Cyanoacrylate Inhibitors of the Hill Reaction
V. The Effect of Chirality on Inhibitor Binding

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Inhibitors of Electron Transport, Hill Reaction, Cyanoacrylates, Optically Active Benzylamino Derivatives

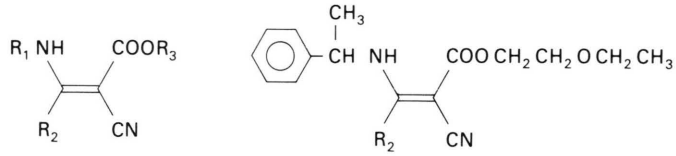
Optically active α-methylbenzylamino 2-cyanoacrylic esters were synthesized and assayed as inhibitors of the Hill reaction in isolated pea chloroplast fragments. The *S*-isomers were more potent inhibitors than the *R*-isomers with discriminations of from ten to greater than 100-fold being observed. A β-alkyl substituent in the cyanoacrylate molecule affected both the level of activity and the difference in activity between the isomers. An α,α-dimethylbenzylamino derivative was also active at about the same level as the corresponding α-methylbenzylamino racemate. This result could be explained in terms of the orientation of the phenyl ring in the receptor site.

Replacement of the α-methylbenzylamino group by other α-alkyl and α-phenyl substituents had little effect on activity. However, an α-benzyl group was beneficial.

Introduction

Recent developments in the study of the photosynthetic electron transport pathway have resulted in an increased knowledge of the mode of action of photosystem II (PS II) inhibitor herbicides at the molecular level [1, 2]. Binding studies have shown that amide-type PS II inhibitors, such as the ureas, the triazines and the uracils, displace a plastoquinone electron acceptor from its binding site on a 32 kDa membrane protein, designated the Q_B or herbicide binding protein, in the PS II complex of chloroplasts [3, 4]. Some insight into the secondary and tertiary structure of Q_B is beginning to emerge [5] and this, coupled with a knowledge of the structure-activity requirements of inhibitor molecules, should lead to the directed synthesis of a new generation of PS II inhibitor herbicides.

2-Cyanoacrylates of general formula **1** are potent PS II inhibitors and appear to act at a receptor site common to the amide-type class of inhibitors [6]. These compounds are particularly suited to probing the topography of the binding site on the Q_B protein since their activity in blocking the Hill reaction in isolated chloroplast fragments was found to be extremely sensitive to minor structural variation [7–12]. The effect of an ether linkage in the ester side-chain (R_3) in enhancing inhibition and the steric effect of an alkyl substituent (R_2) at the β-carbon have been described previously [9, 11, 12]. An investigation of the hydrophobic requirements of the binding domain revealed the high potency resulting from the inclusion of a 4-chlorobenzylamino group (R_1 = 4–Cl–$C_6H_4CH_2$) in the molecule [12]. Compounds bearing a benzylamino or substituted benzyl-



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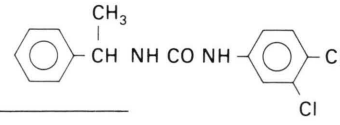

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amino substituent appear to have different binding characteristics to corresponding alkylamino (*e.g.* $R_1 = C_8H_{17}$) derivatives, where interaction with the lipid phase of the binding domain appears to be relatively non-specific depending only on the lipophilicity of the substituent (R_1) attached to nitrogen [9]. The benzyl methylene group was thought to allow the steric mobility necessary for the aryl group to interact with a specific lipophilic region of the binding site [12].

If indeed steric factors are involved in the binding of benzylamino 2-cyanoacrylates, then the replacement of the benzylic methylene group by a group conferring chirality on the molecule (*e.g.* **2**) may prove instructive in revealing the architecture of this region of the binding domain. Compounds containing an optically active α-methylbenzylamino moiety have been examined previously for their effects on, *inter alia,* photosynthetic electron transport. Moreland and Boots [13] studied the *R*- and *S*-isomers and the racemate of 1-(α-methylbenzyl)-3-(3,4-dichlorophenyl)urea (**3**) and found that the PS II receptor was able to discriminate between the enantiomers, with the *S*-isomer being the more active.

2-Cyanoacrylates incorporating the same chiral centre were therefore prepared and their effect on photosynthetic electron transport in isolated pea chloroplast fragments was assessed. For each optical isomer and the racemate, substitution at the β-carbon was varied, the hydrogen atom (**2**, $R_2 = H$) being successively replaced by an alkyl substituent of increasing carbon chain length (**2**, $R_2 = C_1$ to C_4), to determine whether the effect on Hill activity of the size of the β-substituent was similar to that observed with the benzylamino and 4-chlorobenzylamino series examined previously [12]. As in earlier studies [11, 12], all compounds synthesized included an ethoxyethyl ester function, a particularly favourable structural feature for receptor binding [9].

