stirred until a clear supernatant layer of chloroform forms. The chloroform layer is decanted into a flask immersed in a solid carbon dioxide-ethanol bath. The residue is shaken with three 55-ml. portions of cold chloroform and the extracts are decanted into the flask. The combined chloroform extracts are kept cold in the carbon dioxide-ethanol bath until the carbobenzoxy-*l*(−)-leucine azide is ready for use.

l(−)-*Leucine methyl ester hydrochloride.* Dry hydrogen chloride (about 100 g.) is passed for 2 hours with continuous stirring into a mixture containing 250 *g.* of purified *l*(−)-leucine (9) suspended in 1500 ml. of absolute methanol. The solution is distilled under reduced pressure and the residual thick mass of crystals is cooled to 0° and filtered. A second crop of crystals is obtained by treating the filtrate similarly. Both crops of crystals are dried *in vacuo* over NaOH. The first crop is recrystallized once from 6 parts of 1:5 methanol-diisopropyl ether. The second crop is recrystallized once from the mother liquor of the first crop, and once from 6 parts of 1:5 methanol-diisopropyl ether. The total yield of *l*(−)-leucine methyl ester hydrochloride is 260 g. (75%). The product melts at 147–148° and contains about 2% of *l*(−)-leucine hydrochloride as determined by titration with standard base using methyl red indicator. The twice recrystallized product is free of *l*(−)-leucine hydrochloride and melts at 150–151°. Smith and Brown (10) reported the melting point 149–150° for *d*(+)-leucine methyl ester hydrochloride.

Anal. Calc'd for $C_7H_{16}ClNO_2$: C, 46.28; H, 8.88; N, 7.71; Cl, 19.52.

Found: C, 47.07; H, 8.97; N, 7.7; Cl, 19.64.

$[\alpha]_D^{25.3}$ −13.40° (water, c = 5.0775).

[3] The analogous isopropyl ester was prepared by the described procedure except that 2-propanol was substituted for ethanol and the mixture was heated for 60, instead of 10 minutes. The product, m.p. 199–202°, was nearly pure as indicated by titration with standard base and the indicators, alizarine sulfonate and thymolphthalein.

Carbobenzoxy-l(−)-leucine methyl ester (11). Twelve hundred milliliters of chloroform and a solution containing 260 g. (1.43 moles) of *l*(−)-leucine methyl ester hydrochloride dissolved in 650 ml. of water are placed in a 3-l. beaker immersed in an ice-water bath. The electrodes of a *p*H meter, a motor stirrer, and a thermometer are placed in the solution. Two dropping-funnels, one containing 228 ml. (270 g. or 1.4 moles) of a 90% solution of carbobenzoxy chloride (benzyl chloroformate[4]) and the other 250 ml. of 6 *N* NaOH, are clamped so that their outlets extend nearly to the surface of the solution. The solution is stirred and, when the temperature falls to 15°, saturated NaOH (about 79 ml. or 1.43 moles) is added dropwise until the *p*H rises to about 10.0. The carbobenzoxy chloride and 6 *N* NaOH (about 238 ml. or 1.43 moles) are added simultaneously and at such a rate that the *p*H is maintained between 10.0 and 10.5. The temperature is kept between 25° and 35° during the addition (about 60 minutes) of these reagents. When all of the carbobenzoxy chloride has been added the addition of 6 *N* NaOH is discontinued and stirring is continued for 45 minutes. Ten milliliters of pyridine is added[5] and the mixture is acidified to *p*H 2.0 with 6 *N* HCl (about 79 ml. or 1.43 moles). The mixture is transferred to a 3-l. separatory funnel. The chloroform layer is separated and is extracted twice with 600-ml. portions of water, twice with 400-ml. portions of *N* $NaHCO_3$, once with 400 ml. of *N* HCl and once with 600 ml. of water.

The washed chloroform solution is clarified by shaking it with anhydrous Na_2SO_4, the chloroform is removed by distilling the filtrate under reduced pressure and the resulting syrup is purified by adding 100 ml. of water and distilling the solution under reduced pressure. Further purification is effected by adding 50 ml. of ethanol and distilling the solution under reduced pressure. The product is placed for 24 hours in a vacuum desiccator over concentrated sulfuric acid to remove most of the benzyl chloride.[5] The light brown syrupy carbobenzoxy-*l*(−)-leucine methyl ester can be used without further purification for the preparation of the hydrazide. The yield of product is 380 g. (95%).

