Synthesis and Use of Radiolabelled Cyanoacrylate Probes of the Photosystem II Herbicide Binding Site

Helen G. McFadden and John N. Phillips

CSIRO Division of Plant Industry, GPO Box 1600, Canberra, Australia, 2601

Z. Naturforsch. **45c,** 196–202 (1990); received September 20/November 10, 1989

Cyanoacrylate, Photoaffinity Labelling, Photosystem II, Competitive Inhibition, Diuron

Cyanoacrylates are potent inhibitors of photosynthetic electron transport (PET) and are potentially useful probes of the photosystem II herbicide binding site. A series of cyanoacrylates was synthesized and the Hill inhibition activities evaluated in order to select compounds suitable for radioactive synthesis. A cyanoacrylate, 2-(2-nitrophenoxy)ethyl 3-benzylamino-2-cyano-2-pentenoate, was found to displace diuron from the photosystem II herbicide binding site. For this compound the dissociation constant of the inhibitor/binding site complex was found to be 2×10^{-8} M with an active site concentration of 2 nmol/mg chlorophyll. In a similar system the corresponding figures for diuron were 1.2×10^{-8} M and 1.3 nmol/mg chlorophyll. Photoaffinity labelling of PS II thylakoid proteins with 2-(2-azidophenoxy)ethyl 3-[7-^{14}C]-benzylamino-2-cyano-2-pentenoate showed weak binding in the 32 and 28 kD mass regions, consistent with binding to the D_1 peptide.

Introduction

Several commercially important herbicides bind to the photosystem II (PS II) complex of thylakoid membranes in plant chloroplasts. Compounds that bind at this site disrupt photosynthetic electron transport (PET) and hence photosynthesis. They displace plastoquinone Q_B from its site and interact with the 32 kD D_1 peptide and possibly additional peptides in the PS II complex. This particular binding site has been the subject of much interest because it is hoped that, once the structure of the site is well defined, rational design of new herbicides will be possible. Current knowledge of this binding site has been gained from analysis of the positions of mutations in herbicide-resistant plants [1], from the positions of photoaffinity labelling by herbicide analogues [2–5] and by analogy with the X-ray structure of the L-protein from the bacterial photosynthetic reaction centre [6–8].

Cyanoacrylates (**1**) are potent PET inhibitors (Hill inhibitors) with herbicidal potential [9]. Therefore an understanding of the nature of cyanoacrylate interactions with thylakoid peptides will contribute to the overall understanding of the structure of the herbicide-binding site.

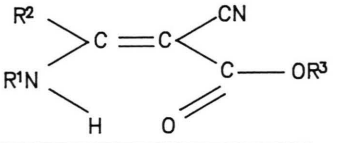
Reprint requests to Helen G. McFadden.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0300–0196 $ 01.30/0

Cyanoacrylates exhibit some properties generally associated with urea/triazine-type inhibitors (hereafter referred to as classical-type inhibitors (see [10] for definitions)). They are less active as herbicides on atrazine-resistant mutants of *Brassica napus* [11] and they show increased potency with increased lipophilicity [12]. However, they also show phenol-type characteristics in their rates of Q_B/inhibitor exchange [13] and in some structural [14] and electronic [15] properties. While both phenol-type PS II inhibitors [5] and classical-type inhibitors bind predominantly to the 32 kD peptide [16–19] it is believed that the two types of inhibitors interact with different portions of the D_1 peptide [5]. Initially, we wished to establish whether cyanoacrylates also bind predominantly to the 32 kD peptide, and then, if possible, determine which region of the D_1 peptide was labelled.

One useful feature of cyanoacrylates is that it is possible to incorporate an aryl azide at either end of the molecule, giving flexibility in the choice of compound suitable for a photoaffinity labelling experiment. An initial series of cyanoacrylates with azidoaryl and precursor nitroaryl substituents on either the ester or the amino function of (**1**) was synthesized. Hill inhibition activities of these compounds were determined to provide an overview of their structure/activity relationships and to allow selection of sufficiently active compounds for synthesis of radioactive analogues (see Table I).

