Biochem. J. (1968) **108**, 155
Printed in Great Britain

7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole: a New Fluorigenic Reagent for Amino Acids and other Amines

By P. B. GHOSH and M. W. WHITEHOUSE*
Departments of Medical Chemistry and Experimental Pathology, John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T., Australia

(*Received* 18 *March* 1968)

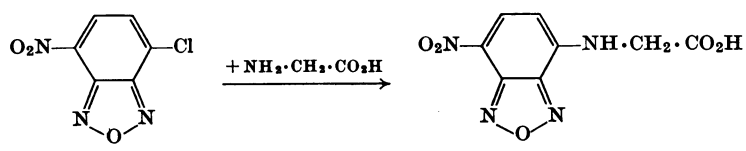
In the course of examining derivatives of 4-nitrobenzo-2-oxa-1,3-diazole for potential anti-leukaemic activity (Ghosh & Whitehouse, 1968) and ability to block thiol groups (Whitehouse & Ghosh, 1968), we noticed that several 7-amino derivatives were highly fluorescent at low dilutions when the amino group was derived from an aliphatic amine. This phenomenon may provide a means of identifying and determining amino acids and peptides if these could be made to react with 7-chloro-4-nitrobenzoxadiazole. Glycine reacts readily with this reagent in ethanol or aqueous solutions in the presence of potassium acetate, and cysteine methyl ester and glutathione reacted in aqueous phosphate buffer solutions at pH 7. The product isolated from cysteine methyl ester was identified by nuclear-magnetic-resonance spectroscopy as the *NS*-bis-(4-nitrobenzo-2-oxa-1,3-diazole) derivative, and it has been established that the strong fluorescence of this compound arises from the substituted amine portion of the molecule.

7-Chloro-4-nitrobenzoxadiazole (I) (also known as 7-chloro-4-nitrobenzofurazan) is a stable non-fluorescent pale-yellow solid, m.p. 97°, readily prepared by nitrating 4-chlorobenzofurazan (Boulton, Ghosh & Katritzky, 1966*a*), obtained from 2,6-dichloroaniline via the dichloronitrosobenzene (Boulton, Ghosh & Katritzky, 1966*b*). For convenience in referring to this promising fluorigenic reagent (I), we propose that its name be abbreviated to 'NBD† chloride'. [There is a good precedent for designating an (activated) chlorobenzene derivative as the aryl chloride implying ready replacement of the chlorine atom (as in acyl chlorides), e.g. the use of 'picryl chloride' for 1-chloro-2,4,6-trinitrobenzene.] The 7-substituted amino-4-nitrobenzoxadiazoles prepared with this reagent are then designated by the prefix 'NBD', e.g. *N*-NBD-glycine is the 7-aminoacetic acid derivative of 4-nitrobenzoxadiazole (II). For other substituents, the abbreviation is best used as a suffix, e.g. the amino derivative is written as 7-amino-NBD.

The strong fluorescence of the NBD-amines is best observed in solvents of low polarity and is excited by visible light (464mμ). Thus quartz cells and a special ultraviolet fluorimeter are not required for its quantitative determination, in contrast with the fluorescence of the DNS-amino acids, which is excited in the region of 254 or 365mμ (Gray, 1967). Since NBD chloride is also more stable (to moisture etc.) and more soluble in aqueous solutions than DNS chloride, there are therefore some distinct advantages in employing NBD chloride for detecting and determining small quantities of amines and amino acids as their fluorescent NBD derivatives. We have been able to detect with a fluorimeter as little as 1mμg. of NBD-glycine/ml. (equivalent to 0·3mμg. of glycine/ml.) dissolved in acetone or ethyl acetate and to detect 1μg. of this glycine derivative on thin-layer-chromatography plates

* Present address: College of Pharmacy, Ohio State University, Columbus, Ohio, 43210, U.S.A.

† Abbreviations: NBD, 4-nitrobenzo-2-oxa-1,3-diazole; DNS, 1-dimethylaminonaphthalene-5-sulphonyl.