Carbobenzoxy-l(−)-leucine hydrazide (11). One hundred fifteen milliliters (2.0 moles) of 85% hydrazine hydrate is added to 380 g. (1.37 moles) of syrupy carbobenzoxy-*l*-(−)-leucine methyl ester. The mixture is allowed to stand for 48 hours at room temperature with occasional shaking. During this time the two-phase liquid system gradually becomes homogeneous and eventually solidifies with the evolution of heat. The white cake of hydrazide is dissolved in 800 ml. of hot ethyl acetate. The solution is filtered and placed overnight in the refrigerator. The suspension is filtered and the crystals are washed with a total of about 2 l. of a 3:8 mixture of peroxide-free diethyl ether and petroleum ether. The product is recrystallized from 2.3 ml. of hot ethyl acetate per gram of crude hydrazide. The yield of carbobenzoxy-*l*(−)-leucine hydrazide, m.p. 118–120°, is 250 g. (68%). An additional 75 g. (20%) may be obtained by combining the filtrates and washings, concentrating the solution to a syrup *in vacuo*, adding 35 ml. of hydrazine hydrate, and treating as described. Further purification may be effected by recrystallizing the crude product from ethyl acetate, hot water, 20% ethanol in water or 20% ethanol in petroleum ether or by dissolving it in HCl and precipitating it with $NaHCO_3$. The melting point of the purified product is 120–122°. Bergmann *et al.* (11) found 121° while Smith and Brown (10) reported that carbobenzoxy-*d*(+)-leucine hydrazide melted at 121°. $[\alpha]_D^{25.2}$ −21.31° (95% ethanol, $c = 4.994$).

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[4] Carter *et al.* (12) have described methods for preparing and determining the strength of carbobenzoxy chloride. An excess of this reagent should be avoided since it introduces objectionable impurities.

[5] If an excess of carbobenzoxy chloride is present, the *p*H of the solution may fall one to two units owing to the formation of carbon dioxide (13) and benzyl chloride from carbobenzoxy chloride by the catalytic action of the pyridine. Benzyl chloride dissolves in the chloroform layer and is responsible for the acrid odor of the final product. Benzyl chloride reacts with hydrazine and tends to contaminate the hydrazide.

Carbobenzoxy-l-(−)-leucine azide (12). A solution of 80 g. (0.29 mole) of carbobenzoxy-*l*(−)-leucine hydrazide in a mixture containing 200 ml. of glacial acetic acid, 100 ml. of concentrated HCl, and 500 ml. of water is placed in a 2-l. round-bottom flask fitted with a mechanical stirrer. The flask is set in a freezing bath and, when the temperature is about −12°, a cold solution of 21.5 g. (0.31 mole) of $NaNO_2$ in 107 ml. of water is added. A yellowish oil forms immediately and rises to the surface. The oily layer is taken up in 600 ml. of cold (−15°) ether and the mixture is poured rapidly into a separatory funnel which has been thoroughly chilled by flushing it with ice-water. The aqueous phase is drawn off as quickly as possible and about 3 l. of ether-saturated ice-water is run through the ether solution (without shaking) as rapidly as possible. The ether solution of the carbobenzoxy-*l*(−)-leucine azide is transferred immediately to a 1-liter flask set in a carbon dioxide-ethanol bath.

Carbobenzoxy-l(−)-leucylglycylglycine ethyl ester (5).[6] A 2-l. Florence flask is fitted with a two-hole cork carrying a thermometer and an outlet to a gas trap (8) to absorb the highly toxic hydrazoic acid. The previously described cold chloroform solution of the glycylglycine ethyl ester and the ether solution of the carbobenzoxy-*l*(−)-leucine azide are transferred in turn to the flask. If necessary to facilitate pouring, these solutions may be thawed in an ice-salt bath. The reaction mixture is heated for 7 hours at about 45°. The chloroform-ether solution, cooled to room temperature, is washed successively with three 250-ml. portions of *N* $NaHCO_3$, one of water, three of *N* HCl, and one of water. The washed solution is dried over Na_2SO_4 and evaporated to a syrup under reduced pressure. Two 30-ml. portions of ether are added and then removed by distillation *in vacuo*. Fifteen hundred milliliters of ether is added and the residue is dissolved by warming the mixture. The solution is filtered if necessary to remove undissolved material, the filtrate is stirred at intervals until crystals form, and the mixture is placed overnight in the refrigerator. The suspension is filtered, the precipitate is suspended in 700 ml. of ether, and the mixture is refluxed for 30 minutes, filtered, and dried at 50°. This product should be redigested with ether if its melting point is below 103°. The yield usually is about 60 g. (50%) based on the carbobenzoxy-*l*(−)-leucine hydrazide. Occasionally lower yields are encountered. Among the side reaction products, an ether-soluble solid melting at 123° and an acidic oil, soluble in *N* $NaHCO_3$ were isolated. The crude product, recrystallized from 50% ethanol, melts at 103–104.5°. Bergmann *et al.* (5) reported 105°. $[\alpha]_D^{25.1°}$ −10.98° (95% ethanol, *c* = 5.010).

