X-Ray Structure Analysis of a Cyanoacrylate Inhibitor of Photosystem II Electron Transport*

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X-ray crystallographic data for the highly potent cyanoacrylate photosynthetic electron transport inhibitor, (*Z*)-ethoxyethyl 3-(4-chlorobenzylamino)-2-cyano-4-methylpent-2-enoate, are presented. This compound has a particularly high affinity for the photosystem II (PS II) herbicide receptor with a pI_{50} value of 9.5 (in the Hill reaction under uncoupled conditions with a chlorophyll concentration of 0.1 μg/ml). Data regarding the structure of small ligands, such as this potent cyanoacrylate, which bind to the site with high affinity may be used to provide the basis for modelling studies of PS II/herbicide complexes. The X-ray data presented confirm the *Z*-stereochemistry of active cyanoacrylates and demonstrate the presence of a planar core stabilized by an intramolecular hydrogen bond between the ester carbonyl oxygen and a benzylamino hydrogen atom. In order to assess the importance of the benzylamino –NH– group in this type of cyanoacrylate, analogues containing a methylene group in its place were synthesized and found to be 100- and 1000-fold less active as Hill inhibitors.

Introduction

The nature and topography of the herbicide binding site in the reaction centre of photosystem II (PS II) have been the subject of intense interest over many years. Several commercially important herbicides of differing structural types are known to disrupt photosynthetic electron transport at a common binding domain on the 32 kDa D_1 polypeptide of the PS II reaction centre [1]. X-ray crystallography has revealed the orientation of a triazine herbicide in the reaction centre of *Rhodopseudomonas viridis* [2]. However, despite significant homology in the amino acid sequences of the corresponding polypeptides in PS II, many herbicides are inactive in the bacterial system. Although current models of the PS II reaction centre account for many of the properties of the PS II herbicide binding domain, the precise architecture of the site is still a subject for speculation. The cyanoacrylate PS II inhibitors have proved useful as probes [3] of this receptor because their activities as inhibitors of photosynthetic electron transport in the Hill reaction are very sensitive to structural variations. Some of these compounds, for example (*Z*)-ethoxyethyl 3-(4-chlorobenzylamino)-2-cyano-4-methylpent-2-enoate (**1a**), are very highly active, indicating a high binding affinity for the receptor site. As with many inhibitor/receptor systems, the preferred conformation of the cyanoacrylate inhibitor molecule may provide insights into the topography of the receptor surface. Therefore the structure of **1a** was determined by X-ray crystallography and possible features of the interaction of this molecule with the receptor inferred. Although X-ray crystallography is a precise tool for determining the structure of compounds in a crystal lattice, the conformation of compounds in aqueous solution, in membranes or on protein surfaces may be different because molecules may adopt higher energy conformations to match the steric requirements of the site. The conformation of the site may also be deformed to accommodate bound inhibitors. However, the crystal structure provides a valuable starting point for further conformational and molecular orbital analysis of compounds of this type.

The crystal structure of this compound was found to contain a cyclic core with a hydrogen bond between the ester carbonyl and benzylamino –NH– groups. Therefore pairs of benzylamino (**1**) and analogous aralkyl cyanoacrylates (**2**) were synthesized in order to assess the influence of the benzylamino –NH– function on the structure and Hill inhibition activity of cyanoacrylates.

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Materials and Methods

PMR spectra were obtained using a JEOL FX90Q NMR spectrometer in $CDCl_3$ with tetramethylsilane as internal standard. Infra-red spectra were determined in CCl_4 solution using an Hitachi 27–30 spectrometer. Satisfactory microanalyses were obtained for all novel compounds.

Hill assays

These were performed as described previously [4] (chlorophyll concentration 8 μg/ml) and are reported 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. Compound **1a** was also assayed under uncoupled conditions using a chlorophyll concentration of 0.1 μg/ml.

