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Thiolysis of *O*-2,4-Dinitrophenyltyrosines

Spectrophotometric Monitoring of the Reaction and Its Use in Peptide Synthesis

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The thiolytic cleavage of *O*-2,4-dinitrophenyl (Dnp) derivatives of phenols was applied to the synthesis of tyrosine-containing peptides. This paper describes the preparation and properties of starting materials for such syntheses and illustrates their use in the synthesis of some peptides containing tyrosine at either the C- or N-terminus. A spectrophotometric method for following the thiolytic removal of Dnp groups from *O*-Dnp-tyrosines was developed and used to establish optimal conditions for quantitative deblockage in aqueous and nonaqueous solvents. The method is based on the fact that upon thiolysis, the colorless solution of *O*-Dnp-tyrosine (λ_{max} at 298 nm, pH 8.5) becomes yellow due to the formation of a dinitrophenylated thiol (for *S*-Dnp-2-mercaptoethanol, λ_{max} at 340 nm, pH 8.5). This gives rise to a difference spectrum with a maximum at 354 nm ($\Delta\epsilon_M$ = + 8680 M^{-1} cm^{-1}), a minimum at 298 nm ($\Delta\epsilon_M$ = −5900 M^{-1} cm^{-1}) and a crossover point at 318 nm, which is different (in the 290–320 nm range) from the difference spectrum obtained upon thiolysis of N^{im}-Dnp-histidine. This method provides a useful analytical tool in peptide and polypeptide synthesis as well as in protein chemistry.

Quite a few functional groups have been suggested for masking the phenolic hydroxyl of tyrosine during peptide and polypeptide synthesis (1, 2). Of these, the most commonly used is the benzyl group (3–5). In the last few years, several reports (6–10) have indicated that this protecting group has two major disadvantages: (a) It is partially removed under the conditions used for deprotection of α-amino groups (e.g., by 50% trifluoroacetic acid in dichloromethane); and (b) its removal by acidolysis is accompanied by an intramolecular rearrangement which results in a modified tyrosine derivative (3-benzyltyrosine). In a multistep synthesis, the partial deprotection and the rearrangement may result in a heterogeneous population of molecules including branched species. In an attempt to avoid these undesirable side reactions the 2,6-dichlorobenzyl and the *m*-bromobenzyl groups have been introduced (6–10). Both certainly represent a considerable improvement over the benzyl group, but do not completely solve the problem. Intramolecular benzylation also does not occur when using the benzyloxycarbonyl group for protection of the phenolic hydroxyl of tyrosine (8). However, this protecting group is not sufficiently stable when exposed to trifluoroacetic acid in dichloromethane and is also removed by nucleophiles such as α-amino groups (8). This instability to nucleophiles also occurs to some extent in the *O*-bromobenzyloxycarbonyl group (9). A few of the other protecting groups that have been suggested for the phenolic hydroxyl of tyrosine seem to have definite advantages. For example, the *tert.*-butyl (11, 12) carbamoyl (13), alkoxycarbonyl (14), 4-picolyl (15), and *o*-nitrobenzyl groups which can be removed photolytically (B. Amit, E. Hazum, M. Fridkin, and A. Patchornik, in preparation). However, each of these has its own limitations and the group chosen must therefore be selected considering the structure of the peptide to be synthesized and the manipulations to be used during its synthesis.

The finding that Dnp[1] derivatives of imidazoles, phenolic hydroxyls, and sulfhydryls can be readily displaced by thiols (16) has potentially made the Dnp group suitable for masking histidine, tyrosine, and cysteine residues during synthesis of peptides and polyamino acids (17). The protection of histidine residues by dinitrophenylation was studied in detail (18–25) and was found suitable for the synthesis of peptides and polyaminoacids containing histidine not only by classical methods but also by solid-phase peptide synthesis.

Here, we report the use of the thiolytic cleavage of *O*-Dnp-phenols during synthesis of peptides containing tyrosine and describe a spectrophotometric method for following the course of thiolysis. Using this method it is possible to establish optimal conditions for unmasking the protected tyrosine side chain in an aqueous or nonaqueous medium.

MATERIALS AND METHODS

The following compounds were prepared according to methods described in the literature: *N*-Z-L-tyrosine (26); *N*,*O*-di-Z-L-tyrosine (27); Z-L-phenylalanine-*N*-hydroxysuccinimide ester (28); N^{Im}-Dnp-L-histidine·2HBr (18). Samples of *N*-Z-L-tyrosyl-L-phenylalanine and *N*-Z-L-tyrosyl-glycine ethyl ester (prepared by a procedure different from the one described here) were kindly provided by Professor M. Wilchek for comparison of melting points and R_f values. *N*-Hydroxysuccinimide and *N*-Boc-*O*-DNP-L-tyrosine were a gift of Mr. J. Jacobson. *O*-Dnp-tyrosine·HCl was obtained from Mann Research Laboratories. All other chemicals were best available grades from commercial sources.

The peptides obtained were characterized by nonaqueous titrations (29, 30) as well as by thin-layer chromatography on silica gel (from Riedel-De Haen AG) using the following solvent systems: (1) chloroform–methanol–acetic acid (9:1:1, v/v/v); (2) chloroform–methanol (9:1, v/v); (3) chloroform–methanol (3:1, v/v); (4) acetonitrile–water (9:1, v/v); (5) *n*-butanol–pyridine–acetic acid–water (15:10:3:12, v/v/v/v); (6) *n*-butanol–acetic acid–water (4:1:1, v/v/v); R_f values are uncorrected. The peptides were revealed by ultraviolet light (with silica gel F plates) or by charring over a flame.

All melting points were measured using a capillary melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter and spectrophotometric measurements were carried out with a Cary Model 15 spectrophotometer.

[1] Abbreviations used: DMF, *N*,*N*-dimethylformamide; FA, formamide; FDNB, 1-fluoro-2,4-dinitrobenzene; N^{Im}, imidazole imino nitrogen; Dnp, dinitrophenyl; Boc, butoxycarbonyl, NSu, succinimido; Ac, acetyl. Abbreviations of amino acid derivatives follow the recommendations of the IUPAC-IUB Commission on Biochemical nomenclature [cf. *Eur. J. Biochem.* 27, 201–207 (1972)].

