BIOORGANIC CHEMISTRY 11, 262–273 (1982)

The Chloro-*o,o'*-Dinitrophenyl Group: Properties and Use in Protein Chemistry[1]

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Received August 31, 1981

The properties of the chloro-*o,o'*-dinitrophenyl group, more suitable as a building block for the design of chromogenic bifunctional protein reagents than the corresponding known *o,p* derivatives, are investigated by model reactions with small molecules and with proteins. This study shows that (1) the chlorine substituent can be easily substituted by protein nucleophiles, (2) arylation of SH and NH_2 groups leads to chromophores in which the differences between the λ_{max} (330 and 430 nm, respectively) are larger than in the case of *o,p*-dinitro compounds, (3) afler substitution by an SH group, transarylation to a vicinal NH_2, whenever possible, occurs faster than in the case of *o,p* derivatives (4) substitution by imidazole or phenol can be reversed by a thiol yielding an alkylthioaryl derivative; however, when mercaptoethanol is used in this reaction, *o,o'*-dinitrophenol is obtained, probably resulting from a *S* to *O* transfer in the initially formed hydroxyethylthiophenol, followed by hydrolysis of the labile alkoxyphenyl derivative.

INTRODUCTION

Aryl halides having two nitro groups in the ortho and para positions such as fluoro- or chloro-2,4-dinitrobenzene can react with different nucleophiles including thiols, imidazole, amines, and phenols. The products formed by displacement of halogen by sulfur or nitrogen are chromophores exhibiting characteristic uv maxima (330 nm, ϵ 10,500 for dinitrophenylated thiols and 360 nm, ϵ 14,500 for amino derivatives) (*1*). *o,p*-Dinitrophenylated amino acids can be identified by suitable analyses (*2*). *o,p*-Dinitrophenyl halides have therefore been widely used in protein chemistry to identify *N*-terminal residues, to study the structure–function relationship in enzymes, etc. (*3*).

While these compounds proved useful as monofunctional protein reagents, their structure hardly leads to the design of bifunctional reagents containing such a chromogenic moiety. Actually, 1,5-difluoro-2,4-dinitrobenzene (and, to some degree, 4,4'-difluoro-3,3'-dinitrodiphenylsulfone) are so far the only examples of parent homobifunctional reagents exhibiting properties similar to those of fluoro-2,4-dinitrobenzene (*4*).

Aryl halides having both nitro groups in the ortho position, however, are easily

[1] Paper IX of the series on New Protein Reagents. Preceeding paper: J. Diopoh and M. Olomucki, *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1257 (1979).

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0045-2068/82/030262-12$02.00/0
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available compounds in which, despite the enhanced steric hindrance, the halogen atom seems to be sufficiently reactive. Some of the known *o,o'*-dinitrophenyl halides have in the para position an additional functional group which offers the opportunity of binding these molecules to another reactive fragment and thus to design a variety of heterobifunctional reagents belonging to the chromogenic dinitrophenyl type.

In the preceding papers are described the synthesis and some examples of the application in protein chemistry of several such reagents, including 4-chloro-3,5-dinitrophenacyl bromide (*5*), *N*-(4-chloromercuriphenyl)-4-chloro-3,5-dinitrobenzamide (*6*), and *N*-(4-chloro-3,5-dinitrobenzoyloxymethyl)maleimide (*7*). We felt, however, that a detailed study of the properties of the halogeno-*o,o'*-dinitrophenyl group as such was necessary. The present paper describes these investigations which were performed on different chloro-*o,o'*-dinitrophenyl derivatives using small model molecules as well as several proteins.

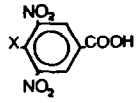
EXPERIMENTAL

4-Chloro-3,5-dinitrobenzoic acid was either commercial (Aldrich) or prepared according to Ref. (*5*). 4-Chloro-3,5-dinitrobenzonitrile, chloro-2,6-dinitrobenzene, and fluoro-2,4-dinitrobenzene were obtained from Aldrich. Ethyl 4-chloro-3,5-dinitrobenzoate and the corresponding amide were prepared according to Refs. (*8*) and (*9*), respectively. Rabbit muscle aldolase was obtained from Worthington Biochemical Corporation and five times crystallized bovine pancreatic ribonuclease from Nutritional Biochemicals Company. Rabbit muscle creatine phosphokinase was a gift from Dr. L.-A. Pradel and Dr. R. Kassab. Thin-layer chromatography was performed on Merck silica gel 60 F_{254} plastic sheet in benzene–methanol–acetic acid 24:4:2 or *n*-butanol–acetic acid–water 15:9:6 v/v. Spectral determinations were performed on the following instruments: ir (KBr disks), Perkin–Elmer 720; uv, Zeiss PMQ II; ^{1}H-nmr, Varian HA 100 in DMSO-d_6,[3] reported in δ values relative to HMDS, unless otherwise stated; mass, AEI MS-30.

