A_B, A_{NaOH}, A_{HClO_4} are the absorbancies of the buffer solution in question and of solutions (b) and (c), respectively, at a chosen wave length.

The measure of the pK_a for each amine was carried out at four wave lengths in the 320–345 mμ region. The values agreed to ±0.03 unit. The average values are listed in Table I.

[CONTRIBUTION FROM THE ORGANIC CHEMICAL RESEARCH SECTION, LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID CO., PEARL RIVER, N. Y.]

The Use of Esters of N-Hydroxysuccinimide in Peptide Synthesis[1]

BY GEORGE W. ANDERSON, JOAN E. ZIMMERMAN, AND FRANCIS M. CALLAHAN

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A number of N-hydroxysuccinimide esters of acylamino acids have been synthesized. These compounds are crystalline solids which react readily with amino acids or peptides or their esters. Peptide formation under aqueous conditions goes well. Because of the water solubility of N-hydroxysuccinimide, these esters appear to be more generally useful than the analogous esters of N-hydroxyphthalimide.

In recent years, *p*-nitrophenyl esters of acylamino acids have been of great value in the synthesis of peptide derivatives.[2] Other activated esters such as cyanomethyl esters[3] have been promising, and most recently esters of N-hydroxyphthalimide[4a,b] have been shown to be valuable intermediates. The latter are particularly interesting because of their high reactivity, and in addition they are readily crystallized. It seemed worthwhile to us to explore the field of hydroxylamine compounds for derivatives with still better properties. We are particularly interested in derivatives which give readily removable by-products; in the field of simple hydroxylamine derivatives, water solubility appeared to be an attractive possibility. In contrast to N-hydroxyphthalimide, N-hydroxysuccinimide[5] is water soluble, and we have found that acylamino acid esters of this compound meet the important requirements of crystallinity and high reactivity. Consequently we have undertaken an extensive investigation of these derivatives.

N-Hydroxysuccinimide has previously been best prepared *via* the O-benzyl derivative[6] or by fusion of succinic anhydride with hydroxylamine.[7] We have employed a simplification of the latter procedure using hydroxylamine hydrochloride to give a 44% yield of pure N-hydroxysuccinimide, with a melting point of 99–100° (see Experimental, example 1).

The dicyclohexylcarbodiimide method of ester synthesis, which has been used for the synthesis of esters of N-hydroxyphthalimide[4] and *p*-nitrophenyl esters,[8,9] has been used for the synthesis of the N-hydroxysuccinimide esters of a number of N-protected amino acids. These are colorless crystalline derivatives with good stability (Tables I and II, Experimental examples 2–5). Exposure to the atmosphere for weeks or months resulted in slight lowering of the melting points in some cases, but storage of the pure compounds in a desiccator gave no change in melting points in 2 months.

(1) Preliminary communication: *J. Am. Chem. Soc.*, **85**, 3039 (1963).
(2) M. Bodanszky, *Ann. N. Y. Acad. Sci.*, **88**, 655 (1960).
(3) R. Schwyzer, M. Feurer, and B. Iselin, *Helv. Chim. Acta*, **38**, 84 (1955).
(4) (a) G. H. L. Nefkens and G. I. Tesser, *J. Am. Chem. Soc.*, **83**, 1263 (1961); (b) G. H. L. Nefkens, G. I. Tesser, and R. J. F. Nivard, *Rec. trav. chim.*, **81**, 683 (1962).
(5) "Beilstein," Vol. 21, 4th Ed., 1935, p. 380.
(6) D. E. Ames and T. F. Grey, *J. Chem. Soc.*, 631 (1955).
(7) R. Wegler, F. Grewe, and K. Mehlhose, U. S. Patent 2,816,111 (1957).
(8) D. F. Elliot and D. W. Russell, *Biochem. J.*, **66**, 49P (1957).
(9) M. Rothe and F. W. Kunitz, *Ann. Chem.*, **609**, 88 (1957).

Acylpeptide esters were conveniently prepared by the reaction of N-hydroxysuccinimide derivatives with equivalent amounts of amino acid or peptide esters in organic solvents at room temperature. Usually a 40-min. reaction time was used (a longer period may improve yields in some cases) and the peptide product was precipitated by the addition of water. Examples 6 and 7 (Experimental), illustrate the procedure used.

