Conjugated Enamino Compounds, a New Molecular Probe for the Mechanism of Photosynthetic Electron Transport

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Aminovinylidenecyclohexanedione, Electron Transport Inhibitor, Inhibitor Binding Site, Molecular Probe

A series of 2-(1-alkylamino)vinylidene-1,3-cyclohexanedione was synthesized and assayed as inhibitors of the Hill reaction in isolated chloroplasts. The pI_{50} value of this series was dramatically changed by modification of the peripheral of its hydrophilic moiety and the maximum pI_{50} value of this series was almost equal to that of DCMU. The results suggest that this series may be a good probe for searching the environment of inhibitors binding site because of its chemical and biological characteristics and its ease of synthesis.

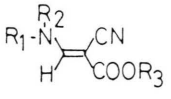
Introduction

A variety of herbicides of different chemical-type are known to interfere with photosynthetic electron transport on the reducing side of photosystem II (PS II) [1, 2]. An unifying feature of these chemicals is their competitive binding at a common receptor site in the thylakoid membrane. Recent advances in molecular biology have revealed the nature of some types of subunits in PS II [3]. In particular, a 32 kDa subunit of PS II has been assigned as the binding site for a large number of herbicides represented by triazines, ureas, and aminotriazinones [4]. Furthermore, the 44 and 51 kDa chlorophyll-peptide subunits of PS II are thought to be the binding site of another group of inhibitors, the phenols, *e.g.* 2-bromo-4-nitrophenol and ioxynil [5]. The essential structural features for DCMU/triazine-type electron transport inhibitors have been proposed [6] and CNDO calculations support these suggestion [7]. Such essential structure might suggest that many inhibitors bound to the common peptide site could have common structural features on the basis of electronic theory of organic chemistry.

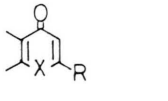
Recently, the alkylaminocyanoacrylates (**1**) have been reported as a new group of Hill reaction inhibitor herbicides [8, 9].

Although these compounds appear to interact with the same receptor site as diuron, the classic PS II inhibitor [10], their functionalities are different from other established inhibitors. They may be regarded as vinylogous amide derivatives and this structural features attracted our attention. Through our synthetic work with the "plastoquinone (PQ) pool" inhibitors (**2**) [11] which block electron transport not by competitive inhibition of the PS II-complex plastoquinone but by a dilution effect at the PQ pool [12], we have noticed some similarity in the structure of **1** and **2**. However, the latter type of compounds has an essentially lower activity consistent with its mode of action.

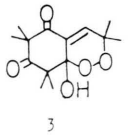
In the course of chemical studies on the plant growth regulators, "G-regulators" of *Eucalyptus* (**3**) [13–15], a new degradative pathway of the 2,3-dioxabicyclo[4.4.0]decene system to form 2-(1-alkylamino)vinylidene-1,3-cyclohexanedione system was recognized. As the latter compounds possessed chemical features in common with **1** (*e.g.* conjugated enamino system), it was of interest to assess their potential as Hill reaction inhibitors. Preliminary assays suggested that the 5,5-dimethyl-2-(1-alkyl-



1



2 X=NH, O



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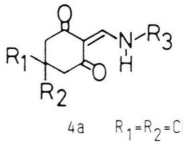

4a $R_1=R_2=CH_3$

4b $R_1=H$, R_2=alkyl

Abbreviations: CNDO, complete neglect of differential overlap; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PET, photosynthetic electron transport; PS, photosystem.

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amino)vinylidene-1,3-cyclohexanedione (**4a**) system was a new functionality for Hill reaction inhibitors.

Herein we wish to report the structural requirements of this type compounds for the inhibition of the Hill reaction.

