Cyanoacrylate Inhibitors of the Hill Reaction
IV. Binding Characteristics of the Hydrophobic Domain

John L. Huppatz and John N. Phillips

CSIRO, Division of Plant Industry, Canberra A.C.T. 2601, Australia

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Inhibitors of Electron Transport, Hill Reaction, Cyanoacrylates, Hydrophobic Binding

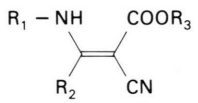
Aryl- and aralkyl-amino derivatives of 2-cyanoacrylic esters were synthesized and assayed as inhibitors of the Hill reaction in isolated pea chloroplast fragments.

Aryl- and aralkyl-amino 2-cyanoacrylates unsubstituted in the β-position were weak inhibitors but activity could be greatly enhanced by inclusion of a β-alkyl group with the increase in potency being dependent on the carbon chain length of the β-substituent. The magnitude of this effect appeared independent of the nature of the aryl- or aralkyl-amino function.

Inclusion of a carbon chain between the phenyl and amino functions of phenylamino-2-cyanoacrylates produced a stepwise increase in activity with increasing chain length, indicating a preferred orientation for the phenyl ring within the hydrophobic binding domain.

Introduction

Earlier papers [1–4] in this series described the inhibition of photosynthetic electron transport by a series of 2-cyanoacrylates of general formula **1**. These compounds appear to act at a receptor site common to other amide-type photosystem II inhibitors [5], although they do not conform with the generally accepted concept of structural requirements associated with this class of Hill inhibitor [6].



The potency of the 2-cyanoacrylates in affecting photosynthetic electron transport was found to be extremely sensitive to minor structural variation, thereby enabling a number of inferences concerning the nature of the binding domains of these inhibitors to be drawn [1, 2, 3, 4]. For example, the inclusion of an ether linkage in the alkyl group (R_3) of the ester function proved particularly favourable in achieving effective inhibition [2], while introduction of an alkyl substituent (R_2) at the β-carbon atom produced changes in Hill inhibition related to the size of the substituent [4]. In contrast, the activity of the long-chain alkylamino derivatives reported previously [1, 2] showed direct dependence on the lipophilicity of the alkyl group (R_1) attached to the nitrogen atom, indicating a relative lack of structural specificity for this portion of the molecule. In the alkylamino-2-cyanoacrylates, R_1 appeared to interact with a mobile lipid phase perhaps associated with the interior of the thylakoid membrane [2]. To probe the nature of this hydrophobic region further, aryl- and aralkyl-amino derivatives were prepared and their activity as inhibitors of the Hill reaction assessed. With each variation in R_1, the effect on activity of alkyl substituents of differing carbon chain length at the β-position in the cyanoacrylate moiety was also determined. All compounds included an ethoxyethyl ester function ($R_3 = CH_2CH_2OCH_2CH_3$) since this structural feature was found to enhance the binding of 2-cyanoacrylate molecules to the receptor site [2].

Materials and Methods

All compounds recorded in Table I gave analytical data within satisfactory limits and the structures were confirmed by PMR spectra which were recorded on a Jeol FX90Q spectrometer using TMS as internal standard and $CDCl_3$ as solvent.

Compounds **2–41** were prepared by reacting an aryl- or aralkyl-amino with the appropriate ethoxy or methoxy 2-cyanoacrylic ester [4]. The reactants were heated at 100–120° for 1 h, except in the case of aniline and 4-chloroaniline where heating at 170–180° was required.

Reprint requests to Dr. J. L. Huppatz.

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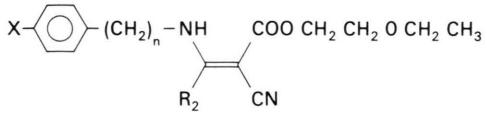
Table I. Physical constants and pI_{50} data for compounds of the following general formula:



