of chelation was given by ashing the products at 1000° and obtaining metal contents that indicated a chelate ratio of 1:1 or 2:1 of organic ligand to metal. Analytically pure products were not obtained.

Synthesis of Chromogenic Substrates for the Assay of Aminopeptidase Activity[1]

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The 2-naphthylamides of six amino acids were prepared as substrates in order to determine if the enzymatic splitting of the amide link is due to a non-specific aminopeptidase or an aminopeptidase specific for each amino acid.

Although the enzymes aminopolypeptidase, aminodipeptidase, and leucyl aminopeptidase have been described[2,3,4] it is not known whether there exists one non-specific aminopeptidase or many aminopeptidases specific for each amino acid. Since a biochemical and histochemical method for leucyl aminopeptidase has been described utilizing L-leucyl-2-naphthylamide as the substrate,[5,6] a series of naphthylamides of other amino acids was prepared and their enzymatic cleavage compared to the leucyl compound. This paper describes the synthesis of the substrates.

The results of the biochemical study[7] may be summarized as follows. The leucyl, methionyl, alanyl, and arginyl amides were assayed at 1×10^{-3} *M*; the glycyl amide at 1×10^{-2} *M*; the phenylalanyl sub-

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strate at 2.5×10^{-4} *M;* and the glutamyl compound at 5×10^{-4} *M*. Poor aqueous solubility and slow enzymatic hydrolysis necessitated these variations in substrate concentration. Rat liver homogenate (2 mg./ml.) and a partially purified preparation of hog kidney were used as the enzyme source. Most of the reactions were run at pH 7.0 and 37° for a 30 min. incubation period. Enzymatic activity was determined by the colorimetric measurement of the azo dye formed, when the enzymatically liberated 2-naphthylamine was coupled with tetrazotized di-*o*-anisidine.[5] Two types of results were obtained. One suggested that aminopeptidase was a single enzyme and the other that there existed more than one aminopeptidase, with various substrate preferences. For example, in comparing the hydrolysis of the substrates with homogenates from four organs of the rat, enzymatic activity decreased in the order of kidney, intestine, liver and brain with all substances other than glycyl naphthylamide. In this case liver was the most active enzyme source. The hydrolytic rates of kidney and intestine homogenates were strikingly similar. The existence of a single non-specific aminopeptidase was favored also by the rate constants, pH optima, temperature activation, aging and fixation effects, the influence of activators and inhibitors and data obtained with combination of substrates. On the other hand the livers of five mammalian species behaved as if there were different concentrations of two or more aminopeptidases. Furthermore, purified leucine aminopeptidase showed a strong preference for the leucyl amide and failed to hydrolyze the glycyl, alanyl and phenylalanyl amides. Even though the present study was confined to a few tissues as enzyme sources, and to substrates which varied only in the structure of the conjugated amino acid, nevertheless, a clear-cut identification of leucine aminopeptidase as a single, characteristic, substrate-specific enzyme species could not be obtained. It was concluded that even though certain of these chromogenic amides may be preferentially hydrolyzed, there is so much overlap that the characterization of the specific aminopeptidases by their amino acid preference serves no more useful function than to label the substrate used. Obviously, it is unrealistic to characterize the enzyme by the amino acid, if a more specific implication is intended.[7]

The alanyl, glycyl, phenylalanyl, methionyl, glutamyl and arginyl derivatives of 2-naphthylamine were prepared in order to obtain substrates representing simple, aromatic, sulfur, acidic and basic amino acids. Generally, the N-carbobenzoxy amino acid was condensed with 2-naphthylamine and the protecting group removed by hydrogenolysis. In the case of the methionine derivative it was found

more convenient to utilize the N-phthaloyl-protected amino acid than the carbobenzoxy compound, since the catalytic removal of the blocking group from the latter was difficult.

Experimental

A.—The 2-naphthylamides of five of the six amino acids described herein were synthesized by hydrogenolysis of the corresponding carbobenzoxy amino acid 2-naphthylamides. The latter compounds were prepared by standard procedures from the corresponding carbobenzoxy amino acids, 2-naphthylamine and the appropriate condensing agent. The physical and analytical data for these substances are given in Tables I and II. Rotations were determined by means of a Kern full-circle polarimeter.

B.—Compounds I, II, VI and VIII were made *via* the mixed anhydride procedure[8,9] using isobutyl chloroformate and tri-*n*-butylamine in dry chloroform or N,N-dimethylformamide. Compounds III and IV were prepared by condensation with dicyclohexylcarbodiimide (DCC).[10] Compounds I and VIII were also obtained by the DCC method but in poorer yield.