(I) (not fluorescent)

(II) (highly fluorescent)

Table 1. *Fluorescence of some aminobenzoxadiazoles and related compounds*

The results were obtained with an Aminco–Bowman spectrofluorometer with ethyl acetate as the solvent unless specified otherwise. Fluorescence intensities are expressed relative to that of NBD-glycine, assigned a value of 1·0.

Compound	Concn. (μM)	Multiplier setting	Excitation wavelength (mμ)	Emission wavelength (mμ)	Trans-mittance (%)	Fluorometric intensity
N-DNS-glycine (piperidine salt) (in water)	0·1	0·03	260x	525	15	0·34
			324	525	42	0·98
(in acetone)	0·1	0·03	260	485	25	0·6
N-NBD-glycine	0·1	0·03	464	512	43	(1·0)
NS-bis-NBD-cysteine methyl ester	0·1	0·03	464	512	4	0·1
O-NBD-(*N*-acetyltyrosine)	500	0·3	464	512	12	$\sim 5 \times 10^{-4}$
S-NBD-thioglycollic acid	500	0·03	464	512	49	$\sim 2{\cdot}2 \times 10^{-3}$
			330	512	30	$\sim 1{\cdot}3 \times 10^{-3}$
O-NBD *p*-nitrophenyl ether	500	0·3	464	524	59	$\sim 2{\cdot}8 \times 10^{-3}$
7-Amino-NBD	0·1	0·03	464	512	30	0·72
7-*N*-Methylamino-NBD	0·05	0·03	464	512	14	0·70
7-Dimethylamino-NBD	0·1	0·03	464	512	10	0·25
7-Acetamido-NBD	500	0·03	464	512	75	0·3
7-Benzylamino-NBD	0·1	0·03	464	504	61	1·4
7-Anilino-NBD	10	0·003	464	524	10	0·02
4-Aminobenzoxadiazole	10	0·03	400	520	42	0·01

with a Chromatavue long-wavelength ultraviolet lamp (U.V. Products Inc., San Gabriel, Calif., U.S.A.).

Table 1 shows that the actual intensity of fluorescence of NBD-glycine in moist ethyl acetate was approximately equal to that of DNS-glycine (as the piperidine salt, from CalBiochem, Los Angeles, Calif., U.S.A.) in water and greater than the fluorescence of DNS-glycine in moist acetone. 4-Aminobenzofurazan (Dal Monte & Sandri, 1964) is about 100-fold less fluorescent than the corresponding 7-nitro derivative: the nitro group evidently not only intensifies the fluorescence but also shifts both the absorption and emission frequencies, which is quite unusual for this group (Jaffe & Orchin, 1965). The data in Table 1 also show that (*a*) substituents on the amino group that restrict its conjugation with the benzoxadiazole nucleus (e.g. acetyl or phenyl) cause a drastic loss of fluorescence, and (*b*) related compounds that are not amine derivatives, but have other groups in the 7-position able to donate electrons to the nucleus, e.g. –OR or –SR ,also fluoresce, but to a much smaller extent than the 7-amino derivatives.

Boulton, A. J., Ghosh, P. B. & Katritzky, A. R. (1966*a*). *J. chem. Soc.* B, p. 1004.

Boulton, A. J., Ghosh, P. B. & Katritzky, A. R. (1966*b*). *Tetrahedron Lett.* no. 25, p. 2887.

Dal Monte, D. & Sandri, E. (1964). *Boll. sci. Fac. Chim. industr. Bologna*, **22**, 33.

Ghosh, P. B. & Whitehouse, M. W. (1968). *J. med. Chem.* **11**, 305.

Gray, W. R. (1967). In *Methods in Enzymology*, vol. 11, p. 137. Ed. by Hirs, C. W. New York: Academic Press Inc.

Jaffe, H. H. & Orchin, M. (1965). *Theory and Application of Ultraviolet Spectroscopy*, p. 544. New York: John Wiley and Sons Inc.

Whitehouse, M. W. & Ghosh, P. B. (1968). *Biochem. Pharm*