Table I. Melting points and Hill inhibition activities of some photoaffinity-labelled cyanoacrylates and precursors of the general formula **1**.



	m.p. [°C]	R_1	R_2	R_3	pI_{50}
1a	130–134	4-$ClC_6H_4CH_2$–	Et–	4-$NO_2C_6H_4OCH_2CH_2$–	5.5
1b	125–128	4-$ClC_6H_4CH_2$–	Et–	3-$NO_2C_6H_4OCH_2CH_2$–	6.9
1c	119–120	4-$ClC_6H_4CH_2$–	Et–	2-$NO_2C_6H_4OCH_2CH_2$–	7.9
1d	103–105	$C_6H_5CH_2$–	Et–	4-$NO_2C_6H_4OCH_2CH_2$–	<4
1e	95– 97	$C_6H_5CH_2$–	Et–	3-$NO_2C_6H_4OCH_2CH_2$–	5.9
1f	99–100	$C_6H_5CH_2$–	Et–	2-$NO_2C_6H_4OCH_2CH_2$–	7.1
1g	oil	$C_6H_5CH_2$–	Et–	2-$N_3C_6H_4OCH_2CH_2$–	6.7
1h	55– 57	$C_6H_5CH_2$–	Et–	3-$N_3C_6H_4OCH_2CH_2$–	<4
1i	120–121	4-$MeOC_6H_4$–$(CH_2)_2$–	Et–	2-$NO_2C_6H_4OCH_2CH_2$–	7.2
1j	70– 73	4-$MeOC_6H_4$–$(CH_2)_2$–	Et–	2-$N_3C_6H_4OCH_2CH_2$–	6.4
1k	108–109	3,4-$(MeO)_2C_6H_4$–$(CH_2)_2$–	Et–	2-$NO_2C_6H_4OCH_2CH_2$–	6.6
1l	oil	3,4-$(MeO)_2C_6H_4$–$(CH_2)_2$–	Et–	2-$N_3C_6H_4OCH_2CH_2$–	5.2
1m	130–132	4-HOC_6H_4–$(CH_2)_2$–	Et–	2-$NO_2C_6H_4OCH_2CH_2$–	5.3
1n	64– 68	4-$NO_2C_6H_4CH_2$–	MeS–	$CH_3CH_2OCH_2CH_2$–	7.6
1o	oil	4-$N_3C_6H_4CH_2$–	MeS–	$CH_3CH_2OCH_2CH_2$–	7.5
1p	114–115	4-$NO_2C_6H_4CH_2$–	Et–	$CH_3CH_2OCH_2CH_2$–	7.2
1q	72– 74	4-$N_3C_6H_4CH_2$–	Et–	$CH_3CH_2OCH_2CH_2$–	7.5
1r	80– 82	3-$NO_2C_6H_4CH_2$–	Et–	$CH_3CH_2OCH_2CH_2$–	6.5

We also considered the ease of radioactive synthesis in the light of commercially available labelled reagents. Previous studies of the structure/activity relationships of cyanoacrylates have demonstrated that cyanoacrylates with an unsubstituted benzylamino function can show up to 100-fold lower Hill inhibition activity than corresponding 4-chlorobenzylamino-substituted compounds [20]. However, as [^{14}C]benzylamine HCl is commercially available, and as incorporation of the amine function occurs at the last step in the synthesis, a series of benzylamino-substituted compounds was made to determine if useful Hill activity was present in benzylamino cyanoacrylates with the required nitro- or azidoaryl substituent on the phenoxyethyl ester function. The use of [^{3}H]methyl iodide, [^{3}H]dopamine and [^{14}C]tyramine could also have provided a route to radioactive cyanoacrylates so compounds that could be made from these reagents were synthesized to evaluate their Hill activities.

Once we had selected the compounds suitable for synthesis of radioactive analogues, we performed competitive binding experiments using [methyl-^{14}C]diuron and the target cyanoacrylates (unlabelled) to confirm that cyanoacrylates displace a classical-type herbicide from the PS II binding site. This also allowed indirect evaluation of the cyanoacrylate-binding parameters for comparison with the parameters obtained using radiolabelled cyanoacrylates. A photoaffinity-labelling experiment was then conducted with the selected [^{14}C]azidocyanoacrylate, using [ethyl-1-^{14}C]azidoatrazine as comparison to allow an estimate of the cyanoacrylate's efficiency as an active site probe.

