benzaldehyde and what appears to be a cyclic condensation product of the monoxime.[2]

The equations below are suggested as possible routes of the main reactions involved.[3]

This series of reactions might be thought of as an interrupted Nef reaction.

The addition of ketones to 2-nitro-1-phenylpropene appears to be fairly general, since both acetone and diethyl ketone give salts similar to I. Pure compounds corresponding to II have not been isolated from these salts, however.

Experimental[4]

Addition of Cyclohexanone to 2-Nitro-1-phenylpropene.—To 65.2 g. (0.4 mole) of 2-nitro-1-phenylpropene in 160 ml. of cyclohexanone was added a solution of 20 g. (0.5 mole) of sodium hydroxide in 50 ml. of water. The mixture was stirred and cooled for about 30 min. during which time it became one phase and the temperature rose to 60°, and then subsided.

Acidification of Addition Product.—The above reaction mixture was poured slowly with stirring and cooling into 300 ml. of methanol containing 60 ml. of acetic acid. After 30 min. the white, crystalline precipitate (II) was filtered and air-dried. There was 47 g. (45%); m.p. 137–139° dec.

Anal. Calcd. for $C_{15}H_{19}NO_4$: C, 68.9; H, 7.33; N, 5.36. Found: C, 69.2; H, 7.49; N, 5.22.

Preparation of 1-(2-Oxocyclohexyl)-1-phenyl-2-propanone (III).—A mixture of 100 ml. of methanol and 26 g. (0.1 mole) of compound II was refluxed for 4 hr. A blue color appeared after a few minutes, then this slowly turned to yellow toward the end of the reaction. The solid went into solution gradually, and a gas was evolved. Analysis by a mass spectrometer showed this gas to be pure nitrous oxide. After evaporation of the methanol, addition of 10 ml. of cyclohexane caused crystallization. After filtration and drying there were 16.4 g. (71%), m.p. 79–80° after recrystallization from isopropyl alcohol.

Anal. Calcd. for $C_{15}H_{18}O_2$: C, 78.2; H, 7.88. Found: C, 78.4; H, 8.02.

Preparation of the Sodium Salt of 1-(2-Oxocyclohexyl)-2-nitro-1-phenylpropene (I).—A solution of 13 g. (0.05 mole) of II in 40 ml. of water plus 2.2 g. (0.055 mole) of sodium hydroxide was filtered into 300 ml. of acetone. A crystalline precipitate formed which weighed 18 g. (92%), m.p. 75–77°. The infrared spectrum of this had a strong band at 1700 cm.$^{-1}$.

Anal. Calcd. for $C_{15}H_{18}NO_3Na\ 6H_2O$; C, 46.0; H, 7.72; N, 3 58. Found: C, 46.6; H, 7.40; N, 3.52.

Bromination of the Sodium Salt of 1-(2-Oxocyclohexyl)-2-nitro-1-phenylpropene (I).—A solution of 13 g. (0.05 mole) of II in 100 ml. of water plus 2.2 g. (0.055 mole) of sodium hydroxide was added slowly with stirring and cooling to 20–25° to 200 ml. of methanol containing 2.5 ml. (0.05 mole) of bromine. The mixture was left in the refrigerator overnight, filtered to give 14 g. of white crystals. After several recrystallizations from isopropyl alcohol the melting point was 113–115°. This compound showed strong bands at 1710 cm.,$^{-1}$ 1550 cm.,$^{-1}$ and 1330 cm.$^{-1}$.

Anal. Calcd. for $C_{15}H_{18}BrNO_3$: N, 4.12; Br, 23.5. Found: N, 4.35; Br, 23.8.

Reaction of the Sodium Salt of 1-(2-Oxocyclohexyl)-2-nitro-1-phenylpropene (I) with Benzyl Chloride.—To 8.0 g. (0.02 mole) of the sodium salt of 1-(2-oxocyclohexyl)-2-nitro-1-phenylpropene in 100 ml. of ethanol was added 2.6 g. (0.021 mole) of benzyl chloride. The mixture was heated in a steam bath for 2 hr., then cooled overnight and filtered to give 1 g. of sodium chloride. The filtrate had the odor of benzaldehyde. It was concentrated to 30 ml., 10 ml. of water was added, and the mixture was cooled overnight. This gave 2.5 g. (42%) of a compound which, after two recrystallizations from isopropyl alcohol, melted at 165–166°. The infrared spectrum of this had intense bands in the regions of 3200 cm.$^{-1}$ and 1615 cm.$^{-1}$.

Anal. Calcd. for $C_{15}H_{20}NO_2$: N, 5.71. Found: N, 5.47.

