Aryldiazonium Salts as Photo-affinity Labelling Reagents for Proteins

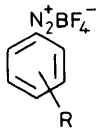
By BRIGITTE L. KIEFFER, MAURICE PH. GOELDNER,* and CHRISTIAN G. HIRTH*

(*Laboratoire de Chimie Organique des Substances Naturelles, associé au C.N.R.S., Université Louis Pasteur, Institut de Chimie, 1 rue Blaise Pascal, 67008 Strasbourg, France*)

Summary Aryldiazonium tetrafluoroborates, substituted in the *para* position by electron donating substituents, are potential photo-affinity labelling reagents for proteins and after light activation irreversibly inhibit acetylcholinesterase; using radiolabelled reagent, the inactivation is shown to be approximately stoicheiometric.

PHOTO-AFFINITY labelling is a well documented method used for the isolation of biological receptors and the characterisation of their active-site[1] amino-acid residues. It involves the design of a chemically inert molecule which can bind reversibly to a biological macromolecule and the subsequent production, by the action of light, of a highly reactive species that irreversibly modifies the protein at the site of interaction. Such a photo-generated species will react rapidly with the amino-acid residues of the protein before it can escape from the active site. Aryldiazonium salts are known to generate photochemically the corresponding aryl cation[2] which is hyper-reactive and not subject to rearrangement.[3] A triplet diradical structure has been recently established for the *para*-(dimethylamino)benzene cation.[4] Thus, aryldiazonium salts which are stable should be good reagents for photo-affinity labelling and they have not been described as such to our knowledge.

We synthesised six aryldiazonium derivatives by diazotisation of the corresponding amines and tested their chemical and physical properties for use as possible photo-affinity labelling reagents. Aryldiazonium salts are stable in aqueous acid conditions but their stability in neutral conditions is directly dependent on their substituents (Table).



(**1**) R = *p*-NMe_2
(**2**) R = *p*-OMe
(**3**) R = *m*-OMe
(**4**) R = *m*-Br
(**5**) R = *p*-I

(**6**)

Photolysis of aqueous solutions of aryldiazonium salts leads to rapid loss of nitrogen and formation of the corresponding phenol as the unique primary product. The more stable derivatives (**1**), (**2**), (**5**), and (**6**) (2×10^{-3} M in water) were photolysed in a Pyrex reactor with a Phillips lamp HPK 125 W type 572033/00. The resulting phenols were identified by comparison with authentic samples. The yields were determined by n.m.r. spectroscopy using an internal reference to be respectively, 92 (**1**); 80 (**2**); 92 (**5**) and 90% (**6**). That smaller amounts of *p*-methoxyphenol (**2**) were obtained is due to its extreme sensitivity to oxygen;[5] photolysis of (**2**) in methanol leads to quantitative formation of *p*-methoxyanisole.

TABLE. Chemical and physical properties of the aryldiazonium salts (**1**)—(**6**).

	Half-time of decomp. in phosphate buffer pH 7·2	λ_{max} (nm)	ϵ	Quantum yields[a]
(**1**)	3·5 h	379	37500	0·59
(**2**)	> 1 day	313	23500	0·46
(**3**)	10 min	273	7300	
		350	1500	
(**4**)	< 5 min	268	7000	
		323	1400	
(**5**)	30 min	326	12500	
(**6**)	> 3 days	338	22500	0·33

[a] C. G. Hatchard and C. R. Parker, *Proc. R. Soc.* (*London*), *Ser. A*, 1956, **235**, 518.

The aryldiazonium salts which are substituted in the *para* position by electron donating groups have very high extinction coefficients at $\lambda > 300$ nm. These strong absorptions, associated with excellent quantum yields (Table), lead to a very efficient photolytic decomposition. One can thus use very short irradiation times and wavelengths where the biological molecule will not be damaged.

Some successful affinity labelling experiments have been described for acetylcholinesterase and the acetylcholine receptor using unstable aryldiazonium derivatives.[6] However, no stoicheiometry of inactivation using a radiolabelled precursor was given for these alkylation experiments. We thus attempted to use aryldiazonium salts to photo-affinity-label acetylcholinesterase (E.C. 3.1.1.7).

Because of their stability in neutral medium we tested compounds (**1**), (**2**), and (**6**) as eventual photo-affinity inactivators of the enzyme. The binding constants determined kinetically by competition with acetylthiocholine as substrate (pH 7·2 at 22 °C) were, respectively, K_I (**1**) 2×10^{-5} M, K_I (**2**) 4×10^{-5} M (competitive inhibitors), and K_I (**6**) 8×10^{-6} M (non-competitive inhibitor). Inactivation of the enzyme in the dark (affinity labelling) was negligible. Photolysis of incubation mixtures containing enzyme and inhibitor (at a concentration five times their K_I values) led to a substantial irreversible loss of enzyme activity in the case of (**1**) and (**2**) (78 and 63% respectively). No inhibition was noticeable with (**6**). The light intensity was modulated so that the number of quanta absorbed by each inhibitor was identical per unit time; the required irradiation times were 2·5 (**1**), 3·5 (**2**), and 4 min (**6**).

A protective effect by NMe_4Br[7] (10^{-2} M) (which is a competitive inhibitor of acetyl cholinesterase, $K_I = 1{\cdot}6 \times 10^{-3}$ M) against enzyme inactivation was observed with both inhibitors (**1**) and (**2**). The percentage of inhibition became, respectively 35 and 11% instead of 78 and 63%.

Using a radiolabelled inhibitor, [*methyl*-^{3}H] (**1**),[8] we determined the stoicheiometry of inactivation to be 1·3. This experiment was done on partially purified enzyme (80% of active sites)[9] at 2×10^{-5} M inhibitor concentration. This value is an average of four measurements for different enzyme inactivations ranging from 22 to 62%. This very efficient and specific inhibition process confirms that aryldiazonium salts have potential as photo-affinity labels.

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