Photosystem II Inhibition by 3-Acyl-5-(1-aminoalkylidene)-4-hydroxy-2 *H*-pyran-2,6(3 *H*)-dione Derivatives

Koichi Yoneyama, Makoto Konnai
Weed Control Research Institute, Faculty of Agriculture, Utsunomiya University, 350-Mine-machi, Utsunomiya 321, Japan

Tadao Asami, Nobutaka Takahashi, and Shigeo Yoshida
The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako 351-01, Japan

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3-Acyl-5-(1-aminoalkylidene)-4-hydroxy-2 *H*-pyran-2,6(3 *H*)-dione derivatives carrying lipophilic amino groups were found to be highly potent inhibitors of photosynthetic electron transport, and both the acyl and the aminoalkylidene groups on the pyran ring appeared to be indispensable for high activity. The structural features needed for activity in the 3-acyl-5-(1-aminoalkylidene)-4-hydroxy-2 *H*-pyran-2,6(3 *H*)-dione derivatives were very similar to those for 3-(1-aminoalkylidene)-2 *H*-pyran-2,4(3 *H*)-diones, except for the acyl group in the former compounds. Thermoluminescence measurements indicated that the 3-acyl-5-(1-aminoalkylidene)-4-hydroxy-2 *H*-pyran-2,6(3 *H*)-dione derivatives bind to the D1 protein in a manner similar to that of DCMU.

Introduction

The receptor site for a wide range of photosynthetic inhibitors has been found to be associated with the D1 protein which, with other polypeptides and pigments, constitutes the reaction center of photosystem II (PS II) [1]. In photosynthetic bacteria, three-dimensional structures of the photochemical reaction centers have been determined by means of X-ray crystallography [2, 3], and this has enabled the investigation of the molecular events involved in the electron transfer process.

However in plants, crystallization of the PS II reaction center has not yet been achieved due to its instability in the isolated state. An alternative approach to study the structure of the PS II reaction center involves probing the binding site with specific and highly active inhibitors. In particular, new PS II inhibitors which differ structurally from well-known inhibitors are promising tools in such studies, since they may interact with the receptor site in a different manner. In fact, studies with new potent PS II inhibitors such as 3-aminocyanoacrylates (**1**) [4, 5], 2-(1-aminoalkylidene)-1,3-cyclohexanediones (**2, AC**) [6, 7] and 3-(1-aminoalkylidene)-2 *H*-pyran-2,4(3 *H*)-diones (**3, AP**) [8, 9] have accumulated novel information on the interaction between these inhibitors and the binding niche.

The carbonyl conjugated enamine structure of these new inhibitors was found to play a major role in their PS II inhibition, and an asymmetric distribution of lipophilicity about the enamine moiety was a further prerequisite for the activity [5, 6, 8]. Therefore, other six-membered ring compounds containing a 1,3-dicarbonyl-2-aminoalkylidene system might also be expected to inhibit PS II. Studies based on this idea led to the syn-

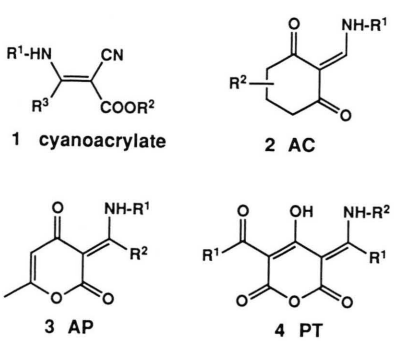

Fig. 1. Chemical structures of new PS II inhibitors. Abbreviations: **AC,** 2-(aminoalkylidene)-1,3-cyclohexanediones; **AP,** 3-(1-aminoalkylidene)-2 *H*-pyran-2,4(3 *H*)-diones; **PT;** 3-acyl-5-(1-aminoalkylidene)-4-hydroxy-2 *H*-pyran-2,6(3 *H*)-diones.

Reprint requests to Dr. K. Yoneyama.

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thesis of 3-acyl-5-(1-aminoalkylidene)tetrahydropyran-2,4,6-trione (**4, PT**).