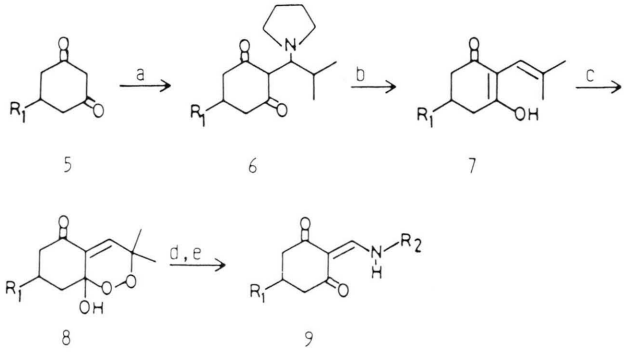
Material and Methods

Hill reaction assay

The compounds were assayed for inhibition of the Hill reaction, using chloroplasts [16] stored at −80 °C after isolation from the leaves of *Spinacia oleracea* (c.v. spinach), with a Clark-type O_2 electrode [17] fitted into a water-jacketed cuvette. The temperature for all assays was at 25 °C.

Chemicals

In this study, 5-alkylcyclohexane-1,3-diones (**5**) were used as starting materials. The synthetic route to compounds **4** is shown in Scheme 1. The method is convenient since the reactions are mild and give high yield [13–15].



Scheme 1. (a) $(CH_3)_2CHCHO$, pyrrolidine, CH_2Cl_2. (b) H_3O^+. (c) O_2,CH_2Cl_2. (d) R_2-NH_2,CH_2Cl_2. (e) H_3O^+.

According to the structural analysis on this series of compounds, the possible keto-enol tautomerism in the 2-(alkylamino)vinylidene-1,3-dione system must be fixed to the ketonic form under normal conditions, as described previously [18].

In contrast, the closely related 2-(1-alkyloxyamino)alkylidene dimedone (Alloxydim type herbicides) are stable in the enolic form at room temperature [19].

^{1}H NMR spectra were obtained with a JEOL MH 100 spectrometer (100 MHz), using tetramethylsilane as an internal standard in $CDCl_3$. IR spectra were recorded with a JASCO A-400 spectrometer. Low and high resolution mass spectra were measured with HITACHI RMU-6 and JEOL DX 303 spectrometers respectively.

*General method for synthesis of 5-alkyl-2-(1-alkylamino)vinylidene-1,3-cyclohexanedione (**4b**)*

i. General synthesis of 5-alkylcyclohexane-1,3-dione (**5**) [20]

In absolute ethanol (50 ml), sodium (0.1 mol), ethyl acetoacetate (0.11 mol) and ethyl 2-alkylacrylate (0.1 mol) were dissolved, and the whole was refluxed for 8 h. After evaporation of the solvent the residue was mixed with 1 M HCl (200 ml) and the mixture was extracted with three portions of hexane (100 ml × 3), and the combined extracts were evaporated under reduced pressure. The residual solid was dissolved in 2 M KOH (100 ml) and the whole was heated to boiling in an open flask for 15 min, then acidified and boiled until no more carbon dioxide was evolved. On cooling, **5** was separated out, filtered and crystallized from hexane (ca. 70% yield).

ii. General synthesis of 5-alkyl-3-hydroxy-2-[(2-methyl-1-piperidino)propyl]cyclohex-2-en-1-one (**6**)

To a stirred solution of 2-methylpropanal (12 mmol) and pyrrolidine (10 mmol) in dichloromethane (25 ml) was added a solution of the 5 (10 mmol) and pyrrolidine (5 mmol) in dichloromethane (25 ml) at 20 °C. After 15 min stirring all volatile materials were removed under reduced pressure and the residual solid was triturated with cold ethyl acetate or acetone and filtered. Yield 95%.

iii. General synthesis of 5-alkyl-2-(2-methyl)propylidene-1,3-cyclohexanedione (**7**)

To a solution of 1 M HCl (50 ml) was added a solution of **6** (5 mmol) in dichloromethane (50 ml). The resulting two layers were agitated vigorously for 10 min, then the organic layer was separated, dried (Na_2SO_4) and evaporated under reduced pressure. The yield of the residual product was nearly quantitative. Most of the products enolized readily on standing, so only enol NMR spectra are given for the compounds obtained in this way.