Materials and Methods

Chemical synthesis

Compounds **1d–f, p** and **1r** gave satisfactory microanalyses. All compounds gave PMR spectra consistent with the assigned structures. Azido-substituted compounds gave satisfactory infra-red (in carbon tetrachloride solution) and ultra-violet (in ethanol) spectra. Recrystallizations were carried out using ethyl acetate/light petroleum (b.p. 40–60 °C), unless otherwise stated.

Synthesis of phenoxyethyl cyanoacetates

2-(2- and 3-Nitrophenoxy)ethanols ([16365-25-6] and [16365-26-7]) were synthesized [21] by condensation of the appropriate phenol with bromo-

ethanol using anhydrous potassium carbonate in refluxing acetone. 2-(4-nitrophenoxy)ethanol [16365-27-8] was synthesized [22] using sodium hydride in dimethyl formamide. Esterification of cyanoacetic acid with an equimolar proportion of the appropriate phenoxyethanol was achieved by refluxing the reagents in toluene in the presence of a catalytic amount of 4-toluenesulphonic acid, using a Dean-Stark water separator. This gave 2-(2-nitrophenoxy)ethyl cyanoacetate (67%) m.p. 51–53 °C (ethanol). PMR spectrum: δ 3.57 (s, 2H, $CNCH_2-$), 4.30–4.47 and 4.54–4.70 (m, 4H, $-(CH_2)_2-$), 6.99–7.93 (4H, aryl H). 2-(3-nitrophenoxy)ethyl cyanoacetate (86%) m.p. 66–68 °C and 2-(4-nitrophenoxy)ethyl cyanoacetate (54%) m.p. 114–116 °C (toluene) gave similar PMR spectra.

The above 2-nitro ester was converted to the corresponding 2-azido ester using standard procedures for reduction (2 atmos. H_2 with palladium on carbon) and conversion to the azide (aqueous $NaNO_2$/HCl then NaN_3), giving 2-(2-azidophenoxy)ethyl cyanoacetate as a brown oil (0.6 g, 54%).

Synthesis of phenoxyethyl 2-cyano-3-ethoxy-2-pentenoates

The required ethoxy compounds were prepared as described previously [23]. While PMR spectra were consistent with the products being predominantly the (*Z*)-isomers, in most cases a signal for the $=CCH_2$ protons in the (*E*)-isomer could also be detected approximately 2.5 ppm upfield from the corresponding (*Z*)-isomer signal. The following compounds were synthesized.

(*Z*)-2-(2-nitrophenoxy)ethyl 2-cyano-3-ethoxy-2-pentenoate (77%), m.p. 74–76 °C. UV spectrum: λ_{max} 260 (ε 20,200). PMR spectrum: δ 1.21 (t, $^3J_{H,H}$ 7.3 Hz, 3H) and 1.43 (t, $^3J_{H,H}$ 7.0 Hz, 3H, $-CH_2C\mathbf{H}_3$), 3.01 (q, $^3J_{H,H}$ 7.6 Hz, 2H, $=CCH_2$), 4.19–4.65 (m, 6H, OCH_2), 6.95–7.89 (m, 4H, aryl H). The corresponding 3-nitrophenoxyethyl compound (87%), m.p. 83–86 °C, and 4-nitrophenoxyethyl compound (81%), m.p. 139–141 °C (ethanol) gave similar PMR spectra.

For synthesis of radiolabelled **1g,** incorporation of the azide function prior to reaction with benzylamine was desired. Attempted reduction of the above 2-nitro vinyl ether was unsuccessful, but synthesis of (*Z*)-2-(2-azidophenoxy)-ethyl 2-cyano-3-ethoxy-2-pentenoate (50%) from the 2-azido ester was achieved by heating the ester with one equivalent of each of triethyl-*ortho*-propionate and acetic anhydride at 120 °C for 20 min only. The product was isolated as a yellow oil which solidified on standing after several months, m.p. 60–62 °C (aqueous ethanol). IR: −CN 2220 cm^{-1}, $-N_3$ 2115. UV spectrum: λ_{max} 258 (ε 24,500). PMR spectrum: δ 1.20 (t, $^3J_{H,H}$ 7.3 Hz, 3H) and 1.43 (t, $^3J_{H,H}$ 7.0 Hz, 3H, $-CH_2C\mathbf{H}_3$), 3.00 (q, $^3J_{H,H}$ 7.6 Hz, 2H, $=CCH_2$), 4.19–4.39 and 4.49–4.65 (m, 6H, OCH_2), 6.85–7.11 (m, 4H, aryl H).