Materials and Methods

Compounds recorded in Tables I and II gave satisfactory analytical data and structures were confirmed by analysis of PMR spectra which were recorded on a Jeol FX90Q spectrometer using TMS as internal standard and $CDCl_3$ as solvent. Optical rotations were recorded on a Bellingham and Stanley polarimeter in methanol.

S(−)-α-Methylbenzylamine ($[\alpha]_D^{20}$ − 30 ± 2°, *c* = 10, EtOH) and the corresponding *R*(+)-isomer ($[\alpha]_D^{20}$ + 30 ± 2°, *c* = 10, EtOH) were purchased commercially (Fluka) and used without further purification.

Compounds **4**–**8** (the *S*- and *R*-isomers and the *RS*-racemate) were prepared by heating the appropriate ethoxy- or methoxymethylene cyanoacrylate [9, 11] with the appropriate α-methylbenzylamine at 130–140° for 1 h. The products were purified by distillation *in vacuo* or by crystallization from ethyl acetate–petroleum ether (b.p. 40–60°).

Compounds **9** [12], **13** and **14** were prepared from ethoxyethyl 3-ethoxy-3-ethyl-2-cyanoacrylate and benzylamine, diphenylmethylamine and 1,2-diphenylethylamine respectively, as described above.

Compounds **10**–**12** were prepared similarly from the appropriate α-substituted benzylamines. These compounds were obtained by lithium aluminium hydride reduction of the corresponding oximes [14, 15].

Compound **15** was obtained as follows. Ethoxyethyl 3-ethoxy-3-ethyl-2-cyanoacrylate (1.2 g) and 2-phenyl-2-propylamine [16] (0.7 g) in toluene (15 ml) were boiled together under reflux for 24 h. Removal of the solvent afforded a brown oil, which was carefully chromatographed on silica gel using chloroform as eluent. Evaporation of appropriate fractions gave the product as a pale yellow oil, which solidified. Crystallization from ethyl acetate–petroleum ether (b.p. 40–60°) gave compound **15** as colourless plates (0.35 g, 21%) m.p. 93–94°. PMR spectrum: δ 0.96 (t, 3H, β−$CH_2\underline{CH_3}$), 1.23 (t, 3H, $OCH_2\underline{CH_3}$), 1.76 (s, 6H, α,α−$(CH_3)_2$), 2.17 (q, 2H, β−$\underline{CH_2}CH_3$), 3.60 (q, 2H, $O\underline{CH_2}CH_3$), 3.65–3.81 (m, 2H, $COOCH_2\underline{CH_2}O$−), 4.25–4.41 (m, 2H, $COO\underline{CH_2}CH_2O$), 7.20–7.50 (m, 5H, aromatic), 10.66 (br, s, 1H, NH).

The urea derivatives **3** were prepared according to Moreland and Boots [13]. Crystallization from ethyl acetate gave the racemate (m.p. 151–153°), the *S*- (m.p. 174–175°) and *R*-isomers (m.p. 174–175°) as colourless needles (Moreland and Boots [13] reported m.p. 149–150°, 171–172° and 171–172° respectively).

Hill reaction assay

Compounds were assayed for inhibition of the Hill reaction using chloroplast fragments isolated from the leaves of 21 day-old plants of *Pisum sativum* (c.v. Victory Freezer), the electron acceptor being the indicator dye 2,3′,6-trichlorophenolindophenol. The

experimental procedure was as described elsewhere [17], with the chlorophyll concentration routinely set at about 8 μg/ml. The uncoupled reactions were performed with 4 mM ammonium chloride in the assay medium. The activity was expressed in terms of pI_{50}, *i.e.*, $-\log_{10} I_{50}$, where I_{50} was the molar concentration required to decrease the rate of dye reduction under illumination of saturating intensity to 50% that obtained in the absence of the compound.

The coupled and uncoupled pI_{50} values recorded in Tables I and II are the mean of at least three separate determinations. The variation in pI_{50} among experiments was less than ± 0.2 for each compound.