4-Imidazol-1-yl-3,5-dinitrobenzoic acid, **1b**

A solution of 2 g (8.1 mmol) of 4-chloro-3,5-dinitrobenzoic acid and 0.56 g (8.1 mmol) of imidazole in 30 ml of tetrahydrofuran was refluxed for 2 hr. The precipitate was filtered, washed with tetrahydrofuran and 0.2 *N* HCl, dried over KOH and P_2O_5, and recrystallized from methanol–chloroform. Yield: 1.4 g (62%); mp 247–248°C; ir 1722 (CO), 1630 (arom.), 1557, 1340 cm^{-1} (NO_2); ^{1}H-nmr 7.09, 7.41, 7.94 (1H and $w_{1/2}$ = 4 Hz each, imidazole), 8.80 (s, 2H, arom.), 10.9 ppm (large signal, COOH).

[3] Abbreviations used: DMSO, dimethyl sulfoxide; DNP, dinitrophenyl group; FDNB, fluoro-2,4-dinitrobenzene; PCMB, *p*-chloromercuribenzoate; CPK, creatine phosphokinase; HMDS, hexamethyldisiloxane; TMS, tetramethylsilane; TLC, thin-layer chromatography.



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X=a,Cl
b,imidazol-1-yl
c,C_6H_5O
d,n-C_3H_7O
e,n-$C_5H_{11}S$
f,n-$C_5H_{11}NH$
g,$HS(CH_2)_2NH$
h,HO

Anal. Calcd for $C_{10}H_6N_4O_6$: C, 43.17; H, 2.17; N, 20.14; O, 34.51. Found: C, 42.9; H, 2.4; N, 20.0; O, 34.3.

4-Phenoxy-3,5-dinitrobenzoic acid, **1c**

A mixture of 5 g (20 mmol) of 4-chloro-3,5-dinitrobenzoic acid, 5.6 g (60 mmol) of phenol, and 11.4 g (40 mmol) of $Na_2CO_3 \cdot 10H_2O$ in 100 ml of tetrahydrofuran was stirred under reflux for 24 hr, filtered, and evaporated. The residual solid was dissolved in water, acidified to pH 3–4 with 2 *N* hydrochloric acid, the precipitate collected, washed with water, dried, and recrystallized from acetone–petroleum ether, yielding 3 g (50%) of product, mp 225°C (reported mp 219–222°C (*10*)); ir 1730 (CO), 1630 (arom.), 1540, 1360 (NO_2), 1220 cm^{-1} (aryl ether); ^1H-nmr 6.94, 7.09, 7.29 (complex m, 5H, phenoxy), 8.75 ppm (s, 2H *o* to NO_2).

Anal. Calcd for $C_{13}H_8N_2O_7$: C, 51, 32; H, 2.65; N, 9.21. Found: C, 51.4; H, 2.6; N, 8.9.

4-Propoxy-3,5-dinitrobenzoic acid, **1d**

Sodium (0.35 g) (15 mmol) was added to 50 ml of *n*-propanol. When the evolution of gas subsided, 2 g (8.1 mmol) of 4-chloro-3,5-dinitrobenzoic acid were introduced and the solution was stirred for 4 days at room temperature. The red viscous reaction mixture was diluted with 1 vol of acetone and filtered, the filtrate evaporated, the residual solid dissolved in acetone, and the solution acidified to pH 3–4 with 0.2 *N* hydrochloric acid. A small amount of solid was filtered off, the filtrate concentrated, diluted with water until turbidity, and chilled. The crystals were filtered, washed with water, and recrystallized twice from acetone–water. Yield: 1.5 g (71%); mp 182°C; ir 1715 (CO), 1625 (arom.), 1540, 1360 (NO_2), 1243, 1103 cm^{-1} (ether); ^1H-nmr 0.88 (t, 3H, CH_3), 1.68 (sext., 2H, CH_2–CH_3), 4.05 (t, 2H, CH_2–O), 8.58 ppm (s, 2H, arom.).

Anal. Calcd for $C_{10}H_{10}N_2O_7$: C, 44.45; H, 3.73; N, 10.37; O, 41, 45. Found: C, 44.5; H, 3.8; N, 10.3; O, 41.6.

