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NEW PROTEIN REAGENTS

II. 4-CHLORO-3,5-DINITROPHENACYL BROMIDE

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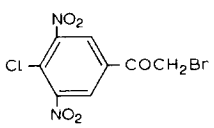
SUMMARY

The new compound 4-chloro-3,5-dinitrophenacyl bromide (CNPB) was studied as a potential polyfunctional protein reagent.

Under mild experimental conditions the bromine atom, more reactive than the chlorine, is easily substituted by thiol groups. The amino groups in amino acids are not attacked, with the exception of phenylalanine, tyrosine and tryptophan, where a charge-transfer complex formed between the reagent and the aromatic nuclei of the amino acids facilitates the substitution of chlorine or bromine by the α-NH_2 groups. A reaction between the bromoacetyl moiety of the reagent and carboxylic acids is also possible. CNPB inactivates ribonuclease by substitution of approximately two histidyl residues accompanied by the formation of a π-complex, presumably with a vicinal aromatic residue.

INTRODUCTION

In the course of investigations of potential protein reagents[1] we were led to synthesize and study a new compound, 4-chloro-3,5-dinitrophenacyl bromide (CNPB).



This product combines a bromoacetyl group frequently used in mono- and bifunctional reagents[2–5,7–9] and a chlorodinitrobenzene moiety connecting it to the well-known family of reagents of the halodinitroaryl type[6]. It seemed interesting to

Abbreviations: CNPB, 4-chloro-3,5-dinitrophenacyl bromide; DTNB, 5,5'-bis-(2-nitrobenzoic acid).

study the properties of a compound having two halogen atoms joined in a chromogenic molecule.

MATERIALS AND METHODS

Chloro-2,4-dinitrobenzene was purchased from Aldrich Chemical Co. Ethyl 4-chloro-3,5-dinitrobenzoate was obtained from the corresponding acid according to the method of Ullman[10].

A-grade soluble yeast ribonucleic acid was supplied by Calbiochem and five times crystallized bovine pancreatic ribonuclease by Nutritional Biochemical Co. Ltd.

Synthesis of 4-chloro-3,5-dinitrophenacyl bromide (CNPB)

4-Chloro-3,5-dinitrobenzoic acid. To a mixture of 4-chloroacetophenone (9.28 g, 0.06 mole) and of concentrated H_2SO_4 (200 g) heated to 70–100 °C, KNO_3 (27.3 g, 0.27 mole) was added in small portions with stirring. The mixture was heated to 120–140 °C for 1.5 h, chilled and poured with stirring into ice (300 g). The precipitated solid was filtered off with suction, washed with water and dried over P_2O_5 and KOH. Recrystallization from chloroform yielded 10 g (67.6%) of a white product, m.p. 160 °C. (Found: C, 34.0; H, 1.3; N, 11.1; Cl, 14.6. $C_7H_3N_2O_6Cl$ requires C, 34.10; H, 1.23; N, 11.36; Cl, 14.38%.)

4-Chloro-3,5-dinitrodiazoacetyl benzene. A solution of 4-chloro-3,5-dinitrobenzoyl chloride (19.8 g, 0.075 mole) (obtained from the corresponding acid according to Lindemann and Wessel[11]) in dry ether (150 ml) was added dropwise with stirring to an ice-chilled solution of diazomethane (approx. 0.22 mole) in ether (500 ml). During the addition a pink solid precipitated. This was filtered off and recrystallized from benzene. Yield: 14.6 g (73%), m.p. 162–163 °C. (Found: C, 35.6; H, 1.3; N, 20.2; O, 28.9; Cl, 13.5. $C_8H_3N_4O_5Cl$ requires C, 35.51; H, 1.12; N, 20.70; O, 29.56; Cl, 13.10%.)