It was of interest to try reactions under aqueous conditions, since older methods of peptide synthesis usually give poorer yields in the presence of water. Illustrative results are given in examples 8–14. Several experiments in the synthesis of Z·gly-L-try·OH (example 11) showed that the use of 2 equivalents of sodium bicarbonate is desirable. Examples 8B and 9B indicate that the analogous use of esters of N-hydroxyphthalimide gave poor results largely because of the water insolubility of N-hydroxyphthalimide.

The results with the aqueous reactions are promising for the development of conditions for the lengthening of peptide chains by the reaction of esters of N-hydroxysuccinimide with salts of peptides or proteins. Further work in this direction is projected.

Experimental

Melting points were determined on a calibrated Fisher-Johns block.

1. **N-Hydroxysuccinimide.**—Succinic anhydride (100 g., 1.0 mole) and hydroxylamine hydrochloride (70 g., 1.0 mole) were combined in a 1-l. flask. The flask was placed on a rotary evaporator and the contents heated by a silicone oil bath. The volatile products which formed were removed under vacuum provided by a water aspirator and, after passage through a cold water condenser, were caught in a Dry Ice trap. The contents of the flask were rapidly heated to about 125° where fusion occurred with evolution of gases. Over the next hour the temperature was increased slowly up to 160°, at which point the formation of water had virtually ceased. The heating was discontinued. When the temperature had dropped to 125°, the amber liquid was poured into a beaker containing 400 ml. of ether which was vigorously stirred. The ether layer was decanted after the product solidified and the residue heated to boiling with 400 ml. of dry 1-butanol. The mixture was filtered and the filtrate rapidly chilled to 0°. After a 1-hr. period, the crystalline material was collected by filtration and the residue was washed carefully with 1-butanol, then ether. The crude product, melting at 93 to 95°, amounted to 75 g. and had a light tan color. This material was treated briefly with 450 ml. of hot ethyl acetate (6 ml./g.) and the mixture was filtered. Cooling and chilling of the filtrate yielded 46 g. of white crystalline material, m.p. 99–100°. The material which had not dissolved in ethyl acetate was retreated with an additional 75 ml. of hot ethyl ace-

TABLE I

N-HYDROXYSUCCINIMIDE ESTERS OF BENZYLOXYCARBONYLAMINO ACIDS[a,10]

Ester of	Recrystn. solvent	Yield, %	M.p., °C.	$[\alpha]^{25}D$ (c 2, dioxane)	—Anal., calcd. %— C	H	N	—Anal., found % C	H	N
Z-Glycine	Methylene chloride–petroleum ether	86	113–114	...	54.90	4.61	9.15	55.08	4.77	8.99
Z-L-Phenylalanine	Chloroform–petroleum ether	76	140–140.5	−17.3	63.63	5.09	7.07	63.63	5.12	7.00
Z-L-Proline	Isopropyl alc.	78	90	−54	58.95	5.24	8.09	58.99	5.45	8.13
Z-L-Isoglutamine[11]	Isopropyl alc.	48	151–153	+1.0	54.11	5.08	11.14	54.37	5.12	11.12
Z-L-Leucine	Isopropyl alc.	51	116–117	−33.6	59.66	6.12	7.73	59.46	6.30	7.69
Z-L-Alanine	Isopropyl alc.	65	123–123.5	−37.2	56.25	5.04	8.75	56.17	5.37	8.59
Z-L-Valine	Isopropyl alc.	53	116–117	−25.1	58.61	5.79	8.04	58.80	5.64	8.14
Z-L-Isoleucine	Isopropyl alc.	56	115.5–116	−15.5	59.66	6.12	7.73	59.71	5.84	7.54
Z_3-L-Arginine[12,c]	Ethyl acetate–petroleum ether	80	85–86	−9.7	60.62	5.24	10.46	59.89	5.31	9.89
Z-L-Methionine[b]	Isopropyl alc.	59	101–102	−15.9	53.71	5.30	7.36	53.51	5.31	7.34
Z-D-Phenylalanine	Methylene chloride–petroleum ether	76	140.5–141	+15.9	63.63	5.09	7.07	63.51	5.17	7.02
Z-L-β-Cyanoalanine[13]	Isopropyl alc.	52	118–119	−36.8	55.65	4.38	12.17	55.46	4.48	12.14
Z-L-Glutamic acid γ-methyl[14] ester	Isopropyl alc.	53	107–108	−23.3	55.10	5.14	7.14	54.95	5.23	7.09

