The Highly Electrophilic Character of 4-Chloro-7-nitrobenzofurazan and Possible Consequences for its Applications as a Protein-Labelling Reagent

By BALDEV S. BAINES,* GEOFFREY ALLEN† and KEITH BROCKLEHURST*‡
*Department of Biochemistry, St. Bartholomew's Hospital Medical College, University of London,
Charterhouse Square, London EC1M 6BQ, U.K., and †National Institute for Medical Research,
The Ridgeway, Mill Hill, London NW7 1AA, U.K.

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4-Chloro-7-nitrobenzofurazan possesses at least one highly electrophilic centre (C-6) that is much more reactive towards nucleophiles than position C-4, the irreversible alkylating site of this reagent. Possible consequences of the electrophilic character of 4-chloro-7-nitrobenzofurazan for its application as a protein-labelling reagent are discussed.

4-Chloro-7-nitrobenzofurazan (I, Scheme 1) has been used as a reactivity probe and as a fluorescent labelling reagent in the study of a number of proteins [see, e.g., Shipton *et al.* (1976) and references therein; Ferguson *et al.* (1976) and references therein]. The nitrobenzofurazan moiety becomes covalently attached to a nucleophilic atom in the protein, which replaces the Cl atom at position C-4 of Nbf-Cl. § One interesting facet of the selectivity characteristics of Nbf-Cl is that 4-mercapto-7-nitrobenzofurazan derivatives can undergo S → N alkyl transfer at position C-4 to a suitably aligned amino group (see Birkett *et al.*, 1970; Price *et al.*, 1975).

The present paper draws attention to another interesting property of Nbf-Cl that may need to be considered when Nbf-Cl is used to label proteins. Evidence is presented that the most electrophilic centre in Nbf-Cl is not at position C-4, the C atom bonded to the replaceable Cl atom, but rather one of the other electrophilic atoms, probably at position C-6. When Nbf-Cl reacts with a protein, rapid but reversible formation of a Meisenheimer (1902) adduct by reaction of a highly nucleophilic centre at position C-6 or N-3 of Nbf-Cl would be expected to occur. In some cases this might help to determine the site in the protein labelled by reaction at position C-4. Also, adduct formation by reaction at positions C-6 or N-3 subsequent to alkylation of another site by reaction at position C-4 would markedly affect the spectral characteristics of the labelled protein.

‡ To whom reprint requests and correspondence should be addressed.

§ Abbreviation: Nbf-Cl, 4-chloro-7-nitrobenzofurazan (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole).

Materials and Methods

Most of the materials and general methods have been described previously (see Allen & Lowe, 1973; Shipton *et al.*, 1976). In the present work a stock solution of Nbf-Cl in water (approx. 900 μM) was prepared without added ethanol. Spectroscopic and kinetic studies were carried out in aqueous buffers at 25°C and I 0.1 by using a Cary 118C spectrophotometer, a Durrum stopped-flow spectrophotometer and an Aminco-Morrow stopped-flow spectrophotometer. In the reactions of Nbf-Cl with OH[−], [Nbf-Cl]₀ was 60 μM and the following buffers were used: pH 9.06–9.38, Tris/HCl; pH 9.58–9.77, H₃BO₃/KCl/NaOH; pH 9.94–11.27, NaHCO₃/NaOH. These were all made up at 3 times the concentrations given for I 0.1 and 25°C (except for borate) (Long, 1971) and diluted 3-fold into the reaction mixture. Kinetic studies were carried out at 315 nm and all reactions accurately obeyed first-order kinetics. The infinity readings from 16 kinetic runs in the pH range 9.06–11.27 were used to provide the equilibrium constant, *K*, for the reaction of Nbf-Cl and OH[−] (see the Results and Discussion section). In the reactions of Nbf-Cl with thiols, [Nbf-Cl]₀ was 56 μM and [thiol]₀ was 40–50 mM.

Results and Discussion

Although in studies of reactions of proteins with Nbf-Cl (I, Scheme 1) attention has been focused on reactions of nucleophiles at position C-4 [see, e.g., Shipton *et al.* (1976) and references therein] it has been recognized that activated benzofurazans may possess

Scheme 1. Reactions of NbF-Cl with a nucleophile RX^-

more than one electrophilic centre [Allen (1971); Shipton *et al.* (1977) and references therein]. In the present paper, previous observations and new results from a kinetic and spectroscopic study of the initial rapid reaction of NbF-Cl with OH^- and with thiolate ions are shown to constitute compelling evidence for at least two electrophilic centres in NbF-Cl, of which one (probably C-6) is much more reactive than the other (C-4).