Synthesis of 2-cyano-3,3-dimethylthio-2-propenoate

Ethoxyethyl cyanoacetate (4 g) was added to a suspension of sodium hydride (2 g) in acetonitrile. Carbon disulphide (1.5 ml) was added to the cooled suspension. After 30 min two equivalents of dimethyl sulphate (5 ml) were added and the reaction mixture was allowed to stir overnight and then washed with water, filtered dry and evaporated. The crude product was purified by chromatography, eluting with 5% acetone in methylene chloride, to give ethoxyethyl 2-cyano-3,3-dimethylthio-2-propenoate as a yellow oil (4.2 g, 65%). PMR spectrum: δ 1.21 (t, $^3J_{H,H}$ 7.0 Hz, 3H, $-CH_2C\mathbf{H}_3$), 2.61 and 2.76 (s, each 3H, $-SCH_3$), 3.57 (q, $^3J_{H,H}$ 7.0 Hz, 2H, $OC\mathbf{H}_2CH_3$), 3.69–3.77 and 4.30–4.40 (m, 4H, $-(CH_2)_2-$).

Synthesis of 4-azidobenzylamine

4-Azidobenzylamine [24] was synthesized from toluidine using a route employed previously [25] for the synthesis of 2-azidobenzylamine. *p*-Toluidine was first converted to the azide and brominated [26] using a catalytic, rather than equivalent, amount of benzoyl peroxide. This product was converted to the amine using the Gabriel synthesis.

Synthesis of phenoxyethyl and ethoxyethyl 2-cyano-3-benzylamino-2-alkenoates (cyanoacrylates)

The required cyanoacrylates were synthesized from equivalent amounts of 3-ethoxy-2-pentenoate or 3,3-dimethylthio-2-propenoate and appropriate benzylamine or phenethanamine (or amine hydrochloride and redistilled triethylamine) either by heating together at 120 °C as described previously [23] or by heating in refluxing acetoni-

trile for 2 h. Azidocyanoacrylates were generally obtained from the appropriate nitro-precursors by reduction and conversion to the azide using standard procedures. Reduction of **1n** was not achieved, so synthesis of the desired azide was achieved using azidobenzylamine. Attempted reduction of *p*-nitrophenoxyethyl esters **1a** and **1d** was also unsuccessful. Compound **1i** was prepared from 4-methoxyphenethanamine and appropriate ethoxy compound, and by methylation of **1m** with methyl iodide using either anhydrous potassium carbonate in refluxing acetone or one equivalent of sodium hydride in acetonitrile overnight at room temperature. We synthesized compounds **1a–r** (see Table I) in moderate yields, for example:

(*Z*)-2-(2-nitrophenoxy)ethyl 2-cyano-3-benzylamino-2-pentenoate **1f** (51%) m.p. 99–100 °C. UV spectrum: λ_{max} 289 (ε 23,000). PMR spectrum: δ 1.25 (t, $^3J_{H,H}$ 7.6 Hz, 3H, $-CH_2CH_3$), 2.64 (q, $^3J_{H,H}$ 7.6 Hz, 2H, $=CCH_2-$), 4.29–4.64 (m, 6H, $-OCH_2-$ and $-NCH_2-$), 6.94–7.88 (m, 9H, aryl H), 10.2 (broad s., 1H, −NH−) and

(*Z*)-2-(2-azidophenoxy)ethyl 2-cyano-3-benzylamino-2-pentenoate **1g** (72%) as a pale brown oil. IR: −CN 2206 cm^{-1}, $-N_3$ 2115 cm^{-1}. UV spectrum: λ_{max} 289 (ε 21,700). PMR spectrum: δ 1.25 (t, $^3J_{H,H}$ 7.6 Hz, 3H, $-CH_2CH_3$), 2.64 (q, $^3J_{H,H}$ 7.6 Hz, 2H, $=CCH_2-$), 4.13–4.62 (m, 6H, $-OCH_2-$ and $-NCH_2-$), 6.66–7.58 (m, 9H, aryl H), 10.3 (broad s., 1H, −NH−).

