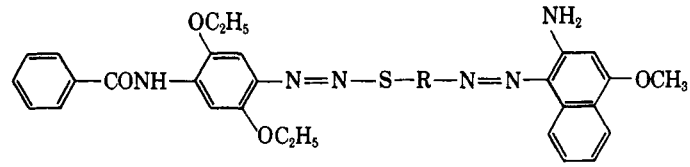
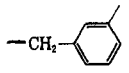
TABLE II

AZO DIAZO THIOETHERS OF II



R	M.p., °C.	λ_{max}^{EtOH} mμ (ε)	Yield, %	Formula	Calcd., % C	Calcd., % H	Calcd., % N	Found, % C	Found, % H	Found, % N
	110 dec.	357, 406 (22,200, 21,400)	70	$C_{35}H_{34}N_6O_4S$	66.23	5.40	13.24	66.39	5.46	13.58
	95–96 dec.	471 (34,900)	88	$C_{34}H_{32}N_6O_4S$	65.80	5.20	13.57	65.43	5.33	13.78
	197–198 dec.	468 (24,800)	86							

Further studies indicated that better yields could be obtained by diazotizing a suspension of the aminodiazo thioether in 80:20 acetic acid–propionic acid mixture with an excess of amyl nitrite. Completion of diazotization was indicated by solution of the aminodiazo thioether (usually about 0.5 hr.). This procedure resulted in less destruction of the diazo thioether linkage. Isolation of the diazonium salt as the zinc chloride double salt was accomplished with the aid of ether.

Azo Diazo Thioethers of 4-Methoxy-2-naphthylamine (II) (Table II).—The cold solution of diazotized aminodiazo thioether or a solution of the zinc chloride double salt was added to a solution of II (or V) in ethanol. Coupling occurred readily without raising the pH of the solution. After adjusting the pH to neutrality by the addition of ammonia, to complete the precipitation of the azo compounds, they were collected by suction filtration. After washing with water and a small quantity of cold ethanol, the compounds were dried under vacuum at room temperature. These azo diazo thioethers decomposed upon attempted recrystallization. After exposure to osmium tetroxide for 5–15 min., the color was replaced by osmium black. This pigment was insoluble in dimethylformamide. Whether the osmium black is osmium metal, lower oxides of osmium, osmium mercaptide, or a mixture of these products has not yet been determined.

Synthesis of Chromogenic Arginine Derivatives as Substrates for Trypsin*,[1]

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The synthesis of 15 chromogenic substrates which are split rapidly by trypsin is described. All are derivatives of arginine and were prepared by the mixed anhydride method utilizing the protonation blocking procedure for masking the guanidine group. The three substrates most rapidly hydrolyzed are N^{α}-carbobenzoxyglycyl-glycyl-L-arginine-2-naphthylamide hydrochloride, β-carboxyproprionyl-L-arginyl-L-arginine-2-naphthylamide dihydrochloride, and N^{α}-carbobenzoxy-L-arginyl-L-arginyl-L-arginine-2-naphthylamide trihydrochloride.

The recent synthesis of N^{α}-benzoyl-DL-arginine-2-naphthylamide hydrochloride (BANA),[2a] L-lysine-*p*-nitroanilide dihydrobromide (LPA),[2b] and N^{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA)[2b] has furnished chromogenic substrates for assaying trypsin activity. After enzymatic hydrolysis the color density of the liberated aromatic amine (*p*-nitroaniline) is measured directly or determined after conversion of the amine (2-naphthylamine) to an azo dye by coupling with an appropriate diazonium salt. Although these substrates provide good assay methods for pure trypsin, they do not compete successfully with antitrypsin and have not been used successfully in assaying tryptic activity in serum because of the presence of antitrypsin. Since we have felt that an elevation of trypsin activity in the serum of patients with pancreatic disease might have diagnostic value, a study was undertaken to learn more about the structural requirements of substrates for trypsin in the hope that a substrate could be devised which would compete favorably for the active sites of trypsin with the trypsin inhibitor in blood serum. While the latter goal has not been attained thus far, we have been successful in synthesizing 15 chromogenic substrates, 10 of which are split more rapidly by trypsin than the aforementioned commercially available chromogenic substrates (BANA, LPA, and BAPA). This paper describes the preparation of a series of amides and peptides of L-arginine-2-naphthylamide. The report of their comparative rates of hydrolysis by trypsin with respect to the effect of chain length, charge, and structure is published elsewhere.[3]

The three substrates which were split most rapidly by crystalline bovine trypsin were carbobenzoxygly-

* To Professor Louis F. Fieser.