iv. General synthesis of 9-alkyl-1-hydroxy-4,4-dimethyl-2,3-dioxabicyclo[4.4.0]dec-4-4en-7-one (**8**)

The solution of **7** (5 mmol) in 50 ml dichloromethane was agitated vigorously for 1 or 2 days under dried air; the oxidation was monitored by TLC (hexane : ethyl acetate = 1:1). After completion of the reaction, evaporation of the solvent gave a solid **8** which was purified by recrystallization from hexane-ethyl acetate.

v. General synthesis of 5-alkyl-2-(2-ethoxy-1-ethylamino)vinylidene-1,3-cyclohexanedione (**9**)

The cyclic peroxide (**8a**) (10 mmol) was dissolved in dichloromethane (25 ml) and slight excess (11 mmol) of ethoxyethylamine in dichloromethane (10 ml) was then added. The mixture was vigorously stirred for 20 min then 1 M HCl (10 ml) was added into the mixture and stirred for 10 min. The organic layer was separated and dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the residual solid was purified with silica gel column (hexane : ethyl acetate = 9:1) to give **4** quantitatively.

Herein only representative analytical data are described below. Melting points were not corrected.

5-Nonyl-1,3-cyclohexanedione (**2**)

M.p. 79–81 °C; IR ν_{max} cm^{-1} (nujol): 1600 (s), 1500 (s); ^1H NMR: 0.88 (3H, br. t), 1.10–1.80 (17H, m), 1.85–2.90 (4H, m), 3.36 (2H, s).

3-Hydroxy-5-nonyl-2-[(2-piperidino)propyl]cyclohex-2-en-1-one

M.p. 88–90 °C; IR ν_{max} cm^{-1} (nujol): 3300 (m), 1580 (s), 1510 (s); ^1H NMR: 0.70–1.05 (CH_3 and $2\times CH_3$, m), 1.04–1.60 ($8\times CH_2$ and $2\times CH$, m), 1.60–2.70 ($4\times CH_2$, m), 2.60–3.40 ($2\times CH_2$ and CH, m), 4.18 (CH, d, J = 14 Hz).

1-Hydroxy-4,4-dimethyl-9-nonyl-2,3-dioxabicyclo-[4.4.0]dec-4-ene-7-one

M.p. 76–79 °C; IR ν_{max} cm^{-1} (nujol): 3370 (s), 1685 (s), 1635 (s); ^1H NMR: 0.88 (CH_3, t), 1.20–1.60 ($8\times CH_2$ and CH, m), 1.32 (CH_3, s), 1.46 (CH_3, s), 1.80–2.90 ($2\times CH_2$, m), 3.32 (OH, broad), 6.50 (vinylic proton, s).

2-(2-Ethoxy-1-ethylamino)vinylidene-5-nonyl-1,3-cyclohexanedione (**48**)

M.p. 82–84 °C; IR ν_{max} cm^{-1} (nujol): 3200 (m), 1670 (s), 1580 (s), 1275 (s), 1120 (s); ^1H NMR: 0.87 (CH_3, t), 1.20 (CH_3, t, J = 7 Hz), 1.10–1.40 ($8\times CH_2$ and CH, m), 1.90–2.70 ($2\times CH_2$, m), 3.30–3.70 (overlapping CH_2, q, J = 7 Hz and $2\times CH_2$, m), 8.10 (CH, d, J = 14 Hz), 10.9–11.2 (NH, br.); HRMS: found, 337.26417; calcd. for $C_{20}H_{35}NO_3$, 337.26667.