Synthesis of (Z)-2-(2-nitrophenoxy)- and (Z)-2-(2-azidophenoxy)ethyl 3-[7-^{14}C]benzylamino-2-cyano-2-pentenoates

A solution of [7-^{14}C]benzylamine hydrochloride (50 μCi, 0.87 μmol, 58 mCi/mmol, Amersham) in water containing 2% ethanol was added to a solution (100 μl, 8.7 mM) of the appropriate 3-ethoxy precursor in acetonitrile. Redistilled triethylamine (10 μl) was added and the reaction refluxed for 1.5 h. A solution of cold benzylamine hydrochloride (10 μl of 0.02 M solution, 0.2 μmol) was added and the reaction refluxed for a further 0.5 h. The solvent was removed with gentle warming under a stream of nitrogen. The residue was dissolved in methylene chloride (1 ml) and washed with water. The organic layer filtered dry and evaporated as above. The residue was dissolved in ethanol and the solution made up to a volume of 10 ml. The presence of desired product and yield were determined by UV spectroscopy. 20 μl aliquots of the solution were added to scintillant and counted to determine the radiochemical yield. (*Z*)-2-(2-nitrophenoxy)ethyl 3-[7-^{14}C]benzylamino-2-cyano-2-pentenoate (**1f**) (0.51 μmol, 59%, radiochemical yield 58%, specific activity 57 mCi/mmol) and (*Z*)-2-(2-azidophenoxy)ethyl 3-[7-^{14}C]benzylamino-2-cyano-2-pentenoate (**1g**) (0.55 μmol, 64%, radiochemical yield 33%, specific activity 30 mCi/mmol) were prepared using this procedure. These compounds were stored in solution at −18 °C until required for bioassay. They were found to be stable under these conditions for about a month.

Hill assays

These were performed using isolated pea thylakoids as described previously [9] and are reported as pI_{50} values where $pI_{50} = -\log_{10} I_{50}$ and I_{50} is the molar concentration of inhibitor giving 50% of the control rate of electron transport.

Binding assays

For competitive binding assays, isolated pea chloroplasts (chlorophyll concentration 50 μg/ml) in 1 ml phosphate buffer (0.3 M sucrose, 0.05 M potassium hydrogen phosphate, 0.01 M potassium chloride, pH 7.3) were incubated with [^{14}C]diuron (spec. activity 27.6 mCi/mmol, concentration range 0.05–0.18 μM) or [^{14}C]-**1f** of [^{14}C]-**1g** (spec. activities as synthesized above, concentration ranges 0.012–0.12 μM and 0.06–0.2 μM respectively). A second control set of tubes as above but without chloroplasts was also prepared. For indirect determination of binding parameters, a third set containing 0.1 μM unlabelled cyanoacrylate **1f** or 1 μM unlabelled **1g** as well as [^{14}C]diuron and chloroplasts was also used. For all tubes, chloroplasts were added last and assays were in duplicate. The tubes were equilibrated at room temperature under normal room light for a few minutes before the thylakoids were precipitated (microcentrifuge, 12,000 rpm). Aliquots (0.9 ml) of the supernatants were added to scintillant for counting. These data were used to calculate the free and bound concentrations of [^{14}C]inhibitors which could then be displayed as a double reciprocal (Lineweaver-Burk) plot [27]. Axis intercepts were calculated using a weighted least squares regres-

sion analysis [28]. For enzyme kinetics the horizontal axis intercept is $-1/K_m$ where, under certain conditions, K_m is the dissociation constant for an enzyme substrate complex. By analogy, the horizontal axis intercept in studies of PS II/inhibitor complexes also gives dissociation constants, K_d. These are often referred to by other authors, *e.g.* [27] as binding constants.

Photoaffinity labelling

Pea thylakoids were suspended in phosphate buffer (chlorophyll concentration 1 mg/ml, total volume 10 ml) and gently stirred in a petri dish in ice under a stream of nitrogen. A solution of ^{14}C-labelled photoaffinity-labelled Hill inhibitor was added and the suspension irradiated with ultraviolet light (wavelength 254 nm, from a UV lamp usually used for thin layer chromatography visualization) for 18 min. Aliquots (1 ml) were transferred to Eppendorf tubes and centrifuged as above. The supernatants were discarded and the pellets stored in liquid nitrogen until required for gel electrophoresis. Photoaffinity labelling was performed with azido[ethyl-1-^{14}C]atrazine (25 mCi/mmol) and [^{14}C]azidocyanoacrylate (30 mCi/mmol), final concentration for each 1 μM.