Carbobenzoxy-l(−)-leucylglycylglycine (5).[7] A solution of 61 g. (0.16 mole) of carbo-

[6] The analogous isopropyl ester was prepared by a similar procedure. The product, m.p. 88–89°, was more difficult to crystallize and purify than the corresponding ethyl ester because of its greater solubility in ether and other solvents.

[7] The tendency of ethyl acetate solutions to spill down the sides of a beaker when poured is counteracted by lubricating the glass under the pouring lip with silicon lubricant (Dow-Corning stop-cock grease).

The relation of yield to hydrolysis time has not been critically studied although prolonged treatment with alkali was avoided to minimize hydrolysis and racemization of the peptide. Even under the mild conditions employed, there was recovered from the combined filtrates from the crystallization of 160 g. of carbobenzoxy-*l*(−)-leucylglycylglycine, 9 g. of a white crystalline substance (m.p. 165–168°) believed to be *dl*-5-isobutylhydantoin-3-acetylglycine as indicated by the following analytical data. The compound had no appreciable specific rotation (−0.03°) in 95% ethanol.

Anal. Calc'd for $C_{11}H_{17}N_3O_5$: C, 48.70; H, 6.32; Eq. wt., 271.3.
Found: C, 49.76; H, 6.43; Eq. wt. by electrometric titration, 267.

The highly acidic character of the compound is indicated by its *p*K value (3.90 in 29% ethanol).

The formation of hydantoins from carbethoxy peptides (14) and the racemization of hydantoins in dilute alkali (15) have been studied by the authors cited.

benzoxy-*l*(−)-leucylglycylglycine ethyl ester in 600 ml. of methanol is filtered into a 4-l. beaker. Ninety milliliters of 2 *N* NaOH is added with stirring. After the solution has stood for 15 minutes, sufficient (about 33 ml.) 6 *N* HCl is added to give a red color with methyl orange paper. One liter of ethyl acetate is added with stirring followed by 1200 ml. of distilled water. The phases are separated with a 4-l. separatory funnel, the aqueous layer is extracted four times with 200-ml. portions of ethyl acetate and discarded, and the five ethyl acetate solutions are combined and shaken in a 4-l. flask with 450 ml. of *N* $NaHCO_3$. The phases are separated, the ethyl acetate layer is extracted three times with 150-ml. portions of *N* $NaHCO_3$ and the ethyl acetate is discarded. The four aqueous extracts are transferred to a 3-l. beaker and immediately acidified with 140 ml. of 12 *N* HCl. The thick oil which separates from the solution is taken up in 250 ml. of ethyl acetate and the water layer is extracted twice with 250-ml. portions of ethyl acetate and discarded. The combined ethyl acetate extracts are dried with anhydrous sodium or magnesium sulfate, decanted or filtered into a 2-l. round-bottom flask and evaporated to a syrup under reduced pressure. Two 30-ml. portions of anhydrous ethyl acetate are added and removed by distillation under reduced pressure. The residue is brought into solution by adding 300 ml. of anhydrous ethyl acetate and warming the mixture. (Occasionally, the product crystallizes as the solvent is being removed and about 1300 ml. of ethyl acetate is required to redissolve it.) Crystallization is induced by scratching the inner surface of the flask with a glass rod at intervals for several hours. When copious crystallization has occurred the flask is placed in the refrigerator for 24 hours. The suspension is filtered, the crystals are washed with 25-ml. of ether and dried at 50°, and the filtrate is placed overnight in the refrigerator. The flask is scratched occasionally to ensure complete crystallization. The yield of crude product, m.p. 133–140°, is 35 g. (62%). The crude product is recrystallized from 2 l. of ethyl acetate. The yield of purified carbobenzoxy-*l*-(−)-leucylglycylglycine, m.p. 144–145°, is 30 g. (85 % recovery). Bergmann *et al.* (5) reported 144° as the melting point and $[\alpha]_D^{22}$ −12.8° (alcohol, c = 4.9).