Allen & Lowe (1973) reported that the electronic absorption spectrum of NbF-Cl in alkaline media undergoes reversible changes characterized by an apparent pK_a value of 9.76 and slow irreversible changes in more strongly alkaline media. Shipton *et al.* (1976) pointed out that the time course of the change in A_{315} consequent upon raising the pH of an NbF-Cl solution from approx. pH 6 to alkaline pH values (a rapid increase followed first by a slow decrease and then by an even slower increase) suggests the existence of two intermediates in the reaction of NbF-Cl with OH^- , one formed very rapidly and one much more slowly. The postulate that the reaction proceeds by deprotonation of an NbF-Cl cation to provide neutral NbF-Cl (the first intermediate), which forms a Meisenheimer (1902) adduct (the second intermediate) by reaction of OH^- at position C-4 before expulsion of Cl^- (Shipton *et al.*, 1976), however, is not consistent with the solubility characteristics of NbF-Cl or with the essentially neutral character of aqueous solutions of NbF-Cl. The Meisenheimer adduct (II, Scheme 1)

formed by nucleophilic attack at position C-4 of NbF-Cl remains an attractive candidate for one of the intermediates in the sequence suggested by Shipton *et al.* (1976), but it is difficult to envisage another intermediate on the pathway between compounds (I) and (III) of Scheme 1. It is argued below that the product of the initial rapid reaction of OH^- with NbF-Cl is compound (IV) of Scheme 1 (with $RX = HO$).

The nitro group on position C-7 of NbF-Cl would be expected to confer electrophilic character on positions C-6, C-4 and N-3. Allen (1971) had proposed that Meisenheimer-adduct formation by reaction of OH^- at position C-6 of NbF-Cl (IV, Scheme 1, $RX = HO$) might account for the reversible changes of the spectrum of this compound in alkali and had cited evidence for this proposal drawn from reactions of analogous compounds. For example, the coloured intermediates in the reactions of sodium methoxide with 1-chloro-2,6-dinitrobenzene derivatives result from attack of methoxide ion at a ring carbon bearing hydrogen rather than that bearing chlorine (Crampton *et al.*, 1971). Shipton *et al.* (1977) have pointed out that another activated benzofurazan (benzofurazan *N*-oxide, benzofuroxan, VI) probably contains three electrophilic centres, N-3, C-4 and C-6, and nucleophilic attack at positions N-3 and C-4 has been demonstrated (Latham *et al.*, 1972*a,b*; Latham, 1973).

The A_{315} -time profile for the reaction of NbF-Cl and OH^- reported by Shipton *et al.* (1976) probably

results therefore from rapid and reversible Meisenheimer-adduct formation by attack of OH^- at position C-6 (or perhaps less likely at N-3), together with slower attack of OH^- at position C-4, which leads to the formation of the substituted product (III) (Scheme 1) by expulsion of Cl^- . The fact that the rate of the rapid spectral change at 315 nm that follows admixture of Nbf-Cl and OH^- is low enough to be easily measured (see below) supports an interpretation that involves O-C (or O-N)-bond formation rather than deprotonation. The monophasic nature of the progress curves of these reactions suggests either that OH^- attacks predominantly only one electrophilic centre (C-6 or N-3) or that the electrophilicities of positions C-6 and N-3 are similar. When the present work had been completed we discovered the report by Di Nunno *et al.* (1975) that methoxide ion reacts rapidly with Nbf-Cl in methanol at position C-6 to provide compound (IV) (Scheme 1, with $\text{RX} = \text{CH}_3\text{O}$).

The apparent $\text{p}K_a$ value of Nbf-Cl determined spectrophotometrically may be used to calculate the equilibrium constant, K (see eqn. 2), for the formation of the 'non-productive' Meisenheimer adduct, BOH^- (IV or V of Scheme 1), which is represented schematically in eqn. (1).

$$K = \frac{[\text{BOH}^-]}{[\text{B}][\text{OH}^-]} = \frac{k_{+1}}{k_{-1}} \quad (2)$$

The value of the ' $\text{p}K_a$ ' of Nbf-Cl determined in the present work (10.1) is a little higher than that (9.76) reported by Allen & Lowe (1973); ' $\text{p}K_a$ ' = 10.1 corresponds to K (eqn. 2) = $7.9 \times 10^3 \text{ M}^{-1}$. Similarly the apparent $\text{p}K_a$ value (9.16) of 4-(*N*-2-aminoethyl-2'-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole (Stuchbury *et al.*, 1975) probably characterizes the reversible reaction of OH^- at position C-6 of this benzofurazan derivative.