4-n-Pentylthio-3,5-dinitrobenzoic acid, **1e**

A solution of 1.3 ml (10 mmol) of *n*-pentanethiol in 20 ml of acetone was added to a solution of 2 g (8.1 mmol) of 4-chloro-3,5-dinitrobenzoic acid in 20 ml of water. The apparent pH was adjusted to 9 with 10 *N* sodium hydroxide and the

mixture was stirred for 2 hr at 40°C under nitrogen, the pH being maintained at 9 by additions of sodium hydroxide. Acetone was evaporated and the remaining solution acidified to pH 3–4 with 0.2 *N* HCl. The precipitate formed was filtered, washed with water, and recrystallized by dissolving in methanol, adding water until turbid and chilling. Yield: 2 g (79%); mp 110°C; uv λ_{max} 330 (ϵ 2050); ir 1720 (CO), 1620 (arom.), 1543, 1360 cm^{-1} (NO_2); ^{1}H-nmr 0.77 (complex, 3H, CH_3), 1–1.5 (broad signal, $3CH_2$), 2.83 (t, 2H, CH_2–S), 8.54 ppm (s, 2H, arom.).

Anal. Calcd for $C_{12}H_{14}N_2O_6S$: C, 45.85; H, 4.49; N, 8.91; S, 10.20. Found: C, 46.0; H, 4.5; N, 8.7; S, 10.3.

This product was also obtained under the same conditions and in the same yields starting either from 4-imidazol-1-yl-3,5-dinitrobenzoic acid (**1b**) or from 4-phenoxy-3,5-dinitrobenzoic acid (**1c**).

4-n-Amylamino-3,5-dinitrobenzoic acid, **1f**

A solution of 2 g (23 mmol) of *n*-amylamine in 10 ml of acetone was added to a solution of 2.5 g (10 mmol) of 4-chloro-3,5-dinitrobenzoic acid in 10 ml of water. The mixture was heated for 7 hr at 40–50°C, concentrated, and acidified to pH 3–4 with 2 *N* HCl. The yellow precipitate was filtered, washed with water, and recrystallized from acetone–water yielding 2.4 g (81%) of **1f**; mp 189°C; uv λ_{max} 430 (ϵ 7100); ir 3355, 1280 (amine), 1700 (CO), 1630 (arom.), 1545, 1362 cm^{-1} (NO_2); ^{1}H-nmr 0.80 (t, 3H, CH_3), 1.21 (m, 4H, CH_3–(CH_2)$_2$), 1.57 (m, 2H, CH_2), 2.90 (quadr., 2H, CH_2–N), 8.47 (t, 1H, NH), 8.52 ppm (s, 2H, arom.).

Anal. Calcd for $C_{12}H_{15}N_3O_6$: C, 48.48; H, 5.09; N, 14.14; O, 32.29. Found: C, 48.4; H, 5.2; N, 14.1; O, 32.3.

4-(2-Mercaptoethyl)amino-3,5-dinitrobenzoic acid, **1g**

A solution of 2 g (8.1 mmol) of 4-chloro-3,5-dinitrobenzoic acid in 6 ml of acetone was mixed with a solution of 0.92 g (8.1 mmol) of cysteamine hydrochloride in 10 ml of water and the pH was adjusted to 9 with 10 *N* sodium hydroxide. The orange mixture was stirred at room temperature under nitrogen, the pH being maintained at 9 by periodical additions of sodium hydroxide; a solid soon began to precipitate. After 6 hr the mixture was acidified with 2 *N* hydrochloric acid, the solid filtered, washed with 1 *N* hydrochloric acid and with water. After three recrystallizations in acetone–water, 1.5 g (65%) of product were obtained in the disulfide form (Toennies and Kolb (*11*) test), mp 253–255°C; ir 3310 (NH), 1700 (CO), 1630 (arom.), 1540, 1360 cm^{-1} (NO_2); ^{1}H-nmr (TMS) 2.97, 3.22 (m, CH_2), 8.59 ppm (s, arom.).

Anal. Calcd for $C_{18}H_{16}N_6O_{12}S_2$: C, 37.76; H, 2.82; S, 11.20. Found: C, 37.9; H, 3.0; S, 11.0.

4-Hydroxy-3,5-dinitrobenzoic acid, 1h

(a) *Alkaline hydrolysis of 4-chloro-3,5-dinitrobenzoic acid.* The pH of a solution of 2 g (8.1 mmol) of 4-chloro-3,5-dinitrobenzoic acid in 20 ml of water was maintained at 9 by periodical additions of 10 *N* NaOH during 12 hr at room temperature. The solution was then acidified, the precipitated solid filtered, washed with

water, and recrystallized from acetone–water; 1.5 g (81%) of product was obtained, mp 251°C (reported, mp 248°C (*12*)); uv λ_{max} 430 nm (ϵ 7100).

Anal. Calcd for $C_7H_4N_2O_7$: C, 36.85; H, 1.77; O, 49.10. Found: C, 36.6; H, 1.8; O, 49.1.

