Cyanoacrylate Inhibitors of the Hill Reaction II. Nature of the Hydrophobic Binding Domains

John N. PHILLIPS and John L. HUPPATZ

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

Received April 4, 1983

A series of alkoxyalkyl-3-alkylamino-2-cyanoacrylates and related compounds have been synthesised and their Hill inhibitory activity determined in isolated pea chloroplasts. The results suggest that the alkyl group attached to the amino function interacts with a mobile lipid phase in the thylakoid membrane whilst the alkoxyalkyl group interacts with a smaller, more ordered, lipid environment. It is suggested that the former region corresponds to the interior of the lipid membrane and the latter to membrane or proteinalkyl chains in the vicinity of the membrane-protein interface. There is evidence of a localized hydrophilic pocket in the more constrained lipid environment.

Differences in the activity of various Hill inhibitor molecules have been related to differences in their binding to the thylakoid membrane.¹⁾ Studies involving the competitive displacement of membrane bound isotopically-labelled inhibitors by a variety of other inhibitors have indicated that such molecules, in spite of their structural differences, appear to interact with the same 32 kD polypeptide on the outside of the membrane. The precise nature of the binding and of the receptor site itself remains unclear, although it has been argued²⁾ that, whilst these inhibitors may interact with the same key grouping in the peptide, they could have different, if overlapping, binding domains.

Recently, a new group of photosynthetic electron transport inhibitors (**1a**), derivatives of 3-amino-2-cyanoacrylic acid (**1a**), have been reported.³⁾ It has been observed that in 3-methylphenylamino derivatives³⁾ (**1a**, $R^1 = C_6H_5$; $R^2 = C_nH_{2n+1}$; $R^3 = CH_3$), the Hill inhibitory activity (pI_{50}) increased with increasing chain length (n) of the alkyl ester group (R^2) until a constant value was reached ($n=3$), whilst in 3-decylamino derivatives⁴⁾ (**1a**, $R^1 = C_{10}H_{21}$; $R^2 = C_nH_{2n+1}$; $R^3 = H$) the inhibitory activity was at first constant ($n=1, 2$) and then declined as the ester chain length increased

($n=3, 4$) (see Fig. 1). Although the pI_{50} -chain length relationship showed an opposite trend in the two series, the change from constant to increasing or decreasing activity occurred in both cases between the ethyl and propyl ester derivatives. It was thought that this region of the alkyl chain, *i.e.*, three to four atoms removed from the ester carbonyl function, might correspond to a discontinuity in the molecular environment around the receptor site.

Accordingly, it was decided to probe for such a discontinuity by measuring the Hill inhibitory activity of alkyl-3-alkylamino-2-cyanoacrylate derivatives in which the molecular architecture of the alkyl ester chain had been modified in the vicinity of the ester group. This was achieved, in the first instance, by incorporating an ether oxygen into the alkyl ester chain (**1b**). The effects of such modifications on the Hill inhibitory activity are reported and their implications as to the nature of the hydrophobic binding domain discussed.

EXPERIMENTAL

Synthesis of the 3-amino acrylic acid derivatives. All the compounds recorded in Table I were prepared by the reaction of the appropriate aryl- or alkylamine with the corresponding ethoxy acrylic acid derivative as previously described.^{3,4} All the compounds in Table I gave analytical data (C, H, N) within satisfactory limits and their structures were confirmed by PMR and mass spectral data.

The 3-ethoxy derivatives of the cyanoacrylates were prepared by reacting the appropriate esters with triethyl orthoformate in the presence of acetic anhydride as previously reported.³ Physical constants for these compounds are: methoxyethyl 3-ethoxy-2-cyanoacrylate, bp 130~132°C/0.05 mm; ethoxyethyl 3-ethoxy-2-cyanoacrylate, bp 158~160°C/0.7 mm; propyloxyethyl 3-ethoxy-2-cyanoacrylate, bp 130~132°C/0.05 mm; isopropyloxyethyl 3-ethoxy-2-cyanoacrylate, bp 121~123°C/0.05 mm; butyloxyethyl 3-ethoxy-2-cyanoacrylate, bp 146~149°C/0.1 mm; pentyloxyethyl 3-ethoxy-2-cyanoacrylate, bp 171~174°C/0.5 mm; ethoxypropyl 3-ethoxy-2-cyanoacrylate, bp 157~160°C/0.5 mm; 3'-methoxybutyl 3-ethoxy-2-cyanoacrylate, bp 172~174°C/0.4 mm; tetrahydrofurfuryl 3-ethoxy-2-cyanoacrylate, bp 175~176°C/0.1 mm; phenoxyethyl 3-ethoxy-2-cyanoacrylate, bp 206~208°C/0.4 mm; cyanoethyl 3-ethoxy-2-cyanoacrylate, mp 96~98°C (ex. EtOH); acetoxyethyl 3-ethoxy-2-cyanoacrylate, bp 162~164°C/0.2 mm.

