Peptide Synthesis *via* Oxidation of N-Acyl-α-amino Acid Phenylhydrazides. III. Dialanyl-Insulin and Diphenylalanyl-Insulin[1]

H. BAYARD MILNE AND FREDERICK H. CARPENTER

Department of Chemistry, Washington State University, Pullman, Washington 99163, and Department of Biochemistry, University of California at Berkeley, Berkeley, California 94720

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t-Butyloxycarbonylamino acid phenylhydrazides were prepared by a papain-catalyzed reaction between the *t*-butyloxycarbonylamino acids and phenylhydrazine. These phenylhydrazides were oxidized with N-bromosuccinimide to give *t*-butyloxycarbonylamino acid phenyldiimides which were used in peptide syntheses. *t*-Butyloxycarbonyl-L-phenylalanine phenyldiimide reacted with glycine phenylhydrazide to give *t*-butyloxycarbonyl-L-phenylalanylglycine phenylhydrazide. *t*-Butyloxycarbonyl-L-alanine phenyldiimide and *t*-butyloxycarbonyl-L-phenylalanine phenyldiimide reacted with insulin in N,N-dimethylformamide and imidazole to give di-*t*-butyloxycarbonyl-L-alanyl- and di-*t*-butyloxycarbonyl-L-phenylalanyl-insulin. Upon treatment of the insulin derivatives with anhydrous trifluoroacetic acid, the *t*-butyloxycarbonyl groups were removed to yield diaminoacylinsulins. These diaminoacyl-insulins were treated with 2,4-dinitrofluorobenzene. Amino acid analysis of the resulting product indicated that the two aminoacyl groups were on the N terminals of the A and B chain and not on the ε-amino group of lysine 29 of the B chain. These diaminoacyl-insulins showed about the same biological activity which amounted to about 50% of that of bovine insulin in the mouse convulsion assay.

Previous communications have demonstrated that the oxidation of acylamino acid phenylhydrazides to yield phenyldiimides may be used as a procedure for carboxyl activation in peptide synthesis.[2] In the earlier studies the benzyloxycarbonyl group was used to protect the amino function. In view of recent developments in peptide chemistry involving extensive use of the *t*-butyloxycarbonyl (*t*-BOC)[3-5] group for amino protection, it was of interest to determine whether this protective group could be used successfully in the phenyldiimide activation.

There have been several procedures reported for the addition of amino acid residues to the amino groups of insulin. Fraenkel-Conrat[6] and Virupaksha and Tarver[7] treated aqueous solutions of insulin with carbamino anhydrides of amino acids (Leuchs' anhydrides). Although the anhydrides reacted to some extent with both of the α-amino groups at the N-terminus of the insulin chains as well as with the ε-amino groups of lysine, in no case did all of the amino groups react completely. Levy and Carpenter have recently reported a procedure by which new amino acid residues can be attached to all of the amino groups in insulin in such a fashion as to put only one amino acid residue on each amino group.[8] The procedure involved the reaction of *t*-butyloxycarbonyl- (*t*-BOC-) amino acid *p*-nitrophenyl esters with insulin in dimethylformamide, followed by removal of the *t*-BOC groups in anhydrous trifluoroacetic acid.

It was of interest to see if N-acyl-α-amino acid phenyldiimides could also be used as reagents for adding amino acid residues to insulin. We now wish to report a procedure by which new amino acid residues can be attached to the two α-amino groups of insulin leaving the ε-amino groups largely unsubstitued. The procedure involves the reaction of *t*-butyloxycarbonyl-amino acid phenyldiimides with insulin in dimethylformamide using imidazole as the base. The *t*-BOC groups are then removed in anhydrous trifluoroacetic acid.

Experimental Section[9,10]

Materials.—Bovine zinc insulin was obtained from Eli Lilly and Co. (lot no. 0LV000). *t*-BOC-amino acids were obtained from Calbiochem, Los Angeles, Calif. Dimethylformamide was purified by refluxing for 2 hr over calcium hydride followed by distillation under vacuum.