Preparation of 1,3-Diphenyl-2-methyl-4,5,6,7-tetrahydroindole.—A mixture of 4.0 g. (0.17 mole) of 1-(2-oxocyclohexyl)-1-phenyl-2-propanone (III), 2 g. (0.21 mole) of aniline, 2 drops of hydrochloric acid, and 25 ml. of ethanol was heated on a steam bath for 2 hr., then cooled 2 hr., and filtered to give 4.3 g. (88%) of white crystals. After recrystallization from ethanol the melting point was 77–79°.

Anal. Calcd. for $C_{21}H_{21}N$: N, 4.87. Found: N, 4.97.

Reaction of Acetone with 2-Nitro-1-phenylpropene.—To 100 ml. of acetone with 16.3 g. (0.1 mole) of 2-nitro-1-phenylpropene was added a solution of 4 g. (0.1 mole) of sodium hydroxide in 10 ml. of water. The mixture was stirred for 1.5 hr. during which a crystalline precipitate formed and the temperature rose to 38° and then dropped. Filtration gave a yellow solid which was dissolved in 50 ml. of 50% aqueous methanol. This was filtered into 300 ml. of acetone to give 11 g. (45%) of a white crystalline product, melting point 110–115° dec.

Anal. Calcd. for $C_{12}H_{14}NO_3Na\cdot 3H_2O$: N, 4.71. Found: N, 4.52.

Acidification of this by acetic acid in methanol led to a blue color, but no crystalline product was recovered.

Diethyl ketone gave a similar salt.

(2) See H. B. Hass and M. L. Bender, *J. Am. Chem. Soc.*, **71**, 3482 (1949).

(3) The mechanism of the Nef reaction is discussed by W. E. Noland, *Chem. Rev.*, **55**, 137 (1955).

(4) Melting points are uncorrected. Infrared data were taken with a Perkin-Elmer Model 21 double beam recording spectrophotometer equipped with sodium chloride optics. Potassium bromide disks were used for all determinations. Gas analysis was done with a Model 21-103-C Consolidated Engineering mass spectrometer.

Peptide Synthesis. An Application of the Esterase Activity of Chymotrypsin

EDWARD WALTON, JOHN OTTO RODIN, CHARLES H. STAMMER, AND FREDERICK W. HOLLY

Merck Sharp & Dohme Research Laboratories, Division of Merck & Co., Inc., Rahway, N. J.

Received December 18, 1961

A frequently required step in peptide synthesis is the hydrolysis of an ester function used to block the terminal carboxyl group. The conventional methods for accomplishing this step may cause side reactions.[1] When we hydrolyzed the methyl ester of isoleucine-5 angiotensin octapeptide[2,3] under either acidic or basic conditions, we obtained low yields of the desired peptide. Chymotrypsin[4]

(1) R. Schwyzer, *Chimia*, **12**, 53 (1958); M. Goodman and G. W. Kenner, "Advances in Protein Chemistry," Vol. XII, Academic Press, New York, 1957, p. 474.

(2) Angiotensin is the present name for the substance formerly called angiotonin and hypertensin: E. Braun-Menendez and I. H. Page, *Science*, **127**, 242 (1958).

(3) H. Schwarz, F. M. Bumpus, and I. H. Page, *J. Am. Chem. Soc.*, **79**, 5697 (1957); R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel, and H. Zuber, *Chimia*, **11**, 335 (1957).

(4) M. Dixon and E. C. Webb, "Enzymes," Academic Press, Inc., New York, 1958, p. 269; and H. Neurath and G. W. Schwert, *Chem. Rev.*, **46**, 69 (1950) review the proteolytic and esterase activities of chymotrypsin.

was found to be an ideal reagent for hydrolysis of this ester without by-product formation.

Isoleucine-5 angiotensin octapeptide[2,3] was synthesized by a sequence requiring, in the final step, hydrolysis of the ester, α-aspartyl-arginyl-valyl-tyrosyl - isoleucyl - histidyl - prolyl - phenylalanine methyl ester. When we hydrolyzed this ester with sodium hydroxide or hydrochloric acid, side reactions took place as shown by paper chromatography and biological assay of the products. We then examined the action of chymotrypsin on this ester. Since the tyrosyl-isoleucine linkage is readily cleaved by chymotrypsin,[5] conditions were required which would allow selective hydrolysis of the ester. It was found that at a chymotrypsin to substrate ratio of 1:1000, both the ester and tyrosyl-isoleucyl bonds were cleaved, while at a ratio of 1:100,000 the ester hydrolyzed at a rate too slow to be practical. But when a ratio of 1:10,000 was used, ester hydrolysis was complete in two hours while no cleavage of a peptide bond or formation of other products was observed. The course of reaction was followed by paper chromatography.