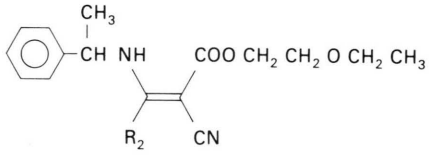
Results

The potency of the *S*- and *R*-isomers and the *RS*-racemates of α-methylbenzylamino-2-cyanoacrylates (**2**) in inhibiting photosynthetic electron transport in isolated pea chloroplast fragments are recorded in Table I. Typically, the pI_{50} values for the uncoupled reaction are higher than those obtained under coupled conditions, the difference being 0.45–0.85 pI_{50} units.

Data for the optically active and racemic urea derivatives (**3**) are also included in Table I. Although the values for the uncoupled reaction are in reasonable agreement with those obtained by Moreland and Boots [13], the level of discrimination between the *R*- and *S*-enantiomers was found to be somewhat less (7-fold, about half the difference reported previously). Nevertheless, the activity of the two isomers was demonstrably different under both sets of experimental conditions, with the *S*-isomer being the more active of the pair in accordance with the previous study [13].

A similar situation pertains with the series of 2-cyanoacrylate derivatives (**2**). In all cases (com-

Table I. Physical constants and effect on coupled and uncoupled photosynthetic electron transport of *R*- and *S*-isomers and *RS*-racemate of general formula **2**.



Compound	R_2	b.p. [mm] or m.p.	Optical rotation*	pI_{50} Coupled	pI_{50} Uncoupled
4*RS*	H	210–212 (0.1)		4.45	5.20
S		210–211 (0.1)	+71	4.80	5.50
R		209–210 (0.1)	−76	3.80	4.50
5*RS*	CH_3	200–202 (0.1)		5.75	6.60
S		199–200 (0.05)	+225	6.20	6.90
R		199–201 (0.05)	−225	4.20	4.95
6*RS*	C_2H_5	196–198 (0.1)		6.65	7.25
S		186–188 (0.01)	+228	7.10	7.55
R		186–188 (0.01)	−230	4.90	5.55
7*RS*	C_3H_7	199–201 (0.1)		5.75	6.60
S		194–196 (0.05)	+230	6.15	7.00
R		194–196 (0.05)	−228	4.35	4.90
8*RS*	C_4H_9	200–202 (0.05)		4.50	5.20
S		200–202 (0.05)	+215	4.85	5.50
R		201–202 (0.05)	−209	3.80	4.25
3*RS*		151–153		4.95	5.70
S		174–175	−64.7#	5.10	5.90
R		174–175	+65.5#	4.60	5.05

* $[\alpha]_D^{25}$ c = 2 (MeOH).
\# Lit. [13]: $[\alpha]_D^{25}$ c = 1.2 (DMSO).

pounds **4**–**8**), the *S*-isomer is significantly more active than the *R*-isomer. However, the level of discrimination between the *S*- and *R*-isomers is, in some cases, considerably higher than that observed with the urea derivatives. For example, compound **6***S* is at least 100-fold more potent than the corresponding enantiomer (compound **6***R*) in both the coupled and uncoupled Hill reactions.

The effect of the β-substituent (R_2) on Hill inhibitory activity is similar to that observed previously with other aralkylamino series [12]. The change in potency with increasing carbon chain length under coupled conditions is presented in Fig. 1. The Hill activity of the racemic compound (**4***RS*) increases by greater than 100-fold in the transition from a β-hydrogen atom to a β-ethyl substituent (**6***RS*). As the carbon chain length of the β-alkyl substituent is further increased (compounds **7***RS* and **8***RS*) activity progressively diminishes such that the β-butyl derivative (**8***RS*) is more than 100-times less active than the optimally-substituted β-ethyl compound (**6***RS*).

The structural requirements and the magnitude of the β-alkyl effect are virtually identical for the *RS*-racemate and the more active *S*-isomer (see Fig. 1). However, while the structural requirements for increasing activity remain the same, the transition from β-hydrogen to β-ethyl in the less active *R*-series produces only a 12-fold increase in potency (compare compounds **4***R* and **6***R*) in contrast with the 200-fold increase observed in the *S*-series (compare compounds **4***S* and **6***S*).