***t*-Butyloxycarbonylamino Acid Phenylhydrazides.**—The *t*-butyloxycarbonylamino acid phenylhydrazides were prepared by a papain-catalyzed method similar to that previously reported for other acylamino acids.[11] The *t*-butyloxycarbonylamino acid (5 mmol) and 35 mmol of sodium acerate were dissolved in a minimum volume (20–200 ml) of pH 4.7, 2 mol/l. acetate buffer. To this solution was added 20 mmol of phenylhydrazine hydrochloride, 10 mmol of cysteine hydrochloride, and 0.2 g of papain. The solution was flushed with nitrogen and incubated for 12–24 hr at 40°. At the end of this time the product was filtered, dried, and recrystallized from ethanol–water or ethyl acetate–petroleum ether (bp 30–60°). The yields, melting points, and elemental analyses are shown in Table I.

***t*-Butyloxycarbonylamino Acid Phenyldiimides.**—To a solution of *t*-butyloxycarbonylamino acid phenylhydrazide (0.01 mol) and pyridine (0.011 mol) in 100 ml of dichloromethane was added 0.01 mol of N-bromosuccinimide. The mixture was stirred for 15 min. The resulting red solution was washed with 100 ml of water, 100 ml of 1% citric acid, 100 ml of 5% sodium bicarbonate, and 100 ml of water. The dichloromethane solution was dried over magnesium sulfate; then the solvent was removed under vacuum to give a red oil. This material was used directly in further reactions without purification.

Reaction of *t*-Butyloxycarbonyl-L-alanine Phenyldiimide with (−)-2-Amino-4-methylpentane.—*t*-Butyloxycarbonyl-L-alanine phenyldiimide, prepared from 0.0279 g (0.1 mmol) of *t*-BOC-L-

(1) Supported in part by U. S. Public Health Service Research Grants AM 00608 and GM 11835 from the National Institutes of Health. For I and II of this series, see ref 2.

(2) (a) H. B. Milne and W. D. Kilday, *J. Org. Chem.*, **30**, 64 (1965); (b) H. B. Milne and Clark Most, Jr., *ibid.*, **33**, 169 (1968).

(3) L. A. Carpino, *J. Amer. Chem. Soc.*, **81**, 955 (1959).

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(7) T. K. Virupaksha and H. Tarver, *Biochemistry*, **3**, 1507 (1964).

(8) D. Levy and F. H. Carpenter, *J. Amer. Chem. Soc.*, **88**, 3676 (1966); *Biochemistry*, **6**, 3559 (1967).

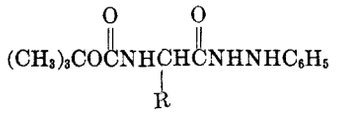
(9) The melting points are corrected. The microanalytical work was done by the Chemistry Department, University of California at Berkeley, Berkeley, Calif. The infrared spectra were determined with a Beckman IR-8 spectrophotometer. Visible and ultraviolet spectra were determined with a Cary Model 15 recording spectrophotometer. Biological assays by the mouse convulsion test and immuno assays were performed at Eli Lilly and Co. Amino acid analyses were performed on a Beckman-Spinco Model 120B automatic amino acid analyzer.[10] All hydrolyses were carried out in 6 *N* HCl in sealed evacuated tubes for 6 hr at 120°. Analysis for N-ε-DNP-lysine was performed on the short column using a pyridine (0.21 *M*) containing buffer at pH 5.28 according to an unpublished procedure of B. Africa.

(10) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(11) E. L. Bennett and C. Niemann, *J. Amer. Chem. Soc.*, **70**, 2610 (1948); H. B. Milne and C. M. Stevens, *ibid.*, **72**, 1742 (1950); H. B. Milne and C. H. Peng, *ibid.*, **79**, 645 (1957).