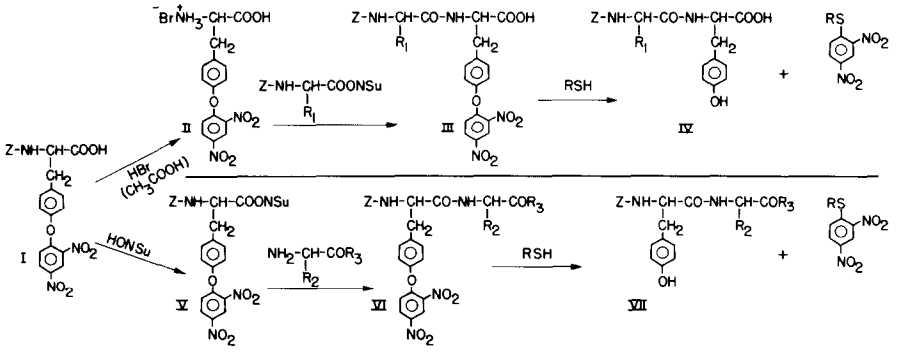
RESULTS

Scheme I illustrates the general procedure used for the synthesis of peptides containing tyrosine at the C- or N-terminus. *N*-Z-*O*-Dnp-L-Tyr (I) was used as a starting material in both cases. Unless otherwise indicated, activation of the carboxyl group was carried out by the NSu-ester method (31).

PREPARATION OF STARTING MATERIALS

N-Z-O-Dnp-L-Tyrosine (I)

Method A. *N*-Z-L-Tyrosine (10 mmol) was dissolved in 30 ml of water containing 30 mmol of $NaHCO_3$. The solution was cooled to 0°C; then FDNB (15 mmol in 30 ml of dioxane) was added. The mixture was stirred for 16 h at 4°C and then concentrated *in vacuo* to about 20 ml. Upon acidification with 4 N HCl, a yellow precipitate was formed which was extracted into ethyl acetate. This solution was washed with water and then with a saturated solution of NaCl, dried over Na_2SO_4, and evaporated to dryness. The oily residue obtained was dissolved in 20 ml of ethyl acetate and the solution was divided into two equal portions. Addition of petroleum ether to one portion yielded a paste-like material which yielded yellowish-white crystals upon trituration with ether. Yield 74%; mp 131–135°C; R_f 0.75 (system 1), 0.32 (system 2), 0.53 (system 3), 0.52 (system 4); $[\alpha]_D^{23}$ + 39.4° (C, 1.0, $CHCl_3$). *Anal.* Calcd. for $C_{23}H_{19}N_3O_9$: C, 57.38; H, 3.98; N, 8.73; neutr. equiv. 481. Found: C, 57.23; H, 3.99; N, 8.50; neutr. equiv. 479 (with $NaOCH_3$ in methanol-benzene). Addition of dicyclohexylamine (5.5 mmol) to the second portion of the ethyl acetate solution yielded a solid dicyclohexylammonium salt of I which was recrystallized as yellow crystals from ethanol–ether (1:1, v/v). Yield 86%; mp 181–183°C; $[\alpha]_D^{23}$ + 19.1° (C, 1.0, DMF). *Anal.* Calcd. for



SCHEME I

$C_{35}H_{42}N_4O_7$: C, 63.37; H, 6.39; N, 8.45. Found: C, 63.29; H, 6.50; N, 8.57.

Method B. *N,O*-Di-Z-L-tyrosine (40 mmol) was dissolved in a mixture of 50 ml of methanol and 120 ml of 1 N NaOH and the solution was allowed to stand at room temperature. After 90 min, the pH was adjusted to 7.0 and the methanol was removed by evaporation *in vacuo*. Upon acidification with 6 N HCl, a precipitate was formed which was extracted into ethyl acetate. This solution was washed with water, dried over Na_2SO_4, and evaporated to dryness. This product was directly reacted with FDNB as described in Method A above. Yield 71%; mp 131–134°C; R_f 0.75 (system 1), 0.32 (system 2); $[\alpha]_D^{23}$ + 36.0° (C, 1.0, $CHCl_3$); *Anal.* Calcd. for $C_{23}H_{19}N_3O_9$: C, 57.38; H, 3.98; N, 8.73. Found: C, 57.18; H, 4.10; N, 8.40.

O-Dnp-L-Tyrosine·HBr (II)

Compound I (2.0 mmol) was dissolved in warm glacial acetic acid (5 ml). The solution was cooled to room temperature and 5 ml of a solution of HBr (45%) in glacial acetic acid was added. The mixture was stirred at room temperature while product II precipitated out. After 60 min, dry ether was added to complete the precipitation and the white precipitate formed was filtered, washed with ether, and dried over NaOH pellets *in vacuo*. Yield, 90%; R_f 0.57 (system 5), 0.50 (system 6); $[\alpha]_D^{23}$ + 9.1° (C, 1.05, DMF). *Anal.* Calcd. for $C_{15}H_{14}N_3O_7Br$: C, 42.07; H, 3,30; Br, 18.69; neutr. equiv. 214 (two acidic groups). Found: C, 42.85; H, 3.48; Br, 18.52; neutr. equiv. 222 (with $NaOCH_3$, in methanol–benzene); 204 (with $HClO_4$ in acetic acid containing $Hg(OAc)_2$).