Compd. No.	n	R_2	X	b.p. [mm] or m.p. [°C]	pI_{50} Coupled	Uncoupled
2	0	H	H	37– 39	<3.4	3.70
3		CH_3		85– 86	3.75	4.70
4		C_2H_5		86– 87	4.80	5.95
5		C_3H_7		203–204 (0.2)	4.15	5.20
6		C_4H_9		182–185 (0.05)	3.95	4.60
7	1	H		81– 82.5	3.95	4.80
8		CH_3		200–203 (0.05)	5.20	6.05
9		C_2H_5		50– 52	6.20	7.15
10		C_3H_7		201–203 (0.01)	5.30	6.25
11		C_4H_9		217–219 (0.05)	4.35	5.10
12	2	H		72– 74	4.15	4.95
13		CH_3		75– 77	5.05	5.85
14		C_2H_5		52– 54	6.00	6.90
15		C_3H_7		205–206 (0.01)	5.50	6.45
16		C_4H_9		209–211 (0.01)	4.45	5.30
17	3	H		72– 74	5.00	6.20
18		CH_3		210–212 (0.01)	6.00	7.20
19		C_2H_5		216–218 (0.05)	6.80	8.00
20		C_3H_7		215–217 (0.01)	6.45	7.45
21		C_4H_9		220–222 (0.01)	5.20	6.30
22	4	H		220–222 (0.05)	5.30	6.15
23		CH_3		231–233 (0.05)	6.15	7.55
24		C_2H_5		218–220 (0.01)	7.00	7.90
25		C_3H_7		226–227 (0.01)	6.20	7.50
26		C_4H_9		236–238 (0.01)	5.40	6.80
27	0	H	Cl	152–154	3.60	4.50
28		CH_3		92– 94	4.55	5.55
29		C_2H_5		85– 86	5.70	6.60
30		C_3H_7		190–193 (0.01)	5.05	6.15
31		C_4H_9		202–204 (0.01)	4.75	5.70
32	1	H		133–134	5.65	6.35
33		CH_3		72– 73	7.20	7.65
34		C_2H_5		65– 66	8.20	8.30
35		C_3H_7		215–217 (0.01)	7.10	7.70
36		C_4H_9		223–224 (0.01)	5.80	6.55
37	2	H		123–125	5.50	6.35
38		CH_3		74– 76	6.65	7.65
39		C_2H_5		67– 69	7.40	8.35
40		C_3H_7		69– 71	7.00	7.95
41		C_4H_9		50– 52	5.85	6.90

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 [7], with the chlorophyll concentration in the chloroplast suspension routinely set at about 8 μg/ml. Uncoupled values were obtained by inclusion of 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 pI_{50} values recorded in Table I 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 compounds recorded in Table I were assayed as inhibitors of the Hill reaction under coupled and uncoupled conditions and the pI_{50} values are assumed to provide a measure of the relative affinity for the binding site of the various aryl- and aralkylamino derivatives.

Compounds incorporating a phenyl ring attached to the nitrogen atom were weak inhibitors of electron flow (compounds **2**–**6**). The low activity of these molecules prompted the introduction of an alkyl chain between the phenyl ring and the NH substituent. This was designed to introduce flexibility into the group interacting with the lipid region of the binding site, as well as increasing the overall lipophilicity of the molecules. In general, lengthening the carbon chain progressively from 1 to 4 atoms resulted in an increase in activity (Table I), though the increase was by no means uniform.

The introduction of a methylene group between the phenyl and NH functions afforded a significant increase in activity (compare compounds **7**–**11** with **2**–**6**). It is noteworthy, however, that a second methylene group (compounds **12**–**16**) provided no further enhancement. An extension of the chain to the phenylpropylamino series (compounds **17**–**21**) resulted in an approximately 10-fold rise in potency, though elongation to a four carbon unit (compounds **22**–**26**) gave less significant changes in activity.

To further amplify the interaction of these molecules with the lipophilic binding domain, a 4-chloro substituent was introduced into the phenyl, benzyl and phenylethyl series. The 4-chloro substituent increased the pI_{50} values in coupled assays of the phenylamino series by 0.8–0.9 pI_{50} units (compounds **27**–**31**) over the unsubstituted series (compounds **2**–**6**). The corresponding benzyl derivatives (compounds **32**–**36**) showed a much greater increase pI_{50} (1.45–2.00 pI_{50} units – coupled) over the parent compounds (**7**–**11**). A slightly lower increase (1.35–1.60 pI_{50} units – coupled) was observed with 4-chloro substitution in the phenylethyl series.

The transition from the 4-chlorophenylamino series to the sterically more flexible 4-chlorobenzylamino series (compounds **32**–**36**) resulted in an increase in potency of more than 100-fold in all compounds except the β-butyl derivative (**36**). Extension of the alkyl chain from one to two carbon atoms (compounds **37**–**41**) produced no significant change in activity, in line with the result obtained with compounds lacking the chlorine atom.