(b) *Reaction of 2-mercaptoethanol with 4-chloro-3,5-dinitrobenzoic acid.* A solution of 0.85 ml (12 mmol) of mercaptoethanol in 8 ml of acetone was added to 2.5 g (10 mmol) of 4-chloro-3,5-dinitrobenzoic acid in 12 ml of water, the pH adjusted to 9 and kept constant by periodical additions of 10 *N* sodium hydroxide at room temperature while the mixture was stirred under nitrogen. When the chloroacid disappeared as shown by thin-layer chromatography (about 5 hr), 0.2 *N* hydrochloric acid was added until pH 3–4 and the solid material was filtered and washed with water, then dissolved in a hot acetone–water mixture, chilled, filtered, and washed with water, yielding 1.79 g (78%) of product, mp 252°C, undepressed by mixture with the product described under paragraph (a); ir 1712 (CO), 1625 (arom.), 1560, 1375 cm^{-1} (NO_2); ^{1}H-nmr (TMS) 5.38 (OH), 8.48 ppm (arom.), no CH_2 signals; mass spectrum (70 eV) *m/e* (rel. intensity) 228 (34), 170 (16), 91 (10), 53 (26), 44 (77), 30 (100).

Anal. Calcd for $C_7H_4N_2O_7$: C, 36.85; H, 1.77; N, 12.28. Found: C, 36.7; H, 1.7; N, 12.1; no S was found in this product.

RESULTS

Model Reactions

The properties of the halogeno *o,o'*-dinitrophenyl group were investigated using 4-chloro-3,5-dinitrobenzoic acid (**1a**) and some parent compounds. Model chlorine-substitution compounds were synthesized using acid **1a** since its acidic character facilitated the isolation of reaction products.

The chlorine in 4-chloro-3,5-dinitrobenzoic acid in a 0.4 *M* aqueous solution, pH 9, is hydrolyzed in 12 hr at room temperature, and the resulting chromophoric (430 nm, ϵ 7100) phenol **1h** can be isolated in a good yield. The chlorine atom can also be substituted by nucleophiles analogous to those present in proteins. Thus, compound **1a** reacts with imidazole, phenol, or an alcohol giving the corresponding derivatives **1b**, **1c**, **1d**, respectively. These products have no characteristic absorptions in the near-uv–visible region.

In contrast, the easy reaction of 4-chloro-3,5-dinitrobenzoic acid with thiols and amines yields chromophoric compounds. Thus, treatment of product **1a** with *n*-pentylmercaptan or *n*-pentylamine leads, respectively, to the alkylthio-derivative **1e** having an absorption peak at 330 nm (ϵ 2050) and to the aniline **1f** having the same uv spectral parameters as the phenol **1h**. The same λ_{max} were found in a series of parent *S*- or *N-o,o'*-dinitroaryl derivatives. When 1 m*M* 4-chloro-3,5-dinitrobenzoic acid, or the corresponding nitrile, amide, ethyl ester, or chloro-2,6-dinitrobenzene, were incubated for 2 hr with 3 m*M* *N*-acetylcysteine in 0.1 *M* bicarbonate, pH 8.8, all the mixtures exhibited a new absorption peak at about 330 nm.

Under the same conditions, when 3 m*M* lysine was used instead of *N*-acetylcysteine, the solutions showed λ_{max} values of 420 to 430 nm.

Reactions of thiols and amines with the chloro-*o,o'*-dinitrophenyl group were further tested using 4-chloro-3,5-dinitrobenzamide. The experiments described in Table 1 were performed under conditions sufficient for substitution of chlorine by thiols (*N*-acetylcysteine or the SH group of glutathione), while amines (glycine, lysine, or the amino group of glutathione) do not react. Amino groups of aromatic amino acids, however, show some reactivity even under these conditions, as exemplified by the test with tryptophan. The unusual ease of substitution of amino groups in aromatic amino acids or of nucleophilic groups vicinal to aromatic residues in proteins by different halogeno-*o,o'*-dinitrophenyl derivatives (4-chloro-3,5-dinitrophenacyl bromide (*5*), ethyl 4-chloro-3,5-dinitrobenzimidate and benzoate (*13*), 4-chloro-3,5-dinitrobenzoic acid (*14*)) has been previously observed. This behavior was explained by preliminary formation of charge-transfer complexes between the reagent and the aromatic ring in the vicinity of the target nucleophile, thus facilitating the substitution of the latter (*5*).