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(2) (a) A. Reidel and E. Wunsch, *Z. Physiol. Chem.*, **316**, 61 (1959); (b) B. F. Erlanger, N. Kokowsky, and W. Cohen, *Arch. Biochem. Biophys.*, **95**, 271 (1961).

(3) M. M. Nachlas, R. E. Plapinger, and A. M. Seligman, *ibid.*, **108**, 266 (1964).

TABLE I

ACYLATED ARGININE ARYLAMIDES[a]

Compound	Formula	Yield, %	M.p., °C.	Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N	Recrystn. solvent (s)
N^{α}-Carbobenzoxy-L-arginine-1-naphthylamide[b] (I)	$C_{24}H_{28}N_5O_3Cl$	80	193	61.33	6.00	14.90	61.26	5.74	15.10	Ethyl alcohol
N^{α}-Benzoyl-DL-arginin-anilide[c] (II)	$C_{19}H_{24}N_5O_2Cl$	76	252	58.53	6.20	17.96	58.70	6.20	17.54	Ethyl alcohol
N^{α}-Benzoyl-DL-arginine-3,4-dichloroanilide[c] (III)	$C_{19}H_{22}N_5O_2Cl_3$	47	212	49.74	4.83	15.27	49.82	4.66	15.00	Ethyl alcohol–ether, then ethyl alcohol–acetone
N^{α}-Benzoyl-DL-arginine-*p*-anisylide[c] (IV)	$C_{20}H_{26}N_5O_3Cl$	43	150–152	57.20	6.24	16.68	57.0	6.09	16.59	N,N-Dimethylformamide–ether, then methyl alcohol–ether
β-Carboxyproprionyl-L-arginine-2-naphthylamide[d] (V)	$C_{20}H_{26}N_5O_4Cl$	95	125[e]	55.10	6.01	16.07	55.08	5.83	16.21	*n*-Butyl alcohol–ether

[a] These substances exist as the hydrochloride. [b] $[\alpha]^{27}D$ −11.5° (*c* 2.0, methyl alcohol). [c] These compounds probably exist as a mixture of D, L, and DL isomers. [d] $[\alpha]^{26}D$ −67.5° (*c* 1.71, methyl alcohol). [e] Softens at 95°, melts at 125°.

cylglycyl-L-arginine-2-naphthylamide hydrochloride, β-carboxypropionyl-L-arginyl-L-arginine-2-naphthylamide dihydrochloride, and carbobenzoxy-L-arginyl-L-arginyl-L-arginine-2-naphthylamide trihydrochloride. Their comparative rates of hydrolysis, compared with BANA taken as 1, were 220, 143, and 113, respectively.[3] Although these substrates provide a sensitive method of assay for pure trypsin and for trypsin added to serum, there was a limit to the amount of trypsin that could be demonstrated in serum in the presence of the inhibitor antitrypsin. The smallest concentration of trypsin which could be assayed was 5 μg./ml. of serum.[3] In order to develop a clinically useful method for trypsin activity in blood serum, considerably less trypsin will need to be measureable.

The synthesis of arginine peptides has always been a difficult task.[4–8] The protonation blocking procedure for protecting the guanidine group of arginine has been demonstrated to work effectively in cases where either the carboxyl group[9] or the amino group[10] of arginine have been joined to other amino acids or peptides. However this report represents the first case in which an arginine molecule is joined to another arginine molecule where both arginine entities are protected by hydrohalide. It represents the first time that three arginine molecules are linked to each other in a stepwise synthesis by this or any other blocking and condensation procedure.

The synthesis of carbobenzoxy-L-arginine-2-naphthylamide hydrochloride from carbobenzoxy-L-arginine hydrochloride and 2-naphthylamine *via* the mixed anhydride procedure[11,12] was described in an earlier publication.[13] Utilizing the same condensing agents and experimental conditions it was possible to prepare the dipeptide carbobenzoxy-L-arginyl-L-arginine-2-naphthylamide dihydrochloride by substituting L-arginine-2-naphthylamide hydrochloride[13] in place of 2-naphthylamine. By catalytic removal of the carbobenzoxy group and condensation with more carbobenzoxyarginine hydrochloride it was possible to obtain carbobenzoxy-L-arginyl-L-arginyl-L-arginine-2-naphthylamide trihydrochloride.