N-Z-O-Dnp-L-Tyrosine-N-Hydroxysuccinimide Ester (V)

Compound I (2.0 mmol) and *N*-hydroxysuccinimide (2.1 mmol) were dissolved in a mixture containing ethyl acetate (5 ml) and DMF (0.2 ml). The solution was cooled to 0°C, and then 2.0 mmol of dicyclohexylcarbodiimide (in 5 ml of ethyl acetate) was added. The reaction mixture was stirred for 1 h at 0°C and then allowed to stand overnight at room temperature. The dicyclohexylurea formed was filtered off and the solution was concentrated *in vacuo* to dryness. Crystallization from 2-propanol yielded yellowish-white crystals. Yield, 95%; mp 130–133°C; R_f 0.90 (system 1), 0.92 (system 3), 0.92 (system 4); $[\alpha]_D^{23}$ + 17.7° (C, 0.95, $CHCl_3$). *Anal.* Calcd. for $C_{27}H_{22}N_4O_{11}$: C, 56.06; H, 3.83; N, 9.69; neutr. equiv. 578. Found: C, 55.93; H, 3.98; N, 9.63; neutr. equiv. 556 (with $NaOCH_3$ in methanol–benzene).

SYNTHESIS OF PROTECTED TYROSINE PEPTIDES

Z-L-Phenylalanyl-O-Dnp-L-Tyrosine (III_a, $R_1 = CH_2—C_6H_5$)

Carbobenzyloxy-L-phenylalanine-*N*-hydroxysuccinimide ester (1 mmol) in 7 ml of dioxane was mixed with 7 ml of an aqueous solution containing 1 mmol of compound II and 3 mmol of $NaHCO_3$. A few drops of DMF were added to clarify the solution. The reaction was allowed to proceed, with stirring, overnight at room

temperature. Then the solution was concentrated *in vacuo* to about 5 ml, cooled to 4°C and acidified with 4 N HCl. The yellow precipitate formed was extracted into ethyl acetate. The extract was then washed with water, dried over Na_2SO_4, and evaporated to dryness. The product, III_a, was crystallized as yellowish crystals by dissolving in ethyl acetate (~15 ml) and precipitation with petroleum ether. Yield, 74%; mp 164–166°C (softening at 128°C); R_f 0.64 (system 3), 0.65 (system 4); $[\alpha]_D^{23}$ −28.4° (C, 1.02, DMF). *Anal.* Calcd. for $C_{32}H_{28}N_4O_{10}$: C, 61.14; H, 4.49; N, 8.91; neutr. equiv. 628. Found: C, 61.77; H, 4.81; N, 8.66; neutr. equiv. 590 (with $NaOCH_3$ in methanol–benzene).

N-Z-O-Dnp-L-Tyrosyl-O-Dnp-L-Tyrosine (III_b, $R_1 = CH_2—C_6H_5—O\text{-}Dnp$)

A solution of compound V (1.0 mmol in 7 ml of dioxane) was mixed with an aqueous solution (7 ml) containing 1.0 mmol of compound II and 3.0 mmol of $NaHCO_3$. The mixture was stirred overnight at room temperature and the product (III_b) was isolated by the procedure described for peptide III_a (see above). Crystallization was achieved by dissolving in a minimum volume of ethyl acetate and precipitation with ether. Yield, 69%; mp 110–115°C; R_f 0.30 (system 2), 0.81 (system 3), 0.84 (system 4); $[\alpha]_D^{23}$ −32.7° (C, 1.02, DMF). *Anal.* Calcd. for $C_{38}H_{30}N_6O_{15}$: C, 56.30; H, 3.73; N, 10.37; neutr. equiv. 810. Found: C, 56.21; H, 3.95; N, 10.02; neutr. equiv. 838 (with $NaOCH_3$ in methanol–benzene).

N-Z-O-Dnp-L-Tyrosyl-L-Phenylalanine (VI_a, $R_2 = CH_2—C_6H_5$, $R_3 = H$)

A solution of compound V (2.0 mmol in 10 ml of dioxane) was mixed with an aqueous solution (10 ml) containing 2.0 mmol of L-phenylalanine and 4.0 mmol of $NaHCO_3$. The homogeneous mixture was allowed to stand overnight at room temperature and the product, VI_a, was isolated by the procedure described for peptide III_a (see above). Crystallization was achieved by dissolving in a minimum volume of ethyl acetate and precipitation with petroleum ether. Yield 71%; mp 96–100°C; R_f 0.18 (system 2), 0.86 (system 3); $[\alpha]_D^{23}$ −19.4° (C, 0.98, DMF). *Anal.* Calcd. for $C_{32}H_{28}N_4O_{10}$: C, 61.15; H, 4.49; N, 8.91; neutr. equiv. 628. Found: C, 61.18; H, 4.72; N, 8.92; neutr. equiv. 664 (with $NaOCH_3$ in methanol–benzene).

N-Z-O-Dnp-L-Tyrosyl-N^{im}-Dnp-L-Histidine (VI_b, $R_2 = CH_2—C_3H_2N_2—Dnp$, $R_3 = H$)

A solution of compound V (0.8 mmol in 5 ml of dioxane) was mixed with an aqueous solution (5 ml) containing 0.8 mmol of N^{im}-Dnp-L-histidine·2HBr and 3.2 mmol of $NaHCO_3$. The reaction was allowed to proceed overnight at room temperature and the product (VI_b) was isolated as described for peptide III_a (see above) and recrystallized from methanol as yellowish-brown crystals. Yield, 80%; mp 194–197°C; R_f 0.15 (system 2), 0.76 (system 3), 0.47 (system 4); $[\alpha]_D^{23}$ +10.0° (C, 0.77, CH_3COOH). *Anal.* Calcd. for $C_{35}H_{28}N_8O_{14}$: C, 53.57; H, 3.60; N, 14.28; neutr. equiv. 784. Found: C, 53.53; H, 3.75; N, 14.48; neutr. equiv. 744 (with $NaOCH_3$ in methanol–benzene).