Compounds unsubstituted at the β-carbon were relatively weak inhibitors (compounds **7**, **12**, **17**, **22**, **27**, **32**, **37**). However, activity was increased by up to 100-fold by alkyl substitution at the β-carbon atom. Maximum inhibition was reached when a β-ethyl substituent was present (compounds **9**, **14**, **19**, **24**, **29**, **34**, **39**) and declined as the chain length of the alkyl group was further increased.

Discussion

The systematic evaluation of aryl- and aralkylamino-2-cyanoacrylates in the Hill reaction has uncovered a number of highly potent inhibitors of photosynthetic electron flow. Compound **34**, for example, is one of the most active inhibitors yet reported, being significantly more active than DCMU [pI_{50} 6.95 (coupled) and 7.80 (uncoupled)] in the assay system used. In fact, the assay procedure routinely used effectively limits the maximum pI_{50} obtainable to 8.2–8.3, and compounds approaching this level may give higher values at lower chlorophyll concentrations.*

When activity in the Hill reaction was measured under uncoupled conditions, a higher pI_{50} value was invariably obtained, the difference between the two determinations being 1.0 pI_{50} unit on average. With very potent compounds (*e.g.*, **34**), the difference between coupled and uncoupled values tended to be less, though this probably reflects the limitations of the assay system. The trends in activity observed with variation in structure of the inhibitors remained the same, regardless of whether the effect on elec-

* The pI_{50} value of compound **34** was found to be 8.70 in an uncoupled spinach thylakoid system at 0.2 μg/ml chlorophyll.

tron transport was measured in a coupled or uncoupled system. Uncoupled Hill activity, however, probably reflects more directly the intrinsic binding affinity of the compounds.

An interesting phenomenon was revealed by focussing attention on the differences in the pI_{50} data obtained for each aryl and aralkyl series of inhibitors. The ΔpI_{50} values calculated for the corresponding compounds in each series as the number of methylene groups between the phenyl ring and the amino function was increased are presented schematically in Fig. 1. Both coupled and uncoupled values were used in the construction of Fig. 1, but data for the β-butyl derivative (compound **6**) in the phenylamino series were omitted since this compound appears anomalous, being more active in both assay systems than would be expected from trends in other series.

Fig. 1 shows that the trend towards increased activity is stepwise. As the carbon chain length increases, significant increase in activity occurs in series with an odd number of carbon atoms between the phenyl and amino groups. The same trend is discernible in series containing a 4-chloro substituent. The overall effect is reminiscent of crystal packing phenomena and would seem to indicate a preferred orientation for interaction of the aryl ring with the hydrophobic region of the binding site. However, the transition from zero carbon atoms between the phenyl and amino groups in the unsubstituted compounds to a two and then a four carbon atom chain results in an increase in pI_{50} of 1.15 units on average for each two carbon atoms. This value is similar to the effect of a two carbon unit on the logarithm of the *n*-octanol-water partition coefficient [9] and is the activity increase expected from a solely lipophilic interaction. The same comment applies to the transition from the benzyl to phenylpropyl series. Here a mean pI_{50} increase of 1.05 is observed, as would be expected from the increase in lipophilicity resulting from addition of two carbon atoms to the chain. The stepwise effect apparent from Fig. 1 must therefore be based only on the different orientation of the phenyl ring in compounds with an even and odd number of carbon atoms intervening between the phenyl and amino functions. Therefore, the greater affinity obtained with the phenyl ring in the correct orientation implies interaction with a specific lipophilic area within the hydrophobic matrix, possibly the lipophilic "spine" associated with the membrane-spanning helices of the 32 kDa herbicide binding protein [8].

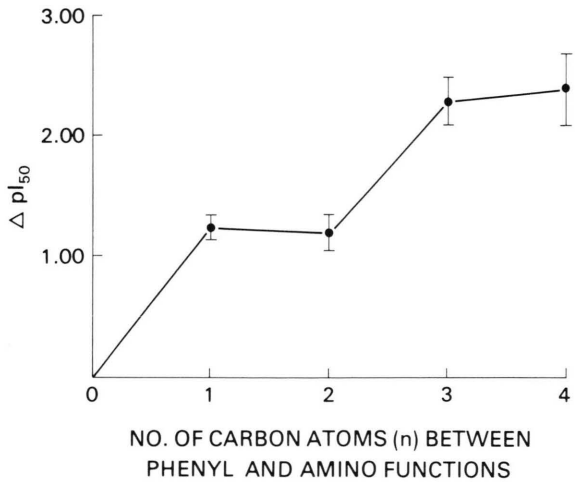


Fig. 1. Diagrammatic representation of the difference in pI_{50} value (ΔpI_{50}) for the corresponding compounds in each series in Table I as the number of methylene groups (*n*) between the phenyl ring and the amino function was increased. The mean ΔpI_{50} and standard deviations were calculated using all values in Table I (both coupled and uncoupled) with the exception of those for compounds **2** (coupled) and **6** (coupled and uncoupled).