Reaction of the mixed anhydride of carbobenzoxy-L-arginine hydrochloride (from isobutyl chloroformate and triethylamine) with either L-arginine-2-naphthylamide hydrochloride or L-arginyl-L-arginine-2-naphthylamide hydrochloride in equimolar amounts yielded the desired peptides contaminated with tertiary amine hydrochloride and both arginine reactants in each case. Utilization of an excess of the aforementioned mixed anhydride caused the reaction to go to completion, triethylamine hydrochloride and carbobenzoxy-L-arginine hydrochloride remaining as the only contaminants. The former was removed by ether extraction of an aqueous solution of the cold reaction mixture at pH 10.6 and the latter by ion-exchange chromatography over Amberlite IR-45 resin in the chloride form at pH 5.5. The carboxylate ion of carbobenzoxy-L-arginine hydrochloride displaced chloride and hydroxide ion and was absorbed onto the column. The peptide as a guanidinium chloride passed through the column.

Most of the arginine amides and peptides herein reported were prepared from the appropriate N-acylated amino acid or N-acylated peptide and the required amino compound *via* the mixed carboxylic–carbonic anhydride method.[11,12] The protonation blocking procedure[9,10] for protecting the guanidine group of arginine was used in every case. However, the purification of several substrates gave great difficulty from contamination with reactants, by-products, and inorganic salts. The exact synthetic procedure is described below for the N^{α}-acylated arginine arylamides. Where experimental difficulties were significantly varied or extensive purification was necessary a detailed description is given.

Three succinyl peptides were prepared to see the effect of charge on the rate of tryptic hydrolysis. The

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TABLE II.—ARGININE PEPTIDES

Compound	Formula	Yield, %	M.p., °C.	C, %	H, %	N, %	Cl, %	C, %	H, %	N, %	Cl, %	$[\alpha]^{25}D$, deg.[a]
N^{α}-Carbobenzoxy-L-arginyl-L-arginine-2-naphthylamide dihydrochloride (VI)	$C_{30}H_{41}N_9O_4Cl_2$	77	133–137	54.38	6.24	19.03	10.70	54.12	6.00	18.71	10.61	−32.50,[b] *c* 2.0
L-Arginyl-L-arginine-2-naphthylamide dihydrochloride (VII)	$C_{22}H_{35}N_9O_2Cl_2$	64	Glass at 100, liquifies at 155	50.00	6.68	23.85	13.42	50.28	6.91	22.48[c]	13.63	−10.75, *c* 2
Carbobenzoxyglycyl-L-arginine-2-naphthylamide hydrochloride (VIII)	$C_{26}H_{31}N_6O_4Cl$	75	162	59.25	5.93	15.95	6.73	59.36	5.79	15.70	6.75	−45.10, *c* 1.75
β-Carboxypropriony-L-arginyl-L-arginine-2-naphthylamide dihydro chloride (IX)	$C_{29}H_{39}N_9O_5Cl_2$	72	Glass at 105, liquifies at 145	49.68	6.25	20.06	11.28	49.68	6.16	20.16	11.02	−28.47, *c* 1.96
N^{α}-Carbobenzoxy-L-arginyl-L-arginine-amide dihydrochloride (X)	$C_{20}H_{35}N_8O_4Cl_2$	71	Softens at 108, liquifies at 120	44.78	6.58	23.50	13.22	44.69	6.61	23.45	12.86	−4.65, *c* 1.40
N^{α}-Carbobenzoxy-L-arginyl-L-arginine-2-naphthylamide trihydrochloride (XI)	$C_{36}H_{54}N_{12}O_5Cl_2$	78	Glass at 135, liquifies at 150	50.35	6.36	21.29	12.44	50.33	6.27	21.31	12.52	−36.0,[b] *c* 2.0
Carbobenzoxyglycylglycyl-L-arginine-2-naphthylamide hydrochloride (XII)	$C_{28}H_{34}N_7O_5Cl$	73	125–130	57.58	5.87	16.79	6.07	57.63	6.29	16.56	5.97	−17.1, *c* 1.4
N-Acetylglycylglycine-L-arginine-2-naphthylamide hydrochloride (XIII)	$C_{22}H_{30}N_7O_4Cl$	87	Glass at 120, liquifies at 140	53.71	6.15	19.93	7.21	53.27	6.52	19.43	7.44	−33.6, *c* 1.84
Carbobenzoxyglycyl-L-arginal-L-arginine-2-naphthylamide dihydrochloride (XIV)	$C_{32}H_{45}N_{10}O_5Cl_2$	66	Glass at 125, liquifies at 135	53.33	6.29	19.44	9.84	53.41	6.23	19.10	10.18	−21.2, *c* 1.52
β-Carboxypropriony lglycylglycyl-L-arginine-2-naphthylamide hydrochloride (XV)	$C_{24}H_{32}N_7O_6Cl$	89	Glass at 108, liquifies at 140	52.41	5.87	17.83	6.45	52.84	5.72	17.93	6.48	−23.92, *c* 1.54