N-Z-O-Dnp-L-Tyrosyl-Glycine Ethyl Ester (VI_c, $R_2 = H$, $R_3 = C_2H_5$)

A solution of compound I, glycine ethyl ester hydrochloride, and triethylamine (4 mmol of each) in 15 ml of chloroform was cooled to 0°C and mixed with a solution (5 ml containing 4 mmol) of dicyclohexylcarbodiimide in chloroform. The reaction mixture was allowed to stand at 0°C for 1 h, then overnight at room temperature. The dicyclohexylurea formed was filtered and the filtrate was washed consecutively with 1 N HCl, 1 N $NaHCO_3$, and water. The washed filtrate was then dried over Na_2SO_4 and evaporated to dryness *in vacuo* and the solid product was triturated with ether to yield yellowish-white crystals. Yield, 72%; mp 113–116°C; R_f 0.89 (system 2), 0.96 (system 3), 0.95 (system 4); $[\alpha]_D^{23}$ −31.6° (C, 0.96, DMF). *Anal.* Calcd. for $C_{27}H_{26}N_4O_{10}$: C, 57.24; H, 4.63; N, 9.89. Found: C, 57.39; H, 4.71; N, 9.68.

Stability of *O*-Dnp Tyrosyl Residues

The stability of *O*-Dnp tyrosyl residues was tested by exposure to a variety of reagents commonly used during peptide

synthesis, or in the course of structural studies of polypeptides and proteins. Subsequently, the occurrence of unmasking or decomposition was assessed by thin-layer chromatography. Thiolysis was then attempted to ascertain that the *O*-Dnp derivative had not undergone an intramolecular rearrangement that would render it nonthiolyzable.

Exposure to 100% Trifluoroacetic Acid

N-Boc-*O*-Dnp-L-tyrosine (0.1 mmol) was dissolved in 0.5 ml of 100% trifluoroacetic acid and allowed to stand at room temperature. Within 10 min, the unmasking of the α-amino group was completed. After 24 h, excess trifluoroacetic acid was evaporated and the product was precipitated with ether and dissolved in 1 ml of DMF. Thiolysis was initiated by adding 6 mmol of 2-mercaptoethanol, monitored spectrophotometrically as described below, and found to proceed to completion. Thin-layer chromatography yielded only one product which was identical in its R_f values to a tyrosine marker. The above experiment was repeated with *N*-Z-*O*-Dnp-L-tyrosyl-glycine ethyl ester and it was found that in this case also the *O*-Dnp-tyrosyl residue was stable for 24 h in 100% trifluoroacetic acid and that the masking group could subsequently be removed in quantitative yield by thiolysis.

Exposure to Anhydrous Hydrogen Fluoride

N-Z-*O*-Dnp-L-tyrosine (2 mmol) was dissolved in a solvent composed of anhydrous hydrogen fluoride (10 ml) and anisole (1 ml) at 0°C. After 30 min, the product was precipitated with ether and shown to be identical with a marker of *O*-Dnp-L-Tyr · HBr on thin-layer chromatography. The product could be quantitatively thiolyzed in DMF (by addition of 60 mol of 2-mercaptoethanol per mole of the *O*-Dnp derivative) to yield tyrosine.

Exposure to Triethylamine

N-Z-*O*-Dnp-L-tyrosine and triethylamine (0.1 mmol of each) were dissolved in 1 ml of DMF and allowed to stand at room temperature for 24 h. Thin-layer chromatography of an aliquot of this solution indicated the presence of no decomposition product, and the Dnp derivative could be quantitatively thiolyzed in DMF by addition of 60 mol of 2-mercaptoethanol per mole of *N*-Z-*O*-Dnp-L-tyrosine to yield *N*-Z-L-tyrosine.

Exposure to Free α-Amino Groups

A solution containing *N*-Z-*O*-Dnp-L-tyrosine, glycine ethyl ester · HCl, and triethylamine (0.1 mmol of each) in DMF (1 ml) was allowed to stand at 4°C, and a similar solution was allowed to stand at 23°C. After 1 h, the extent of aminolysis (determined by disappearance of *N*-Z-*O*-Dnp-L-tyrosine and formation of *N*-Z-L-tyrosine) was found to be negligible (<0.1% at 4°C and <1% at 23°C). After 3 h, the extent of aminolysis was still low (<1% at 4°C and <5% at 23°C) but after 24 h considerable aminolysis took place (~40% at 4°C and ~70% at 23°C).

Exposure to Extreme pH Values in Aqueous Solutions

To test the stability of *O*-Dnp-tyrosines in aqueous media we incubated (for 20 h at 22°C) solutions of *O*-Dnp-L-tyrosine at strongly basic and acidic pH values and monitored their spectra at various times. As seen in Fig. 1 (right) the Dnp group on the phenolic hydroxyl appears to be stable

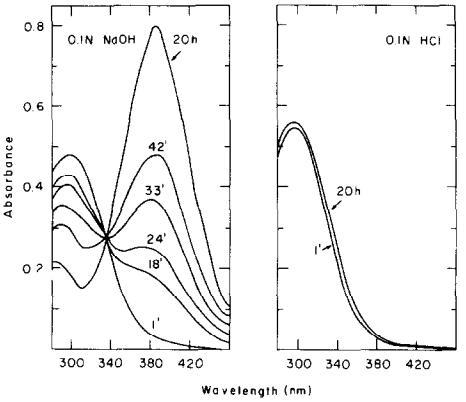


FIG. 1. Stability of *O*-Dnp-L-tyrosine at extreme basic and acidic pH values. *O*-Dnp-L-Tyrosine · HCl was dissolved in DMF (1×10^{-3} M) and then diluted with 0.1 N NaOH (left) or with 0.1 N HCl (right) to final concentrations of 4.15×10^{-5} and 4.5×10^{-5} M, respectively. The spectra of the solutions were recorded 1 min after dilution or after standing in the dark (22°C) for the indicated time.

under very acidic conditions, but hydrolyzes in 0.1 N NaOH within 20 h (Fig. 1, left). Nevertheless, at pH 10.2 the extent of decomposition (under the same conditions) does not exceed 6% (Table I). These results were confirmed by chromatography of aliquots from the incubation mixture at the beginning and at the end of the incubation period.