4-Chloro-3,5-dinitrophenacyl bromide. To a suspension of 4-chloro-3,5-dinitrodiazoacetyl benzene (11.7 g, 0.043 mole) in a mixture of acetic acid (35 ml) and dry ether (20 ml), 48% HBr (10 ml) was added dropwise at room temperature. During the addition the solid gradually dissolved, followed by the appearance of a precipitate. The solid was collected and purified by dissolution in benzene and addition of cyclohexane. Yield: 12.1 g (86%), m.p. 98–99 °C. (Found: C, 30.3; H, 1.4; N, 8.6; Br, 24.8; Cl, 11.3. $C_8H_4N_2O_5BrCl$ requires C, 29.69; H, 1.24; N, 8.66; Br, 24.70, Cl, 10.96%.)

Thin-layer chromatography was carried out on Eastman Chromagram sheet (No. 6060 silica gel with fluorescent indicator) using *n*-butanol–acetic acid–water (4:1:5, by vol.) (Solvent A) or benzene–methanol–acetic acid (90:16:8, by vol.) (Solvent B). Whenever possible, spots were detected by viewing the plates in ultraviolet light. Ninhydrin was used to reveal amino compounds and a 10^{-3} M solution of DTNB in 0.2 M phosphate buffer, pH 7.5, to detect thiols.

Ultraviolet spectra were recorded using a Gilford 240 spectrophotometer. The values of λ_{max} for derivatives obtained after the substitution of the chlorine atom in CNPB and in some similar aryl chlorides by an amino(lysine) or sulfhydryl-(cysteine) function were: 420–425 nm for N-derivatives of CNPB, ethyl 4-chloro-3,5-dinitrobenzoate and chloro-2,6-dinitrobenzene, and 320–330 nm for the corresponding S-derivatives.

The activity of ribonuclease was measured according to the method of Kunitz[12]. The protein concentration was determined by the microbiuret method[13]. Titration of histidyl residues was performed as described by Barnard and Stein[14].

Properties of CNPB

The hydrolysis of CNPB was followed in 0.05 M solutions of the reagent in a 1:1 (v/v) mixture of acetone and 0.3 M sodium carbonate buffer, pH 8.8, at 20 °C; halide ions were periodically titrated on 10-ml aliquots and the absorption at 420 nm was recorded. The reactivity of CNPB towards amino acids was studied by incubating for 2 h at 20 °C solutions of final concentration $3 \cdot 10^{-3}$ M with respect to the reagent and 10^{-3} M with respect to the amino acid in the same solvent as above; the solutions were then analyzed by thin-layer chromatography and by spectroscopy.

RESULTS AND DISCUSSION

Under the experimental conditions used, the chlorine atom of CNPB is stable towards hydrolysis (no absorption at 420 nm shown after 6 h); the bromine hydrolyzes with a half-reaction time of 25 min. Similarly, CNPB reacts easily through its bromine atom with cysteine: while the –SH groups disappear in the reaction mixture, the ultraviolet spectrum of the latter shows no band in the 330 nm region. Thin-layer chromatography indicates the presence of a new main compound having a higher R_F than cysteine and giving a positive ninhydrin and a negative DTNB reaction. The ready substitution of the thiol of cysteine is followed by a reaction of the chlorodinitrophenyl group with the amino function of cysteine which takes also place to some extent as evidenced by a weak absorption at 420 nm and the presence of a small amount of a ninhydrin- and DTNB-negative derivative detectable by thin-layer chromatography on viewing the plates in ultraviolet light.