Although **AC** and **AP** compounds were very similar in their structural requirements for inhibition [8], they seemed to differ in their binding behavior with the D1 protein. In particular, it was suggested that **AP** compounds could be classified as a new type of inhibitor which bound to the site in a manner totally different from that of other PS II inhibitors [9]. Accordingly, in these two types of inhibitors, structural features surrounding the conjugated enamine moiety greatly affected both the activity and the binding behavior with the D1 protein. Presumably, thermoluminescence (TL) analysis [9, 10] of the binding mode of **PT** derivatives, which are structurally related to **AP,** would afford some information on the unique mode of action of the **AP** compounds.

In this paper, we wish to describe the structural requirements for PS II inhibition of the **PT** derivatives and discuss their binding behavior with the D1 protein based on the results obtained by TL measurements [10] and the use of mutant chloroplasts [11].

Materials and Methods

PS II inhibition

Spinach (*Spinacia oleracea* L.) and rape (wild type and atrazine-resistant mutant *Brassica napus* L.) [11] chloroplasts were obtained by conventional methods [12] and were stored in liquid nitrogen. The PS II activity was measured as reported previously [13], chlorophyll concentration being adjusted to 0.5 μg/ml for spinach, and 5 μg/ml for both wild type (atrazine-susceptible) and mutant (atrazine-tolerant) *Brassica napus,* respectively. The PS II inhibitory activities of the compounds are expressed by pI_{50} values, which indicate the negative logarithms of the concentration (M) of the compounds giving 50% inhibition of electron transport.

Thermoluminescence measurement

Thylakoids, diluted to give 0.25 mg Chl/ml in 25% (v/v) glycerol, 10 mM $MgCl_2$ and 50 mM HEPES–NaOH (pH 7.0), were illuminated with orange light (>500 nm) for 45 sec before being left in the dark for 5 min at room temperature. Thermoluminescence was measured as described in [14], the samples being illuminated with xenon flashes, rapidly cooled to −196 °C, and then heated at a rate of 0.8 °C/sec.

Chemicals

All melting points (m.p.) are given as uncorrected values. Structures of the synthesized compounds were confirmed by ^{1}H NMR and IR spectra, which were obtained with a Hitachi R 1200 and a SHIMADZU IR-400 spectrometer, respectively. Mass spectra were recorded on a Finnigan MAT INCOS 50 spectrometer. Satisfactory analytical data have been obtained for all the tested compounds.

Several preparative methods for the 3-acyl-5-(1-aminoalkylidene)tetrahydropyran-2,4,6-trione (**PT**) derivatives have been reported [15, 16], and the compounds having *N*-phenyl groups were synthesized according to Snader *et al.* [16]. The other compounds were synthesized according to Kiang *et al.* [15].

Synthesis of 3-acetyl-5-[1-(1-decylamino)ethylidene]-4-hydroxy-2H-pyran-2,6(3H)-dione (7)

In a 100 ml flask, 1.06 g (5 mmol) of 3,5-diacetyltetrahydropyran-2,4,6-trione [15], 0.79 g (5 mmol) of 1-decylamine, and 3 ml of acetic acid were heated at 95–100 °C for 20 min. After evaporating the acetic acid *in vacuo,* 30 ml of toluene was added to the residue, and the mixture was heated under reflux for 4 h. The toluene was removed *in vacuo,* and the residue was purified by silica gel column chromatography ($CHCl_3$–acetone = 95:5, v/v) to give 1.19 g (68%) of **7.**

M.p.: 83–84 °C (recrystallized from ethanol). IR: 1730, 1650, 1595, 1560, 1280, 1120 (nujol). ^{1}H NMR δ (ppm): 0.88 (CH_3, t, br.), 1.16–2.10 (8 × CH_2, m), 2.61 (CH_3, s), 2.68 (CH_3, s), 3.40–3.75 (CH_2, m), 12.12 (NH, br.), 18.88 (OH, br.). M^+ (*m/z*): 351.

$C_{19}H_{29}NO_5$ (351.4)
Calcd C 64.94% H 8.32% N 3.99% O 22.76%,
Found C 64.64% H 8.20% N 3.95% O 23.21%.