Separation of chloroplast peptides was achieved by SDS polyacrylamide gel electrophoresis on 10–16% polyacrylamide gels overlaid with a 5% stacking gel as described previously [29]. Samples and standards (Pharmacia low molecular weight calibration kit) were solubilized in 0.125 M Tris-HCl (pH 6.6), 20% glycerol, 0.002% bromophenol blue, 5% SDS, 10% mercaptoethanol at 70 °C for 3 min. Each sample lane was loaded with sample containing 25 μg chlorophyll. The gels were run until the chlorophyll pigments just reached the end of the gel. Gels were then fixed and stained as before [29], fixed (40% ethanol, 25% glacial acetic acid), soaked in enhancer (12.5 g polyphenoxazole, 125 g 2-methylnaphthalene, 700 ml ethanol and 1670 ml glacial acetic acid), rinsed in water, dried onto Whatman 3 MM paper and exposed to Fuji medical X-ray film at −80 °C for 3 months. The X-ray films were then developed automatically and scanned using a Joyce-Loebl microdensitometer.

Results and Discussion

The Hill inhibition activities of the cyanoacrylates synthesized are presented in Table I. An interesting trend may be noted in the comparison of

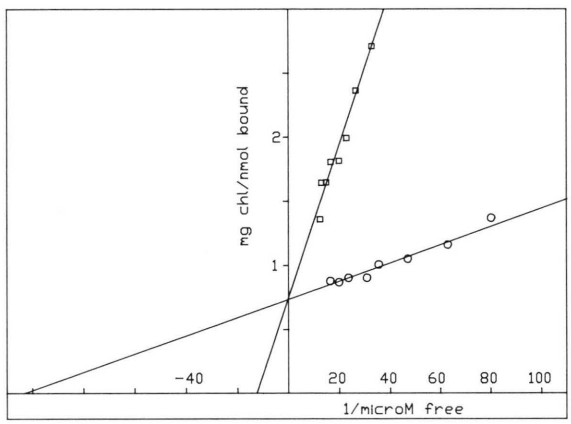


Fig. 1. Lineweaver-Burk plot of [^{14}C]diuron binding to isolated pea chloroplasts alone (open circles) and in the presence of 0.1 mM (*Z*)-2-(2-nitrophenoxy)ethyl 2-cyano-3-benzylamino-2-pentenoate (**1f**) (open squares).

Table II. Binding parameters determined directly ([^{14}C]inhibitors) and indirectly (unlabelled inhibitors displacing [^{14}C]inhibitors) and Hill inhibition activities of cyanoacrylates (*Z*)-2-(2-nitrophenoxy)ethyl 2-cyano-3-benzylamino-2-pentenoate (**1f**), (*Z*)-2-(2-azidophenoxy)-ethyl 2-cyano-3-benzylamino-2-pentenoate (**1g**) and diuron.

Compound	Binding parameters		Hill inhibition		Concentration of binding sites nmol/mg Chl
	pK_d direct	pK_d indirect	pI_{50} coupled	pI_{50} uncoupled	
Diuron	7.9	–	7.0	8.0	1.3
1f	7.7	7.5	7.1	8.2	2.1
1g	6.5	7.3	6.7	7.1	9.0

the Hill inhibition activities of the nitrophenoxyethyl ester series, where the *ortho*-substituted esters are more active than the *meta*-substituted esters, while the *para*-substituted esters are even less active. This may be explained in terms of steric hindrance to receptor protein binding by groups in the *para* position. This phenomenon has been examined in greater detail and will be discussed elsewhere [30]. On the basis of Hill activity and ease of synthesis, radioactive analogues of **1f** and **1g** were synthesized.

As expected, cyanoacrylate **1f** showed competitive binding with [^{14}C]diuron (see Fig. 1), and **1g** behaved similarly. The binding parameters obtained in both competitive binding of unlabelled cyanoacrylates and direct binding of labelled analogues are presented in Table II. The values for the dissociation constants of the inhibitor/PS II complex for diuron and the cyanoacrylates are of the same order as the Hill inhibition values obtained under uncoupled conditions, indicating a probable 1:1 relationship between the binding of a cyanoacrylate molecule and inhibition of electron transport. While there is good agreement between the binding parameters for **1f** determined directly and indirectly, the values obtained for **1g** (particularly the value for the concentration of binding sites) by these two procedures do not agree. A similar phenomenon was noted for the binding of azidomonuron [19], where this anomaly was attributed to non-specific binding of the azido-substituted inhibitor which complicated interpretation of the double reciprocal plots. We obtained similar values for the binding parameters of [^{14}C]azidocyanoacrylate for experiments carried out in dim room light or in the dark, demonstrating that the interference was not due to light-activated binding of the azide function.