Anal. Calc'd for $C_{18}H_{25}N_3O_6$: C, 56.98; H, 6.64; N, 11.08; Eq. wt., 379.4.
Found: C, 57.89; H, 6.74; N, 10.59, 10.60; Eq. wt. by electrometric titration, 376.4.
$[\alpha]_D^{25}$ −14.28° (95% ethanol, c = 4.955).

l(−)-*Leucylglycylglycine.* To a solution of 88 g. (0.23 mole) of carbobenzoxy-*l*(−)-leucylglycylglycine in 880 ml. of methanol in a 2-l. flask are added 0.4 g. of activated palladium oxide (16) and 44 ml. of glacial acetic acid. The flask is fitted with inlet and outlet tubes and is shaken in a mechanical shaker while hydrogen is bubbled through the solution at room temperature and pressure. The hydrogenation is continued (1 to 6 days) until no more carbon dioxide is evolved (determined by passing the gas through barium hydroxide solution in a trap) upon the addition of 0.05-g. portions of fresh catalyst. The precipitated tripeptide is dissolved by adding 175 ml. of water and heating the mixture on a steam-bath. The suspension of catalyst is filtered, washed with 80% ethanol and preserved for reactivation. The combined clear filtrate and washings are distilled under reduced pressure and the residual crude *l*(−)-leucylglycylglycine is dissolved by adding 120 ml. of water and heating the mixture on a steam-bath. The solution is cooled to room temperature, 80 ml. of absolute 2-propanol is added and the solution is filtered. Five hundred sixty milliliters of 2-propanol is added to the filtrate and the mixture is heated over a steam-bath until crystals form. The mixture is placed for 24 hours in the refrigerator. The suspension of crystals is filtered and the precipitate is dried *in vacuo*. The yield of crude product is 56 g. (98%). The crude product is recrystallized by dissolving it in 125 ml. of water and adding 670 ml. of 2-propanol to the solution. The yield of purified *l*(−)-leucylglycylglycine, obtained as bulky, finely crystalline powder, is 50 g. (89% recovery).

The tripeptide is nearly insoluble in cold 88% 2-propanol, slightly soluble in methanol, and soluble in 1.6 parts of boiling water, and 2.5 parts of water at 5°. The following approximate solubilities at 5° (given in the parentheses in g. per 100 g. of solvent) were found at the indicated percentage concentrations of 2-propanol in water: 0 (39.2), 66 (5.8), 78 (0.6), 84

(0.15), and 88 (0.0). Precipitation of the tripeptide from 90 and higher percentage concentrations of 2-propanol is incomplete in 20 hours and the precipitates are extremely fine and difficult to filter. Purification is more satisfactory with an 81% solution of 2-propanol. The tripeptide liquefies in 30 seconds when it is placed in an open Pyrex capillary tube and the latter is plunged into a bath at 220° (uncorr.). The apparent specific volume of 0.728 ml./g. was calculated from the densities of 0.206 molar solution at 15°, 20°, 25°, and 30°. The apparent molal volume, 178 ml., is the same as that found by Greenstein and Wyman (17) for *dl*-leucylglycylglycine in 0.04 to 0.08 molal solutions. Specific rotations determined in a 4 dm. tube at temperatures (t) from 20° to 30° from concentrations (c) of 1.27 to 5.11 % are summarized in the following equation:

$$[\alpha]_D^{t^\circ} = +65.30^\circ - 0.46\ c + 0.017\ c^2 - 0.35\ t + 0.0046\ t^2.$$

The values reported by Abderhalden and Fodor (4) are 12° to 15° lower than those found for our product due, probably, to partial racemization during amination. The probable high purity of our tripeptide preparation was established by the further observation that the specific rotations of 5.000% solutions at 25° were + 57.56° for the original purified product (A), +57.72° for the product (B) obtained by recrystallization of (A) from water, and +57.56° for the product obtained by precipitating it with alcohol from the filtrate of (B).

Anal. Calc'd for $C_{10}H_{19}N_3O_4$: N, 17.13. Found: N, 17.18, 17.22.

SUMMARY

Methods have been described for the synthesis of 50 g. of purified *l*(−)-leucylglycylglycine by the following reactions: Glycine → glycine anhydride → glycylglycine hydrochloride monohydrate → glycylglycine ethyl ester hydrochloride → glycylglycine ethyl ester. *l*(−)-Leucine → *l*(−)-leucine methyl ester hydrochloride → carbobenzoxy-*l*(−)-leucine methyl ester → carbobenzoxy-*l*(−)-leucine hydrazide → carbobenzoxy-*l*(−)-leucine azide. Glycylglycine ethyl ester + carbobenzoxy-*l*(−)-leucine azide → carbobenzoxy-*l*(−)-leucylglycylglycine ethyl ester → carbobenzoxy-*l*(−)-leucylglycylglycine → *l*(−)-leucylglycylglycine.

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