TABLE I

t-BUTYLOXYCARBONYLAMINO ACID PHENYLHYDRAZIDES



t-BOC amino acid phenylhydrazide	Registry no.	Yield,[a] %	Mp, °C	Formula	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N
L-Ala	17790-84-0	74[b]	151–152	$C_{14}H_{21}N_3O_3$	60.17	7.58	15.04	60.25	7.53	15.34
Asp-β-benzyl ester	17790-85-1	57[b]	109–110	$C_{22}H_{27}N_3O_5$	63.89	6.58	10.16	63.98	6.59	9.95
Gly	17790-86-2	56[c]	122–123	$C_{13}H_{19}N_3O_3$	58.83	7.22	15.84	58.52	6.89	16.09
L-Leu	17790-87-3	74[c]	134–135	$C_{17}H_{27}N_3O_3$	63.53	8.47	13.07	63.39	8.33	12.99
L-Phe	17790-88-4	68[b]	134–135	$C_{20}H_{25}N_3O_3$	67.59	7.09	11.82	67.87	6.87	12.03
L-Trp	17790-89-5	66[b]	168–169	$C_{22}H_{26}N_4O_3$	66.94	6.65	14.20	66.82	6.68	14.42
L-Phe-Gly	17790-90-8	48[c]	145.5–146.5	$C_{22}H_{28}N_4O_4$	64.06	6.84	13.58	63.99	6.77	13.82

[a] After one recrystallization. [b] Recrystallized from ethanol–water. [c] Recrystallized from petroleum ether–ethyl acetate.

alanine phenylhydrazide in the usual manner, was mixed with a solution of 0.05 g (0.2 mmol) of (−)-2-amino-4-methylpentane tartrate and 0.04 ml of triethylamine in 10 ml of dichloromethane. After 15 hr the light yellow solution was diluted to 20 ml with dichloromethane. This solution was washed with 30 ml of water, 30 ml of 1% citric acid, 30 ml of 5% sodium bicarbonate, and 30 ml of water. The solution was then dried with anhydrous magnesium sulfate. The dichloromethane was removed under vacuum and the residue was redissolved in 5 ml of dichloromethane. This solution was analyzed by the glpc method of Halpern, *et al.*[12] The chromatogram indicated the presence of a large amount of *t*-BOC-L-alanine (−)-4-methyl-2-pentylamide and showed no evidence for *t*-BOC-D-alanine (−)-4-methyl-2-pentylamide.

Reaction of *t*-Butyloxycarbonyl-L-leucine Phenyldiimide with (−)-2-Amino-4-methylpentane.—*t*-Butyloxycarbonyl-L-leucine phenyldiimide, prepared from 0.0323 g (0.1 mmol) of *t*-BOC-L-leucine phenylhydrazide in the usual manner, was mixed with a solution of 0.05 g (0.2 mmol) of (−)-2-amino-4-methylpentane tartrate and 0.04 ml of triethylamine in 10 ml of dichloromethane. After 15 hr the yellow solution was diluted to 20 ml with dichloromethane and washed with 30 ml of water, 30 ml of 1% citric acid, 30 ml of 5% sodium bicarbonate, and 30 ml of water. The solution was dried over anhydrous magnesium sulfate, and the dichloromethane was removed under vacuum. The residue was dissolved in 5 ml of dichloromethane, and the resulting solution was analyzed by Halpern's glpc method. The resulting chromatogram showed the presence of *t*-BOC-L-leucine (−)-4-methyl-2-pentylamide but showed no evidence of *t*-BOC-D-leucine (−)-4-methyl-2-pentylamide.