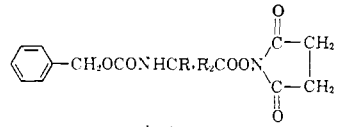
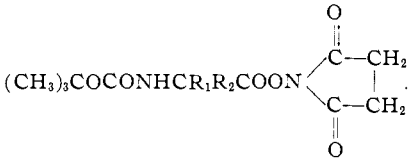
[a] General formula:  The benzyloxycarbonylamino acids used were obtained from commercial sources unless otherwise indicated. [b] *Anal.* Calcd.: S, 8.43. Found: S, 8.78. [c] *Anal.* Calcd. for 0.5 mole of water: C, 59.98; H, 5.32; N, 10.25; H_2O, 1.3. Found: H_2O, 0.7.

TABLE II

N-HYDROXYSUCCINIMIDE ESTERS OF *t*-BUTYLOXYCARBONYLAMINO ACIDS[a,10]

Ester of	Recrystn. solvent	Yield, %	M.p., °C.	$[\alpha]^{25}D$ (c 2, dioxane)	—Anal., calcd. %— C	H	N	—Anal., found % C	H	N
BOC-L-Alanine	Isopropyl alc.	71	143–144[f]	−49	50.34	6.34	9.79	50.45	6.60	9.90
BOC-S-Benzyl-L-cysteine[b]	Isopropyl alc.	49	117–117.5	−54.0	55.87	5.92	6.86	55.93	6.04	6.73
BOC-Glycine	Isopropyl alc.	62	168–170	....	48.52	5.92	10.29	48.99	6.34	9.88
BOC-L-Isoleucine[e]	Diisopropyl ether	61	92–93	−26.5	54.86	7.37	8.53	55.13	7.39	8.69
BOC-L-Leucine	Diisopropyl ether	48	116	−41.8	54.86	7.37	8.53	54.84	7.44	8.62
BOC-DL-Methionine[c]	Isopropyl alc.	65	118–119	....	48.54	6.40	8.09	48.46	6.50	7.93
BOC-L-Methionine[d]	Isopropyl alc.	59	128–129	−20.6	48.54	6.40	8.09	48.82	6.34	8.05
BOC-D-Phenylalanine	Isopropyl alc.	86	152–153	+20.9	59.66	6.12	7.73	59.55	6.10	7.91
BOC-L-Phenylalanine	Diisopropyl ether–isopropyl alc.	81	152–153	−19.0	59.66	6.12	7.73	59.53	6.11	7.64
BOC-L-Proline	Isopropyl alc.	74	135–136	−55.3	53.84	6.45	8.97	53.71	6.77	8.93
BOC-L-Tryptophan	Isopropyl alc.	37	153–154	−22.4	59.84	5.78	10.47	59.78	5.68	10.60
BOC-L-Valine	Methylene chloride–methylcyclohexane	74	128–129	−37.0	53.49	7.05	8.91	53.54	6.98	8.93

[a] General formula:  The BOC-amino acids used were prepared by the method of Anderson and McGregor.[15] [b] Calcd.: S, 7.85. Found: S, 7.92. [c] Calcd.: S, 9.26. Found: S, 9.46. [d] Calcd.: S, 9.26. Found: S, 9.25. [e] Prepared from anhydrous BOC-ileu-OH, m.p. 70–71°, which was obtained by recrystallization from ethyl ether–petroleum ether. Calcd.: C, 57.12; H, 9.15; N, 6.06. Found: C, 57.55; H, 9.41; N, 5.97. The compound was previously reported as a hemihydrate,[15] m.p. 49–57°. [f] Another form, m.p. 167°, has been obtained.

tate to yield an additional 4 g. with the same melting point; total yield 50 g. (44%). Wegler, *et al.*,[7] reported m.p. 98–98.5° and 53% yield by a comparable reaction with the free base of hydroxylamine.