[a] Rotations were determined in methyl alcohol. [b] Determined at 25°. [c] We were unable to obtain a satisfactory nitrogen analysis for this compound.

succinyl peptides were obtained by reaction of the particular amino acid amide or peptide with succinic anhydride.[14]

The physical and analytical data for all the substances reported herein are presented in Tables I and II.

All of the asymetric centers in the peptides prepared have the *levo* configuration since the peptide chain was built up by stepwise addition of carbobenzoxyamino acid to the free amino end of each successive precursor. By this procedure the mixed anhydride method could be used without loss of optical activity.[15,16] Proof of stereochemical homogeneity of carbobenzoxy-L-arginyl-L-arginyl-L-arginine-β-naphthylamide trihydrochloride was established by treatment of a decarbobenzoxylated sample of this substance with leucine aminopeptidase[17] (LAP), followed by two-dimensional paper chromatography of the digest. Only one product, L-arginine hydrochloride, could be detected indicating the absence of racemization during synthesis.

Experimental[18]

N^{α}-Acylated Arginine Arylamides.—A solution of 0.025 mole of either N^{α}-carbobenzoxy-L-arginine hydrochloride[13,20] or N^{α}-benzoyl-L-arginine hydrochloride[1,20] and triethylamine (2.53 g., 0.025 mole) in 75 ml. of dry N,N-dimethylformamide (DMF) was cooled to −10° and allowed to react with isobutyl chloroformate (3.41 g., 0.025 mole) at −10°. This reaction mixture was maintained at −10° for 20 min., after which a solution of 0.025 mole of the appropriate amine in 50 ml. of dry DMF was added. The reaction mixture was permitted to come to room temperature. After 24 hr. triethylamine hydrochloride was removed by filtration and the solvent was removed *in vacuo* on the steam bath leaving an oily residue. Purification was effected by recrystallization from appropriate solvents. The physical and analytical data for these substances (compounds I–IV) are given in Table I.

β-Carboxypropionyl-L-arginine-2-naphthylamide Hydrochloride (V).—Succinic anhydride (3.0 g., 0.030 mole) and L-arginine-2-naphthylamide hydrochloride (6.72 g., 0.02 mole) were dissolved in 40 ml. of dry DMF and kept at 60–70° for 8 hr. The cooled homogenous solution was poured into dry ether with stirring yielding a gum. This gum was dissolved in ethanol. *n*-Butyl alcohol was added and the ethyl alcohol was removed *in vacuo*. Precipitation with ether gave the desired product which was washed with ether and dried.

N^{α}-Carbobenzoxy-L-arginyl-L-arginine-2-naphthylamide Dihydrochloride (VI).—The mixed anhydride formed from N^{α}-

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(15) M. B. North and G. T. Young, *Chem. Ind.* (London), 1597 (1955).

(16) N. A. Smart, G. T. Young, and M. W. Williams, *J. Chem. Soc.*, 3902 (1960).

(17) R. L. Hill and E. L. Smith, *J. Biol. Chem.*, **228**, 577 (1957).

(18) All melting points were performed on a Fisher-Johns melting point apparatus and are uncorrected. Microanalyses were performed by Clark Microanalytical Laboratory, Urbana, Ill. Rotations were determined by means of a Kern full circle polarimeter. The homogeneity of the peptides was determined by ascending two-dimensional paper chromatography on Whatman No. 1 filter paper at room temperature. R_f^1 values refer to the solvent system *t*-butyl alcohol–water–90% formic acid in a ratio of 70:15:15 (by volume). R_f^2 values refer to the system *n*-butyl alcohol–water–ethanol in a ratio 2:2:1 (by volume). R_f^3 values refer to the system *n*-butyl alcohol–methyl ethyl ketone–ammonia (14.8 *N*)–water in a ratio of 5:3:1:1 (by volume). Systems 1 and 2 were utilized for those peptides containing only arginine. Systems 1 with 2 and 2 with 3 were used for the glycine peptides. The location of the peptide spots was revealed by either the Sakaguchi reagent or the NaOCl–KI–starch procedure.[19] All samples were first dried in a vacuum desiccator over calcium chloride at room temperature. The last traces of butyl alcohol were removed from those samples recrystallized from butyl alcohol–ether by continuous extraction of the sample in a Soxhlet extractor with ether for 6 hr. Prior to microanalysis all samples were dried in an Abderhalden drying pistol at 78 or 100° for 4 hr. at 0.2 mm.