2-(2-Ethoxy-1-ethylamino)vinylidene-5,5-dimethyl-1,3-cyclohexanedione (**18**)

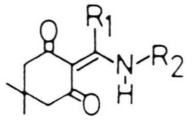
M.p. 121–123 °C; IR ν_{max} cm^{-1} (nujol): 3200 (m), 1665 (s), 1580 (s), 1280 (s), 1120 (s); ^1H NMR: 1.02 ($2\times CH_3$, s), 1.18 (CH_3, t, J = 7 Hz), 2.34 (CH_2, s), 2.36 (CH_2, s), 3.40–3.70 (overlapping CH_2, q, J = 7 Hz and $2\times CH_2$, m), 8.02 (CH_2, d, J = 14 Hz), 10.90–11.20 (NH, broad); HRMS: found, 239.15394; calcd. for $C_{13}H_{21}NO_3$, 239.15214.

Results and Discussion

To design the structure of the new series of photosynthetic electron transport inhibitors **4**, there is valuable information given by the results on **2** in which the total number of methylene groups in both the aminoalkyl (R^1) and ester alkyl (R^2) chains should be in the range 10–13 to show optimum activity [8, 9]. It was also suggested that Hill inhibitory activity might depend not so much on overall lipophilicity, as it would depend on the distribution of lipophilicity within the molecule as represented by the methylene group ratio (R^1/R^2) or by the disparity in alkyl group size (R^1-R^2); *viz*, the optimum inhibitory activity is associated with an assymmetric distribution of lipophilic groups (R^1, R^2) about the aminocyanoacrylate moiety. Furthermore, reduction of the lipophilicity of the R^2 group by replacement of a methylene group with an ether oxygen atom, enhance inhibition activity [9].

Modification on the amino group of **4** derivatives was carried out as listed in Table I. In this modification, the results described above were fully utilized, *i.e.*, the combination of some N-substituents was selected based on this information. Without an ether linkage in the side chain, **4** derivatives are inactive or less active except for propyl and allyl derivatives. Four compounds containing an ether linkage

Table I. PET inhibitory activity of 2-[1-(1-alkylamino)alkylidene]-5,5-dimethyl-1,3-cyclohexanedione.



Compd. No.		R_1	R_2	pI_{50}	Mp. (°C)
Group[a]	10	H	$-(CH_2)_2CH_3$	3.1	152-154
	11	H	$-CH_2CH{=}CH_2$	3.3	100-102
	12	H	$-(CH_2)_5CH_3$	3 >	123-125
	13	H	$-(CH_2)_7CH_3$	3 >	115-118
A	14	H	$-(CH_2)_9CH_3$	3 >	104-106
	15	H	$-CH_2Ph$	3 >	189-190
	16	H	$-CH_2CH_2Ph$	3 >	144-146
	17	H	$-(CH_2)_2OCH_3$	4.7	118-120
	18	H	$-(CH_2)_2OCH_2CH_3$	4.8	122-123
	19	H	$-(CH_2)_3OCH_3$	3.6	144-146
	20	H	$-(CH_2)_3OCH_2CH_3$	3 >	110-112
	21	H	$-(CH_2)_2OPh$	3 >	141-143
B	22	H	$-CH_2-$	4.5	146-148
	23	H	$-CH_2-$	3 >	115-117
	24	H	$-CH_2CH(CH_3)OCH_3$	3 >	90-91
	25	H	$-CH(CH_2CH_3)CH_2OCH_3$	3 >	
	26	H	$-(CH_2CH_2O)_2CH_3$	3.2	74-75
	27	H	$-(CH_2CH_2O)_3CH_3$	3 >	oil
	28	H	$-CH_2COOCH_2CH_3$	3 >	126-127
	29	H	$-(CH_2)_2N(CH_3)_2$	3 >	141-144
	30	H	$-CH_2CONHCH_2COOCH_2CH_3$	3 >	104-105
	31	H	$-OCH_3$	3 >	55-58
C	32	H	$-OCH_2Ph$	3 >	66-68
	33	H	geranyl	3 >	97-99
	34	H	$-(CH_2)_2Br$	3 >	197-199
	35	H	$-(CH_2)_2OH$	3 >	124-126
	36	CH_3	$-(CH_2)_2OCH_3$	3 >	oil
	37	CH_3	$-(CH_2)_2OCH_2CH_3$	3 >	oil
D	38	CH_3	$-(CH_2)_3OCH_3$	3 >	oil
	39	CH_3	$-(CH_2)_9CH_3$	3 >	oil

[a] All the compounds listed here are classified into four groups according to their structural features as follows. A: R_2 is an alkyl or alkyl-related group. B: R_2 includes C–O–C bond. C: R_2 is a miscellaneous functional group. D: R_1 is a methyl group.