2. N-Hydroxysuccinimide Ester of Benzyloxycarbonylglycine.—N,N-Dicyclohexylcarbodiimide (26 g., 0.126 mole) was added to a solution of benzyloxycarbonylglycine (26.3 g., 0.126 mole) and 14.5 g. (0.126 mole) of hydroxysuccinimide in 250 ml. of dioxane with cooling. The reaction mixture was allowed to stand in the refrigerator overnight. The formed dicyclohexylurea was filtered and washed with dioxane. The filtrate was concentrated *in vacuo* to yield a yellow oil which soon crystallized. The compound was triturated with ether and filtered to obtain 37.5 g. (97%), m.p. 108–11°. Recrystallization from methylene chloride and petroleum ether yielded 33 g. (86%), m.p. 113–114°.

(10) Amino acid abbreviations (where used) are those of E. Brand and B. F. Erlanger, *J. Am. Chem. Soc.*, **73**, 3508 (1951); Z is benzyloxycarbonyl, BOC is *t*-butyloxycarbonyl, T is trityl, F is formyl.

(11) Z·glu·NH_2 (L) was prepared by the procedure of H. Gibian and E. Klieger, *Ann.*, **640**, 145 (1961).

(12) Z_3-arg·OH (L) was prepared from the sodium salt (Aldrich Chemicals Co.) by the method of L. Zervas, M. Winitz, and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).

(13) Z·ala·OH (L) was prepared by the method of C. Ressler and H. Ratzkin, *ibid.*, **26**, 3356 (1961).

(14) Z·glu·OH (L) was prepared by the method of G. Losse, H. Jeschkeit, and W. Langenbeck, *Ber.*, **96**, 204 (1963).

(15) G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, **79**, 6180 (1957).

Other benzyloxycarbonylamino acid derivatives were prepared in a similar manner (Table I). Dimethoxyethane and dimethylformamide were occasionally used in place of dioxane.

3. N-Hydroxysuccinimide Ester of *t*-Butyloxycarbonyl-L-alanine.—Butyloxycarbonyl-L-alanine[15] (1.32 g., 0.0070 mole) and N-hydroxysuccinimide (0.805 g., 0.0070 mole) were mutually dissolved in 10 ml. of anhydrous dimethoxyethane at 0°. Then dicyclohexylcarbodiimide (1.59 g., 0.0070 mole + 10%) was dissolved with stirring and the solution kept at 0–5° for a period of 20 hr.

The urea which formed was separated by filtration and the filtrate evaporated to dryness in an open dish leaving a crystalline residue of 2.33 g. of crude product. Two successive recrystallizations from isopropyl alcohol gave the pure product, 1.42 g. (71%), melting at 143–144°. The other compounds in Table II were similarly prepared.

4. N-Hydroxysuccinimide Ester of Phthaloylglycine.—Phthaloylglycine[16] (4.10 g., 0.020 mole) and hydroxysuccinimide (2.30 g., 0.020 mole) were dissolved together in 25 ml. of anhydrous dimethoxyethane. To the ice-cold solution was added dicyclohexylcarbodiimide (4.53 g., 0.020 mole + 10%) with stirring. After a period of 20 hr. at 0° the urea was removed by filtration and the filtrate concentrated to dryness by evaporation of the solvent in an open vessel. The residue was recrystallized from isopropyl alcohol to yield the analytically pure product, m.p. 182–183°, yield 4.21 g. (70%). *Anal.* Calcd. for $C_{14}H_{10}N_2O_6$: C, 55.63; H, 3.34; N, 9.27. Found: C, 55.86; H, 3.36; N, 9.11.