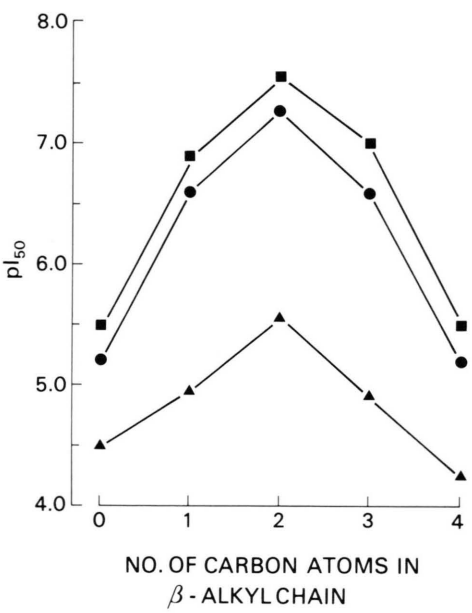
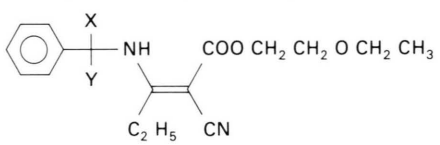


Fig. 1. Variation in pI_{50} (uncoupled) with the number of carbon atoms in the β-substituent (R_2) of general structure (**2**); ● *RS*-racemates, ■ *S*-isomers, ▲ *R*-isomers.

Table I also reveals differences in the discrimination of the binding site for the optical isomers. While the *S*-isomer is the more active of each optical pair, the level of discrimination appears to be determined by the β-substituent. Compounds **4** and **8** show a 10-fold difference between the *S*- and *R*-isomers, while the β-methyl (**5**), β-ethyl (**6**) and β-propyl (**7**) derivatives show differences of the order of 100-fold, irrespective of whether the assay is run under coupled or uncoupled conditions.

Compounds recorded in Table II show the effect on Hill inhibition of variation in the α-substituent in the benzylamino moiety. Apart from a slight increase in activity by inclusion of an α-alkyl substituent (compare compounds **6** and **9**) and a further slight increase as the length of the alkyl chain increases to three carbon atoms (compound **11**), α-substitution has little effect on the ability of these compounds to inhibit electron transport. Even compound **13**, in which a benzylic hydrogen of compound **9** is replaced by a phenyl ring, shows comparable potency to the parent molecule. The only exception is compound **14**, in which inclusion of an α-benzyl substituent produces a significant increase in activity. Somewhat surprisingly, the α,α-dimethyl derivative **15** showed

Table II. Physical constants and effect on photosynthetic electron transport of compounds of general formula:



Compound	X	Y	b.p. [mm] or m.p.	pI_{50} (coupled)
9	H	H	50– 52	6.20
6*RS*	CH_3	H	196–198 (0.1)	6.65
10*RS*	C_2H_5	H	174–176 (0.01)	6.60
11*RS*	C_3H_7	H	178–180 (0.01)	6.85
12*RS*	$C_3H_7^i$	H	185–188 (0.01)	6.40
13*RS*	C_6H_5	H	Oil*	6.30
14*RS*	$CH_2C_6H_5$	H	73– 74	7.50
15	CH_3	CH_3	93– 94	6.40

* Purified by chromatography on silica gel using chloroform as eluent.

comparable activity to the compound carrying a single α-methyl substituent (**6**).

Discussion

2-Cyanoacrylates derived from *S*-α-methylbenzylamine (compounds **4***S*–**8***S*) are more potent inhibitors of photosynthetic electron transport than either the corresponding *RS*-racemates (**4***RS*–**8***RS*) or the *R*-isomers (**4***R*–**8***R*). The data obtained with this series of compounds confirms the observation of Moreland and Boots [13] that ureas derived from the same optically active precursors showed different levels of inhibition in the Hill reaction. In fact, the discrimination shown by the *S*- and *R*-isomers of compound **4**, unsubstituted in the β-position, was of the same order as observed with the ureas (**3***S* and **3***R*). However, substitution of the β-hydrogen atom of compound **4** with a methyl, ethyl or propyl group affords a dramatic widening in the potency difference observed between the *S*- and *R*-isomers. A discrimination of the order of 100-fold is shown by the enantiomers of compounds **5**, **6** and **7**.

As previously noted [12] with other series in which β-substitution is varied, maximum activity is associated with a β-ethyl group. Although this effect is observed with both the *S*- and *R*-isomers and the *RS*-racemates, the increase in activity shown by replacement of the β-hydrogen with a two carbon unit is an order of magnitude higher with the *S*-isomer than with the less effective *R*-isomer (Fig. 1). This would seem to imply that, while the β-ethyl function is able to exert a beneficial effect on the binding of the *S*-isomer and the *RS*-racemate to the receptor, this property is much less in evidence with the *R*-isomer. This phenomenon is reminiscent of the relatively low increase in potency associated with β-alkyl substitution of long chain alkylamino-2-cyanoacrylates compared with benzylamino and 4-chlorobenzylamino derivatives [12]. The suggestion [12] that the former compounds interact with the lipid phase non-specifically, whereas compounds of the benzylamino type bind to a specific region of the hydrophobic domain may well provide an explanation for the lower sensitivity of the activity of the *R*-isomer to β-substitution. Presumably, the preferred orientation of the phenyl ring in the *R*-isomer is conformationally incompatible with the requirements of the specific hydrophobic binding region.