Results and Discussion

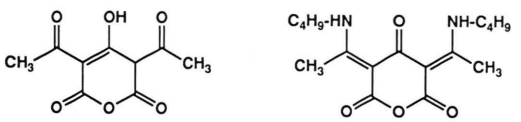
Essential structural features for PS II inhibition

In order to examine the essential structural features for PS II inhibition, several types of tetrahy-

dropyran-2,4,6-trione derivatives were synthesized and their PS II inhibitory activities in isolated spinach chloroplasts determined (Fig. 2). Among the compounds shown in Fig. 2, only the compound having both an acyl (acetyl) and an aminoethylidene group (**7**) was a highly active PS II inhibitor. Weak activity of the di-enamine (**6**) and the lack of activity of the diacyl-pyrantrione (**5**) clearly demonstrated that the carbonyl-conjugated enamine structure is necessary for inhibition. Further lengthening of the *N*-alkyl group in the di-enamine led to the loss of the activity (data not shown), and thus both the acyl group and the enamine moiety on the nucleus were needed for higher activity. Therefore, we tried to optimize the structure of the **PT** derivatives (**4**) for PS II inhibition by varying the two substituents, R^1 and R^2 (Fig. 1).

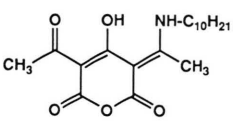
It has been reported that the major tautomers of 3,5-diacyltetrahydropyran-2,4,6-triones and their mono-enamines are 4-hydroxy-2*H*-pyran-2,6-(3*H*)-diones [16, 17], and thus, the 3-acyl-5-(1-aminoalkylidene)tetrahydropyran-2,4,6-trione derivatives (**4**) should be described as 3-acyl-5-(1-aminoalkylidene)-4-hydroxy-2*H*-pyran-2,6(3*H*)-dione derivatives. However, we will use the term **PT** and **AP** for the compounds **4** and **3,** respectively.

In designing active **PT** derivatives, information on the structure-activity relationships of the closely related PS II inhibitors, **AP,** was available [8, 9]. In the case of **AP,** active compounds carry lipophilic amino groups. A similar trend was also observed with the **PT** derivatives as shown in Table I. Among the *N*-alkyl derivatives listed in the Table (**7–16**), the most active one, carrying the *N*-dodecyl group (**14**), was much more potent than DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Accordingly, structural features for PS II inhibition of the **PT** derivatives seemed to be very similar to those of **AP.**



5 (<5.0)

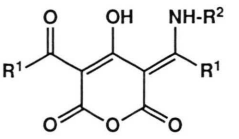
6 (5.9)



7 (7.5)

Fig. 2. Chemical structures and PS II inhibitory activity of pyran-trione derivatives Figures in parentheses are pI_{50} values.

Table I. PS II inhibitory activity of the *N*-alkyl derivatives.

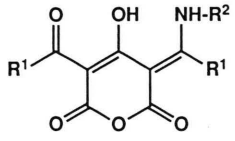


Compd. No.	R^1	R^2	pI_{50}	m.p. [°C]
8	Methyl	Butyl	<5	115–116
9	Methyl	Pentyl	5.0	125–126
10	Methyl	Hexyl	5.9	91–92
11	Methyl	Heptyl	6.4	84–85
12	Methyl	Octyl	6.8	85–86
13	Methyl	Nonyl	7.0	86–87
7	Methyl	Decyl	7.5	83–84
14	Methyl	Undecyl	7.6	72–73
15	Methyl	Dodecyl	7.9	74–75
16	Methyl	Pentadecyl	7.4	78–79
DCMU*			7.3	

* 3-(3,4-Dichlorophenyl)-1,1-dimethylurea.

