Structure-Activity Relationships and Physiological Aspects of New Photosynthetic Electron Transport Inhibitors, 3-Alkylaminoalkyliden-2*H*-pyran-2,4(3*H*)-diones (APs)

Tadao Asami, Hiroyuki Koike*, Yorinao Inoue*,
Nobutaka Takahashi, and Shigeo Yoshida

Chemical Regulation of Biomechanisms Laboratory and * Solar Energy Research Group,
The Institute of Physical and Chemical Research 2-1 Hirosawa, Wako, Saitama 351-01, Japan

Z. Naturforsch. **43c**, 857–861 (1988); received July 22, 1988

Conjugated Enamine, Photosynthetic Electron Transport Inhibitor, 3-Alkylaminoalkyliden-2*H*-pyran-2,4(3*H*)-diones, Inhibitor Binding Site of D1 Protein, Thermoluminescence

3-Aminoalkylidene-2*H*-pyran-2,4(3*H*)-diones (APs), possessing a conjugated enamino moiety which is common to cyanoacrylates and 2-aminoalkylidenecyclohexane-1,3-diones (ACs), were found as a new class of photosynthetic electron transport inhibitors. Although the structural requirement of APs for photosynthetic electron transport inhibition was very similar to that of cyanoacrylates and ACs, thermoluminescence measurements indicated that the binding manner of APs to D1 protein was totally different from that of other inhibitors.

Introduction

There are many chemical classes of photosynthetic electron transport inhibitor herbicides, and some of them have played a great role in physiological investigation on the photosynthetic electron transport system. Designing of new type of such inhibitors which block the electron flow at a specific site, is still a very hard task, although the researches on the classical inhibitors have revealed their binding modes and/or site(s) at the *inter*-protein of chloroplast level. The behavior of the inhibitors at the *intra*-protein level and/or the actual form of the binding niche have been retained as very curious problems. In order to elucidate the interaction between those type inhibitors and their binding sites, various new approaches will come in the next decade, *e.g.* topological analyses for a dynamic state of receptor (binding) protein, modification of peptide unit(s) by gene technology and computer simulation to optimize the effect of inhibitors. Discovery of new classes of photosynthetic inhibitors and subsequent activity-structure studies may support to accumulate more information for such new approaches.

Cyanoacrylates (**1**) [1, 2] and aminoalkylidenecyclohexane-diones (ACs) (**2**) [3] are new type of potent inhibitors for the photosynthetic electron transport. Both involves a conjugated enamine system as an essential functionality for their activity [4]. Furthermore, the character of substituents around the conjugated enamine moiety of those two types show similar trends in lipophilicity to reach the optimum activity, namely they need the suitable disparity of lipophilic and relatively hydrophilic functionalities. These facts indicates that both chemical classes might have a similar binding niche on D1 protein of the PS II judging from the structural similarity.

According to the structural relation to AC series, other six membered ring compounds carrying a 1,3-dicarbonyl-2-aminoalkylidene system were examined with the photosynthetic electron transport inhibition assay. Among them 3-alkylaminoalkylidene-2*H*-pyran-2,4(3*H*)-dione (AP) derivatives (**3**) were found as potent photosynthetic electron transport inhibitors [4]. In this paper we describe the effects of lipophilic substituents around the conjugated enamine part of AP molecules and the binding site of this inhibitor series analyzed by the thermoluminescence band measurements.

Materials and Methods

The structures of synthetic chemicals were confirmed by various ^{1}H NMR spectra which were recorded on instrumental analyses: ^{1}H NMR (JEOL

Abbreviations: AP(s), 3-aminoalkylidene-2*H*-pyran-2,4(3*H*)-dione(s); AC(s), 2-aminoalkylidenecyclohexane-1,3-dione(s); bromopyridinol, 2-(α-bromooctyl)-3,5-dibromo-2-methylpyridine-4-ol; PS, photosystem; TL, thermoluminescence.

Reprint requests to Dr. S. Yoshida.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/1100–0857 $ 01.30/0

MH-100 and FX-100 spectrometers, 100 MHz), ^{13}C NMR spectra (FX-100, 25.5 MHz), IR spectra (JASCO A-400) and UV spectra (HITACHI MODEL 200-20).

In this study, 3-acyl-6-methyl-2*H*-pyran-2,4(3*H*)-dione and 6-methyl-2*H*-pyran-2,4(3*H*)-dione were used as starting materials for synthetic procedures described in the previous paper [4].

Hill reaction assay

Spinach thylakoids were prepared as described in [5]. Thylakoids (final concentration 0.5 μg Chl/ml) were suspended in 50 mM HEPES-NaOH (pH 7.0), 10 mM NaCl, 20 mM methylamine, 50 μM DCIP, and inhibitors if added. Electron transport activity was measured by monitoring DCIP photoreduction at 600 nm with Shimadzu UV-300 spectrophotometer.

Thermoluminescence measurement

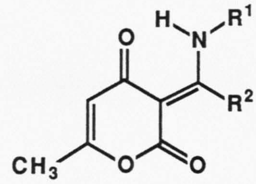
Thylakoids were diluted (0.25 mg Chl/ml) with 25% (v/v) glycerol, 10 mg $MgCl_2$, and 50 mM HEPES-NaOH (pH 7.0), and were illuminated with orange light for 45 sec then dark adapted for 5 min at room temperature. Thermoluminescence was measured as described in [6]. The samples were illuminated with xenon flashes, rapidly cooled to −196 °C, then heated at a rate of 0.8 °C/sec.