(methoxyethyl (**17**), ethoxyethyl (**18**), methoxypropyl (**19**) and furfuryl (**22**) derivatives) inhibited the photosynthetic electron transport at 10^{-5} M level.

These facts suggest that the substituted amino group of the **4** series must be corresponding to the less lipophilic part of the aminocyanoacrylates. The enhancement of the activity due to an ether group is remarkable, however the ether bond in the side chain must be precisely located to show higher inhibition; the ether oxygen and the amino groups need to be separated by three atoms. The whole size of the side chain is presumably limited to the range between four to five carbons. Since the ether group is relatively inert in a chemical bonding sense, it seems that its effect on activity is related to its polar character and possibly to its ability to interact with hydrogen bond donor groups of receptor site peptides. It is noteworthy that a substitution at the peripheral of ether methylene causes total loss of the activity (*e.g.* **23**, **24**, **25**) except in the case of **22** which provides a very flat feature around the ether group. A substitution of the vinylic proton by a methyl group also causes loss of the activity. Increasing the number of ether groups in the side chain drastically reduces the activity.

The above results indicate that methoxyethyl and/or ethoxyethyl amino-groups are the most suitable for such dimedone derivatives in producing inhibitory activity. According to the study on the lipophilic effects of side chains in **1** as "the Hill reaction inhibitors", there must be the optimum distribution of lipophilicity within a molecule or the preferable disparity in the side chain size to attain the maximum level of inhibition. This idea encouraged us to make various kinds of cyclohexane-1,3-dione derivatives to examine the lipophilic effect of this part in the series based on compound **4**.

Results listed in Table II reveal remarkable improvement in potency associated with more lipophilic substituents at C-5 of the ring structure. Varying the chain length of the C-5 substituent leads to an increase in Hill reaction inhibitory activity over the range C_0 to C_{15}. The pI_{50} value increases with increasing chain length (Fig. 1), reaching a maximum with the nonyl and undecyl chain, to the same level of DCMU. This activity enhancement would be attributed to increased lipophilicity, however C_{15} alkyl group (**53**) seems too long to possess a high activity. Although the directions of the long chain alkyl and the alkoxyalkyl about the conjugated enamino system of **4** are opposite to those of **1**, the similar trends of the side chain effects and binding displacement studies [8] and fluorescent transients studies [21] suggest that **4** and **1** interact with the same receptor site in the photosynthetic electron transport system.

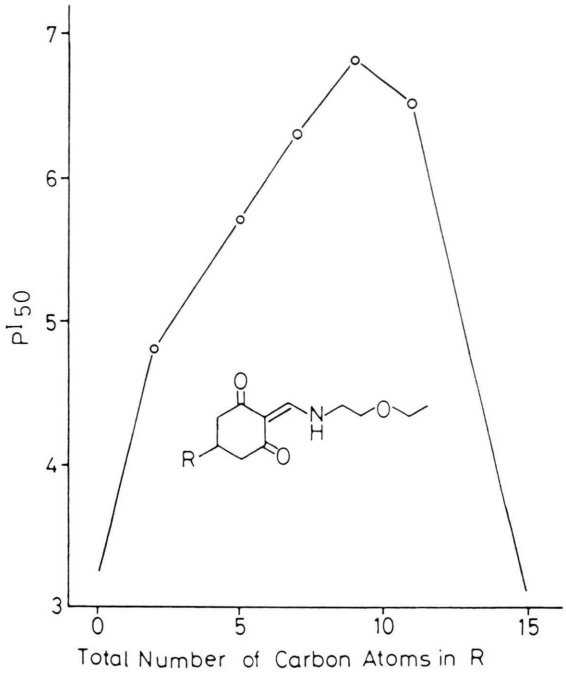


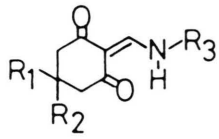
Fig. 1. Relationship between inhibitory activity and the number of carbon atoms in substituent (R).

