The Coupling of 4-Methoxy-2-naphthylamine with Diazotized Aminodiazo Thioethers*,[1]

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The preparation of a new series of compounds, aminodiazo thioethers, is reported. Their diazotization and coupling with 4-methoxy-2-naphthylamine (II) is demonstrated. Three improved synthetic routes for the preparation of 4-methoxy-2-napthylamine in good yield are also described. The new osmiophilic diazonium compounds make possible the demonstration of sites of aminopeptidase activity in the electron microscope.

The L-leucyl amide[2-4] or L-alanyl amide[5] (I) of 4-methoxy-2-naphthylamine have proved to be superior substrates for the histochemical localization of aminopeptidase.

For the electron microscopic demonstration of aminopeptidase it would be desirable to incorporate an osmiophilic moiety[6] such as the mercapto, thiocarbamyl, or diazo thioether group in the diazonium salt which couples with 4-methoxy-2-naphthylamine (II) produced by the action of the enzyme. Attempts to prepare diazonium compounds from mercaptoanilines with retention of the thiol group were unsuccessful. Attempts to prepare diazonium compounds by diazotization of arylamines containing a thiocarbamyl group also resulted in loss of the osmiophilic property of this functional group.

We found, however, that the mercapto group of a mercaptoaniline could be coupled preferentially on the thiol group in acid solution. This is not surprising owing to the strong nucleophilic character of the thiol as compared to the anilinium group. The aminodiazo thioether formed was then diazotized to give a diazonium salt containing the osmiophilic diazo thioether group. Thus, diazotized 4′-amino-2′,5′-diethoxybenzanilide (III) was coupled into *o*-, *m*-, and *p*-mercaptoaniline and *m*-aminobenzylthiol (IV) in acid solution. The immediately resulting aminodiazo thioethers which precipitated were isolated, redissolved, and diazotized. These diazonium salts could then be coupled into II (or 2-naphthylamine, V) to give coupled products which reacted readily with OsO_4 vapor. The rate of coupling[3] with II is much greater than with V. The aminodiazo thioether (VI) resulting from the coupling of III into IV, when diazotized and coupled into II (or V), gave products which reacted most readily with OsO_4 vapor. This is not unexpected since the diazo thioethers resulting from alkylthiols are usually less stable than those derived from thiophenols.[7] In histochemical preparations, the localization obtained was most precise when this diazotized aminodiazo thioether (VI) was used with the substrate I. This finding justifies proceeding to examination of tissue in the electron microscope with this new method in order to determine the exact intracellular sites of aminopeptidase activity.

Three new synthetic routes for the preparation of II are described which eliminate the step involving the selective reduction of 1,3-dinitronaphthalene (VII) which handicapped the previously reported syntheses.[2,8] This step always gave a mixture of isomeric nitronaphthylamines from which 3-nitro-1-naphthylamine (VIII) could not be obtained in good yield. The first route employs the readily available VII[9] as the starting material. Upon treatment with sodium methoxide in methanol under pressure, a good yield of 1-methoxy-3-nitronaphthalene (IX) is obtained by nucleophilic displacement which is then reduced to II in the usual manner.[2] The second route depends on the preparation of VIII from 3-nitro-1-naphthoic acid (X)[10] by a Schmidt reaction. VIII is then converted to II in the usual manner.[2] The third route employs the readily available 1,3-naphthalenediol as the starting material. Upon treatment with ammonia under pressure according to the directions of Friedlaender, and Rüdt,[11] 3-amino-1-naphthol (XI) resulted which was not isolated but was acetylated *in situ*. The resulting 3-acetamido-1-naphthyl acetate (XII) was hydrolyzed yielding 3-acetamido-1-naphthol (XIII), which was then methylated in the usual manner. The resulting 3-acetamido-1-methoxynaphthalene (XIV) gave II upon hydrolysis.

It is of interest to note that all samples of II prepared by us melted at 75–76° as opposed to the previously reported values[2,8] of 57.5–58.5° and 59–60°. The infrared spectra of the lower melting samples were identical with the infrared spectra of the higher melting samples.

Experimental[12]

1,3-Dinitronaphthalene (VII).—This was prepared in 75% yield by the deamination of N-2,4-dinitro-1-naphthyl-*p*-toluenesulfonamide[13] according to the procedure of Hodgson and Birt-

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(11) P. Friedlaender and H. Rüdt, *Ber.*, **29**, 1609 (1896).

(12) All melting points are uncorrected. Analyses were by Mr. Joseph A. Walter and Clark Microanalytical Laboratories.

(13) H. H. Hodgson and E. W. Smith, *J. Chem. Soc.*, 1854 (1935).

well.[9] The crude product containing copper oxide was purified by chromatography of an ethylene dichloride solution on neutral alumina to yield yellow needles, m.p. 148–150°.