A Spectrophotometric Method for Following the Extent of Thiolysis of *O*-Dnp-Tyrosines

In aqueous solutions and at neutral pH, *O*-Dnp-L-tyrosine has an absorption maximum at 298 nm with a molar extinction of 11,300 M^{-1} cm^{-1}. These spectral properties are slightly changed by lowering the pH of the solution to 0.7, by raising it to 9.0, or by transferring the compound into nonaqueous solvents such as ethanol, dioxane, or DMF (Table II). Upon thiolysis, the colorless solution of *O*-Dnp-L-tyrosine becomes yellow due to the formation of a dinitrophenylated thiol [for *S*-Dnp-2-mercaptoethanol, λ_{max} is at 340 nm in water, pH 7.0; at 332 nm in ethanol; at 333 nm in dioxane; and at 341 in DMF (3)]. This gives rise to a difference spectrum with a maximum at 354 nm ($\Delta\epsilon_M$ = + 8680 M^{-1} cm^{-1}), a minimum at 298 nm ($\Delta\epsilon_M$ = −5900 M^{-1} cm^{-1}), and a crossover point at 318 nm. The extent of thiolysis can therefore be followed either directly (Fig. 2) or by difference spectroscopy (Fig. 3).

TABLE I

Effect of pH on the Cleavage of *O*-Dnp-L-Tyrosine[a]

pH	Time (h)	Extent of cleavage (%)
9.2	1.5	<0.5
	4.5	<0.5
	21.0	<0.5
10.2	1.5	<0.5
	4.5	<0.5
	21.0	6
11.2	1.5	3
	4.5	10
	21.0	61

[a] A solution of *O*-Dnp-L-tyrosine in DMF (1 × 10^{-3} M) was prepared. Aliquots of this solution were diluted into three carbonate buffers (0.05 M) of pH 9.2, 10.2, and 11.2 to a final concentration of 5 × 10^{-5} M. These solutions were allowed to stand at 22°C in the dark and their spectra at various times were recorded. The extent of cleavage was calculated from the increase in absorption at 385 nm due to the formation of 2,4-dinitrophenol.

Optimal Conditions for the Thiolytic Cleavage of *O*-Dnp-Tyrosines

The thiolytic cleavage of *O*-Dnp-tyrosines is dramatically affected by the nature of the solvent used. As seen in Fig. 4,

TABLE II

Spectral Properties of *O*-Dnp-L-Tyrosine in Various Solvents[a]

Solvent	λ_{max} (nm)	ϵ_M (M^{-1} cm^{-1})
Water		
~ 0.1 M HCl, pH 0.7	297	12200
0.05 M sodium phosphate, pH 7.2	298	11300
0.05 M sodium bicarbonate, pH 9.0	298	11600
Ethanol	293	12000
Dioxane	296	11650
DMF	298	11400

[a] A solution of *O*-Dnp-L-tyrosine in DMF (1 × 10^{-3} M) was diluted 1:20 into each of the indicated solvents and the spectra were recorded. Values of λ_{max} and ϵ_M given are averages of duplicates in each case.

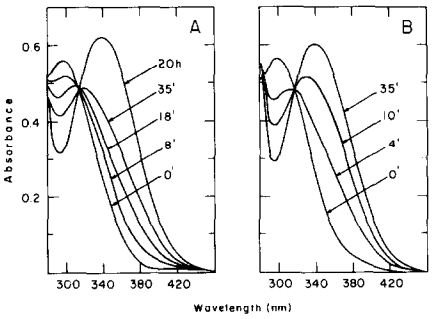


Fig. 2. Spectrophotometric monitoring of the thiolytic cleavage of *O*-Dnp-L-tyrosine in aqueous solution (pH 8.5) using 100 mol/mol (A) or 1000 mol/mol (B) of 2-mercaptoethanol. *O*-Dnp-L-Tyrosine·HCl was dissolved in DMF (1 × 10^{-3} M) and then diluted with 0.05 M sodium pyrophosphate (pH 8.5) to a final concentration of 5 × 10^{-5} M. The spectrum of the diluted solution was recorded as t = 0. Thiolysis was initiated by addition of 2-mercaptoethanol (2 μl (A) or 20 μl (B) to 6 ml of the solution of *O*-Dnp-L-tyrosine). The reaction was allowed to proceed at 22°C in the dark and its progress with time was followed.

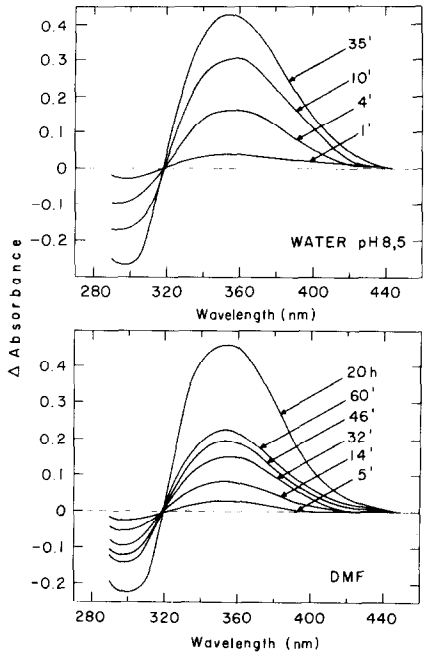


FIG. 3. Monitoring the thiolytic cleavage of O-Dnp-L-tyrosine in aqueous and in nonaqueous solvents by difference spectra. A solution of O-Dnp-L-tyrosine·HCl in DMF (1×10^{-3} M) was diluted 1:20 with either 0.05 M sodium pyrophosphate, pH 8.5 (top) or DMF (bottom). The reaction mixture cell as well as the reference cell contained 3 ml of the same solution of O-Dnp-L-tyrosine. Thiolysis was initiated in the reaction mixture cell by addition of 10 μl of 2-mercaptoethanol (1000 mol of thiol/mol of Dnp derivative). The reaction was allowed to proceed in the dark and at 22°C. Difference spectra were recorded at the indicated times.

there is essentially no thiolysis in water at acidic pH values, while there is a marked increase in the rate of thiolysis with increasing pH above neutrality. Considerable differences in the rate of thiolysis are observed in different organic solvents (Fig. 5): Under reaction conditions which brought about no thiolysis in dioxane or ethanol within 60 min there was 43 and 50% thiolysis in formamide and DMF, respectively. It is therefore advisable to establish (by spectrophotometric monitoring) the optimal solvent and reaction conditions for thiolysis in each particular case.