***t*-Butyloxycarbonyl-L-phenylalanylglycine Phenylhydrazide.**—To a solution of 3.55 g (0.01 mol) of *t*-butyloxycarbonyl-L-phenylalanine phenylhydrazide and 0.81 ml of pyridine in 100 ml of dichloromethane was added 1.78 g (0.01 mol) of N-bromosuccinimide. The mixture was shaken for 15 min. The resulting red solution was washed with 100 ml of water, 100 ml of 1% citric acid, 100 ml of 5% sodium bicarbonate, and 100 ml of water. The solution was dried over magnesium sulfate; then the solvent was removed under vacuum to give a red oil. This oil was added to a solution of 2.70 g (0.0165 mol) of glycine phenylhydrazide[2b] in 75 ml purified dioxane. After 15 min the color had turned from red to yellow. At this time the solution was evaporated to dryness. The residue was dissolved in 100 ml of dichloromethane and washed with 100 ml of water, two 100-ml portions of 1% citric acid, and 100 ml of water. The dichloromethane solution was dried over magnesium sulfate, 250 ml of petroleum ether (bp 30–60°) was added, and the solution was stored overnight in a refrigerator. The mixture was filtered, yielding 1.53 g of white crystals, mp 144–145°. The filtrate was evaporated to dryness and the residue was dissolved in 30 ml of dichloromethane. About 200 ml of petroleum ether was added and the solution was stored in a refrigerator. An additional crop of crystals was obtained (0.48 g), total yield 2.01 g (50.2%). The *t*-butyloxycarbonyl-L-phenylalanylglycine phenylhydrazide was recrystallized from dichloromethane–petroleum ether to give a product: mp 144–145°; λ 280 mμ (ε 1480), 235 (10,700).

Anal. Calcd for $C_{22}H_{28}N_4O_4$: C, 64.06; H, 6.84; N, 13.58. Found: C, 63.99; H, 6.77; N, 13.82.

Insulin Derivatives.—*t*-Butyloxycarbonyl-L-alanine phenylhydrazide (0.1–1.2 mmol), 1 equiv of pyridine, and 1 equiv of purified N-bromosuccinimide were mixed in 100 ml of dichloromethane. After the reaction was complete (10–20 min) the solution was washed with 100 ml of water, 100 ml of 1% citric acid, 100 ml of 5% sodium bicarbonate, and 100 ml of water. The solution was then dried over magnesium sulfate. The solvent was removed under vacuum to give a red crystalline solid. This solid was added to a solution of 60 mg of insulin hydrochloride[13] and 0.01 ml of triethylamine or 50 mg of imidazole in 5 ml of purified dimethylformamide. The mixture was allowed to react at room temperature for the time indicated in Table II. At this time the insulin was precipitated and washed with ether. The resulting product was dried over phosphorus pentoxide under vacuum and then dissolved in 2.0 ml of anhydrous trifluoroacetic acid. The solution was kept for 2 hr at room temperature; then the insulin derivative was precipitated and washed with ether and dried. The residue was dissolved in 7 ml of 0.5 mol/l. acetic acid (containing 1 mg of zinc acetate/ml) and precipitated by adjusting the pH to 5.0 with ammonium hydroxide. The final precipitate was collected by centrifugation, washed with 10 ml of water, 25 ml of acetone, and 25 ml of ether, and dried over phosphorus pentoxide under vacuum.

Acid hydrolysis and amino acid analysis revealed the incorporation of 2.0 to 3.0 alanine residues and the disappearance of 0 to 1 histidine residues as indicated in Table II.

TABLE II

INSULIN ALANYL DERIVATIVES

Sample no.	Diimide, g (μmol)	Reaction time, hr	Triethylamine, ml (imidazole, g)	Amino acid analysis Alanine	Amino acid analysis Histidine
1	0.10 (300)	18.5	0.01	5.25	1.62
7	0.40 (1200)	60	0.01	5.5	1.00
14	0.279 (1000)	4.0	(0.10)	4.90	1.97
12	0.20 (600)	4.5	(0.05)	5.00	1.85
11	0.20 (600)	8.5	(0.05)	5.53	1.93
10	0.20 (600)	20	(0.25)	5.7	2.12

Dinitrophenyl-insulins.—Insulin hydrochloride or the insulin derivative (1 mg each) along with 10 mg of sodium bicarbonate in 1 ml of water was added to drawn out combustion tubes. Dinitrofluorobenzene in ethanol (2 ml of 5% w/v) was added to each tube. The tubes were shaken at room temperature for 21 hr. At this time 0.1 ml of concentrated hydrochloric acid was added to each tube. Each solution was extracted with two 7-ml portions of ether. The remaining ether was removed from the aqueous solution with an aspirator. Concentrated hydrochloric acid (1 ml) was added to each tube and the contents were frozen and degassed. The tubes were sealed under vacuum. The hydrolysis proceeded for 6 hr at 120°. The results of the amino acid analysis are shown in Table III.