A study of the kinetics of the reaction of Nbf-Cl with OH^- provided values of the apparent first-order rate constant, $k_{\text{obs.}}$, at 16 pH values in the range 9.06–11.27. These values of $k_{\text{obs.}}$ together with the value of K ($7.9 \times 10^3 \text{ M}^{-1}$) and eqns. (2) and (3) were used to calculate values of k_{+1} ($72 \pm 2 \text{ M}^{-1} \cdot \text{s}^{-1}$) and k_{-1} ($8.6 \times 10^{-3} \pm 0.2 \times 10^{-3} \text{ s}^{-1}$).

$$k_{\text{obs.}} = k_{+1}[\text{OH}^-] + k_{-1} \quad (3)$$

The values of k_{+1} and k_{-1} (see eqn. 1) are means $\pm$ S.E.M. of the 16 sets of values provided by determination of $k_{\text{obs.}}$.

It seems reasonable to assume that the high electrophilicity at position C-6 displayed by Nbf-Cl towards OH^- and towards methoxide ion may be displayed also towards other nucleophiles. In the case of thiolate ions, this is supported by spectroscopic evidence. Allen & Lowe (1973) reported that the u.v. spectrum of 4-(2'-hydroxyethylthio)-7-nitrobenzofurazan undergoes striking spectral changes in the presence of excess of 2-mercaptoethanol, involving decrease in A_{425} . Shipton (1976) studied the activation by 2-mercaptoethanol of the catalytically inactive papain derivative of nitrobenzofurazan (papain-S-Nbf), prepared by reaction of papain and Nbf-Cl and separated from low-molecular-weight material by gel filtration on Sephadex G-25. The nitrobenzofurazan moiety is bonded at position C-4 to the active-centre thiol group of papain (see Allen & Lowe, 1973; Shipton *et al.*, 1976). Shipton (1976) demonstrated that, when papain-S-Nbf (λ_{max} , 405 nm) is mixed with excess of 2-mercaptoethanol at pH 8, the 405 nm band collapsed within 15 s and a shoulder formed at approx. 335 nm on the 280 nm band. This spectral change occurred very much more rapidly than the regeneration of enzymic activity. In the present work it has been demonstrated that reactions of Nbf-Cl with 2-mercaptoethanol and 1-mercapto-propane result in a rapid shift of the absorption band of Nbf-Cl (λ_{max} , 343 nm) to shorter wavelengths (λ_{max} , 325 nm) before the much slower development of the absorption band with λ_{max} , 425–430 nm characteristic of 4-mercapto-7-nitrobenzofurazans (see Birkett *et al.*, 1970).

All of these spectral changes are consistent with rapid and reversible formation of Meisenheimer adducts by reaction of the thiol at position C-6 of the nitrobenzofurazan.

When Nbf-Cl is used as a protein-labelling reagent it seems probable, in view of the results discussed above, that reaction at position C-4 will be preceded by rapid and reversible reaction at another electrophilic centre. Although the model reactions indicate attack at position C-6, the reactions of benzofuroxan discussed by Shipton *et al.* (1977) suggest that with some proteins reaction at position N-3 of Nbf-Cl may be an alternative possibility. Initial Meisenheimer-adduct formation involving protein and either positions C-6 or N-3 of Nbf-Cl may have consequences for the nature of the resulting protein derivative. For example, addition of Nbf-Cl to a highly nucleophilic centre may trigger conformational changes that direct alkylation by position C-4 of another Nbf-Cl molecule to a nucleophilic centre other than the one that is most reactive in the

native protein. Another possibility might involve attack of a second nucleophilic centre (Y) in the protein at position C-4 during the breaking of the (C-6)-X bond of compound (IV) (Scheme 1 with R = protein). The diminished electrophilicity of position C-4 in such a transition state might be compensated for by favourable orientation of Y with position C-4.

Even when position C-4 of the Nbf label has become bonded to the protein, Meisenheimer-adduct formation at either position C-6 or N-3 could occur subsequently if a nucleophilic centre in the protein happens to be correctly aligned. Such adduct formation would cause the labelled protein to have spectral characteristics markedly different from those expected from the study of simple 4-substituted nitrobenzofurazans.

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