Microdensitometer traces of the fluorogram obtained from the SDS-polyacrylamide gel of thylakoid peptides with azidoatrazine and azidocyanoacrylate are shown in Fig. 2. As expected, azidoatrazine labels a peptide in the 32 kD mass region. Labelling in the 28 kD mass region is also observed, probably due to binding to the protein-rich light harvesting chlorophyll/protein complex band as has been noted by others [15, 31]. [^{14}C]Azidocyanoacrylate also shows tagging in the 28 kD and 32 kD region. No binding in the 40–45 kD region was detected, though considering the low density of the fluorogram for this compound, this observation does not preclude the presence of binding in this region. For this compound the predominant radioactive band was observed in a region near the front of the gel associated with rapidly moving pigments. This phenomenon has also been noted previously [32, 33] and is probably due to lipid or pigment-bound or non-covalently bound azidocyanoacrylate derivative. The observation of only very weak binding of the photoaffinity-labelled cyanoacrylate is disappointing. Previous structure/activity studies of cyanoacrylates have provided evidence for the presence of a hydrophilic interface within a lipophilic domain in the ester binding re-

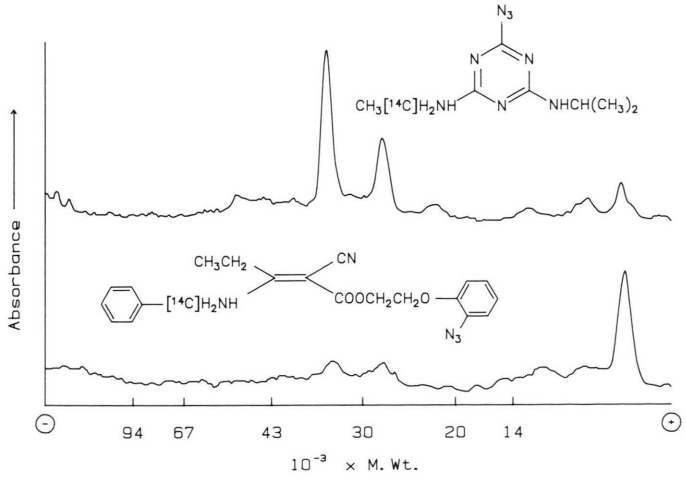


Fig. 2. Microdensitometer traces of fluorogram of SDS-PAGE gel of thylakoid peptides labelled with [^{14}C]azidoatrazine (upper trace) and (*Z*)-2-(2-azidophenoxy)ethyl 3-[7-^{14}C]-benzylamino-2-cyano-2-pentenoate (**1g**) (lower trace).

gion of the cyanoacrylate binding site [34]. If this interface is with water or if the lipophilic binding domain lies in the lipid membrane then there may be no protein close enough for efficient reaction with the activated azide. The azide then tags lipid fragments or, because it is an *ortho*-substituent, reacts intramolecularly. These low molecular weight products then move at the front of the gel. Therefore, although some specific labelling in the 32 kD region was observed, it is unlikely that this compound would make an efficient active site probe at the subpeptide level. The corresponding *meta*- and *para*-substituted compounds were less active as Hill inhibitors than the *ortho* compounds, so they are also not suitable. Compounds **1o** and **1q** are active and should be more effective as active site probes, once synthetic routes for radioactive analogues have been developed.

Acknowledgements

We thank Dr. J. M. Anderson for the use of gel electrophoresis equipment and Ms. S. Hossack-Smith and Ms. C. Edgerly for expert technical assistance. We thank Mr. L. McCaffery for synthesis of compounds **1c** and **1d.** We also thank Dr. J. L. Huppatz for helpful discussions. The financial support of the Australian Reserve Bank Rural Credits Research Fund is gratefully acknowledged.