(12) B. Halpern, L. F. Chew, and J. W. Westley, *Anal. Chem.*, **39**, 399 (1967).

(13) F. H. Carpenter, *Arch. Biochem. Biophys.*, **78**, 539 (1958).

TABLE III
AMINO ACID ANALYSES OF INSULIN DERIVATIVES

Amino acid	Insulin	Control insulin	Di-Ala-insulin	Di-Phe-insulin	DNP-insulin	DNP-di-Ala-insulin
Asp	3	3.12	3.09	2.96	3.08	2.91
Thr	1	1.06	0.94	0.92	1.10	0.95
Ser	3	2.85	2.90	2.93	2.55	2.69
Glu	7	7.15	6.85	6.97	6.91	7.00
Pro	1	1.06	0.78	0.87	0.79	0.81
Gly	4	4.05	4.18	4.15	**3.08**	**3.98**
Ala	3	3.01	**5.01**	3.06	2.78	2.86
Cys	6	5.50	5.60	5.56	4.60	4.55
Val	5	4.75	4.77	4.81	4.75	4.80
Ile	1	0.75	0.80	0.91	0.69	0.68
Leu	6	6.03	5.85	5.80	6.07	6.05
Tyr	4	4.17	3.90	4.02		
Phe	3	3.00	2.93	**4.85**	**1.93**	**2.82**
Lys	1	1.04	1.06	1.09	**0.09**	**0.20**
His	2	1.90	2.01	1.99		
Arg	1	1.06	0.94	.96	0.73	1.04
N-ϵ-DNP-Lys					**1.01**	**0.85**

Discussion

***t*-Butyloxycarbonylamino Acid Derivatives.**—*t*-Butyloxycarbonylamino acid phenylhydrazides were used in these studies because Levy and Carpenter[8] had demonstrated that the *t*-butyloxycarbonyl group may be coupled to insulin and then removed by treating the insulin derivative with anhydrous trifluoroacetic acid with very little reduction in the activity of the regenerated insulin.

The *t*-BOC-amino acid phenylhydrazides were prepared by a papain-catalyzed reaction[12] between the *t*-BOC-amino acids and phenylhydrazine. The *t*-BOC-amino acids proved to be effective substrates for this reaction. In each case the reactions were rapid, the yields were high, and the resulting *t*-BOC-amino acid phenylhydrazides were easily purified.

The *t*-BOC-amino acid phenylhydrazides were oxidized with N-bromosuccinimide in dichloromethane to give *t*-BOC-amino acid phenyldiimides which were used directly in the coupling reactions without purification.

Absence of Racemization.—The recently developed gas chromatographic technique developed by Halpern, Chew, and Westley[12] was used to determine the extent of racemization when *t*-BOC-amino acid phenyldiimides were used in peptide synthesis. *t*-BOC-L-alanine phenyldiimide and *t*-BOC-L-leucine phenyldiimide were allowed to react with (−)-2-amino-4-methylpentane in dichloromethane and the reaction mixtures were used in the glpc analyses. The analysis indicated that the resulting *t*-BOC-L-alanine (−)-4-methyl-2-pentylamide and the *t*-BOC-L-leucine (−)-4-methyl-2-pentylamide were uncontaminated with any of the D-(−) diastereoisomers. Halpern, *et al.*, have shown that in this method 2% of the D-(−) diastereoisomers could be detected.