Chemical synthesis

Ethoxyethyl 3-(4-chlorobenzylamino)-2-cyano-4-methylpent-2-enoate (**1a**) was synthesized as follows. Ethoxyethyl cyanoacetate was first acylated using a modification of a known procedure [5]. The ester (7.8 g) and redistilled triethylamine (10.1 g) were mixed in dry acetonitrile (50 ml) and anhydrous magnesium chloride (4.8 g) added. The mixture was stirred, protected from moisture, at room temperature for 15 min. *Iso*-butyryl chloride (5.3 g) was added dropwise and stirring continued overnight. 5 N HCl (100 ml) was added followed by water (100 ml) and the mixture extracted with ether (2 × 100 ml). The ether layers were dried (Na_2SO_4) and evaporated and the residue distilled giving predominantly (*Z*)-ethoxyethyl 2-cyano-4-methyl-3-oxopentanoate (8.2 g, 72%, b.p. 92–95° (0.01 mm Hg)). PMR spectrum: δ 1.25 (d, $^3J_{H,H}$ 6.7 Hz, 6H, CH_3-), 2.97–3.40 (m, 1H, $-CHMe_2$), 3.41–3.84 (m, 7H, $-CH_2OCH_2CH_3$), 4.32–4.51 (m, 2H, $-COOCH_2-$). This intermediate was added dropwise in ether solution to an ice-cooled ethereal solution containing 3-fold excess diazomethane. After 30 min, remaining diazomethane was quenched with acetic acid, the volatiles were removed and the residue distilled giving (*Z*)-ethoxyethyl 2-cyano-3-methoxy-4-methylpent-2-enoate (6.9 g, 80%, b.p. 107–110°(0.01 mm Hg)). PMR spectrum similar to that for the above compound, but in addition δ 4.33 (s, 3H, $-OCH_3$, (*Z*)-isomer, 70%), 4.03 (s, 3H, $-OCH_3$, (*E*)-isomer, 30%). This vinyl ether (1.8 g) was heated with 4-chlorobenzylamine (1.0 g) at 120° for 1 h as described previously [6] to give the desired product, (*Z*)-ethoxyethyl 3-(4-chlorobenzylamino)-2-cyano-4-methylpent-2-enoate (**1a**) (1.6 g, 60%, m.p. 126–128° (EtOAc/light petroleum b.p. 40–60)). PMR spectrum: δ 1.20 (t, $^3J_{H,H}$ 7.0 Hz, 3H, ester-CH_3), 1.21 (d, $^3J_{H,H}$ 7.3 Hz, 6H, *iso*-propyl-CH_3), 3.06–3.30 (m, 1H, *iso*-propyl-CH), 3.40–3.75 (m, 4H, $-OCH_2-$), 4.29 (t, $^3J_{H,H}$ 5.7 Hz, 2H, $-COOCH_2-$), 4.58 (d, $^3J_{H,H}$ 6.1 Hz, 2H, $-NCH_2-$), 7.00–7.45 (m, 4H, aryl-H), 10.6 (broad s, 1H, NH). The compound was recrystallized from aqueous ethanol for X-ray crystallography. Cyanoacrylates (**1b–e**) were prepared similarly from appropriate vinyl ethers [6].

Cyanoacrylate analogues (**2**) were synthesized by condensation of appropriate ketones with ethoxyethyl cyanoacetate as described previously [7]. The required ketones were prepared by alkylation of ethylacetoacetate followed by hydrolysis and decarboxylation using the following procedure. Sodium hydride (0.8 g) (60% oil suspension) was washed with light petroleum (b.p. 40–60) to remove oil and suspended in toluene (50 ml). Ethyl acetoacetate (2.6 g) dissolved in toluene (10 ml) was added dropwise to the sodium hydride suspension. When evolution of hydrogen ceased, after about 20 min, 3-bromobenzylbromide was added and the mixture refluxed overnight. The reaction mixture was cooled, filtered to remove sodium bromide, and evaporated. A solution of 10% potassium hydroxide in 5% aqueous ethanol was added to the residue and the mixture was stirred at room temperature overnight. It was then acidified cautiously with concentrated HCl when immediate evolution of carbon dioxide was observed. The product was extracted into methylene chloride. The organic layer was separated, filtered dry and evaporated giving 4-(3-bromophenyl)-2-butanone [3506-70-5] as a colourless oil (3.7 g, 80%). The ketone, ethoxyethyl cyanoacetate (2.5 g), piperidine (0.1 ml) and glacial acetic acid (1 ml) were refluxed in 100 ml toluene overnight using a Dean-Stark water separator. The reaction mixture was washed with water and the organic layer separated, dried and evaporated. The crude product was purified by chromatography to give (*E*)- and (*Z*)-ethoxyethyl 2-cyano-3-methyl-5-(3-bromophenyl)-2-pentenoate (isomer ratio 1:1) as a colourless oil (3.8 g,

68%). PMR spectrum: δ 1.21 (t, $^3J_{H,H}$ 7.3 Hz, 3H, $-CH_2CH_3$), 2.28 and 2.39 (both s, together 3H, $=CCH_3$, (*Z*)- and (*E*)-isomers respectively), 2.80–3.10, 3.44–3.77, 4.28–4.48 (all m, 10H, $-CH_2-$), 7.09–7.43 (m, 4H, aryl-H).