C. Compound V.—A solution of 13.0 g. of N-phthaloyl-L-methionine[11] and 3.7 ml. of thionyl chloride in 50 ml. of dry benzene was heated at reflux for 15 min. after which the reaction mixture was concentrated to half its volume under reduced pressure to remove all traces of thionyl chloride. The acid chloride thus prepared was not isolated. This reaction mixture was added directly to a solution of 7.15 g. of 2-naphthylamine and 20 ml. of pyridine in 50 ml. of benzene and heated at 100° for 30 min. The yellow reaction mixture was concentrated to half its volume and cooled in an ice bath for 30 min. The solid pyridine hydrochloride was filtered and the oil filtrate crystallized from ethyl acetate. A small amount of methanol aids in the crystallization if the product persists as an oil. The product (6.3 g.) was obtained as white needles.

D. Compound VII.—A solution of 8.5 g. of carbobenzoxy-L-glutamic anhydride[12,13] and 5.60 g. of 2-naphthylamine in 120 ml. of toluene was heated at 100° for 3 hr. A white gelatinous precipitate formed immediately and was present throughout the course of the reaction. The reaction mixture was cooled to room temperature, filtered and washed with several portions of ether to remove traces of starting material. Recrystallization of the resulting solid (10.8 g.) from ethyl acetate gave white needles.

E. Compound VIII.—N^{α}-Carbobenzoxy-L-arginine hydrochloride[14] (15.85 g.), triethylamine (4.6 g.), and isobutyl chloroformate (6.28 g.) reacted at −10° in 120 ml. of N,N-dimethylformamide (DMF) and were kept at −10° for 20 min. 2-Naphthylamine dissolved in 50 ml. of DMF at −10° was added to the cold solution of the mixed anhydride with stirring. The reaction mixture was allowed to come to room temperature. After 24 hr., precipitated triethylamine hydro-

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TABLE I

CARBOBENZOXY AMINO ACID-2-NAPHTHYLAMIDES

	Compound	Formula	Yield, %	M.p., °C.[a]	Analyses, % Calculated C	Calculated H	Found C	Found H	$[\alpha]^{25}D$
I	Carbobenzoxyglycine-2-naphthylamide	$C_{20}H_{18}N_2O_3$	65[b], 18[c]	181.5[d]	72.0	5.47	71.8	5.43	
II	Carbobenzoxy-L-alanine-2-naphthylamide	$C_{21}H_{20}N_2O_3$	67[b]	193.5[d]	72.4	5.75	72.5	5.86	
III	Carbobenzoxy-L-phenylalanine-2-naphthylamide	$C_{27}H_{24}N_2O_3$	70[c]	175[d]	76.5	5.66	76.2	5.82	−4.42 (*c* 1.4, EtOAc
IV	Carbobenzoxy-DL-phenylalanine-2-naphthylamide	$C_{27}H_{24}N_2O_3$	66[c]	172[d]	76.5	5.66	76.39	5.76	
V	N-phthaloyl-L-methionine-2-naphthylamide	$C_{23}H_{20}N_2O_3S$	34	147[d]	68.4	5.18	68.2	5.09	−2.28 (*c* 1.1, EtOAc
VI	Carbobenzoxy-DL-methionine-2-naphthylamide	$C_{22}H_{24}N_2O_3S$	15[b]	162.5[d]					
VII	Carbobenzoxy-N-α-glutamic acid 2-naphthylamide	$C_{23}H_{22}N_2O_5$	66	221[d]	67.8	5.17	68.0	5.43	−0.71 (*c* 1.7, N,N-dimethylformamide)
VIII	N^{α}-Carbobenzoxy-L-arginine-2-naphthylamide hydrochloride	$C_{24}H_{28}ClN_5O_3$	80[b], 41[c]	187[e]	61.34	6.00	61.10	5.69	−20.0[f] (*c* 3.0, MeOH)

[a] Fisher-Johns melting point block. [b] Prepared by mixed anhydride procedure. [c] DCC procedure. [d] Recrystallized from ethyl acetate. [e] Recrystallized from ethanol–ether. [f] Determined at 24°.