5. N-Hydroxysuccinimide Ester of Tritylglycine.—Tritylglycine[17] (6.34 g., 0.020 mole) and hydroxysuccinimide (2.30 g., 0.020 mole) were mutually dissolved in 125 ml. of anhydrous dimethoxyethane (solvent). The solution was cooled to 5° and dicyclohexylcarbodiimide (4.52 g., 0.020 mole +10%) added. After a period of 20 hr. at 5°, the urea was separated by filtration and the filtrate evaporated to dryness leaving a residue of 8.85 g. Recrystallization from isopropyl alcohol yielded 6.90 g. (83%) of the pure product, m.p. 145.5–146.5°. *Anal.* Calcd. for $C_{25}H_{22}N_2O_4$: C, 72.45; H, 5.35; N, 6.76. Found: C, 72.54; H, 5.39; N, 6.95.

6. Ethyl Benzyloxycarbonyl-L-phenylalanyl-L-tyrosinate.—A solution of 1.98 g. (0.005 *M*) of the N-hydroxysuccinimide ester of carbobenzoxy-L-phenylalanine in 10 ml. of dimethoxyethane was added to a solution of 1.045 g. (0.005 *M*) of ethyl tyrosinate[18] (L) in 10 ml. of the same solvent. The reaction was allowed to stand for 40 min. at room temperature and was then diluted with 60 ml. of water. The crystals which formed immediately were collected and washed with 10% sodium bicarbonate solution, water, *N* hydrochloric acid, and water. The crude yield after drying was 2.62 g. (100%), m.p. 151–157°. Two recrystallizations from ethanol–water yielded 2.08 g. (85%), m.p. 156–158°, $[\alpha]^{26}D$ −9.1 ± 0.5° (*c* 10.0, ethanol). These results compare favorably with the literature values of m.p. 159–160°, $[\alpha]^{24}D$ −9.1 ± 0.1° (*c* 10, ethanol), and 46% yield by the mixed anhydride procedure.[19]

7. Ethyl *t*-Butyloxycarbonylglycyl-L-phenylalanylglycinate.—Ethyl L-phenylalanylglycinate hydrobromide (2.48 g., 75 mmoles), the hydroxysuccinimide ester of *t*-butyloxycarbonylglycine (2.04 g., 75 mmoles), and triethylamine (1.05 ml., 75 mmoles) were mutually combined in 25 ml. of anhydrous dimethoxyethane. The mixture was stirred for a period of 20 min. after which it was added to 100 ml. of cold water. The product crystallized and was collected by filtration. The crude product (2.58 g.) melted at 98–99°. Recrystallization from alcohol–water yielded the pure tripeptide, m.p. 100.5–101.5°, with a net yield of 79%; $[\alpha]^{25}D$ −9.75 ± 2.46° (*c* 2.05, methanol). Anderson and McGregor[15] reported a yield of 57% by the pyrophosphite method and a melting point of 98–99°, $[\alpha]^{26}D$ −10.9° (*c* 2.00, methanol).

8. Benzyloxycarbonylglycyl-L-proline. A. *Via* the N-Hydroxysuccinimide Intermediate.—A solution of 1.53 g. (0.005 mole) of the N-hydroxysuccinimide ester of benzyloxycarbonylglycine in 10 ml. of dimethoxyethane was added to a solution of 0.865 g. (0.0075 mole) of proline and 0.63 g. (0.0075 mole) of sodium bicarbonate dissolved in 8 ml. of water at room temperature. After 1 hr., 5 ml. more water was added and the solution was acidified with concentrated hydrochloric acid to pH 2. After chilling for 30 min., 0.93 g. of crystals, m.p. 157–159°, was collected. From the filtrate with further chilling two small crops were collected which totaled 0.2 g., m.p. 157–158°. The three fractions were combined and recrystallized from 50 ml. of hot ethyl acetate to give 0.97 g., m.p. 158–159°, and with cooling, a second crop of 0.18 g., m.p. 157–158°; total yield 75%. Rydon and Smith[20] obtained a 68% yield, m.p. 155°, using the thiophenyl ester method.