Despite the higher reactivity of thiols, in the case of aminothiols having the two functional groups closer than in glutathione, such as in compounds of the aminoethanethiol structure (cysteamine, cysteine), only amino substitution derivatives are formed (Table 1). Also, the model reaction of 4-chloro-3,5-dinitrobenzoic acid with aminoethanethiol yields the aniline **1g** (isolated in the disulfide form). Similar results have been found for the reaction of halogeno-*o,p*-dinitrobenzene with cysteine, in which a primary attack on the thiol group is followed by a migration of the aryl substituent to the neighboring amine (*15*). This *S–N* shift seems to occur faster in the case of *o,o'*-dinitroaryl derivatives than in the *o,p*-dinitrobenzene

TABLE 1

REACTIONS OF 4-CHLORO-3,5-DINITROBENZAMIDE WITH SOME AMINO ACIDS AND PARENT COMPOUNDS

Compound tested[a]	Derivatives formed (mol%)[b]	
	S-aryl	*N*-aryl
Glycine	—	0
Lysine	—	0
Glutathione	94	0
N-Acetylcysteine	78	—
Tryptophan	—	20
Cysteamine	0	71
Cysteine	0	82

[a] 1 m*M* incubated 30 min at pH 9, room temperature, with 0.1 m*M* 4-chloro-3,5-dinitrobenzamide.

[b] Determined photometrically (*S*-aryl 330 nm, *N*-aryl 430 nm).

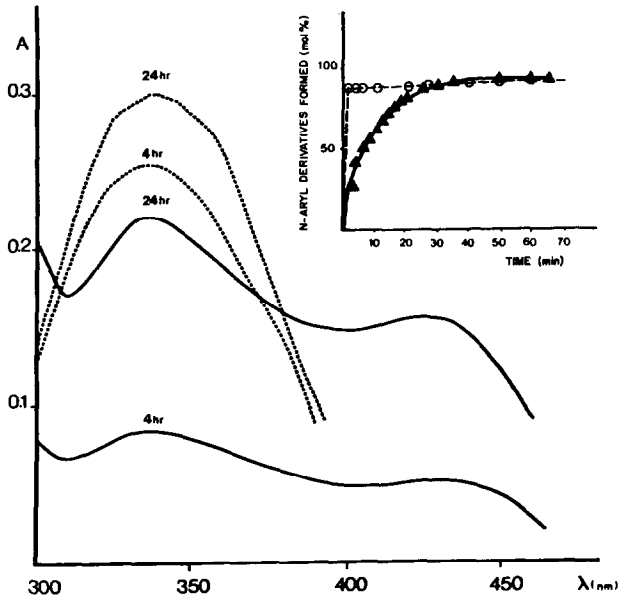

FIG. 1. Ultraviolet spectra of mixtures of cysteine and 4-chloro-3,5-dinitrobenzoic acid or fluoro-2,4-dinitrobenzene. Solutions 3×10^{-4} *M* of dinitrophenyl halide and 3×10^{-3} *M* of cysteine in 0.1 *M* acetate buffer, pH 5.2, containing 10% of ethanol, were incubated at 25°C under nitrogen. Spectra were recorded against blanks of the corresponding dinitroaryl halide at the indicated times in samples diluted 10 times with the buffer. —, 4-chloro-3,5-dinitrobenzoic acid; ···, fluoro-2,4-dinitrobenzene. *Inset:* Rate of *S–N* transfer of *o,o'*- and *o,p*-dinitroaryl derivatives. Each of the above solutions after 24 hr of incubation at pH 5.2 was diluted 10 times in 0.1 *M* bicarbonate, pH 8.4, and the absorbance was followed at 430 nm for the *o,o'*-dinitrophenyl compound (○) and at 360 nm for the *o,p*-dinitrophenyl derivative (▲).

system. Figure 1 shows that in acidic (pH 5.2) medium only the SH group of cysteine reacts with fluoro-2,4-dinitrobenzene, and no *S–N* transfer (no absorption at 360 nm) is observed. In contrast, under the same conditions such a transfer, although slow, takes place in the case of product **1a**, since in addition to the absorption peak of the *S*-derivative at 330 nm a significant absorbance at 430 nm due to the *N*-derivative appears in the reaction mixture. In both cases raising the pH to 8.4 promotes the *S–N* rearrangement which, however, requires 0.5 hr in the case of the *o,p*-DNP derivative while it is quasi-instantaneous in the case of the *o,o'*-dinitroaryl compound (Fig. 1, inset).

The *o,o'*-dinitrophenylation of imidazole or phenol, but not of an amine, can be reversed by a thiol. Thus, compound **1e** can be obtained under the same experimental conditions by the reaction of pentanethiol not only with the chloride **1a** but also with the imidazolyl or phenoxy derivative **1b** or **1c** (see under Experimental and Fig. 2, curve C').