(19) S. C. Pan and J. D. Dutcher, *Anal. Chem.*, **28**, 836 (1956).

(20) These substances have become available recently through Cyclo Chemical Corp., Los Angeles 1, Calif.

carbobenzoxy-L-arginine hydrochloride[13,20,21] (34.82 g., 0.101 mole), triethylamine (10.2 g., 0.101 mole), and isobutyl chloroformate (13.79 g., 0.101 mole) in 275 ml. of dry DMF was allowed to react with L-arginine-2-naphthylamide hydrochloride[13,20,22] (33.58 g., 0.100 mole) in 100 ml. of dry DMF in the manner described above for compounds I–IV. After filtering triethylamine hydrochloride (5.6 g.) and concentrating the solvent the sirupy residue was dissolved in 500 ml. of distilled water. This solution (pH 2.3) was cooled to 0–5°, brought to a pH of 10.6 by addition of dilute sodium hydroxide, and extracted several times with ether, the pH of the cooled solution being adjusted to 10.6 after each ether extraction. The pH was then adjusted to 7.0 and the water was removed at 40–45° on a Buchler flash evaporator. The residue was dissolved in ethanol and taken to dryness on the evaporator. This operation was repeated twice. Acetone was then added and also removed on the evaporator. The residue was finally dissolved in ethyl alcohol and n-butyl alcohol was added. The ethyl alcohol was removed leaving a butyl alcohol solution containing just enough ethyl alcohol to dissolve the product. The sodium chloride remained suspended in the solution. After 24 hr. the sodium chloride was filtered over Celite and the butyl alcohol was removed *in vacuo*, leaving 80.7 g. of wet residue. This was dissolved in 400 ml. of water, and the pH was adjusted to 5.5 with hydrochloric acid. This solution was poured over a column 2.50 cm. in diameter and 84.0 cm. high containing 350 g. of Amberlite IR-45 resin (20–50 mesh) at a pH of 5.5[23] in the chloride form. (The resin was activated by washing with 1 *N* hydrochloric acid, water, 1 *N* sodium hydroxide, and water, and then neutralizing with 1 *N* hydrochloric acid and washing till the wash water gave a negative halogen test and a pH of 5.5). The liquid was allowed to flow through the column at a rate of about 6 ml./min. The effluent liquid was checked for pH, Sakaguchi color test, and chloride ion (with silver nitrate). The column data is presented in Table III.

TABLE III

Volume eluted, ml.	pH	Chloride test	Sakaguchi test
0	5.7	−	−
50	6.1	−	−
100	6.2	−	−
125	6.3	−	−
150	8.5	+	+
175	8.5	+	+
200	9.1	+	+
300	9.6	+	+
400	9.1	+	+
500	9.1	+	+
600	8.2	+	+
700	7.8	Faint	Faint
850	7.6	Faint	Faint
900	7.5	−	Faint

Fractions, 150–700 ml., were combined, pH was adjusted to 7.0, and solvent was removed at 40–45° on the Buchler flask evaporator. The residue was treated as described earlier in this experiment with ethyl alcohol, acetone, and ethyl alcohol–butyl alcohol. The final butyl alcohol solution was poured into absolute ether with stirring. The solid was allowed to settle, the solvent above the solid was removed by suction with a pipet and replaced with dry ether, and the mixture was stirred vigorously. This operation was repeated twice. The highly hygroscopic and electrostatically charged solid was filtered rapidly, packed down tightly with a rubber dam, and transferred as quickly as possible to a vacuum desiccator containing anhydrous calcium chloride; R_f^1 0.83[24]; R_f^2 0.84.[24]