[1] J. Rochaix and J. Erickson, Trends Biol. Sci. **13,** 56–59 (1988).
[2] P. K. Wolber and K. E. Steinback, Z. Naturforsch. **39c,** 425–429 (1984).
[3] P. K. Wolber, M. Eilmann, and K. E. Steinback, Arch. Biochim. Biophys. **248,** 224–233 (1986).
[4] R. Dostatni, H. E. Meyer, and W. Oettmeier, FEBS Lett. **239,** 207–210 (1988).
[5] W. Oettmeier, K. Masson, J. Höhfeld, and H. Meyer, Z. Naturforsch. **44c,** 444–449 (1989).
[6] J. Deisenhofer, O. Epp, K. Miki, R. Huber, and H. Michel, J. Mol. Biol. **180,** 385–398 (1984).
[7] H. Michel, O. Epp, and J. Deisenhofer, EMBO J. **5,** 2445–2451 (1986).
[8] H. Michel and J. Deisenhofer, Biochemistry **27,** 1–7 (1988).
[9] J. L. Huppatz and J. N. Phillips, Z. Naturforsch. **42c,** 684–689 (1987) and references cited therein.
[10] A. Trebst, Z. Naturforsch. **42c,** 742–750 (1987).
[11] J. N. Phillips and J. L. Huppatz, Z. Naturforsch. **42c,** 670–673 (1987).
[12] J. N. Phillips and J. L. Huppatz, Advs. in Photosynth. Res. **IV,** 1–4 (1984).
[13] W. F. J. Vermaas, Thesis, Landbouwhogeschool Wageningen (1984).
[14] A. Trebst and W. Draber, Photosynth. Res. **10,** 381–396 (1986).
[15] U. Bühmann, E. C. Herrmann, C. Kötter, A. Trebst, B. Depka, and H. Wietoska, Z. Naturforsch. **42c,** 704–712 (1987).
[16] G. Gardner, Science **211,** 937–940 (1981).
[17] K. Pfister, K. E. Steinback, G. Gardner, and C. J. Arntzen, Proc. Natl. Acad. Sci. U.S.A. **78,** 981–985 (1981).
[18] W. Oettmeier, K. Masson, H. J. Soll, and W. Draber, Biochim. Biophys. Acta **767,** 590–595 (1984).
[19] A. Boschetti, M. Tellenbach, and A. Gerber, Biochim. Biophys. Acta **810,** 12–19 (1985).
[20] J. L. Huppatz and J. N. Phillips, Z. Naturforsch. **42c,** 679–683 (1987).
[21] C. F. H. Allen and J. W. Gates, Org. Synth. Coll. **Vol. 3,** 140–141 (1955).
[22] I. G. C. Coutts, N. J. Culbert, M. Edwards, J. A. Hadfield, D. R. Musto, V. H. Pavlidis, and D. J. Richards, J. Chem. Soc. Perkin Trans. I **1985,** 1829–1836.
[23] J. L. Huppatz, J. N. Phillips, and B. M. Rattigan, Agric. Biol. Chem. **45,** 2769–2773 (1981).
[24] P. I. Pozdnyakov, N. I. Sokolova, and Z. A. Shabarova, Zh. Obschei. Khim. **46,** 2743–2744; Engl. trans. 2617–2618 (1976).
[25] P. A. S. Smith, G. F. Budde, and S. P. Chou, J. Org. Chem. **50,** 2062–2066 (1985).
[26] D. H. R. Barton, P. G. Sommes, and G. G. Weingarten, J. Chem. Soc. (C) **1971,** 721–736.
[27] W. Tischer and H. Strotmann, Biochim. Biophys. Acta **460,** 113–125 (1977).
[28] G. N. Wilkinson, Biochem. J. **80,** 324–332 (1961).
[29] J. M. Anderson, Biochim. Biophys. Acta **806,** 145–153 (1985).
[30] J. L. Huppatz, H. G. McFadden, and L. McCaffery, Z. Naturforsch., submitted.
[31] W. Oettmeier, K. Masson, and U. Johanningmeier, Biochim. Biophys. Acta **679,** 376–383 (1982).
[32] W. Oettmeier, K. Masson, and U. Johanningmeier, FEBS Lett. **118,** 267–270 (1980).
[33] U. Johanningmeier, E. Neumann, and W. Oettmeier, J. Bioenerg. Biomembr. **15,** 44–66 (1983).
[34] J. N. Phillips and J. L. Huppatz, Agric. Biol. Chem. **48,** 55–58 (1984).