By consideration of the effect of the ether group and its environment and of the effect of methyl group substitution of the vinylic proton, it is clear that the conjugated enamino group with a ether chain plays an important role in the binding of **4** to receptor site polypeptides, and it is supposed that a hydrophilic pocket with which the ether group and enamino group can interact exists, and it is surrounded by a lipophilic area. The etherenamino pocket is extremly localized, such that modification of the structure in this part of the molecule causes loss of the activity.

Conclusion

It is clear that the conjugated N-alkyl enamino moiety forms a new type of the Hill reaction inhibitors. These compounds show a high level of activity *in vitro* similar to that of DCMU. The optimum Hill inhibitory activity is associated with the conju-

Table II. PET inhibitory activity of 5-substituted 2-(1-alkylamino)vinylidene-1,3-cyclohexanedione.



Compd. No.	R_1	R_2	R_3	pI_{50}	Mp. (°C)
40	H	H	$-(CH_2)_2OCH_3$	3 >	90-92
41	H	H	$-(CH_2)_2OCH_2CH_3$	3 >	61-62
42	H	H	$-(CH_2)_3OCH_3$	3 >	75-77
43	H	H	$-(CH_2)_3OCH_2CH_3$	3 >	59-60
17	CH_3	CH_3	$-(CH_2)_2OCH_3$	4.7	118-120
18	CH_3	CH_3	$-(CH_2)_2OCH_2CH_3$	4.8	122-123
19	CH_3	CH_3	$-(CH_2)_3OCH_3$	3.6	144-146
44	H	$-(CH_2)_4CH_3$	$-(CH_2)_2OCH_3$	5.4	80-83
45	H	$-(CH_2)_4CH_3$	$-(CH_2)_2OCH_2CH_3$	5.7	88-90
46	H	$-(CH_2)_6CH_3$	$-(CH_2)_2OCH_3$	6.1	76-77
47	H	$-(CH_2)_6CH_3$	$-(CH_2)_2OCH_2CH_3$	6.3	86-87
48	H	$-(CH_2)_6CH_3$	$-(CH_2)_3OCH_3$	6.2	100-103
49	H	$-(CH_2)_8CH_3$	$-(CH_2)_2OCH_3$	6.4	78-79
50	H	$-(CH_2)_8CH_3$	$-(CH_2)_2OCH_2CH_3$	6.8	82-84
51	H	$-(CH_2)_8CH_3$	$-(CH_2)_3OCH_2CH_3$	3 >	88-90
52	H	$-(CH_2)_{10}CH_3$	$-(CH_2)_2OCH_2CH_3$	6.5	73-75
53	H	$-(CH_2)_{14}CH_3$	$-(CH_2)_2OCH_2CH_3$	3 >	62-65
		DCMU		6.7	
		cyanoacrylate		6.8	

Cyanoacrylate = ethoxyethyl-2-cyano-3-n-decylaminoacrylate, kindly provided by Dr. Phillips [9].

gated enamino system being connected to well balanced lipophilic groups. Both our results and the studies on compounds of type **1** suggest that the binding domain of the receptor site contains an asymmetric distribution of hydrophobic regions with which lipophilic groups of these compounds interact.

The above results suggest that these conjugated enamino compounds should be a suitable probe for the purpose of searching the environment of the Hill reaction inhibitors binding sites. More detailed studies of the structure-activity relationships of the conjugated enamino compounds should provide further information concerning the environment on the binding site of these and other PS II inhibitors.

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