The hydroxyethyl ester (compound **14**) was prepared from the corresponding acetoxy derivative (**22**) as follows. Acetoxyethyl 3-ethoxy-2-cyanoacrylate (4.7 g) and decylamine (3.4 g) were heated together at 100~120°C for 1 hr. Concentrated hydrochloric acid (50 ml) was added to the crude product and the mixture heated on a steam-bath for 1 hr. Water (150 ml) was then added and the product extracted with chloroform (3 × 50 ml). The combined extracts were washed well with water, dried and evaporated. *Hydroxyethyl 2-cyano-3-decylaminoacrylate* (5.2 g, 82%) was obtained as a pale yellow oil. Attempted distillation resulted in extensive decomposition and an analytical sample was prepared by chromatography through a short silica column using chloroform as the eluent.

Hill reaction assay. Each compound was assayed for its Hill inhibition activity as previously reported.⁵ The activity of a compound as a Hill inhibitor is expressed in terms of its pI_{50} value, 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% of that obtained in the absence of the compound.

RESULTS AND DISCUSSION

Table I records the Hill inhibitory activity, expressed in terms of pI_{50} values, for a series of 2-cyanoacrylic acid esters. In general, the com-

pounds have been grouped into series (A, B, C, D, E) in which only one structural feature has been varied.

Although 3-arylamino and 3-alkylamino cyanoacrylate inhibitors appear to interact with the same receptor site,⁴ it is evident from the opposite trends of the alkyl group effect in Fig. 1 that their orientations at the receptor site, i.e., their binding domains, are quite different. Further evidence of this is shown by the different effects on Hill inhibitory activity of replacing a pentyl ester by an ethoxyethyl ester group. As Table I-A shows, this leads to a five hundred fold depression of activity in the arylamino series (cf. **2** and **3**), but a ten thousand fold enhancement of activity in the alkylamino series (cf. **4** and **5**). Such spectacular differences lend support to the hypothesis that there is a discontinuity in the molecular environment close to the receptor site. Since the ether group is isosteric with the methylene group and relatively inert in a chemical bonding sense, it is likely that its effect on activity is related to its polar character and possibly to its ability to interact with hydrogen bond donor groups of water or peptide molecules. This would be consistent with a molecular discontinuity in the vicinity of the receptor site arising from a hydrophilic pocket buried in a lipophilic matrix.

The effect of varying the position of the ether link in a series of decylamino alkoxyalkyl ester derivatives containing the same total number of atoms in the ester group is indicated in Table I-B. The data show that the derivative (**6**), in which the ether oxygen is four atoms removed from the carbonyl group, is fifty fold more active than derivatives (**7**, **8**), where the oxygen is five atoms removed from the carbonyl group. This indicates the relatively localized nature of the hydrophilic pocket with which the ether group can interact.

Increasing the chain length of the alkylamino (R^1) group in the ethoxyethyl ester series leads to an increase in Hill inhibitory activity over the range $C_4 \rightarrow C_{10}$ (compds **5**, **9**~**13**, Table I-C). The almost linear relationship between pI_{50} and alkyl chain length over

TABLE I. PHYSICAL DATA AND pI_{50} VALUES FOR COMPOUNDS OF THE GENERAL FORMULA

	Compound No.	R ¹	R ²	R ³	bp (°C)	mp (°C)	pI_{50}
A	2	C ₆ H ₅	C ₅ H ₁₁	CH ₃		63~64 ³⁾	5.9 ³⁾
	3		(CH ₂) ₂ OC ₂ H ₅			63~65	3.2
	4	C ₁₀ H ₂₁	C ₅ H ₁₁	H	192~194 (0.01) ⁴⁾		<3.4
	5		(CH ₂) ₂ OC ₂ H ₅		212~215 (0.2)	60~61	7.3
B	6	C ₁₀ H ₂₁	(CH ₂) ₂ OC ₃ H ₇	H	199~201 (0.1)	63~64	6.5
	7		(CH ₂) ₃ OC ₂ H ₅			59~60	4.8
	8		(CH ₂) ₂ CH(CH ₃)OCH ₃			48~50	4.7
C	9	C ₄ H ₉	(CH ₂) ₂ OC ₂ H ₅	H	165~170 (0.2)		4.5
	10	C ₆ H ₁₃			184~188 (0.2)	63~65	5.5
	11	C ₈ H ₁₇			202~205 (0.2)	64~65	6.4
	5	C ₁₀ H ₂₁					7.3
	12	C ₁₁ H ₂₃				63~64	7.2
	13	C ₁₂ H ₂₅				61.5~63	6.9
D	14	C ₁₀ H ₂₁	(CH ₂) ₂ OH	H	Oil*		5.2
	15		(CH ₂) ₂ OCH ₃		204~206 (0.1)	60~61	6.6
	5		(CH ₂) ₂ OC ₂ H ₅				7.3
	6		(CH ₂) ₂ OC ₃ H ₇				6.5
	16		(CH ₂) ₂ OC ₃ H ₇ (i)		216~219 (0.1)		6.8
	17		(CH ₂) ₂ OC ₄ H ₉			59~61	5.5
	18		(CH ₂) ₂ OC ₅ H ₁₁			63~65	5.1
E	19	C ₁₀ H ₂₁	CH ₂ -	H		73~74	7.0
	20		(CH ₂) ₂ OC ₆ H ₅			70~72.5	6.0
	21		(CH ₂) ₂ CN			66~67	6.0
	22		(CH ₂) ₂ OCOCH ₃		Oil*		4.8

* Compound decomposed on attempted distillation.