In order to demonstrate the usefulness of the *t*-BOC-amino acid phenyldiimides in peptide synthesis, *t*-BOC-phenylalanine phenyldiimide was allowed to react with glycine phenylhydrazide to give *t*-BOC-L-phenylalanylglycine phenylhydrazide in 50% yield.

$$t\text{-BOC-L-Phe-N=N-C}_6\text{H}_5 + \text{H}_2\text{N-Gly-NHNHC}_6\text{H}_5 \longrightarrow t\text{-BOC-L-Phe-Gly-NHNHC}_6\text{H}_5 + \text{C}_6\text{H}_6 + \text{N}_2$$

Insulin Derivatives.—The *t*-BOC-L-alanine phenyldiimide was used for the initial reaction with insulin. A large excess of *t*-BOC-L-alanine phenyldiimide was allowed to react with insulin hydrochloride in dimethylformamide using triethylamine as the base. After an appropriate time the coupled product was separated from the reaction mixture by precipitation with ether. Amino acid analysis of these products indicated that when the time of reaction was long (60 hr) there was a loss of histidine equivalent to one residue (Table II). This product was light brown. The *t*-BOC group was removed in TFA and the derivative was precipitated at pH 4.9. Amino acid analysis of the resulting insulin showed a total of 5.5 residues of alanine and 1 residue of histidine which indicated the addition of 2.5 residues of alanine and the loss of 1 residue of histidine. This material assayed at 3.6 ± 0.7 units/mg in the mouse convulsion assay (Table IV).

Because it seemed probable that the loss of histidine in this reaction was either due to a reaction between the acylamino acid phenyldiimide or some active species formed in the reaction with the imidazole ring of histidine, the reaction between *t*-BOC-L-alanine phenyldiimide and imidazole was studied. The ultraviolet spectrum of a mixture of *t*-BOC-alanine phenyldiimide and imidazole in dimethylformamide was unchanged after 24 hr indicating that any reaction between *t*-BOC-L-alanine phenyldiimide and imidazole was too slow to interfere with the reaction with insulin and that imidazole could be used as a base in these reactions. It was hoped that, if there were an active species formed in the reaction, it would react with the imidazole and not the histidine in the insulin. This proved to be the case. When imidazole was used as a base there was no loss of histidine in the amino acid analysis of the resulting insulin derivatives.

However, the use of imidazole as a base in these reactions should be used with caution. Benzyloxycarbonylglycine phenyldiimide reacted with imidazole in dioxane to give a 1:1 addition product rather than the expected N-benzyloxycarbonylglycylimidazole. The nature of this reaction is being investigated further.

The reaction between *t*-BOC-alanine phenyldiimide and insulin was followed by precipitating the insulin from samples of the reaction mixture with ether and testing the resulting insulin with ninhydrin. The results indicated that two amino groups of the insulin were covered within 4.5 hr and that further reaction was much slower. The *t*-BOC group was removed with anhydrous TFA from the insulin which had reacted for 4.5 hr with the *t*-BOC-L-alanine phenyldiimide. After an isoelectric precipitation the amino acid analysis of the product indicated the addition of two alanines with no loss of histidine (Table II).

In order to obtain evidence for the location of the two new alanines in the dialanyl-insulin, the derivative was treated with 2,4-dinitrofluorobenzene. Amino acid analysis of the resulting product indicated 3.98 Gly, 2.82 Phe, 0.20 Lys, and 0.85 N-ϵ-DNP-Lys. If there were a random distribution of two alanines on the three amino groups of insulin, the values expected would be 3.66 Gly, 2.66 Phe, 0.66 Lys, and 0.33 N-ϵ-DNP-Lys. On the other hand, if the analines were exclusively on the α-amino groups, the expected values would be 4 Gly, 3 Phe, 0 Lys, and 1 N-ϵ-DNP-Lys.

TABLE IV
BIOLOGICAL ASSAYS

Amino acid added	Reaction conditions	Amino acid residues added	Histidine residues in product	Mouse assay, units/mg	Immuno assay, units/mg
Control		0	1.83	21.4 ± 4.3	23.8
Alanine	60 hr (triethylamine)	2.5	1.02	3.6 ± 0.7	10.38
Alanine	4 hr (imidazole)	2.2	2.06	11.8 ± 2.1	15.6
Phenylalanine	5 hr (imidazole)	1.8	2.00	10.2 ± 1.7	15.4

The above results indicate that about 90 ± 5% of the two added alanines are on the α-amino groups of the insulin and about 10 ± 5% on the ε-amino group of the insulin. Also little or no substitution took place on tyrosine or histidine which were lost on dniitrophenylation. If they had been substituted with alanine, they would have been recovered upon hydrolysis of the DNP derivative.