COMPARISON WITH THE THIOLYTIC CLEAVAGE OF N^{Im}-Dnp-HISTIDINES

Figure 6 illustrates the difference spectrum obtained upon thiolysis of N^{Im}-Dnp-L-histidine·2HBr, which has a maximum around 350 nm ($\Delta\epsilon_M = +8800\ M^{-1}\ cm^{-1}$). In contrast to the case of O-Dnp-L-tyrosine, the molar absorptivity difference does not attain negative values at 298 nm (compare with Fig. 3, top).

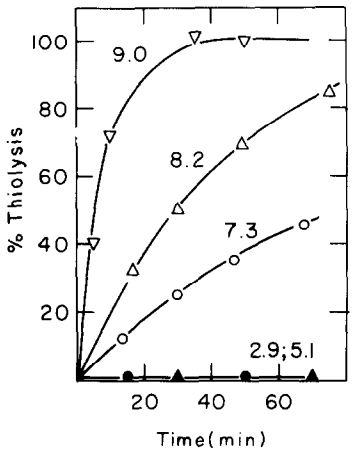


FIG. 4. Effect of pH on the rate of thiolysis of O-Dnp-L-tyrosine. The reaction was initiated and carried out as described in the legend to Fig. 3 (1000 mol of thiol/mol of O-Dnp-L-tyrosine) and the extent of thiolysis was determined spectrophotometrically by the increase in absorption at 355 nm. Buffers used were: 0.1 M phosphate-citrate for pH 2.9 (▲), 5.1 (●) and 7.3 (○); 0.1 M phosphate for pH 8.2 (△) and 0.1 M carbonate-bicarbonate for pH 9.0 (▽).

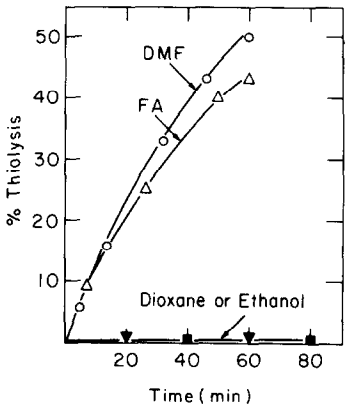


FIG. 5. Rates of thiolysis of O-Dnp-L-tyrosine in nonaqueous solvents. The reaction was initiated and carried out as described in the legend to Fig. 3 (1000 mol of thiol/mol of O-Dnp-L-tyrosine) and the extent of thiolysis was determined spectrophotometrically by the increase in absorption at 355 nm. Solvents used were: dioxane (■), ethanol (▼), formamide (△), and DMF (○).

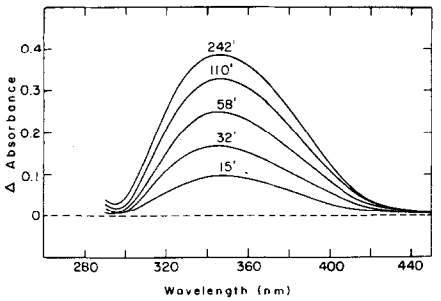


FIG. 6. Monitoring the thiolytic cleavage of N^{Im}-Dnp-L-histidine by difference spectra. N^{Im}-Dnp-L-histidine·2HBr was dissolved in DMF (10^{-3} M) and then diluted with a 0.1 M phosphate buffer (pH 8.5) to a final concentration of 5×10^{-5} M. Thiolysis was initiated, carried out, and monitored as described in the legend to Fig. 3. Difference spectra were recorded at the indicated times.

DEBLOCKAGE OF PROTECTED TYROSINE PEPTIDES

Z-L-Phenylalanyl-L-Tyrosine (IV_a, $R_1 = CH_2—C_6H_5$)

To a solution of 314 mg (0.5 mmol) of compound III_a in 1 ml of DMF, we added 14 mmol of 2-mercaptoethanol. The reaction was followed by subjecting aliquots of the reaction mixture at various times to thin-layer chromatography (system 3). After 1 h (22°C) thiolysis was completed. The DMF was then evaporated under high vacuum and the product was crystallized from ethyl acetate-petroleum ether. After two recrystallizations from ethanol-petroleum ether the product melted at 182–185°C [lit. 181.5–183°C, (14)]: yield 91%; R_f 0.59 (system 3); 0.64 (system 4).

N-Z-L-Tyrosyl-L-Tyrosine (IV_b, $R_1 = CH_2—C_6H_5—OH$)

To a solution of 81 mg (0.1 mmol) of compound III_b in 0.5 ml of DMF we added 5.6 mmol of 2-mercaptoethanol. After ~30 min (22°C) the product started to precipitate out. The reaction mixture was allowed to stand for an additional hour, then ether was added to ensure complete precipitation of the dipeptide. The product melted at 151–153°C [lit. 148°C (32)]: yield 95%; R_f 0.23 (system 2), 0.45 (system 3).

N-Z-L-Tyrosyl-L-Phenylalanine (VII_a, $R_2 = CH_2—C_6H_5$, $R_3 = H$)

Compound VI_a (314 mg, 0.5 mmol) was thiolyzed at 22°C in DMF (2 ml) using 14 mmol of 2-mercaptoethanol. After 1 h the reaction mixture was concentrated under high vacuum and the residual oil was crystallized from ethyl acetate-ether. After three recrystallizations from ethyl acetate, the product had a mp of 168°C (compared with 169°C; M. Wilchek, unpublished results); yield 92%; R_f 0.64 (system 3), 0.62 (system 4) (the product was identical in its R_f values with a marker of VII_a prepared by M. Wilchek by a different procedure); neutr. equiv.: calcd. 462, found 466.