B. *Via* **the N-Hydroxyphthalimide Intermediate.**—A solution of 1.77 g. (0.005 mole) of the N-hydroxyphthalimide ester of benzyloxycarbonylglycine[4b]) in 25 ml. of dimethoxyethane was added to a solution of 0.575 g. (0.005 mole) of proline and 0.42 g. (0.005 mole) of sodium bicarbonate in 8 ml. of water at room temperature. The solution remained clear, although a bright red color formed immediately. After 10 min., another 15 ml. of water was added. The solution was acidified very slowly with concentrated hydrochloric acid and fractions were collected at various pH values: pH 6, 0.25 g., m.p. >160°; pH 4, 0.05 g., m.p. >160°; pH 3, 0.98 g., m.p. 145–148°. The change of color in the solution could be observed as the pH decreased. The solution was colorless at pH 3. The last fraction was redissolved in 10 ml. of 10% sodium bicarbonate solution and the slow acidification was repeated. At pH 4–4.5 the solution was slightly yellow and a small fraction (0.15 g.), m.p. 223–226°, was collected. The filtrate was now acidified to pH 2 and 0.65 g., m.p. 151–154°, was collected. This filtrate on chilling yielded another 0.05 g., m.p. 145–150°. The total yield of still impure dipeptide was 0.70 g. (45%).

9. Benzyloxycarbonyl-L-prolylglycine. A. *Via* the N-Hydroxysuccinimide Intermediate.—A solution of 1.73 g. (0.005 mole) of the N-hydroxysuccinimide ester of benzyloxycarbonyl-L-proline in 17 ml. of ethanol was added to a solution of 0.38 g. (0.005 mole) of glycine and 0.84 g. (0.010 mole) of sodium bicarbonate in 12 ml. of water. After 18 hr., the solution was concentrated under vacuum. Acidification to pH 2 with concentrated hydrochloric acid gave an oil which crystallized. After chilling, this was collected and dried; wt. 1.4 g., m.p. 116–124°. Recrystallization from ethyl acetate–petroleum ether gave 1.12 g. (72% yield), m.p. 124–125°. Grassman and Wunsch[21] obtained an 85% yield and m.p. 124–125° by saponification of the ethyl ester. Ondetti[22] has recently obtained the compound in 82% yield by the reaction of the *p*-nitrophenyl ester of benzyloxycarbonyl-L-proline with glycine in a water–pyridine–sodium hydroxide medium.

B. *Via* the N-Hydroxyphthalimide Intermediate.—A solution of 1.97 g. (0.005 mole) of the N-hydroxyphthalimide ester of benzyloxycarbonyl-L-proline[4b] in 9 ml. of dimethoxyethane was added to a solution of 0.375 g. (0.005 mole) of glycine and 0.42 g. (0.005 mole) of $NaHCO_3$ in 7 ml. of water with stirring at room temperature. An additional 2 ml. of dimethoxyethane was added to maintain a clear solution. After 50 min. the solution was concentrated under vacuum to remove dimethoxyethane. The aqueous reaction mixture was acidified to pH 5 with concentrated hydrochloric acid and filtered to yield 0.72 g., m.p. 188°. The filtrate was acidified further to pH 2 and filtered to give 0.43 g. (28% of the theoretical 1.53 g. of peptide), m.p. 106–108°.

Thin layer chromatography indicated that both fractions were mixtures of benzyloxycarbonyl-L-prolylglycine and N-hydroxyphthalimide. An attempt was made to separate the two using fractional crystallization from chloroform. Both fractions were treated with chloroform and the insoluble portions filtered off. The first gave 0.44 g., m.p. 225–226°, and the second 0.03 g., m.p. 224–226°. The chloroform filtrates were evaporated to dryness and chromatographed to show that both fractions were still mixtures. N-Hydroxyphthalimide melts at 225–226° and benzyloxycarbonyl-L-prolylglycine at 124–125°.