In the course of this work we examined the action of chymotrypsin on four other peptide esters: L-prolyl-L-phenylalanine methyl ester, carbobenzyloxy-L-valyl-L-tyrosine methyl ester, N^{α}-carbobenzyloxy-N^{g}-nitro-L-arginyl-L-valyl-L - tyrosine methyl ester, and carbobenzyloxy methylene L-asparaginyl-L-tyrosine methyl ester. When an aqueous solution of chymotrypsin was added to a solution of L-prolyl-L-phenylalanine methyl ester in methanol, L-prolyl-L-phenylalanine crystallized rapidly in a 90% yield. Carbobenzyloxy-L-valyl-L-tyrosine was obtained in 80% yield from its methyl ester. In this case a solution of the peptide ester in dimethylformamide was added to an aqueous solution of chymotrypsin. Even though the ester had precipitated from the solution, hydrolysis was complete in two hours. Similarly, the tripeptide derivative, N^{α}-carbobenzyloxy-N^{g}-nitro-L-arginyl-L-valyl-L-tyrosine methyl ester was converted to the corresponding acid in 95% yield, and carbobenzyloxy methylene-L-asparaginyl-L-tyrosine methyl ester was hydrolyzed in 80% yield to the corresponding acid. In these cases the blocking groups did not interfere with enzymic hydrolysis and the peptides were obtained rapidly in pure form by procedures readily adapted to large-scale preparations.

These examples point out the preparative practicality of enzymic peptide ester hydrolysis. Depending upon the nature of the C-terminal amino acids, enzymes other than chymotrypsin would be used.

Experimental

Melting points were taken on a Kofler micro hot stage.

Paper chromatograms were done on 32-cm. Whatman No. 1 circles with a 1-cm. center hole.[6]

The compounds were located on the paper by means of ninhydrin (N), diazotized sulfanilic acid (P), or Sakaguchi reagent (S). A compound which has an R_f value of 0.5 in the MPW[7] system and was located with ninhydrin reagent is reported as R_f^{MPW} 0.5 (N).

Hydrolysis of L-Prolyl-L-phenylalanine Methyl Ester.—A solution of 18 mg. of α-chymotrypsin (crystallized, Worthington Biochemical Corp.) in 50 ml. of 0.5 *M* ammonium acetate (*p*H 6.4) was added rapidly to a stirred solution of 1 g. of L-prolyl-L-phenylalanine methyl ester hydrochloride[8] in 5 ml. of methanol.

Crystallization of L-prolyl-L-phenylalanine occurred almost immediately. After the suspension had been stirred at 22° for 15 min., the product was filtered, washed with water and with methanol; 0.9 g. of L-prolyl-L-phenylalanine was obtained. No impurities were detected by radial paper chromatography in two solvent systems: R_f^{BAW} 0.64 (N); R_f^{BAm} 0.26 (N).

A sample crystallized from water and dried at 110° had the following properties: m.p. 234–238°; $[\alpha]^{22}D$ −42° (*c* 2.1, 20% hydrochloric acid).[9]

Hydrolysis of Carbobenzyloxy-L-valyl-L-tyrosine Methyl Ester.—A solution of 0.5 g. of carbobenzyloxy-L-valyl-L-tyrosine methyl ester[10] in 2 ml. of dimethylformamide was added dropwise during a 5-min. period to a stirred solution of 20 mg. of α-chymotrypsin in 22 ml. of 0.5 *M* ammonium acetate to which ammonium hydroxide had been added to adjust the solution to *p*H 8. A precipitate formed immediately. The *p*H of the solution gradually dropped, and after 10 min., the solution was readjusted to *p*H 8 with ammonium hydroxide. After the mixture had been stirred at 22° for 2 hr., a clear solution had formed. The solution was acidified to *p*H 1 with hydrochloric acid, the resulting crystalline precipitate was filtered, washed with water, and dried; 0.4 g. of carbobenzyloxy-L-valyl-L-tyrosine[10] was obtained, R_f^{BAm}[7] 0.69 (P).

A sample was recrystallized from aqueous methanol: m.p. 163–169°; $[\alpha]^{22}D$ +26.5° (*c* 2.1, pyridine).

A similar reaction, but at *p*H 6.4, gave incomplete hydrolysis. A control experiment at *p*H 8 in the absence of enzyme showed no hydrolysis.