L-Arginyl-L-arginine-2-naphthylamide Dihydrochloride (VII).—N^α-Carbobenzoxy-L-arginyl-L-arginine-2-naphthylamide dihydrochloride (23.85 g., 0.036 mole) in 210 ml. of absolute methanol containing 3.0 g. of 10% palladium-on-charcoal catalyst was shaken with hydrogen at 60 p.s.i. for 16 hr. The catalyst was filtered and replaced by 3 g. of fresh catalyst, and the solution was hydrogenated again for 16 hr. The catalyst was removed, and the filtrate was concentrated to dryness at reduced pressure. The residue was dissolved in absolute ethyl alcohol and *n*-butyl alcohol was added. The ethyl alcohol was removed by concentration *in vacuo*. The product was isolated and dried as for VI to yield a highly electrostatically charged and hygroscopic solid. When subjected to two-dimensional paper chromatography, it appeared as a single spot positive to the Sakaguchi reagent; R_f^1 0.31; R_f^2 0.64; R_f^3 0.23.

Carbobenzoxyglycyl-L-arginine-2-naphthylamide Hydrochloride (VIII).—This compound was prepared from carbobenzoxyglycine[25] (2.09 g., 0.01 mole) and L-arginine-2-naphthylamide hydrochloride[13,20,22] (3.36 g., 0.01 mole) by the procedure described for I–IV. Purification was effected by recrystallization from hot water and then from ethyl alcohol; R_f^1 0.83; R_f^2 0.76; R_f^3 0.76.

β-Carboxyproprionyl-L-arginyl-L-arginine-2-naphthylamide Dihydrochloride (IX).—This compound was prepared from succinic anhydride (0.4 g., 0.004 mole) and L-arginyl-L-arginine-2-naphthylamide dihydrochloride (1.0 g., 0.0018 mole) by the procedure described for V. Two-dimensional paper chromatography indicated only one spot, Sakaguchi positive; R_f^1 0.55; R_f^2 0.73.

N^α-Carbobenzoxy-L-arginyl-L-argininamide Dihydrochloride (X).—This compound was prepared by the procedure described for VI from N^α-carbobenzoxy-L-arginine hydrochloride[13,20,21] (20.68 g., 0.060 mole), triethylamine (6.06 g., 0.060 mole), isobutyl chloroformate (8.19 g., 0.060 mole), and L-argininamide hydrochloride (0.055 mole). The latter compound was liberated in DMF from L-argininamide dihydrochloride monohydrate[5,26,27] (14.53 g., 0.055 mole) by addition of triethylamine (5.55 g., 0.055 mole). L-Argininamide dihydrochloride monohydrate was insoluble when placed in either cold or hot DMF. It dissolved when a suspension of the compound in hot DMF was cooled with stirring. The purification procedure described for VI was utilized. A maximum column pH of 8.2 was reached on elution of this product from the Amberlite IR-45 column. Elemental analysis indicated that the sample first isolated (22.1 g.) was contaminated with about 5% triethylamine hydrochloride. This was removed by dissolving a 2-g. sample in absolute ethyl alcohol and adding dry acetone with stirring. The dipeptide dihydrochloride precipitated as an oil. Triethylamine hydrochloride remained in the supernatent liquid which was decanted. The oil was dissolved in ethyl alcohol to which *n*-butyl alcohol was added. Removal of the ethyl alcohol *in vacuo* was followed by precipitation of the product from *n*-butyl alcohol with ether. It was washed several times with ether and transferred rapidly to a vacuum desiccator, yielding a hygroscopic, electrostatically charged substance. Two-dimensional chromatography indicated the presence of only one spot which gave a positive reaction with the Sakaguchi reagent; R_f^1 0.43; R_f^2 0.68.

N^α-Carbobenzoxy-L-arginyl-L-arginyl-L-arginine-2-naphthylamide Trihydrochloride (XI).—This compound was prepared, isolated, and purified by the procedure described for VI. The mixed anhydride prepared from N^α-carbobenzoxy-L-arginine hydrochloride[13,20,21] (0.044 mole), triethylamine (0.044 mole), and isobutyl chloroformate (0.044 mole) was allowed to react with L-arginyl-L-arginyl-2-naphthylamide dihydrochloride (0.0407 mole). A maximum column pH of 8.0 was reached on elution of this product from the Amberlite IR-45 resin column. The isolated product contained two contaminants in trace amounts, N^α-carbobenzoxy-L-arginine hydrochloride and a second component with R_f^1 of 0.28 and R_f^2 of 0.57[28] which we believed to be unreacted L-arginyl-L-arginine-2-naphthylamide dihydrochloride. Part of the isolated product (21.8 g.) was dissolved in

(21) R_f^1 0.79; R_f^2 0.82.