1-Methoxy-3-nitronaphthalene (IX).—In a typical run, 7 g. of sodium methoxide was added to a suspension of 15 g. of VII in 500 ml. of methanol. The mixture was shaken in a bomb (glass lined) at 105 ± 5° for 24 hr. After cooling to room temperature, water was added to the reaction mixture, and the product was steam distilled. Ether extraction of the steam distillate followed by evaporation of the ether yielded 6 g. (43%) of IX, m.p., 103–104°, not depressed when mixed with an authentic sample.[2] The infrared spectra of the two samples were identical.

3-Nitro-1-naphthylamine (VIII).—To a well-stirred suspension of 32.55 g. of 3-nitro-1-naphthoic acid (Aldrich Chemical Co., Inc., Milwaukee 10, Wis.) in 493 g. of concentrated sulfuric acid was added 11.4 g. of sodium azide in small portions at 50° so that the addition took approximately 1.5 hr. Upon reaction the naphthoic acid dissolved and the mixture darkened considerably. After stirring at 50° for 4 hr., the mixture was allowed to come to room temperature. It was then poured with stirring into a large quantity of ice, whereupon a suspension of the white amine sulfate appeared. Upon neutralization of the amine sulfate with alkali, a copious precipitate of the free base appeared which was collected on a filter and washed with water. This material, 24.4 g. (87%), m.p. 135–137°, could be used without further purification. Upon recrystallization from methanol–water, 23 g. (81%) of yellow needles, m.p. 137–137.5°, was obtained. Its melting point was not depressed on mixture with an authentic sample and the infrared spectra of the two were identical.

3-Acetamido-1-naphthyl Acetate (XII).—To 28 g. of finely powdered 1,3-naphthalenediol in a steel bomb was added 100 ml. of liquid ammonia. The bomb was sealed and shaken for 12 hr. at 133 ± 5°. After cooling to room temperature with shaking, the bomb was immersed in a Dry Ice–chloroform bath for further cooling before opening. The bomb was carefully opened in a hood and immediately flushed with nitrogen to remove excess ammonia and prevent oxidation of the 3-amino-1-naphthol formed. After removal of the ammonia, 150 ml. of acetic anhydride was added, the bomb sealed again and shaken until the the evolution of heat had ceased (approximately 0.5 hr.). The contents of the bomb, after cooling, was added slowly to a cold saturated sodium acetate solution. Concomitantly with the hydrolysis of excess anhydride, 28.2 g. (67%) of a brown precipitate appeared, m.p. 146–147.5°. The analytical sample, m.p. 150–150.5°, was obtained by recrystallization from methanol with little loss.

Anal. Calcd. for $C_{14}H_{13}NO_3$: C, 69.12; H, 5.39; N, 5.76. Found: C, 69.29; H, 5.58; N, 6.07.

3-Acetamido-1-naphthol (XIII).—A suspension of 10 g. of XII in 300 ml. of 10% potassium hydroxide was stirred at room temperature. Solution occurred as the hydrolysis progressed and the mixture became very dark. The solution was filtered after approximately 2 hr. and the naphthol precipitated with concentrated hydrochloric acid. This was filtered, washed with water, and air dried. It was recrystallized from acetone–ligroin to yield 7.5 g. (90%) of XIII, m.p. 209–210°. The analytical sample, m.p. 214–215°, was obtained by recrystallization from ethanol.

Anal. Calcd. for $C_{12}H_{11}NO_2$: C, 71.63; H, 5.51; N, 6.96. Found: C, 71.21; H, 5.52; N, 6.77.

3-Acetamido-1-methoxynaphthalene (XIV).—To a solution of 6.15 g. of XIII in 100 ml. of acetone was added 15 g. of anhydrous potassium carbonate and 6.5 ml. of dimethyl sulfate. The mixture was refluxed for 3 hr. during which time it became progressively lighter in color. After the addition of 200 ml. of water and the removal of the acetone on a steam bath, the precipitate which appeared was collected on a filter and washed with water. The product, 6.25 g., melting at 199.8–200°, was obtained in 95% yield.

Anal. Calcd. for $C_{13}H_{13}NO_2$: C, 72.54; H, 6.09; N, 6.51. Found: C, 72.27; H, 6.28; N, 6.49.

4-Methoxy-2-naphthylamine (II). Method A. Catalytic Reduction of III.—The previously reported synthesis[2] was improved by precipitation of the product with water from the methanol solution obtained after filtration of the catalyst. The product which melted at 72–73° was obtained in 90% yield. Recrystallization from aqueous methanol yielded II as colorless prisms, m.p. 75–76°.