N-Z-L-Tyrosyl-L-Histidine (VII_b, $R_2 = CH_2—C_3H_3N_2$, $R_3 = H$)

Compound VI_b (397 mg, 0.5 mmol) was thiolyzed at 22°C in DMF (4 ml) using 28 mmol of 2-mercaptoethanol. The reaction was followed by thin-layer chromatography and found to be completed after 1.5 h. The mixture was concentrated *in vacuo* and the residue was crystallized twice from DMF-ether, twice from DMF-ethyl acetate and finally by dissolving the peptide in 0.1 N NaOH and precipitation by lowering the pH to 6.5, with 0.5 N HCl. Yield 90%; mp 193°C [lit. 199°C (33)]; R_f 0.07 (system 3); 0.78 (system 5).

N-Z-L-Tyrosyl-Glycine Ethyl Ester (VII_c, $R_2 = H$, $R_3 = C_2H_5$)

Compound VI_c (283 mg, 0.5 mmol) was dissolved in DMF (2 ml) and thiolyzed by addition of 14 mmol of 2-mercaptoethanol. After 1 h, the solution was concentrated *in vacuo* and the product was precipitated by addition of ether. Yield, 93%; mp 162–165°C (compared with 163–165°C; M. Wilchek, unpublished results); R_f 0.81 (system 2), 0.90 (system 3), 0.84 (system 4). The product was identical in its R_f values with a marker of VII_c prepared by M. Wilchek by a different procedure.

DISCUSSION

The use of the Dnp group for protection of phenolic hydroxyls during synthesis of peptides and poly-amino acids containing tyrosine combines the following useful features.

(a) The protected starting materials for such syntheses can be easily prepared as crystalline well-defined compounds.

(b) The protected tyrosine peptides are stable enough to withstand exposure to reagents used during peptide synthesis, such as 100% CF_3COOH, anhydrous HF, and triethylamine, and retain their protecting group during the manipulations used for their purification, such as washing under acidic and basic conditions for the removal of excess starting materials. However, prolonged exposure to free amino groups should be avoided, to prevent unmasking by aminolysis.

(c) The thiolytic unmasking of protected tyrosine residues is a quantitative process, readily completed under very mild reaction conditions which are not apt to cause removal of other protecting groups used in peptide synthesis (e.g., benzyloxycarbonyl, *tert.*-butyloxycarbonyl, or *p*-toluenesulfonyl groups on amines; methyl, ethyl, or benzyl ester groups on carboxyls; *O*-benzyl groups on serines; *S*-benzyl groups on cysteines; etc.) Therefore, the Dnp group may be useful for differential protection of functional groups during synthesis of peptides and copolymers of amino acids.

(d) The *O*-Dnp group of protected tyrosine residues can be removed by thiols under nonaqueous as well as aqueous conditions. Therefore, it can be used not only in classical peptide synthesis but also for solid-phase synthesis (34–38) and for aqueous synthesis of peptides using *N*-carboxyanhydrides of amino acids (39). In this connection, it should be mentioned that we have recently prepared the *N*-carboxyanhydride of *O*-Dnp-L-tyrosine.

(e) Simple and rapid analytical methods are available for following the removal of the masking group. Thiolytic cleavage of *O*-Dnp-tyrosines can be continuously monitored by difference spectroscopy or measured by acid hydrolysis of the peptide and quantitative determination of *O*-Dnp-tyrosine using paper chromatography and electrophoresis (40).

The spectrophotometric method described here for monitoring the thiolytic cleavage of *O*-Dnp-tyrosines is useful not only for optimizing the synthesis of tyrosine peptides and poly-amino acids, but also for structure–function studies of proteins. It is well known that dinitrophenylation of proteins results in the labeling of N-terminal α-amino groups as well as lysine, cysteine, histidine, and tyrosine side chains. We have previously shown that, at neutral pH and room temperature, Dnp derivatives of α- and ϵ-groups are not displaced by thiols but the Dnp derivatives of cysteine, histidine, and tyrosine side chains are thiolyzed, yielding of *S*-Dnp derivative of the thiol used (16). While thiolysis of the *S*-Dnp derivative of cysteine does not result in a spectral change (when 2-mercaptoethanol is used as a thiolyzing agent), the thiolytic cleavage of N^{Im}-Dnp-histidine (40) and *O*-Dnp-tyrosine results in a difference spectrum with a maximum at 350–355 nm. In the present paper, we have shown that the difference spectrum obtained upon thiolysis of *O*-Dnp-tyrosine has a negative $\Delta\epsilon_M$ at 298 nm (Fig. 3, top) which does not occur with N^{Im}-Dnp-histidine (Fig. 6). This observation forms the basis for determining spectrophotometrically which Dnp derivative is being thiolyzed.

Dinitrophenylation and subsequent thiolysis are used for differential labeling of "exposed" and "buried" functional groups in proteins (17, 41), for the reversible labeling, and for the mapping of enzyme and antibody active sites (17, 40, 42–44) and even for establishing spatial relationships between protomers in multi-subunit enzymes (17, 45). In such studies, it is extremely important to establish optimal reaction conditions for carrying out the labeling and unmasking steps with high specificity and a minimum of undesirable side reactions. Therefore, the availability of a continuous and rapid method for monitoring the thiolytic cleavage and identifying the functional groups involved is most valuable.

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REFERENCES

1. SCHRÖDER, E., AND LÜBKE, K. (1965) The Peptides, pp. 220–226, Academic Press, New York/London.
2. RIDGE, B., AND JONES, J. H. (1975) *in* Amino Acids, Peptides and Proteins, Vol. 5, pp. 284–