A different result, however, is obtained when mercaptoethanol is used as the thiol. When compound **1a**, **1b**, or **1c** is treated with mercaptoethanol at pH 9, no absorption at 330 nm due to *S*-aryl derivatives can be observed in the reaction mixture (Fig. 2), although in the case of products **1b** and **1c**, TLC shows that

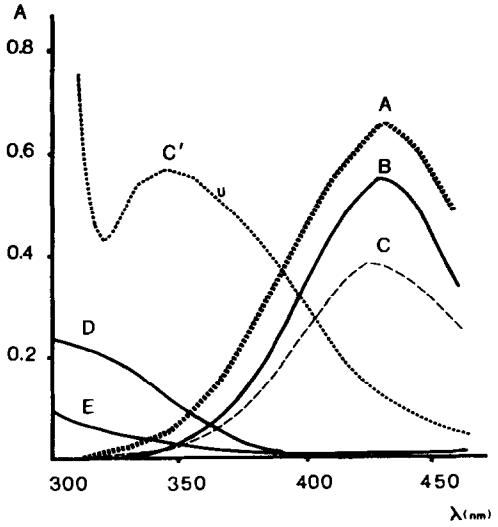

FIG. 2. Reactions of thiols with X-*o,o'*-DNP derivatives. Solutions 10^{-2} *M* of compound **1a**, **1b**, or **1c** and 2×10^{-2} *M* of mercaptoethanol in 0.2 *M* bicarbonate, pH 9, were incubated at room temperature for 20 min (**1a**) or 2 hr (**1b** and **1c**) and the uv spectra were recorded on aliquots diluted 100 times: A, **1a**; B, **1c**; C', **1b** treated under the same conditions with 2×10^{-2} *M* pentanethiol instead of mercaptoethanol; D, untreated 10^{-4} *M* **1c** in acetonitrile; E, untreated 10^{-4} *M* **1b** in acetonitrile.

imidazole or phenol are formed, respectively. Instead, a peak at 430 nm appears rapidly, and a yellow product forms which has the same TLC characteristics as 4-hydroxy-3,5-dinitrobenzoic acid **1h.** The latter was isolated in high yield and carefully identified when the chloro derivative **1a** was treated with mercaptoethanol on a preparative scale.

Compound **1h** formed in the experiments described in Fig. 2 does not result from hydrolysis of the starting product **1a**, **1b**, or **1c**, since the latter are stable under the conditions used. On the other hand, a comparison of the stability of the ether **1d** and the thioether **1e** showed that, as could be expected (*16*), product **1d** at room temperature hydrolyzes rather easily at pH 9, as shown by TLC and uv analysis of the reaction mixture, while the thioether **1e** remains unchanged even after 5 days under the same conditions. Therefore, formation of compound **1h** by hydrolysis of the intermediate hydroxyethylthioaryl derivative does not seem probable either. Instead, in view of the ease of *S–N* transfer in the *o,o'*-dinitrophenyl series it might be assumed that a similar *S–O* shift occurs here, which in this case is followed by an additional hydrolysis step since the *O*-aryl derivative thus formed is less stable than the *N*-aryl compound resulting from the *S–N* rearrangement. (See Fig. 3.)

Reactions with Proteins

The same characteristics of the reactivity of Cl-*o,o'*-DNP derivatives are observed in the reactions of these compounds with proteins. Thus, native bovine pancreatic ribonuclease which has no SH groups does not react with ethyl 4-

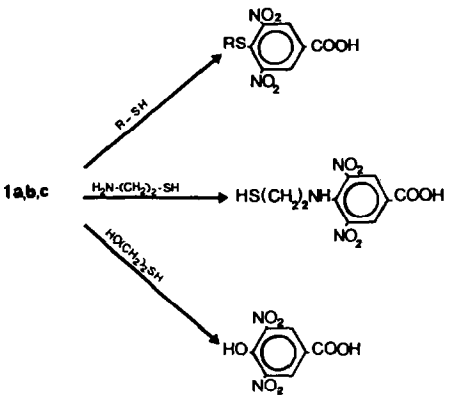

FIG. 3. Reactions of 4-chloro-3,5-dinitrobenzoic acid or some of its chlorine-substitution derivatives with three types of thiols.

chloro-3,5-dinitrobenzoate under the experimental conditions described in Table 2, but in the reduced protein not only the SH but also the NH_2 groups are arylated. Obviously *N*-substitution becomes possible when thiol groups are liberated and is therefore probably due to a *S–N* transfer. Under the same conditions fluoro-2,4-dinitrobenzene, which is likewise inactive with native ribonuclease, arylates only SH groups in the reduced enzyme. A similar effect is observed in the case of aldolase. This result again shows that the *S–N* transfer is faster in the *o,o'*-DNP than in the *o,p*-DNP series.