Method B. By Acid Hydrolysis of XIV.—A mixture of 4 g. of XIV, 50 ml. of concentrated hydrochloric acid, 50 ml. of water, and 50 ml. of ethanol was refluxed for 2 hr. The clear solution was cooled in ice and poured slowly into 250 ml. of 10% sodium hydroxide solution. The precipitated product, 3 g. (93%), m.p. 70–72°, was collected on a filter and washed with water. Recrystallization from methanol–water gave colorless prisms, m.p. 75–76°.

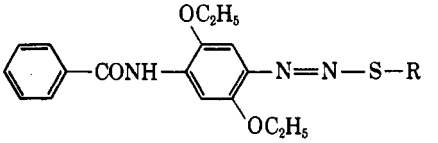
L-Alanyl-4-methoxy-2-napthylamide Hydrochloride (I).[14a]—This was prepared in methanol containing 1 equiv. of hydrogen chloride in 90% yield by hydrogenolysis of carbobenzoxy-L-alanyl-4-methoxy-2-naphthylamide prepared by reaction of 4-methoxy-2-naphthylamine with a mixed anhydride formed from carbobenzoxy-L-alanine and ethyl chlorocarbonate in the usual manner. After filtration of the 10% palladium-on-charcoal catalyst, the product was precipitated with ether and recrystallized from methanol–acetone–ether. The product melted completely at 188° with softening at 135°, $[\alpha]^{28}D$ +17.5° (*c* 1, 2.5 *N* hydrochloric acid). Paper chromatography (Whatman No. I) in 88% w./v. phenol–water or 1-butanol–acetic acid–water (18:2:5) revealed only one ninhydrin-positive spot. In recent experiments in which we have prepared 50-μ sections for study with the electron microscope, we have noted that the alanyl derivative not only was hydrolyzed more rapidly but penetrated thick sections to a greater depth than the leucyl substrate. This makes the alanyl substrate more useful for electron microscopy.

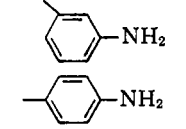
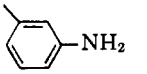
Anal. Calcd. for $C_{14}H_{17}ClN_2O_2$: N, 9.98. Found: N, 9.76.

Aminodiazo Thioethers (Table I).—To a filtered aqueous solution (1 g./10 ml.) of the commercially available diazonium salt was added 20 ml. of ethanol. The solution was chilled in an ice bath and the precipitated salts removed by filtration. This solution was then added to a solution of an equimolar amount of mercaptoaniline hydrochloride or *m*-aminobenzylthiol hydrochloride[14b] in 10 ml. of water. The yellow aminodiazo thioether which precipitated immediately was filtered, washed with water, and dried *in vacuo* at room temperature for a few hours. Like diazo thioethers[15] they were very unstable and decomposed with the evolution of nitrogen upon attempted recrystallization. These amines were not stored but were promptly diazotized.

TABLE I

AMINODIAZO THIOETHERS[a]



Compd.	R	M.p., °C.	Yield,[b] %
VI		87–89 dec.	12
		75–76 dec.	17
		79–80 dec.	18

[a] All of the compounds in this table had elemental analyses in agreement with theory. [b] Based on mercaptoaniline.

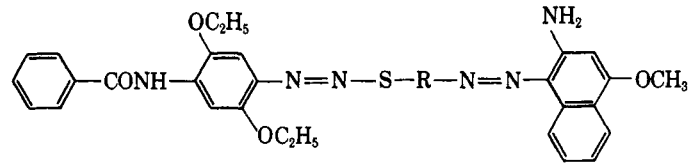
Diazotization of Aminodiazo Thioethers.—The aminodiazo thioethers were suspended in 10% hydrochloric acid and diazotized at −5° with a slight excess (starch–iodide test) of 20% sodium nitrite solution. The excess nitrite was decomposed by the addition of urea. If isolation of the diazonium salt was desired, it was accomplished by the addition of excess zinc chloride and sodium chloride (when necessary) to precipitate the zinc chloride double salt. They were collected by suction filtration, washed with a small quantity of alcohol and ether, and stored in the freezer at −20°.

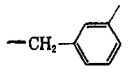
(14) (a) This compound was prepared by Dr. Herman Plaut, Cyclo Chemical Corp., Los Angeles, Calif. (b) Cyclo Chemical Corp., Los Angeles, Calif.

(15) (a) A. Hantzsch and H. Freese, *Ber.*, **28**, 3237 (1895); (b) J. T. Dunn, Jr., and W. A. Fletcher, *Trans. Kansas Acad Sci.*, **37**, 123 (1934).

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-

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