Anti-allergic activity of the **PT** derivatives, in particular that of *N*-phenyl derivatives, was extensively studied by Snader *et al.* [16]; the compounds being described as pyranenamines in their reports [16, 18]. Cramer *et al.* applied the quantitative structure-activity relationship analysis to obtain an optimal structure for the anti-allergic activity, and found that the introduction of hydrophilic substituents in the aniline moiety enhanced the activity [18]. In contrast, the *N*-phenyl derivatives were not highly active PS II inhibitors, and the introduction of hydrophilic substituents like the hydroxyl group greatly reduced their activity as shown in Table II. Thus the 4-OH derivative (**18**), which has been reported to be an active anti-allergic compound [16, 18], was inactive on the PS II system. On the other hand, compounds carrying lipophilic substituents on the benzene ring (**19, 22** and **23**) showed weak to moderate PS II inhibitory activities. These results indicated that the structural requirements of the **PT** derivatives for anti-allergic activity clearly differed from those for PS II inhibition, and that increased lipophilicity enhanced the PS II inhibitory activity.

Table II. PS II inhibitory activity of the *N*-phenyl and *N*-phenylalkyl derivatives.



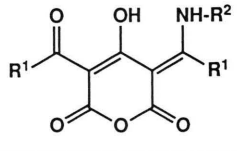
Compd. No.	R^1	R^2	pI_{50}	m.p. [°C]
17	Methyl	Phenyl	<5	189–190
18	Methyl	4-OH-Phenyl	≪5	221–223
19	Methyl	4-Cl-Phenyl	5.0	205–206
20	Methyl	4-NO_2-Phenyl	<5	231–232
21	Methyl	4-Me-Phenyl	<5	197–198
22	Methyl	4-Pr-Phenyl	5.4	163–164
23	Methyl	4-Phenoxyphenyl	6.2	182–183
24	Methyl	Benzyl	5.7	138–139
25	Methyl	2-Phenethyl	5.4	146–147
26	Methyl	3-Phenylpropyl	6.6	119–120
27	Methyl	4-Phenylbutyl	5.9	120–122
28	Methyl	5-Phenylpentyl	6.6	72–74
29	Methyl	(*R*)-1-Phenethyl	4.3	131–132
30	Methyl	(*S*)-1-Phenethyl	6.0	130–131
DCMU*			7.3	

* 3-(3,4-Dichlorophenyl)-1,1-dimethylurea.

Although **PT** derivatives having the *N*-phenyl group were only weakly active PS II inhibitors, the introduction of a methylene group between the benzene ring and the amino nitrogen atom enhanced the activity (**17** *vs.* **24**). Further lengthening of the carbon chain produced a stepwise increase in the activity until the chain length reached C_5. More precisely, the compounds having an even number of methylene groups between the phenyl and amino moieties (**17, 25** and **27**) were less active than those having an odd number (**24, 26** and **28**). A similar effect has been observed with the phenylalkylamino-cyanoacrylate derivatives, and Huppatz and Phillips explained this in terms of a preferred orientation of the benzene ring for interaction with a specific region within the hydrophobic matrix; the compounds showed higher activities when the benzene rings were in the correct orientations resulting from interaction with a lipophilic region in the D1 protein [19]. In addition, the difference in the PS II inhibitory activity of the enantiomers of the 3-acetyl-5-[1-(1-phenethyl)amino]-ethylidene-4-hydroxypyran-2,6-dione derivative (**29** and **30**) may also be caused by the different orientations of the benzene rings in these compounds. The *S*-isomer (**30**) was more active than the *R*-isomer (**29**) as in the case of other PS II inhibitors [20, 21], suggesting a common site of chiral recognition prefering an *S*-configuration for optimal interaction.

Effects of the alkylidene part of the **PT** derivatives on the activity are shown in Table III. Among the *N*-decyl derivatives, the compound containing the propylidene structure (**31**) shows the highest activity, which drops dramatically when the carbon number of the alkylidene moiety exceeds 3 (**32–34**). In this study, only **PT** deriva-

Table III. Effects of alkylidene moiety on the PS II inhibitory activity.



Compd. No.	R^1	R^2	pI_{50}	m.p. [°C]
7	Methyl	Decyl	7.5	
31	Ethyl	Decyl	7.6	74–75
32	Propyl	Decyl	5.0	42–43
33	Butyl	Decyl	5.0	46–47
34	Pentyl	Decyl	<5	amorphous

tives having the same substituent (R^1) on both the 2-position of the aminoalkylidene and the carbonyl carbon attached to the 5-position of the pyran ring were examined, so that increasing R^1 by one methylene group was associated with a two methylene contribution to overall lipophilicity. However, the size of R^1 would seem to be more important than the overall lipophilicity because of the rather sudden decrease in activity between propylidene and butylidene analogues.