We attempted to isolate the reaction product of CNPB with *N*-acetylcysteine instead of cysteine itself in order to avoid side reactions on the $-NH_2$ group. 10 ml of an aqueous solution containing *N*-acetylcysteine (0.65 g, 0.004 mole), made alkaline to pH 8–9 with 1 M NaOH, were added dropwise with stirring at room temperature to a solution of CNPB (1.3 g, 0.004 mole) in acetone (8 ml). The pH was maintained at 5–6 by adding 1 M NaOH. After 30 min the precipitated solid was filtered off with suction and washed with water. After two recrystallizations from acetone–light petroleum, 0.5 g of a white solid was obtained; its sharp m.p. 183 °C remained unchanged after further recrystallization. $R_F = 0.61$ (thin-layer chromatography in Solvent B): DTNB reaction negative, the spot was visible in ultraviolet light. The product becomes yellow on standing. It gives no derivatives with mineral acids. The infrared spectrum (nujol or KBr) indicates the absence of a carboxylic OH group. The NMR spectrum shows two pairs of non-equivalent aromatic protons at 8.68 and 8.72 ppm. When the solid was dissolved in 2 M NaOH the solution became yellow and exhibited an absorption peak at 420 nm. In the presence of hydroxylamine a hydroxamic acid was formed as shown by thin-layer chromatography in 95% ethanol–dioxane–water–acetic acid (60:20:19:1, by vol.)[15]; the spot was detected by $FeCl_3$ according to the method of Bergmann and Segal[15]. These properties could correspond to a product formed by the reaction of the bromoacetyl groups of two molecules of CNPB with the SH and the COOH group of one molecule of *N*-acetyl-

cysteine*. The elemental analysis seems to confirm this supposition, although it does not entirely fit the required values. (Found: C, 40.0; H, 2.8; N, 10.2; S, 4.5; Cl, 10.4. $C_{21}H_{15}N_5O_{13}SCl_2$ requires C, 38.90; H, 2.33; N, 10.80; S, 4.94; Cl, 10.93%.) For a 1:1 reaction product of CNPB with *N*-acetylcysteine, $C_{13}H_{12}N_3O_8SCl$ would require S, 7.90; Cl, 8.74%.

Among other amino acids lysine seems to react slightly with CNPB; thin-layer chromatography showed a weak new spot of a higher R_F, detectable by ninhydrine. Histidine and methionine do not react. In contrast, phenylalanine, tyrosine and tryptophan react completely through their amino group. The chromatograms showed in each case yellow spots of a higher R_F than the starting amino acid and no ninhydrin-positive compound. Detection by means of the Ehrlich and Pauly reagents, although perturbed by the original coloration of the spots, indicated that the derivatives of tyrosine and tryptophan contained phenol and indol groups, respectively. Absorption spectra of mixtures of CNPB with tryptophan, phenylalanine and tyrosine are shown in Fig. 1. All of them exhibit a significant absorption band at 425 nm showing that in each case a considerable proportion of the amino acid was substituted on the α-nitrogen by the chlorodinitrophenyl group of the reagent**.

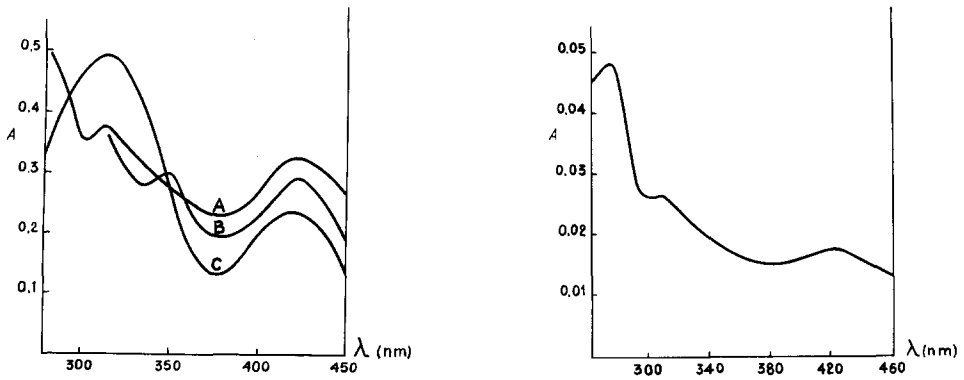


Fig. 1. Absorption spectra of mixtures of CNPB with aromatic acids. Aliquots of the solutions described in Materials and Methods were diluted 20 times. A, tyrosine; B, tryptophan; C, phenylalanine.