1-(4-Chlorophenyl)-3-pentanone [95416-62-9] was synthesized similarly from methyl 3-oxypentanoate (1.3 g) and 4-chlorobenzyl bromide (2 g) and condensed with ethoxyethyl cyanoacetate (1 g) as above to give (*E*)- and (*Z*)-ethoxyethyl 2-cyano-5-(4-chlorophenyl)-3-ethyl-2-pentenoate as a colourless oil (15 g, 64%), isomer ratio not discernible from PMR but presumed to be 1:1). PMR spectrum: δ 1.00–1.40 (overlapping triplets, 6H, $-CH_2CH_3$), 2.26–2.97, 3.42–3.97 and 4.28–4.43 (all m, 12H, $-CH_2-$), 7.02–7.40 (m, 4H, aryl-H).

Structure determination

Reflection data were measured with an Enraf-Nonius CAD-4 diffractometer in θ/2θ scan mode using graphite monochromatized molybdenum radiation (λ 0.7107 Å). Further details of data collection and additional data are presented in the Appendix (for lodgment with the journal as supplementary material).

Results and Discussion

Crystallography

Crystal data for **1a**. $C_{18}H_{23}ClN_2O_3$, M 350.8, monoclinic, space group $P2_{1/c}$, a = 14.875(8), b = 9.253(3), c = 15.768(6) Å, β = 120.33(2)°, V = 1873(1) Å^3, D_c = 1.24 g cm^{-3}, Z 4, μ_{Mo} = 2.18 cm^{-1}. The determined structure of **1a** is shown in Fig. 1. Coordinates for non-hydrogen atoms are given in Table I and selected bond lengths are given in Table II.

Table I. Atomic coordinates for non-hydrogen atoms of **1a.** Esd* in parentheses. B_{eq} (Å^2) is the isotropic equivalent of the anisotropic temperature factor.

Atom	No.	*x*	*y*	*z*	B_{eq}
Cl	1	0.8821(1)	0.5357(1)	1.0336(1)	7.61(5)
O(1)	2	0.4499(2)	0.6313(3)	0.5892(2)	5.16(9)
O(2)	3	0.2896(2)	0.6814(2)	0.4660(1)	4.76(9)
O(3)	4	0.1110(2)	0.4999(3)	0.4120(2)	5.73(10)
N(1)	5	0.6051(2)	0.7070(3)	0.5654(2)	4.49(11)
N(2)	6	0.2894(2)	0.8424(3)	0.2777(2)	6.05(13)
C(1)	7	0.8378(2)	0.5914(4)	0.9134(2)	4.59(13)
C(2)	8	0.8211(3)	0.4909(4)	0.8436(3)	5.25(15)
C(3)	9	0.7845(3)	0.5359(4)	0.7479(2)	5.05(15)
C(4)	10	0.7629(2)	0.6800(4)	0.7225(2)	4.19(13)
C(5)	11	0.7803(2)	0.7792(4)	0.7945(3)	4.67(14)
C(6)	12	0.8179(3)	0.7357(4)	0.8904(2)	4.90(14)
C(7)	13	0.7193(2)	0.7251(4)	0.6168(2)	5.40(14)
C(8)	14	0.5357(2)	0.7460(3)	0.4748(2)	3.69(13)
C(9)	15	0.5761(2)	0.8038(3)	0.4104(2)	4.28(13)
C(10)	16	0.5312(3)	0.7238(4)	0.3130(2)	6.14(16)
C(11)	17	0.5628(3)	0.9666(4)	0.3977(3)	7.30(22)
C(12)	18	0.4298(2)	0.7350(3)	0.4432(2)	3.69(12)
C(13)	19	0.3526(3)	0.7934(3)	0.3507(3)	4.33(14)
C(14)	20	0.3942(3)	0.6778(3)	0.5065(2)	4.15(14)
C(15)	21	0.2519(3)	0.6381(4)	0.5308(2)	5.68(16)
C(16)	22	0.1360(3)	0.6251(5)	0.4705(3)	6.08(18)
C(17)	23	0.0015(3)	0.4843(6)	0.3467(3)	7.89(20)
C(18)	24	−0.0197(3)	0.3590(6)	0.2819(3)	9.23(23)

* Esd, Estimated standard deviation (of the least significant digit).