TABLE II

AMINO ACID-2-NAPHTHYLAMIDES

	Compound	Formula	Yield, %	M.p., °C[a]	Analyses, % Calculated C	Calculated H	Found C	Found H	$[\alpha]^{26}D$
IX	Glycine-2-naphthylamide	$C_{12}H_{12}N_2O$	63	115[c, d]					
X	L-Alanine-2-naphthylamide	$C_{13}H_{14}N_2O$	53	97.5[d]	72.9	6.54	73.3	6.61	
XI	L-Phenylalanine-2-naphthylamide	$C_{19}H_{18}N_2O$	48	129[d]	78.6	6.21	78.5	6.54	−1.43 (*c* 1.8, EtOAc)
XII	DL-Phenylalanine-2-naphthylamide	$C_{19}H_{18}N_2O$	59	113[d]	78.6	6.21	78.3	6.26	
XIII	L-Methionine-2-naphthylamide	$C_{15}H_{18}N_2OS$	27	83[d]	65.6	6.61	65.5	6.61	−4.37 (*c* 2.4, EtOAc)
XIV	*N*-α-Glutamic acid-2-naphthylamide	$C_{15}H_{16}N_2O_3$	40	66[b]	61.1	6.11	60.9	6.24	
XV	L-Arginine-2-naphthylamide hydrochloride	$C_{16}H_{22}ClN_5O$	86	184[e]	57.21	6.55 N, 20.83	57.12	6.87 N, 20.46	+31.2 (*c* 3.0, MeOH)

[a] Fisher–Johns melting point block. [b] Recrystallized from ethyl acetate. [c] No depression of the melting point was observed with this substance and an authentic sample prepared by reaction of ammonia with chloroacetic acid 2-naphthylamide.[19] [d] Recrystallized from 90–100° petroleum ether. [e] Recrystallized from methanol–ether.

chloride (4.24 g.) was filtered and the solvent removed under vacuum. The orange oily residue was dissolved in warm water and then cooled in an ice bath. To this vigorously stirred mixture was added an equal volume of ether. The resulting solid which formed at the interface was filtered, washed with ether and recrystallized from ethanol–ether. When DDC was tried as condensing agent the yield was halved. N^{α}-Carbobenzoxy-L-arginine hydrochloride,[14] utilized above, was formed from the free base and gaseous hydrogen chloride. It melted at 155°.

Anal. Calcd. for $C_{14}H_{21}ClN_4O_4$: C, 48.77; H, 6.14; N, 16.26. Found: C, 48.89; H, 6.01; N, 15.92.

N^{α}-Carbobenzoxy-L-arginine[14] was synthesized by modification of a procedure described by Gish and Carpenter[15] for preparing N^{α}-(*p*-nitrocarbobenzoxy)-L-arginine. L-Arginine or its monohydrochloride (0.15 mole) was dissolved in a mixture of 45 ml. of 0.67 *N* sodium hydroxide and 120 ml. of 1 *N* sodium bicarbonate. To this cooled solution (0–10°) carbobenzoxy chloride (25.56 g.) dissolved in 65 ml. of dioxane and then 37.5 ml. of 4 *N* sodium hydroxide were added with stirring in small portions over a 45 min. period, keeping the pH of the solution about 9.0–9.5. Stirring was continued for an additional 45 min. at 0–10°. The white solid precipitating from solution was washed with ice water, acetone and then ether. Yield was 29.5 g. (67%), m.p. 175°.[16] Recrystallization from water did not raise the melting point.

F.—Compounds IX, X, XI, XII and XIV were derived from their respective carbobenzoxy derivatives by hydrogenolysis in ethanol containing 1.1 equivalents of anhydrous hydrogen chloride utilizing 10% palladium on charcoal as catalyst. These compounds were isolated as their free bases by neutralization of the resulting product with sodium bicarbonate followed by recrystallization from an appropriate solvent. To obtain XV hydrogenolysis of VIII was carried out in methanol.

G. Compound XIII.—To 2.4 g. of N-phthaloyl-L-methionine-2-naphthylamide in 40 ml. of ethanol was added 20 ml. of 1 *M* alcoholic hydrazine hydrate. On heating, the solid went into solution but shortly afterward a white voluminous precipitate formed. After 1 hr. at reflux the reaction mixture was cooled to room temperature and the white solid filtered. This solid was heated at 50° with 25 ml. of 2 *N* HCl for 10 min. and then cooled to room temperature. After filtering the solid phthaloyl hydrazide the clear filtrate was neutralized with sodium bicarbonate, precipitating L-methionine-2-naphthylamide. This was extracted with benzene, washed twice with water and dried over sodium sulfate. After removal of solvent, the amorphous white solid was recrystallized from 90–100° petroleum ether to give slender white needles.

Attempts to prepare DL-methionine-2-naphthylamide from carbobenzoxy-DL-methionine-2-naphthylamide were unsuccessful. Cleavage of the blocking group with sodium in liquid ammonia[11] resulted in hydrolysis of the amide, making isolation difficult. The use of phosphonium iodide[17] or hydrogen bromide[18] in nitromethane or acetic acid was not attempted.

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