The change from phenyl- and phenylalkyl-amino compounds to the corresponding 4-chloro derivatives also produced an unexpected result. A 4-chloro substituent in the phenylamino series (compounds **27–31**) increased the pI_{50} values by 0.65–1.1 pI_{50} unit over the unsubstituted series (compounds **2–6**). This effect is consistent with the increase in lipophilicity of the molecule afforded by the presence of the chlorine atom ($\pi = 0.71$ as measured [9] using the octanol-water system) and suggested the interaction of the 4-chlorophenyl substituent with a relatively mobile lipid environment. However, the effect of the chlorine atom in the benzylamino series particularly, is much more pronounced (compare compounds **32–36** with compounds **7–11**) and cannot be ascribed solely to extra lipophilicity introduced by the presence of the substituent. The higher potency of this series may be at least partly attributable to a reinforcing of binding with the specific lipophilic area postulated above. The increase observed with the phenylethyl series (compare compounds **37–41** with compounds **12–16**) is somewhat less, probably reflecting the inability of these molecules to adopt the precise orientation necessary to maximize interaction with the site.

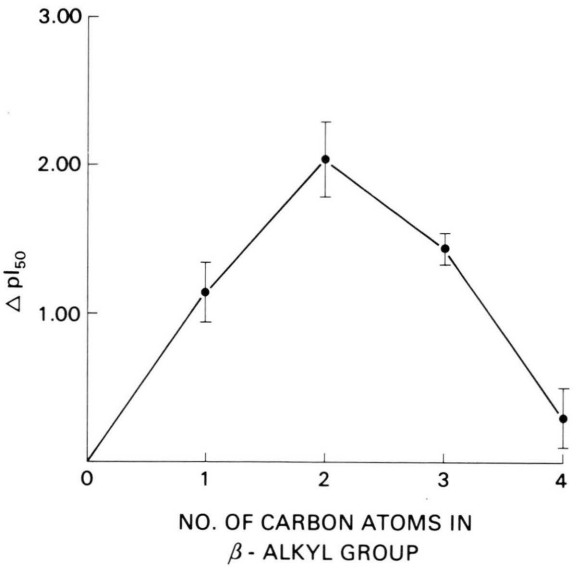


Fig. 2. Diagrammatic representation of the change in pI_{50} (ΔpI_{50}) for compounds of increasing β-alkyl chain length relative to the corresponding β-unsubstituted compounds. The mean ΔpI_{50} values and standard deviations were calculated for all series. Compounds **2** (coupled), **6** and **31** were omitted.

In all series studied, activity was increased by alkyl substitution at the β-carbon, reaching a maximum when a β-ethyl substituent was present in the molecule and declining as the chain length of the group was further increased. A remarkable consistency was observed in the quantitative change in activity with variation in the length of the alkyl chain. The change in pI_{50} relative to the β-unsubstituted molecules has been calculated for the different β-alkyl chain lengths in all series and the results presented schematically in Fig. 2. Again, the anomalous β-butyl derivatives (**6** and **31**) in the phenyl- and 4-chlorophenyl-amino series have been omitted. Fig. 2 emphasizes the parabolic nature of the β-alkyl effect and the consistent change in pI_{50} effected by the chain length of the β-alkyl substituent. It is evident that an increase in activity of about 100-fold results from the transition from compounds unsubstituted in the β-position to the corresponding β-ethyl derivatives, irrespective of the nature of the aryl or aralkyl group attached to nitrogen. This contrasts strongly with the 6-fold increase in pI_{50} observed for the same change in a series of octylamino compounds studied previously [4], although qualitatively the effect of β-substitution was the same. The large and consistent amplification of potency by the presence of a favourable β-alkyl substituent in aryl- and aralkyl-substituted compounds implies a high degree of spatial specificity in the interaction of the aryl ring with the binding domain. A well-fit β-alkyl group obviously plays an important role in determining the orientation of the aryl and aralkyl groups on the receptor. In addition, as discussed above, a more subtle effect on the positioning of the aryl ring, influenced by the length of the carbon chain in an aralkyl group also appears to operate. The steric requirements of the hydrophobic region of the binding domain are therefore critical factors in determining optimum Hill inhibition.

Acknowledgements

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