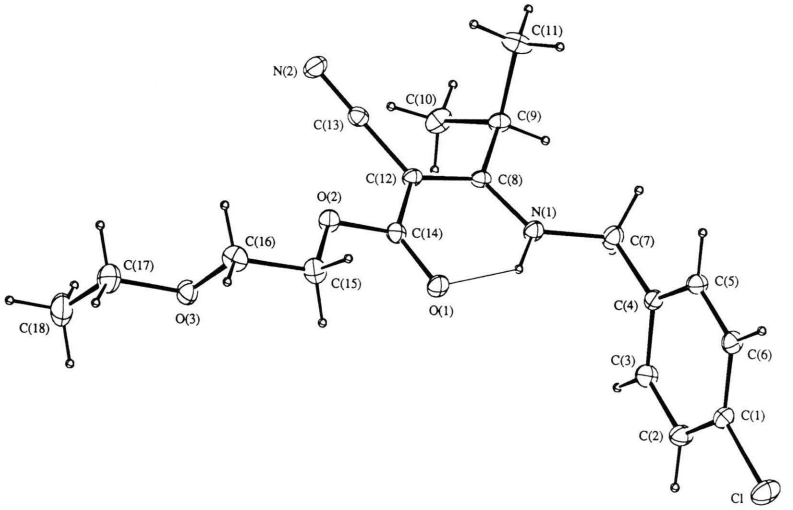


Table II. Selected bond lengths (Å) for **1a.** Esd in parentheses.

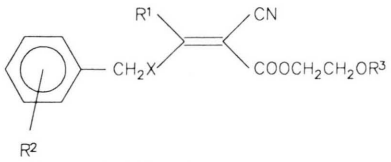
Bond	Bond length
C(7)–N(1)	1.476(4)
N(1)–C(8)	1.322(3)
C(8)–C(9)	1.515(4)
C(9)–C(10)	1.522(4)
C(9)–C(11)	1.519(5)
C(8)–C(12)	1.396(4)
C(12)–C(13)	1.430(4)
C(13)–N(2)	1.150(4)
C(12)–C(14)	1.446(4)
C(14)–O(1)	1.217(3)
C(14)–O(2)	1.351(4)
C(15)–O(2)	1.447(3)

The main feature of note in the structure of **1a** (Fig. 1) is the delocalization of π electrons around the double bond (C(8)–C(12)) giving a six-membered ring structure that incorporates a hydrogen bond between the benzylamino –NH– (N(1)) and the ester carbonyl oxygen (O(1)). Delocalization is indicated by the bond lengths for N(1)–C(8) and C(12)–C(14), which are shorter than expected for pure single bonds (compare C(7)–N(1) and C(8)–C(9) respectively (Table II), and for C(8)–C(12) which is long for a double bond. The central core of the molecule is largely planar, with the maximum deviation from the least squares plane comprising C(7)–N(1)–C(8)–C(12)–C(14)–O(1) being only 0.05 Å.

Another feature of note in the X-ray structure of **1a** is the orientation of the *iso*-propyl group with the two methyl groups oriented away from the benzylamino function. In a previous study [10] it was found that replacement of the *iso*-propyl group in a close analogue of **1a,** namely **1b,** with a *tert*-butyl group to give **1c,** resulted in a 10,000-fold drop in Hill inhibition activity (Table III). This was taken to imply extremely adverse steric interference in this region of the site in that an additional methyl group virtually abolished activity. Careful re-examination of infra-red and NMR spectra for **1c** reveals that this compound is in fact the alternative (*E*)-isomer. Evidence for this is provided by the higher frequency of the NH and carbonyl stretching signals [8] in the infra-red spectrum consistent with an absence of hydrogen bonding and π delocalization, and in the shift of the $-NCH_2-$ signal in the proton NMR spectrum from a clearly defined doublet at δ 4.57 to an obscured signal in the region δ 4.24–4.45. Thus the bulk of the *tert*-butyl group is sufficient to destabilize the highly active (*Z*)-configuration giving the (*E*)-isomer which obviously cannot conform to the steric requirements imposed by the receptor. Although it is therefore tempting to speculate that the spatial orientation of the *iso*-propyl group in **1a,** as determined by X-ray crystallography, conforms closely to the topography of the site, it is not possible to test this hypothesis because the (*Z*)-isomer bearing a β-*tert*-butyl group is too unstable for synthesis and testing under normal conditions. However, we can conclude that active cyanoacrylates are in the (*Z*)-conformation when bound to the receptor site.