The somewhat surprising activity shown by the α,α-dimethylbenzylamino derivative (**15**) provides further clarification of the inter-relationship of specific groups involved in the binding of these molecules. Normally, the spatial orientation of the hydrogen atom (the least bulky substituent) would determine which enantiomer is repulsed sterically by the receptor. The comparable activity of compound **15** with the corresponding racemate **6***RS* would indicate that this is not the case here. Thus, the activity of the *S*-isomer and the relative inactivity of the *R*-isomer must involve only the orientation of the phenyl ring and its capacity to interact with the specific hydrophobic binding region. It may be that the presence of a second α-methyl group effectively ensures that the phenyl ring is in contact with the preferred hydrophobic area. Inspection of space-filling models (Fisher Scientific) provides a reasonable conception of the orientation of the inhibitor molecules on the receptor site. It is assumed that the amino and ester functions adopt a *cis* orientation [11] and that the ester, nitrile, amino and double bond fragments of the molecule are planar (or nearly so, given the steric constraints inherent in the molecule). It is possible for both α-methyl groups of compound **15** to locate on one side of this pseudo-planar system. The phenyl ring would then preferably be in an orientation above the plane of the cyanoacrylate system. The benzylamino derivatives (**9**) could also adopt this conformation, as could the *S*-isomers of compounds **4**–**8**, where a benzylic hydrogen atom is adjacent to the β-hydrogen or β-alkyl substituent. The alternative *R*-enantiomer, however, requires the benzylic α-methyl group to abut the β-substituent if the same orientation of the phenyl ring is to be achieved. This is sterically a very unfavourable conformation. Thus, the *R*-isomer is energetically far less likely to be able to conform to the steric requirements of the phenyl binding region, particularly when strong steric interference is offered by a β-alkyl substituent. This explanation also accounts for the level of discrimination between the *S*- and *R*-isomers being similar for compounds **5**, **6** and **7**, even though compound **6** is clearly the most active. The level of discrimination is determined by the presence of a β-alkyl substituent, whereas activity is determined by the size of the group. This again highlights the importance of the presence and size of the β-alkyl substituent for effective binding. Both compound **4**, with no β-alkyl substituent, and compound **8**, with a β-

alkyl substituent which is too large, are less well accommodated by the architecture of the binding domain than other compounds in the series with more favourable β-substitution.

Further evidence for the specific nature of the binding of the benzyl phenyl ring is provided by studies of the qualitative rates of inhibitor-Q_B (the secondary electron-accepting quinone) exchange at the common binding site in the thylakoid membrane [18]. Significantly, the *S*-isomer (**6***S*) exchanges slowly with Q_B, in common with the classic inhibitors diuron and atrazine [19]. However, the rate of exchange of the *R*-isomer is relatively rapid and similar to the phenol-type PS II inhibitors. The long-chain alkylamino derivatives studied previously (*e.g.* **1**, $R_1 = C_{10}H_{21}$; $R_2 = H$; $R_3 = CH_2CH_2OCH_2CH_3$) also exchange rapidly with the site [19]. While the relative residence times of a particular inhibitor on the site shows little relationship to the pI_{50} of the molecule, it may provide an indication of the class of inhibitor involved [18]. It may be premature to speculate further, but the evidence at this stage would suggest that the specific site occupied by the phenyl ring of the *S*-benzylamino series overlaps, at least partly, the binding region of the aromatic portion of the urea and triazine inhibitor molecules.

The activity of benzylamino derivatives bearing α-substituents other than methyl (compounds **10**–**14**) presents an interesting departure from previous experience with 2-cyanoacrylate inhibitors [8–12]. Increasing bulk or lipophilicity of the substituent resulted in only marginal change in Hill activity. Even an α-phenyl substituent (compound **13**) was readily accommodated in this position. This lack of sensitivity to structural variation in this region could indicate that the α-substituent extends partly beyond the binding protein into aqueous space [20]. The exception to this pattern is compound **14**, where the benzyl group confers a significant activity increase. Presumably, the flexibility introduced by the methylene group allows the phenyl ring to avoid the hydrophilic region. The enhanced activity of compound **14** may indicate interaction with the bulky, non-specific lipophilic region of the site [9, 21].

Acknowledgement

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