The role played by vicinal thiol groups in *N*-arylation by ethyl 4-chloro-3,5-dinitrobenzoate also appears clearly when the action of the reagent on proteins having SH groups is compared with its behavior toward the same proteins in which the thiols are blocked by iodoacetate or tetrathionate or when the distance between SH and NH_2 groups is modified by denaturation: in both cases substitution of amino groups is decreased or completely stopped.

Blocking of thiols by *p*-chloromercuribenzoate, however, does not have the same effect. In this case *N*-arylation is not only possible but can even be easier. The favorable influence of vicinal SH groups is probably replaced here by a possibility for ethyl 4-chloro-3,5-dinitrobenzoate to form a charge-transfer complex with the aromatic benzoate rings introduced in the proximity of the NH_2 groups. In mixtures of ethyl 4-chloro-3,5-dinitrobenzoate or some other aromatic compounds with cysteine and PCMB new absorption peaks appear, at 455 nm for aqueous mixtures containing ethyl 4-chloro-3,5-dinitrobenzoate (465 nm in 66% aqueous acetonitrile), at 400 nm for FDNB in water, and at 320 and 400 nm for aqueous solutions containing 2,4-dinitrophenol (365 nm in aqueous acetonitrile). Such shifts, depending on substituents in the ring and on the polarity of the solvent, are characteristic of bands arising from formation of π-complexes (*17*).

DISCUSSION

The investigation of the chloro-*o,o'*-dinitrophenyl group was undertaken in or-

TABLE 2

REACTIONS OF ETHYL 4-CHLORO-3,5-DINITROBENZOATE AND FDNB WITH PROTEINS

Experiment[a]	Time of incubation (min)	Number of *N*-DNP derivatives per mole of enzyme	Number of *S*-DNP derivatives per mole of enzyme
Native ribonuclease + CNB[b]	30	0	0
Reduced ribonuclease[c] + CNB	30	1.4	4
Native ribonuclease + FDNB	30	0	0
Reduced ribonuclease[c] + FDNB	90	0	4.9
Native aldolase + CNB	30	1.3	4
Native aldolase + FDNB	30	0	3.9
Iodoacetate-treated aldolase[d] + CNB	30	0.6	2.9
PCMB-treated aldolase[e] + CNB	45	2.2	0
CPK + CNB	30	1.1	2.6
CPK + CNB	45	1.6	2.9
7 *M* guanidine-treated CPK + CNB	30	0	2.3
Tetrathionate-treated CPK[f] + CNB	30	0	0
Tetrathionate-treated CPK[f] + CNB	150	0.5	0
PCMB-treated CPK[e] + CNB	30	0.95	0
PCMB-treated CPK[e] + CNB	50	1.5	0

[a] 10^{-4} *M* reagent and 10^{-5} *M* protein in 0.1 *M* bicarbonate, pH 8.8, room temperature.
[b] CNB, ethyl 4-chloro-3,5-dinitrobenzoate.
[c] Denatured with 8 *M* urea and reduced with $NaBH_4$ according to Cavallini *et al.* (*18*).
[d] Incubated for 45 min with 2×10^{-5} *M* iodoacetate.
[e] Incubated for 30 min with 2×10^{-3} *M* PCMB.
[f] Incubated for 30 min with 1.5×10^{-3} *M* tetrathionate.

der to get more information about this system, which is considered as a better building block for the design of bifunctional protein reagents than *o,p*-dinitrophenyl halides. Compared with the latter, in the *o,o'*-DNP system steric hindrance around the halogen is enhanced by the presence of two nitro groups in adjacent positions. This situation has two consequences:

1. The reactivity of chlorine in the chloro-*o,o'*-DNP group is lower than in the *o,p*-DNP system. This decrease, however, is moderate (*19*), and Cl-*o,o'*-DNP derivatives are still able to react with protein nucleophiles efficiently. On the other hand, in several circumstances "proximity effects" can compensate for the lower reactivity at chlorine:

(i) If the Cl-*o,o'*-DNP group, as a part of a heterobifunctional protein reagent,

is involved in the second step of crosslinking, substitution of chlorine is facilitated by the intramolecular character of the reaction. Thus, when yeast alcohol dehydrogenase was treated with *N*-(4-chloromercuriphenyl)-4-chloro-3,5-dinitrobenzamide (*6*) or *N*-(4-chloro-3,5-dinitrobenzoyloxymethyl)maleimide (*7*) or when papain was treated with 4-chloro-3,5-dinitrophenacyl bromide (*5*), the chlorine was smoothly displaced by NH_2 groups in these enzymes after binding of the other end of the reagents to SH groups.