Hydrolysis of Carbobenzyloxy Methylene-L-asparaginyl-L-tyrosine Methyl Ester.—A solution of 230 mg. of carbobenzyloxy methylene-L-asparaginyl-L-tyrosine methyl ester[11] in 2 ml. of methanol was added dropwise to a stirred solution of 10 mg. of α-chymotrypsin in 20 ml. of 0.5 *M* ammonium acetate to which ammonium hydroxide had been added to adjust the solution to *p*H 8. The precipitate which formed went rapidly into solution. After the solution had been stirred about 2 hr. at 22°, it was acidified to *p*H 1 with hydrochloric acid. The oily precipitate which formed crystallized slowly on standing overnight to give 177 mg. of carbobenzyloxy methylene-L-asparaginyl-L-tyrosine,[11] m.p. 118–122°, $[\alpha]^{22}D$ −25° (*c* 1.0, pyridine), R_f^{MPW} 0.85 (N).

---

(5) A. A. Plentl and I. H. Page, *J. Biol. Chem.*, **163**, 49 (1946) describe cleavage of angiotensin by chymotrypsin.

(6) E. Lederer and M. Lederer, *Chromatography*, 2nd ed., Elsevier Publishing Co., New York, 1957, p. 134.

(7) BAW, butanol–acetic acid–water—4:1:5. The upper phase was used. BAm, butanol–1.5 *N* ammonium hydroxide—1:1. The upper phase was used. MPW, methyl ethyl ketone–pyridine–water—4:1:1.6.

(8) W. Rittel, B. Iselin, H. Kappeler, B. Riniker, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 614 (1957).

(9) E. Fischer and A. Luniak, *Ber.*, **42**, 4752 (1909), report m.p. 252°, $[\alpha]$ −40.9° (*c* 5, 20% hydrochloric acid).

(10) H. Schwarz and F. M. Bumpus, *J. Am. Chem. Soc.*, **81**, 890 (1959).

(11) C. H. Stammer, *J. Org. Chem.*, **26**, 2556 (1961).

Hydrolysis of N^{α}-Carbobenzyloxy-N^{g}-nitro-L-arginyl-L-valyl-L-tyrosine Methyl Ester.—A solution of 500 mg. of N^{α}-carbobenzyloxy-N^{g}-nitro-L-arginyl-L-valyl-L-tyrosine methyl ester[8] in 5 ml. of dimethylformamide was added dropwise to a solution of 50 mg. of α-chymotrypsin in 40 ml. of 0.5 *M* ammonium acetate to which ammonium hydroxide had been added to adjust the solution to *p*H 7.5. The reaction mixture was stirred at 22° for 15 min. during which time a precipitate formed and redissolved. Acidification of the solution to *p*H 2 with hydrochloric acid gave 480 mg. of N^{α}-carbobenzyloxy-N^{g}-nitro-L-arginyl-L-valyl-L-tyrosine,[10] R_f^{BAm} 0.55 (N), $[\alpha]^{22}D$ −13.4° (*c* 0.82, methanol). A sample recrystallized from ethanol melted at 178–182°.

Isoleucine-5 Angiotensin Methyl Ester.—Isoleucine-5 angiotensin has been synthesized[9] by (a) condensation of carbobenzyloxy-β-methyl ester-L-aspartyl-N^{g}-nitro-L-arginine with L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester, (b) alkaline hydrolysis of the condensation product, and (c) removal of the carbobenzyloxy and nitro groups by catalytic hydrogenation. Similarly, we condensed carbonbenzyloxy-β-benzyl ester-L-aspartyl-N^{g}-nitro-L-arginine[12] with the hexapeptide ester using dicyclohexyl-carbodiimide[13] as the condensing agent: hydrogenation of the product over a palladium catalyst yielded a mixture containing isoleucine-5 angiotensin methyl ester. This ester was carried through a 96-plate countercurrent distribution in the system butanol–propanol–acetic acid–water (30:15:5:50) followed by a 196-plate distribution of the peak fractions in butanol–acetic acid–water (4:1:5). The product from the major peak showed only one component, R^{BAW_7} 0.61 (N); $R_f^{MPW_7}$ 0.69 (N); $R_f^{BAm_7}$ 0.40 (N).

Chymotrypsin Hydrolysis of Isoleucine-5 Angiotensin Methyl Ester. Method A. (1–1000).—A 5.3-mg. sample of isoleucine-5 angiotensin methyl ester was added to 0.8 ml. of 0.5 *M* ammonium acetate containing 5.3 γ of chymotrypsin. Samples were examined periodically by radial paper chromatography in MPW; ninhydrin was used to locate the spots. In 2 min. considerable hydrolysis had occurred and chromatography showed that the free octapeptide, $R_f^{MPW_7}$ 0.48 (N,P,S), was being produced. In 40 min., however, cleavage of the peptide chain to give the tetrapeptides α-L-aspartyl-L-arginyl-L-valyl-L-tyrosine, $R_f^{MPW_7}$ 0.39 (N,P,S) and L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine, $R_f^{MPW_7}$ 0.62 (N, P), had occurred, while an observable amount of octapeptide ester $R_f^{MPW_7}$ 0.69 (N,P,S) remained unhydrolyzed.