Fig. 2. Absorption spectrum of CNPB-treated ribonuclease. A solution of ribonuclease (60 mg, approx. $0.4 \cdot 10^{-5}$ mole) in 0.2 M citrate–phosphate buffer, pH 6.8 (10 ml) containing CNPB (130 mg, $0.4 \cdot 10^{-3}$ mole) was incubated 5 h at 37 °C, dialyzed 24 h against distilled water and the spectrum measured on a 10-times diluted aliquot.

The unusual reactivity of CNPB towards these three amino acids which are aromatic derivatives could be explained in terms of a charge-transfer interaction with the reagent facilitating the substitution. Actually, as can be seen in Fig. 1, new absorption bands which can be ascribed to π-complexes[8] appear in the spectra of solutions of CNPB containing tryptophan (345 nm), tyrosine (315 nm) and phenyl-

* Substitution of COOH group in proteins by reagents having a bromoacetyl moiety has been described[79].

** Since the amino acids disappear almost entirely in the reaction mixtures, other reactions such as substitution of the α-NH_2 groups by the bromine or the carbonyl of the bromoacetyl moiety of CNPB must also be considered. As attempts to isolate the formed derivatives were unsuccessful their exact structure could not be determined.

alanine (312 nm). The same peaks at 315 and 312 nm, respectively, appeared when CNPB was hydrolyzed by a 24 h treatment with 2 M NaOH to remove the halogen atoms and tyrosine or toluene were added to the solutions.

Reaction of CNPB with ribonuclease results in an almost complete (95%) inactivation of the enzyme. This cannot be due to an arylation by the chlorodinitrophenyl group of the reagent since, as shown in Fig. 2, only a weak absorption at 425 nm is observed. On the other hand, a new peak appears at 312 nm which is characteristic of a π-complex of the reagent with a benzene nucleus. Alkylation of histidyl residues is also observed: titration with diazobenzene–sulphonic acid[14] shows a 36% difference in the absorbance at 498 nm between the native and the CNPB-treated enzyme ($A_{498\ nm}$ = 0.840 and 0.618, respectively) which, compared to the predicted difference of 21% for 1 His blocked[14], indicates a substitution of 1.7 residues. Thus, it could be assumed that CNPB reacts with ribonuclease through two parts of its molecule: formation of a charge-transfer complex between the dinitrophenyl ring of the reagent and an aromatic residue of the enzyme would facilitate the substitution of a vicinal histidyl residue. It is known that in ribonuclease the essential His 119 and His 12 are located in the proximity of phenylalanine residues (Phe 120 and 8).

A similar effect has been observed by Sigman and Blout[8] in the reactions of α-bromo-4-nitroacetophenone and α-bromo-2,4-dinitroacetophenone with model aromatic compounds and with chymotrypsin. New absorption bands characteristic of charge-transfer complexes have appeared in the spectrum of the treated enzyme in which one methionine residue was simultaneously substituted. These reagents performing two functions, though having only one chemically reactive group, have been named by the authors chemical-optical bifunctional reagents. According to these considerations CNPB, a composite alkylating–arylating reagent giving π-complexes, could be termed a chemical-optical trifunctional reagent.

We tried to see whether in the reaction of CNTB with ribonuclease an additional substitution of the chlorine atom of the reagent by a vicinal (*e.g.* lysyl?) residue could occur, thus manifesting the third possible action of CNPB. The fact that the absorption at 425 nm arising from this substitution was weak even after 5 h incubation of the CNPB-treated ribonuclease at pH 8.8 and 37 °C shows that this reaction does not take place to a great extent, perhaps because of an inadequate orientation of the reagent or a wrong distance to the neighbouring amino acid residues.

Other modifications of various proteins with CNPB may prove possible. Thus, our preliminary assays with papaine showed that $-NH_2$ groups reacted with the chlorine atom and that arylation is considerably decreased when the –SH group of the protein is blocked by iodoacetate. These findings could be consistent with a cross-linking of the –SH and $-NH_2$ groups by a reaction of the two halogen atoms of CNPB.

Thus it appears that CNPB can react in many ways, and its application as a protein reagent seems to be promising.

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