It is possible that the planar hydrogen-bonded core revealed by the X-ray structure is also present when the compound is in solution and on the surface of the protein, but one of the main mechanisms of D_1 peptide-ligand interactions for other herbicides is believed to be hydrogen bonding to serine 264 or histidine 215 [9]. Therefore, in order to assess the importance of the benzylamino –NH– group in cyanoacrylate-peptide interactions, the effect of replacing this –NH– group with a methylene ($-CH_2-$) group on Hill inhibition activity and compound structure was examined. Two pairs of structures, analogous except for the replacement of the –NH– (**1d** and **1e**) with $-CH_2-$ (**2a** and **2b**) were synthesized and tested as Hill inhibitors. Physical data and Hill inhibition activities for the compounds tested are presented in Table III. The methylene analogues **2a** and **2b** are oils, precluding the use of X-ray crystallography. Comparison of the infra-red stretching frequencies for the ester carbonyls reveals that the frequencies for the benzylamino compounds **1d** and **1e** are considerably lower than those for the methylene analogues **2b** and **2c,** reflecting a greater degree of π delocalization and hydrogen bonding in **1d** and **1e.** The activities of the methylene analogues **2a** and **2b** are 100- and 1000-fold lower than that of the respective benzylamino compounds. A similar reduction in Hill inhibition activity was noted previously [7] when the alkylamino –NH– group in ethoxyethyl 2-cyano-3-octylaminopropenoate was replaced with a methylene group.

Table III. Physical data for cyanoacrylates (**1a–1e**), and methylene analogues (**2a** and **2b**).



Compd No	X	R^1	R^2	R^3	m.p./b.p.	$\nu_{C=O}$ (cm^{-1})	ν_{CN} (cm^{-1})	ν_{NH} (cm^{-1})	pI_{50}
1a	NH	Pr^i	4-Cl	Et	126-128	1662	2207	3100	9.5*
1b	NH	Pr^i	4-Cl	Me	ref[10]	1662	2204	3212 3140	8.2
1c	NH	Bu^t	4-Cl	Me	ref[10]	1694	2200	3440 3340	4.3
1d	NH	Me	3-Br	Et	79-81	1665	2208	3260 3200	7.2
2a	CH_2	Me	3-Br	Et	204-206**	1728	2225	-	4.9
1e	NH	Et	4-Cl	Et	51-54	1664	2208	3210 3140	8.1
2b	CH_2	Et	4-Cl	Et	178-182**	1725	2220	-	5.1

* Hill inhibition determined under uncoupled conditions, chlorophyll = 0.1 μg/ml. Under high chlorophyll concentrations (as used for the other compounds) the limiting value of 8.2 was obtained.
** B.p. determined at 0.5 mm · Hg.

The presence of 50% of the inactive (*E*)-isomer in the methylene compounds **2a** and **2b** will contribute to the reduced Hill inhibition activities observed. However, 50% of an inactive compound will only cause a reduction in Hill inhibition activity of 0.3 ($\log_{10}2$) units, considerably less than the differences observed here. The reduction in Hill inhibition activity in the methylene analogues may be because the $-CH_2-$ group has reduced participation in π delocalization and because it does not participate in hydrogen bonding. Thus the main factors contributing to stabilization of the planar six-membered core of the molecule are removed. The stabilized core structure may be responsible for the very high affinity of benzylamino cyanoacrylates for the receptor peptide and disruption of this stabilization may account for the reduced Hill inhibition activities of the methylene analogues. Alternatively, absence of an $-NH-$ group may result in the absence of an important peptide-ligand hydrogen bond which reduces the strength of the interactions between the inhibitor and the binding site. If hydrogen bonding is important for cyanoacrylate binding to the D_1 peptide, it may not be *via* the ester carbonyl, as this structural feature is present in both active cyanoacrylates and less active methylene analogues. This observation is inconsistent with the observations of Bühmann *et al.* [9] who propose that ester carbonyl-serine 264 interaction is important in cyanoacrylate/D_1 binding. It therefore appears that more work is required before it is possible to determine the precise nature of cyanoacrylate binding to the PS II herbicide binding site.

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