Binding mode to D 1 protein

On the basis of TL measurement, the **AP** derivatives were shown to behave differently from other classical PS II inhibitors in their binding to the D 1 protein [9]. Since the **PT** derivatives were found to be very similar to **AP** in the structural features required for PS II inhibition, they might also have been expected to bind similarly to the D 1 protein. Thermoluminescence analysis of the **PT** derivative (**7**), however, revealed that this compound should be classified as a DCMU type PS II inhibitor based on the TL emission band temperature and the normal oscillation pattern observed (Table IV). Thus, the **PT** and **AP** derivatives appear to differ in their binding mode to the D 1 protein. This may be due to the presence of the acyl group in the former compounds, which could form an intramolecular hydrogen bond with the carbonyl group at the 4- or 2-position of the nucleus or act as a hydrogen bond acceptor to some hydrogen bond donor on the receptor site.

Table IV. Thermoluminescence (TL) glow peak temperatures of new and standard PS II inhibitors.

Inhibitors	TL [°C]	Oscillation pattern
7	+ 6	normal
AC[a]	+ 6	normal
AP[b]	+18	abnormal
DCMU[c]	+ 6	normal
Atrazine[d]	+ 2	normal
Ioxynil[e]	− 7	normal

[a] 2-(1-Ethoxyethylaminomethylidene)-5-nonyl-1,3-cyclohexanedione.
[b] 2-(1-Dodecylaminopropylidene)-6-methyl-2 *H*-pyran-2,4(3 *H*)-dione.
[c] 3-(3,4-Dichlorophenyl)-1,1-dimethylurea.
[d] 2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine.
[e] 3,5-Diiodo-4-hydroxybenzonitrile.

Table V. PS II inhibitory activities of new and standard inhibitors in thylakoids isolated from atrazine-resistant and susceptible *Brassica napus*.

Inhibitors	pI_{50} (resistant)	pI_{50} (susceptible)
7	6.3	7.5
AC[a]	4.3	7.1
AP[b]	6.4	7.3
DCMU[c]	6.8	7.2
Atrazine[d]	<3	6.4
Ioxynil[e]	7.1	6.6

[a] 2-(1-Ethoxyethylaminomethylidene)-5-nonyl-1,3-cyclohexanedione.
[b] 2-(1-Dodecylaminopropylidene)-6-methyl-2 *H*-pyran-2,4(3 *H*)-dione.
[c] 3-(3,4-Dichlorophenyl)-1,1-dimethylurea.
[d] 2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine.
[e] 3,5-Diiodo-4-hydroxybenzonitrile.

As mentioned before, major tautomers of the **PT** derivatives are the 4-hydroxy form, *i.e.*, 3-acyl-5-(1-aminoalkylidene)-4-hydroxy-2 *H*-pyran-2,6(3 *H*)-dione structures [16]. On the other hand, active **AP** as well as **AC** inhibitors exist only in the 4-keto form when the amino nitrogen is substituted with an alkyl group [9, 10]. These observation suggest that the 5-(1-aminoalkylidene)-4-hydroxypyran-2,6-dione derivatives use the carbonyl functionality on the 3-position of the pyran ring to stabilize the active 4-hydroxy (or 2-hydroxy) structure by forming an intramolecular hydrogen bond.

Table V shows the inhibitory activities of the **PT** derivative (**7**) on PS II in chloroplasts isolated from atrazine-resistant and susceptible biotypes of *Brassica napus*. The **PT** derivative was less active against atrazine-resistant than against atrazine-susceptible biotypes, indicating that this compound could be classified as an inhibitor of the urea/triazine type [11]. These results were in agreement with those obtained by TL measurements.

Consequently, the **PT** derivatives were found to be good molecular probes for photosynthetic research because of their high inhibitory activities. Further synthetic and biochemical studies on these readily prepared compounds are in progress.

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