The biological activity of the dialanyl-insulin was 11.8 ± 2.1 units/mg by the mouse convulsion assay. This value may be compared with 10 ± 0.8 units/mg reported by Levy and Carpenter for the trialanyl-insulin.[8]

In the trialanyl-insulin, amino acid residues were added to the ε-amino group as well as to the N-terminal α-amino groups. It was impossible to ascertain whether the decreased biological activity exhibited by this derivative was due to covering the N-terminal groups or of the ε-amino group of lysine at position 29 or perhaps to a combination of both. The fact that the dialanyl derivatives prepared here, which involves primarily the substitution of the N-terminal amino groups with very little reaction on the ε-amino group, have approximately the same biological activity as the trialanyl-insulin indicates that the N-terminal groups are relatively more important for biological activity than the ε-amino group. However, as the dialanyl-insulin was prepared by the phenyldiimide method and the trialanyl-insulin was prepared *via* *p*-nitrophenyl ester, one or the other reaction could have caused a change in the insulin which would be reflected in the assay but not in the amino acid analyses. Diphenylalanyl-insulin prepared by the same method assayed 10.2 ± 1.7 units/mg.

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Aromatic Boronic Acids. Synthesis of *o*-Boronophenylalanine[1]

J. R. KUSZEWSKI, W. J. LENNARZ, AND H. R. SNYDER

Noyes Chemical Laboratory, University of Illinois, Urbana, Illinois 61801

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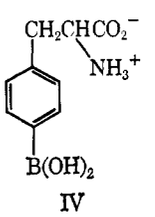
Syntheses of N-acetyl-*o*-boronophenylalanine, α-amino-*o*-boronobenzylmalonic anhydride, and *o*-boronophenylalanine anhydride are described. Alkylation of diethyl acetamidomalonate with *o*-(bromomethyl)benzeneboronic anhydride yields *o*-(2-carbethoxy-2-acetamidoethyl)benzeneboronic acid rather than the expected *o*-(2,2-dicarbethoxy-2-acetamidoethyl)benzeneboronic acid. It is postulated that decarbethoxylation occurs through participation of the boronic acid function in ester hydrolysis. Decarboxylation of α-amino-*o*-boronobenzylmalonic anhydride requires an unusually high temperature; this observation is interpreted in terms of a bridged, polycyclic structure. The decarboxylation product, the boronic anhydride related to *o*-boronophenylalanine, gives no indication of the zwitterionic structure, presumably because of interaction between the nitrogen and boron atoms.

The synthesis of *p*-boronophenylalanine (IV) has been reported.[2] *p*-(Bromomethyl)benzeneboronic acid



IV

(I) was condensed with sodio diethyl acetamidomalonate, and the product (II) was saponified and decarboxylated to give the acetyl derivative (III) which was hydrolyzed. The general method was that of Snyder, Shekleton, and Lewis.[3] The infrared spectrum of IV indicated the zwitterionic structure common to amino acids.

The above procedure has now been applied in an attempt to prepare *o*-boronophenylalanine. Condensation of *o*-(bromomethyl)benzeneboronic anhydride with sodio diethyl acetamidomalonate does not yield the expected *o*-(2,2-dicarbethoxy-2-acetamidoethyl)-benzeneboronic acid, but rather *o*-(2-carbethoxy-2-acetamidoethyl)benzeneboronic acid (IX). Evolution of carbon dioxide occurs when the alkylation mixture is acidified and warmed to 50°. Decarbethoxylation, with concomitant formation of diethyl carbonate, is known to occur sometimes as a side reaction in the alkylation of malonic esters;[4] however, decarbethoxylation *via* diethyl carbonate formation is considered unlikely, since alkylation of the *p*-bromomethyl analog proceeded normally, and also since, under the

(1) This work was supported in part by a grant from the Atomic Energy Commission, Report No. COO-314-11.

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