(22) R_f^1 0.62; R_f^2 0.80; R_f^3 0.60.

(23) It was established that Amberlite IR-45 resin under these experimental conditions would retain carbobenzoxy-L-arginine hydrochloride.

(24) As both arginine reactants have similar R_f^1 and R_f^2 values this in itself is not too significant. However the decarbobenzoylated product VII was also homogeneous in these solvent systems. L-Arginine hydrochloride (R_f^1 0.26; R_f^2 0.27), L-arginine-2-naphthylamide hydrochloride,[22] and L-arginyl-L-arginine-2-naphthylamide dihydrochloride (VII) gave separate spots.

(25) R_f^1 0.85; R_f^2 0.87; R_f^3 0.38.

(26) Purchased from Schwarz Bioresearch, Inc., Mount Vernon, N. Y.

(27) R_f^1 0.20; R_f^2 0.23.

(28) The mixed anhydride formed from N^α-carbobenzoxy-L-arginine hydrochloride, triethylamine, and isobutyl chloroformate in DMF as solvent was allowed to come to room temperature. After removal of solvent the residue was dissolved in water and spotted on Whatman No. 1 paper. This mixed anhydride decomposed to carbobenzoxy-L-arginine hydrochloride and an unidentified arginine component (possibly N^α-carbobenzoxy-L-arginine isobutyl ester) of R_f^1 0.21 and R_f^2 0.25.

DMF and allowed to react with 0.002 mole of the mixed anhydride of N^{α}-carbobenzoxy-L-arginine hydrochloride. The reaction mixture was worked up and purified as described earlier in this preparation again using the base extraction, the Amberlite IR-45 column, and *n*-butyl alcohol–ether precipitation. The product was filtered and extracted in a Soxhlet apparatus with hot tetrahydrofuran for several hours. This trihydrochloride was then recrystallized again from *n*-butyl alcohol–ether as a highly electrostatically charged white solid, difficult to handle when wet, which could be handled and stored readily when dry. A sample (52 μg.) subjected to two-dimensional paper chromatography revealed only one spot, Sakaguchi positive; R_f^1 0.60; R_f^2 0.74.

LAP Digest of Decarbobenzoxylated XI.—Compound XI was hydrogenated over 10% palladium-on-charcoal catalyst in methyl alcohol. After removal of solvent and catalyst a solid of R_f^1 0.17 and R_f^2 0.46 was obtained by *n*-butyl alcohol–ether precipitation. This solid (2.0 mg.) was dissolved in 1.0 ml. of tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.5, 0.01 *M* Mg^{2+}), 0.2 ml. of a LAP solution (5 mg. of Worthington LAP in 1 ml. of water) was added, and the solution was maintained at 37° for 24 hr. This solution (30 μl.) was then spotted on Whatman No. 1 paper. Only one spot (R_f^1 0.21; R_f^2 0.30), corresponding to arginine hydrochloride, Sakaguchi and ninhydrin positive, was detected.

Carbobenzoxyglycylglycyl-L-arginine-2-naphthylamide Hydrochloride (XII).—This compound was prepared from carbobenzoxyglycylglycine[29] (13.32 g., 0.05 mole) and L-arginine-2-naphthylamide hydrochloride[13,20,22] (16.79 g., 0.05 mole) by the procedure described for I–IV. The residue obtained after removal of solvent was purified by dissolving in hot water, cooling, adding ether, and agitating. The flocculent solid obtained was washed with acetone–ether (1:1) and recrystallized from ethyl alcohol–ether. Two-dimensional chromatography showed a single spot, chlorine and Sakaguchi positive; R_f^1 0.83; R_f^2 0.84; R_f^3 0.71.

Acetylglycylglycyl-L-arginine-2-naphthylamide Hydrochloride (XIII).—This compound was prepared from acetylglycylglycine[20,30] (5.22 g., 0.03 mole) and L-arginine-2-naphthylamide hydrochloride[13,20,22] (10.07 g., 0.03 mole) by the procedure described for I–IV. The residue obtained after removal of solvent was dissolved in water and the solution was cooled to 0–5° and extracted at pH 10.6 with ether several times. The water layer, adjusted to a pH of 7, was concentrated to dryness at 40–45°. The residue obtained was dried by dissolving in ethyl alcohol several times and concentrating under reduced pressure to dryness. It was finally dissolved in *n*-butyl alcohol containing just enough ethyl alcohol to dissolve it. After standing overnight, sodium chloride was removed by filtration and the hygroscopic electrostatic solid was precipitated with dry ether. Two-dimensional chromatography showed a single spot positive to both the chlorine and Sakaguchi reagents; R_f^1 0.69; R_f^2 0.79; R_f^3 0.47.