(ii) Even if the reaction is intermolecular, it can be favored by the presence of an aromatic electron donor ring in the proximity of the attacked nucleophile because of the ability of the Cl-*o,o'*-DNP group to form charge transfer complexes. This circumstance explains the preferential reactivity of Cl-*o,o'*-DNP derivatives with NH_2 groups of aromatic amino acids (see Table 1 and Ref. (*5*)), with protein NH_2 groups vicinal to mercuribenzoate-substituted SH groups (Table 2), or with some lysine residues vicinal to phenylalanine in cytochrome *c* (*14*).

(iii) Substitution of chlorine can also be assisted by a neighboring SH group, the primary attack directed toward this highly nucleophilic function being followed by a fast transarylation to the final nucleophile (Tables 1 and 2).

2. The influence of the greater steric hindrance around position 4 also appears in the interconversion of different *o,o'*-DNP derivatives. Although *S*-aryl compounds of this type are easily formed, a transarylation to a smaller atom, whenever possible, occurs more readily than in the *o,p*-DNP series. Thus, the *S–N* transfer is faster for the *o,o'*-DNP compounds, probably because in this case it results in a larger steric relaxation. This could also be the reason for a possible *S–O* shift observed in the reaction of some *o,o'*-DNP derivatives with mercaptoethanol; such a transarylation to the OH group, less reactive than NH_2, does not take place in the *o,p*-DNP series, but could be favored in the case of *o,o'*-compounds because it relieves increased steric strain. In addition, the carbonyl group, present in the *o,o'*-dinitroaryl derivatives investigated here, as well as in the formerly described bifunctional protein reagents (*5–7*), should also have some effect on the ease of these transfers.

Finally, while *o,o'*-DNP halides have the disadvantage of possessing lower extinction coefficients than those of the *o,p*-compounds (particularly those of the *S*-aryl derivatives), a desirable feature of *o,o'*-DNP halides is that the difference between the λ_{max} of their *S*- and *N*-aryl derivatives (90–100 nm) is larger than in the case of the corresponding *o,p*-DNP derivatives (30 nm). This difference can facilitate the quantitative determination of dinitrophenylated thiol and amino groups in proteins.

ACKNOWLEDGMENTS

We are indebted to Mr. L. Lacombe for nmr spectra and to Mr. J. Y. Le Gall for skillful technical assistance.

REFERENCES

1. M. SOKOLOVSKY, T. SADEH, AND A. PATCHORNIK, *J. Amer. Chem. Soc.* **86**, 1212 (1964).

2. L. KESNER, E. MUNTWYLER, G. E. GRIFFIN, AND P. QUARANTA, *Methods Enzymol.* **11,** 94 (1967).
3. C. H. W. HIRS, *Methods Enzymol.* **11,** 548 (1967).
4. F. WOLD, *Methods Enzymol.* **25,** 623 (1972).
5. J. DIOPOH AND M. OLOMUCKI, *Biochim. Biophys. Acta* **263,** 220 (1972).
6. J. DIOPOH AND M. OLOMUCKI, *Eur. J. Biochem.* **75,** 441 (1977).
7. J. P. LAUTIE AND M. OLOMUCKI, *Hoppe-Seyler's Z. Physiol. Chem.* **359,** 211 (1978).
8. F. ULLMAN, *Ann. Chem.* **366,** 93 (1909).
9. H. LINDEMANN AND W. WESSEL, *Chem. Ber.* **58,** 1221 (1925).
10. J. H. BARNES, J. ELKS, F. F. STEPHENS, AND G. J. WALLER, *J. Chem. Soc.* 764 (1953).
11. G. TOENNIES AND J. J. KOLB, *Anal. Chem.* **23,** 823 (1951).
12. J. NEVOLE, *Chem. Ber.* **77,** 61 (1944).
13. J. DIOPOH, Doctoral thesis, Paris, 1971.
14. D. L. BRAUTIGAN, S. FERGUSON-MILLER, AND E. MARGOLIASH, *J. Biol. Chem.* **253,** 130 (1978).
15. K. WALLENFELS AND B. EISELE, "Chemical Reactivity and Biological Role of Functional Groups in Enzymes" (R. M. S. Smellie, Ed.), p. 21. Academic Press, New York, 1970.
16. L. BRANDSMA AND J. F. ARENS, "The Chemistry of the Ether Linkage" (S. Patai, Ed.), p. 553. Interscience, New York/London, 1967.
17. E. M. KOSOWER, "An Introduction to Physical Organic Chemistry," p. 183. Wiley, New York, 1968.
18. D. CAVALLINI, M. T. GRAZIANI, AND S. DUPRE, *Nature (London)* **212,** 294 (1966).
19. M. E. C. BIFFIN, J. MILLER, AND R. ROPER, *Aust. J. Chem.* **22,** 2555 (1969).