286, The Chemical Society, London.
3. WÜNSCH, E., FRIES, G., AND ZWICK, A. (1958) *Chem. Ber.* **91**, 542–547.
4. CHILLEMI, D., SCARSO, L., AND SCOFFONE, E. (1957) *Gazz. Chim. Ital.* **87**, 1356–1366.
5. ECKSTEIN, H., SIEVERS, R. E., AND BAYER, E. (1973) *Annalen* 1467–1470.
6. ERICKSON, B. W., AND MERRIFIELD, R. B. (1972) *in* Chemistry and Biology of Peptides (Meienhofer, J., ed.), pp. 191–195, Ann Arbor Science Publishers, Ann Arbor, Mich.
7. YAMASHIRO, D., NOBLE, R. L., AND LI, C. H. (1972) *in* Chemistry and Biology of Peptides (Meienhofer, J., ed.), pp. 197–202, Ann Arbor Science Publishers, Ann Arbor, Mich.
8. ERICKSON, B. W., AND MERRIFIELD, R. B. (1973) *J. Amer. Chem. Soc.* **95**, 3750–3756.
9. YAMASHIRO, D., AND LI, C. H. (1973) *J. Org. Chem.* **38**, 591–592.
10. YAMASHIRO, D., AND LI, C. H. (1973) *J. Amer. Chem. Soc.* **95**, 1310–1315.
11. BEYERMAN, H. C., AND BONTEKOE, J. S. (1961) *Proc. Chem. Soc. (London)* 249.
12. CALLAHAN, F. M., ANDERSON, G. W., PAUL, R., AND ZIMMERMAN, J. E. (1963) *J. Amer. Chem. Soc.* **85**, 201–207.
13. JÄGER, G., GEIGER, R., AND SIEDEL, W. (1968) *Chem. Ber.* **101**, 2762–2770.
14. GEIGER, R., JÄGER, G., VOLK, A., AND SIEDEL, W. (1968) *Chem. Ber.* **101**, 2189–2196.
15. YOUNG, G. T., AND KOTAI, A. (1973) *in* Peptides–1972 (Hanson, H., and Jakubke, H. D., eds.), p. 128, North-Holland, Amsterdam/London.
16. SHALTIEL, S. (1967) *Biochem. Biophys. Res. Commun.* **29**, 178–183.
17. SHALTIEL, S. (1974) *Israel J. Chem.* **12**, 403–419.
18. SHALTIEL, S., AND FRIDKIN, M. (1970) *Biochemistry* **9**, 5122–5127.
19. FRIDKIN, M., AND SHALTIEL, S. (1971) *Arch. Biochem. Biophys.* **147**, 767–771.
20. SHALTIEL, S., MOZES, E., AND SELA, M. (1972) *Israel J. Chem.* **10**, 627–633.
21. CHILLEMI, F., AND MERRIFIELD, R. B. (1969) *Biochemistry* **8**, 4345–4346.
22. STEWART, J. M., KNIGHT, M., PAIVA, A. C. M., AND PAIVA, T. (1972) *in* Peptides (Lande, S., ed.), pp. 59–64, Gordon and Breach, New York.
23. POLZHOFER, K. P., AND NEY, K. H. (1971) *Tetrahedron* **27**, 1997–2001.
24. LIN, M. C., GUTTE, B., CALDI, D. G., MOORE, S., AND MERRIFIELD, R. B. (1972) *J. Biol. Chem.* **247**, 4768–4774.
25. MARSHALL, G. R., VINE, W., AND NEEDLEMAN, P. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1624–1630.
26. WÜNSCH, E., AND JENTSCH, J. (1964) *Chem. Ber.* **97**, 2490–2496.
27. KATCHALSKI, E., AND SELA, M. (1953) *J. Amer. Chem. Soc.* **75**, 5284–5289.
28. ANDERSON, G. W., ZIMMERMAN, J. E., AND CALLAHAN, F. M. (1964) *J. Amer. Chem. Soc.* **86**, 1839–1842.
29. FRITZ, J. S., AND LISKICKI, N. M. (1951) *Anal. Chem.* **23**, 589–591.
30. PATCHORNIK, A., EHRLICH-ROGOZINSKI, S., AND FRIDKIN, M. (1975) *in* Peptides–1974 (Wolman, Y., ed.), pp. 257–269, Wiley, New York.
31. ANDERSON, G. W., ZIMMERMAN, J. E., AND CALLAHAN, F. M. (1963) *J. Amer. Chem. Soc.* **85**, 3039.
32. BERGMANN, M., ZERVAS, L., SOLZMANN, L., AND SCHLEICH, H. (1934) *Z. Physiol. Chem.* **224**, 17–33.
33. FISCHER, R. F., AND WHETSTONE, R. R. (1954) *J. Amer. Chem. Soc.* **76**, 5076–5080.
34. MERRIFIELD, R. B. (1963) *J. Amer. Chem. Soc.* **85**, 2149–2154.
35. GUTTE, B., AND MERRIFIELD, R. B. (1969) *J. Amer. Chem. Soc.* **91**, 501–502.
36. FRIDKIN, M., PATCHORNIK, A., AND KATCHALSKI, E. (1966) *J. Amer. Chem. Soc.* **88**, 3164–3165.
37. FRIDKIN, M., PATCHORNIK, A., AND KATCHALSKI, E. (1968) *J. Amer. Chem. Soc.* **90**, 2953–2957.
38. KALIR, R., WARSHAWSKY, A., FRIDKIN, M., AND PATCHORNIK, A. (1975) *Eur. J. Biochem.* **59**, 55–61.
39. DENKELWALTER, R. G., SCHWAM, H., STRACHAM, R. G., BEESLEY, T. E., VEBER, D. F., SCHOENEWALDT, E. F., BARKEMEYER, H., PALEVEDA, W. J., JACOB, T. A., AND HIRSCHMANN, R. (1966) *J. Amer. Chem. Soc.* **88**, 3163–3164.
40. SHALTIEL, S., AND SORIA, M. (1969) *Biochemistry* **8**, 4411–4415.
41. STALLCUP, W. B., AND KOSHLAND, D. E. (1973) *J. Mol. Biol.* **80**, 41–62.
42. GIVOL, D., AND SHALTIEL, S. (1968) *Israel J. Chem.* **6**, 108.
43. SHALTIEL, S., AND ZAIDENZAIG, Y. (1970) *Biochem. Biophys. Res. Commun.* **39**, 1003–1009.
44. SHALTIEL, S., CORTIJO, M., AND ZAIDENZAIG, Y. (1972) *in* Metabolic Interconversion of Enzymes (Wieland, O., Helmreich, E., and Holzer, H., eds.), pp. 73–101, Springer Verlag, Berlin.
45. REISLER, E., TAUBER-FINKELSTEIN, M., AND SHALTIEL, S. (1975) *FEBS Lett.* **54**, 315–318.