10. Tritylglycyl-N$^{\epsilon}$-formyl-L-lysine.—ε-Formyl-L-lysine[23] (0.87 g., 0.005 mole) and sodium bicarbonate (0.42 g., 0.005 mole) were dissolved in 18 ml. of dimethoxyethane. A solution of the N-hydroxysuccinimide ester of tritylglycine (2.07 g., 0.005 mole) in 8 ml. of water was added with stirring at room temperature. A precipitate began to form so another 10 ml. of dimethoxyethane was added to maintain a clear solution. This was allowed to

(16) J. H. Billmann and W. F. Harting, *J. Am. Chem. Soc.*, **70**, 1473 (1948).
(17) Purchased from Aldrich Chemicals Co.
(18) E. Fischer, *Ber.*, **34**, 433 (1901).
(19) J. R. Vaughan, Jr., and R. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1956).
(20) H. N. Rydon and P. W. G. Smith, *J. Chem. Soc.*, 3643 (1956).
(21) W. Grassmann and E. Wünsch, *Ber.*, **91**, 449 (1958).
(22) M. A. Ondetti, *J. Med. Chem.*, **6**, 10 (1963).
(23) Purchased from Cyclo Chemical Corporation.

stand at room temperature for 45 min. and then kept at 0° for 16 hr. The solution was then acidified with glacial acetic acid to pH ~3, giving a white gummy amorphous product which was collected, dried, and triturated with hot diisopropyl ether, leaving 2.17 g. (75%) of crystals, m.p. 148–151°. Three successive recrystallizations from chloroform–diisopropyl ether yielded 1.0 g. (42%), m.p. 161–162°, $[\alpha]^{25}D$ +11.9 ± 2.5 (*c* 2.03, ethanol). *Anal.* Calcd. for $C_{28}H_{31}N_3O_4 + {}^1/_4H_2O$: C, 70.30; H, 6.64; N, 8.78; H_2O, 0.9. Found: C, 70.09; H, 6.64; N, 8.57; H_2O, 0.6.

11. **Benzyloxycarbonylglycyl-L-tryptophan.**—L-Tryptophan, (2.04 g., 10 mmoles), 1.68 g. (20 mmoles) of sodium bicarbonate, and 25 ml. of water were mixed, giving partial solution and a pH about 8. Then a solution of 3.06 g. (10 mmoles) of the N-hydroxysuccinimide ester of benzyloxycarbonylglycine in 15 ml. of acetonitrile was added at room temperature. All materials were in solution in a short time. After half an hour, the solution was concentrated to about three-fourths of the original volume on a rotary evaporator under vacuum and with slight warming by a water bath. Acidification with concentrated hydrochloric acid to pH about 1 precipitated a gum. This was extracted into 25 ml. of ethyl acetate, and the resulting solution was dried briefly over sodium sulfate. The clear solution was then diluted to cloudiness with petroleum ether (about 35 ml.) and refrigerated. The resulting crystalline solid was collected; weight 2.38 g., m.p. 140–142°. Concentration of the filtrate yielded 0.90 g. with somewhat lower m.p.; recrystallization of this from about 100 ml. of ethanol–water (1:9) gave 0.77 g., m.p. 141–142°. The combined products were recrystallized from 115 ml. of ethanol–water (1:2) to yield 2.75 g. (70% yield) of pure product, m.p. 142–143°, $[\alpha]^{25}D$ + 32.9 ± 2.17° (*c* 2.3, absolute alcohol). Use of lesser amounts of sodium bicarbonate gave slightly lower yields. Weygand and Steglich[24] prepared the compound in 56% yield, m.p. 141–142°, $[\alpha]^{24}D$ +33.3° (*c* 2.34, ethanol) and Erlanger and Kokowsky[25] in 50% yield in a two-step process.

12. **Benzyloxycarbonyl-L-isoglutaminyl-L-asparagine.**—As paragine monohydrate (0.75 g., 0.005 mole) was dissolved in 10 ml. of water with heating. The solution was cooled to room temperature and 0.7 ml. (0.005 mole) of triethylamine was added. Then 1.89 g. (0.005 mole) of the N-hydroxysuccinimide ester of benzyloxycarbonyl-L-isoglutamine dissolved in 20 ml. of tetrahydrofuran was added with stirring at room temperature. After 30 min. the reaction was diluted with 20 ml. of water and acidified to pH 2 with hydrochloric acid. After overnight chilling, 0.87 g., m.p. 179-185°, was collected. Chilling the filtrate gave 0.5 g., m.p. 171–173°. The two fractions were recrystallized separately from DMF–acetonitrile (1:2) over a 6-day period. When gel-like material formed, it was redissolved by gentle warming; finally crystals formed on cooling. The two fractions yielded 0.95 g., m.p. 187-190°, and 0.3 g., m.p. 184–187° (total yield 63%). Ressler and du Vigneaud[26] report 41% yield, m.p. 187.5–188°, by a mixed anhydride procedure.