Carbobenzoxyglycyl-L-arginyl-L-arginine-2-naphthylamide Dihydrochloride (XIV).—This compound was prepared from carbobenzoxyglycine[26] (1.04 g., 0.005 mole) and L-arginyl-L-arginine-2-naphthylamide dihydrochloride (2.64 g., 0.005 mole) by the procedure described for I-IV. Isolation of this hygroscopic electrostatic solid was effected by the procedure used for XIII. Two-dimensional chromatography showed a single spot positive to the chlorine and Sakaguchi reagents; R_f^1 0.80; R_f^2 0.87; R_f^3 0.39.

β-Carboxypropionylglycylglycyl-L-arginine-2-naphthylamide Hydrochloride (XV).—This compound was prepared from succinic anhydride (1.0 g., 0.01 mole) and glycylglycyl-L-arginine-2-naphthylamide hydrochloride[31] (3.30 g., 0.0075 mole) by the procedure described for V. The product was recrystallized from ethyl alcohol–ether. It exhibited only one spot positive to the chlorine and Sakaguchi reagents when subjected to two-dimensional chromatography; R_f^1 0.65; R_f^2 0.82; R_f^3 0.37.

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(29) R_f^1 0.84; R_f^2 0.83; R_f^3 0.30.

(30) R_f^1 0.56; R_f^2 0.42; R_f^3 0.06.

(31) Obtained by catalytic hydrogenolysis of XII with 10% palladium on carbon in methanol. The crude solid obtained by removal of catalyst and solvent was used without further purification; R_f^1 0.65; R_f^2 0.72; R_f^3 0.59.

Olefinic Cyclizations. VII. Formolysis of *cis*- and *trans*-5,9-Decadienyl *p*-Nitrobenzenesulfonate and of Some Isomeric Monocyclic Esters*,[1,2]

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Solvolysis of *trans*-5,9-decadienyl *p*-nitrobenzenesulfonate in 100% formic acid containing sodium formate yields, among other products, the formates of the *trans* monocyclic alcohol II and the *trans*-2-decalols III and IV. Solvolysis of the *cis* isomer VIII (R = $SO_2C_6H_4NO_2$) gave a very similar product distribution except that the cyclic products belonged exclusively to the *cis* series, *i.e.*, IX, X, and XI. The stereospecificity of these processes has interesting mechanistic implications which are given consideration. The formolysis of the *p*-nitrobenzenesulfonates of the monocyclic alcohols II, VI, and IX has been examined. These reactions are not stereospecific, and the mechanistic implications are discussed. It was discovered that butenylcyclohexene (VII), as well as the tertiary alcohol V, on treatment with 100% formic acid containing sodium formate undergoes stereospecific cyclization to give *cis-syn*-2-decalol (XI) in over 20% yield.

The solvolysis of *trans*-5,9-decadienyl *p*-nitrobenzenesulfonate (I, R = $SO_2C_6H_4NO_2$) in 80% formic acid containing sodium formate has been examined.[3] The most striking feature of this reaction was the fact that the products of ring closure were formed highly stereoselectively, perhaps even stereospecifically. Thus *trans*-2-(Δ³-butenyl)cyclohexanol (II) was produced (as the formate) to the exclusion of the *cis* isomer, and the decalols that formed were exclusively those with *trans*-fused rings, namely III and IV. As previously noted,[3,4] this stereoselective *trans* addition to the internal olefinic bond is in accord with the concepts advanced by Stork[5] and Eschenmoser[6] regarding the biogenesis of natural polycycloisoprenoids. There is, however, an alternative rationalization which must be considered, namely that the solvolysis proceeds to give

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(1) Part VI: W. S. Johnson and R. Owyang, *J. Am. Chem. Soc.*, **86**, 5593 (1964).

(2) A portion of this work has been disclosed in a preliminary communication, W. S. Johnson and J. K. Crandall, *ibid.*, **86**, 2085 (1964).

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