Method B. (1–100,000)—A sample was treated as described in A above, except that 0.053 γ of chymotryspin was used. Under these conditions both ester and amide hydrolyses were slow—after 2 hr. only a small amount of ester hydrolysis and no amide hydrolysis was observed.

Method C. (1–10,000).—A 10.2-mg. sample of isoleucine-5 angiotensin methyl ester in 0.8 ml. of 0.05 *M* ammonium acetate was added to 0.28 ml. of water containing 1.0 γ of chymotrypsin. The solution was *p*H 6. In 2 hr. the ester was completely hydrolyzed as indicated by a single spot at R_f^{MPW} 0.48 (N,P,S). No cleavage of the peptide chain was observed. Only after 22 hr. was formation of a small amount of the tetrapeptides, R_f^{MPW} 0.39 (N,P,S) and R_f^{MPW} 0.62 (N,P), detected. The product isolated by lyophilization after 2 hr. of hydrolysis under the above conditions had a high order of biological activity. In normal anesthetized dogs, a dose of 1 γ/kg. produced a 60-mm. mean arterial pressure rise, while the starting octapeptide ester was essentially inactive at dosages up to 100 γ/kg.

The biological assays were carried out by Dr. L. S. Watson in these laboratories.

(12) L. T. Skeggs, Jr., K. E. Lentz, J. R. Kahn, and N. P. Shumway, *J. Exp. Med.*, **108**, 283 (1958).

(13) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

o-Trifluoromethyl- and Some *ortho, meta*-Disubstituted Benzeneboronic Acids and Anhydrides

L. SANTUCCI, L. TAVOLETTI, AND D. MONTALBANO

Department of General Chemistry, University of Rome and Chemical Laboratory, Istituto di Patologia del Libro, Rome, Italy

Received December 18, 1961

In connection with a reaction mechanism study, the organolithium procedure followed by boronation with tri-*n*-butyl borate was satisfactorily employed to prepare a series of known boronic acids and anhydrides[1] and the new ones reported herein.

Except for *o*-trifluoromethylbenzeneboronic acid and its anhydride, these compounds are analogs or isomers of others described in another work,[2] reference to which should be made for general remarks on difficulties of purification and characterization. Unfortunately, no infrared spectral analysis was available to us: we regret the lack of such a facility for improving characterization of our compounds, and because of some discussion[3] on previous observations of one of us.[2]

Dehydration of acids to anhydrides, as it was pointed out,[2,3] in some cases is accomplished simply by moderate heating in anhydrous solvents, but sometimes does require temperatures over 100°.[4] The application of vacuum, even in the presence of dehydrating agents, in our experience was seldom effective.

Another striking example of the influence of substituents on ease of dehydration is offered by the comparison of the two isomeric hydroxybromobenzeneboronic anhydrides reported herein: they both have a bromine atom in the *meta* position with respect to the borono group, but whereas the one brominated *para* to the hydroxyl required a long time of heating above 100° to be obtained from the acid, the other one with bromine *ortho* to hydroxyl is so much more stable that the acid could not even be detected in the product crystallized from water. A hydroxyl *ortho* to the borono group is known to favor stabilization of the anhydride, as compared with the unsubstituted benzeneboronic compound[5]: apparently, the disturbing action of bromine is neutralized when this substituent is *ortho* to the hydroxy group, possibly by hydrogen-bonding.

The only other trifluoromethyl derivative of a boronic acid hitherto reported appears to be the *m*-

(1) L. Santucci, *Boll. Ist. patol. libro*, **20**, 123 (1961).

(2) L. Santucci and H. Gilman, *J. Am. Chem. Soc.*, **80**, 193 (1958).

(3) H. R. Snyder, M. S. Konecky, and W. J. Lennarz, *J. Am. Chem. Soc.*, **80**, 3611 (1958).

(4) See also H. G. Kuivila and A. R. Hendrickson, *J. Am. Chem. Soc.*, **74**, 5068 (1952).

(5) H. Gilman, L. Santucci, D. R. Swayampati, and R. O. Ranck, *J. Am. Chem. Soc.*, **79**, 3077 (1957).