FIG. 1. pI_{50} Data as a Function of Increasing Alkyl Chain Length of the Ester Group for the 3-Methylphenylamino Series (1a, R¹=C₆H₅; R²=C_nH_{2n+1}; R³=CH₃) ○—○, and the Decylamino Series (1a, R¹=C₁₀H₂₁; R²=C_nH_{2n+1}; R³=H) ●—●.

this range corresponds to an increase of ~0.45 pI_{50} unit per methylene group. This value is similar to the effect of a methylene group on the logarithm of the *n*-octanol-water partition coefficient⁶⁾ and points to the alkylamino group interacting with a relatively mobile hydrocarbon phase such as the lipid chains in the interior of the thylakoid membrane. It will be noted that the pI_{50} values reach an optimum for the C₁₀ alkyl group and then decline. A similar optimum around C₁₀–C₁₂ has been observed⁴⁾ for both the alkylamino methyl ester series (1a, R¹=C_nH_{2n+1}; R²=CH₃; R³=H) and the alkylester methylamino series (1a, R¹=CH₃; R²=C_nH_{2n+1}; R³=H). This

would suggest that chain lengths in excess of C_{10} – C_{12} were unable to fully interact with mobile membrane lipids.

Varying the chain length (n_2 —structure **1b**) of the alkoxy group in the decylamino alkoxyethyl ester series (Table I-D) gives rise to a parabolic relationship between pI_{50} and n_2 . Inhibitory activity initially increases (**14**, **15**), reaches an optimum value with the ethoxyethyl derivative (**5**), and then declines (**6**, **16**–**18**). Such changes in the marginal effectiveness of a methylene group on the activity imply that the alkoxyethyl group is interacting with a somewhat ordered lipid environment and one quite different from the mobile lipid phase associated with the amino-alkyl group, where the marginal effectiveness per methylene group tended to be constant over the range $C_4 \rightarrow C_{10}$.

Data for compounds in which the alkoxyalkyl ester function was replaced by other polar groups are recorded in Table I-E. The cyclic ether analogue (**19**) of the propoxyethyl ester (**6**) and the phenoxyethyl compound (**20**) are both potent Hill inhibitors, indicating that a cyclic structure *per se* does not greatly interfere with the hydrophobic binding of these molecules. The greater activity of the cyanoethyl ester (**21**) ($pI_{50}=6.0$) as compared with the analogous butyl ester (**1a**, $R^1=C_{10}H_{21}$; $R^2=C_4H_9$; $R^3=H$; $pI_{50}=3.45$)⁴ shows that groups other than alkoxy groups can enhance binding to the receptor site and is consistent with the involvement of a polar type interaction with a hydrophilic region. The highly localised nature of such a hydrophilic pocket has been demonstrated by the fifty fold greater activity of compound (**6**), where the ether oxygen is four

atoms away from the ester carbonyl, as compared with (**7**) and (**8**), where it is five atoms away. It may also account for the weak activity of the acetoxyethyl compound (**22**) ($pI_{50}=4.8$), where the polar acetonyl oxygen is six atoms removed from the ester carbonyl.

The preceding study⁴ based on the alkyl esters of 3-alkylamino-2-cyanoacrylic acid had indicated that optimum Hill inhibitory activity was associated with the cyanoacrylate moiety being flanked by lipophilic groups of differing size. It was assumed that there was a corresponding asymmetric distribution of hydrophobic regions in the vicinity of the receptor site with which these lipophilic groups interacted. The present data highlight differences in the nature of these discrete hydrophobic regions and suggest that the larger, more mobile, lipid region is associated with the interior of the lipid membrane and the smaller, more constrained, lipid environment with membrane or protein lipid chains close to the membrane-protein interface. There are indications of a highly localised hydrophilic pocket within the smaller hydrophobic region.

REFERENCES

- 1) W. Tischer and H. Strotmann, *Biochim. Biophys. Acta*, **460**, 113 (1977).
- 2) K. Pfister and C. J. Arntzen, *Z. Naturforsch.*, **34c**, 996 (1979).
- 3) J. L. Huppatz, J. N. Phillips and B. M. Rattigan, *Agric. Biol. Chem.*, **45**, 2769 (1981).
- 4) J. N. Phillips and J. L. Huppatz, *Agric. Biol. Chem.*, **48**, 55 (1984).
- 5) B. T. Brown, J. N. Phillips and B. M. Rattigan, *J. Agric. Food Chem.*, **29**, 719 (1981).
- 6) C. Hansch, A. Leo, S. H. Unger, K. H. Kim, D. Nikaitani and E. J. Lien, *J. Med. Chem.*, **16**, 1207 (1973).