13. **Benzyloxycarbonylglycyl-L-phenylalanylglycine.**—L-Phenylalanylglycine hydrate[27] (0.775 g., 3.2 mmoles) and 0.252 g. (3 mmoles) of sodium bicarbonate were dissolved in 8 ml. of water. A solution of 0.6125 g. (2 mmoles) of the N-hydroxysuccinimide ester of benzyloxycarbonylglycine in 8 ml. of dimethoxyethane was added with stirring at room temperature. After 40 min., 10 ml. more water was added and the solution was acidified to pH 2 with hydrochloric acid. Crystals formed immediately. After overnight chilling, 0.75 g. (94%) of white crystals, m.p. 155–160°, was collected. The product was recrystallized from hot ethyl acetate to give, in two fractions, 0.74 g. (92%), m.p. 157–158°. Kenner and Stedman[28] obtained a glass in 60% yield by a mixed anhydride procedure; two recrystallizations gave a product, m.p. 155.5–157.9° (yield not given). We have obtained a yield of 85% by saponification of the ethyl ester, m.p. 157–158°.

14. **Benzyloxycarbonyl-L-prolylglycyl-L-phenylalanylglycine.**—A solution of 2.79 g. (10 mmoles) of glycyl-L-phenylalanylglycine[29] and 1.68 g. (20 mmoles) of sodium bicarbonate in 50 ml. of water plus 25 ml. of ethanol was made by warming, then cooling to room temperature. To this was added a solution of 3.46 g. (10 mmoles) of the N-hydroxysuccinimide ester of benzyloxycarbonyl-L-proline in 25 ml. of ethanol, also made by warming and cooling to room temperature; 5 ml. of wash ethanol was also used. The resulting solution was allowed to stand for 18 hr. Then it was acidified to pH about 1.5 by the addition of hydrochloric acid. Some of the ethanol was removed by vacuum distillation, and an oil precipitated from the remaining solution. This solidified on refrigeration, and it was filtered off; dry weight 4.53 g. (89%), m.p. 148–152°. Recrystallization from 105 ml. of water plus 35 ml. of alcohol gave the pure tetrapeptide derivative, wt. 3.87 g. (76% yield), m.p. 154–155°. Working up the filtrate gave 0.25 g. more, making 4.12 g. in all (80% yield), $[\alpha]^{25}D$ −27.6 ± 2.5° (*c* 2, dioxane). *Anal.* Calcd. for $C_{26}H_{30}N_4O_7$: C, 61.16; H, 5.92; N, 10.98. Found: C, 61.45; H, 6.11; N, 11.01.

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The Betaines of 3-Hydroxyproline. Assignment of Configuration and Inhibition of Acetylcholinesterase

BY F. SAKIYAMA,[1] F. IRREVERRE, S. L. FRIESS,[2] AND B. WITKOP

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The conversion of *trans*-3-hydroxy-L-proline and of *cis*-3-hydroxy-L-proline to their betaines under nonepimerizing conditions leads to the so-called 3-hydroxystachydrine a and the optical antipode of 3-hydroxystachydrine b, respectively, from *Courbonia virgata*. Weak competitive inhibitory activities in the system acetylcholinesterase–acetylcholine (AChE–ACh) are shown by *cis*- as well as *trans*-hydroxystachydrines. The differences in activities are discussed in terms of two-point attachment of the inhibitors to the catalytic surface of the enzyme.

We have recently described the isolation of *trans*-3-hydroxy-L-proline from marine sponge[3] and *cis*-3-hydroxy-L-proline from the antibiotic telomycin.[4] The stereochemical assignments rest on the mode of synthesis, *i.e.*, stereoselective hydroboration of 3,4-dehydroproline,[3] and on the oxidative conversion of the

(1) Fellow in the Visiting Program of the U. S. Public Health Service.

(2) U. S. Naval Medical Research Institute.

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