Results and Discussion

Structure-activity relationships

The chemical modification of the *N*-substituent (R^1) and the alkylidene part (R^2) in AP series were carried out and the bioassay results are listed in Table I. When R^2 is fixed to $-CH_3$ or $-C_2H_5$ groups, a proper range of lipophilicity in R^1 caused very high inhibition, however, only negligible activity was observed in the case of $-H$ or $-C_4H_9$ groups for R^2. Thus, the suitable combination of R^1 and R^2 is necessary for AP to show the inhibition on the photosynthetic electron flow, as well as the case of cyanoacrylates and AC. The lipophilic effect of R^1 was demonstrated by the change of the alkyl chain length in the AP series which carries a ethyl group as R^2 (Fig. 1). The effect was optimized with the dodecyl group and the rather wide range (from C_8 to C_{14} or more) of lipophilicity was shown to be effective for the high level (more than 6.0 in pI_{50} values) of activity.

Table I. Photosynthetic electron transport inhibition of various APs.



Compounds	R^1	R^2	pI_{50}
4	$-(CH_2)_2OCH_2CH_3$	$-H$	3.5>
5	$-(CH_2)_5CH_3$	$-H$	3.5>
6	$-(CH_2)_9CH_3$	$-H$	3.5>
7	$-(CH_2)_{13}CH_3$	$-H$	3.5>
8	$-(CH_2)_5CH_3$	$-CH_3$	5.6
9	$-(CH_2)_7CH_3$	$-CH_3$	5.8
10	$-(CH_2)_9CH_3$	$-CH_3$	6.4
11	$-(CH_2)_{11}CH_3$	$-CH_3$	7.3
12	$-(CH_2)_{13}CH_3$	$-CH_3$	7.2
13	$-(CH_2)_5CH_3$	$-CH_2CH_3$	5.4
14	$-(CH_2)_7CH_3$	$-CH_2CH_3$	6.6
15	$-(CH_2)_9CH_3$	$-CH_2CH_3$	7.4
16	$-(CH_2)_{11}CH_3$	$-CH_2CH_3$	7.5
17	$-(CH_2)_{13}CH_3$	$-CH_2CH_3$	7.1
18	$-(CH_2)_5CH_3$	$-(CH_2)_2CH_3$	5.0
19	$-(CH_2)_9CH_3$	$-(CH_2)_2CH_3$	6.2
20	$-(CH_2)_{13}CH_3$	$-(CH_2)_2CH_3$	5.0
21	$-(CH_2)_3CH_3$	$-(CH_2)_2CH_3$	3.5>
22	$-(CH_2)_5CH_3$	$-(CH_2)_2CH_3$	3.5>
23	$-(CH_2)_9CH_3$	$-(CH_2)_2CH_3$	3.8
24	$-(CH_2)_{13}CH_3$	$-(CH_2)_2CH_3$	3.5>
DCMU			7.3

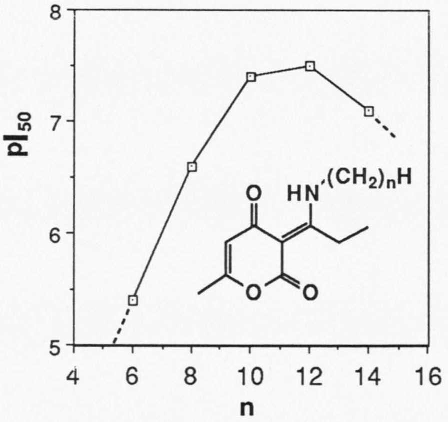


Fig. 1. Diagrammatic representation of the change of pI_{50} for APs of increasing alkyl chain length in R^1.

On the contrary, R^2 of AP was critically limited to the narrow range (between C_1 and C_3) of the lipophilicity as illustrated in Fig. 2. Since this pecul-

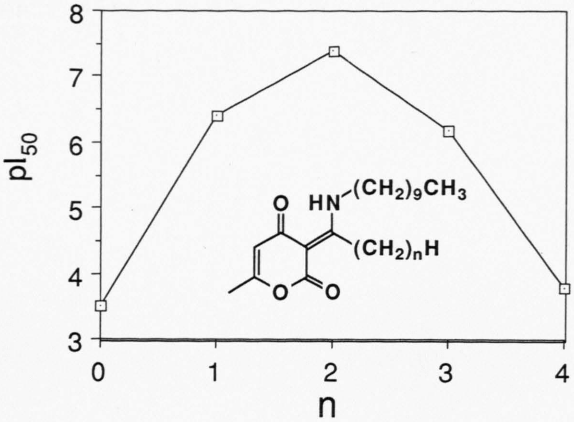


Fig. 2. Diagrammatic representation of the change of pI_{50} for APs of increasing alkyl chain length in R^2.

iar nature of R^2 in AP is similar to that in cyanoacrylates [7, 8], it may be possible to think about a common domain for the enamine functions of both types in the D1 protein.

Consequently the combination of dodecyl for R^1 and ethyl for R^2 has been recognized as the optimum distribution for the lipophilicity around the enamine functionality for AP series to inhibit the photosynthetic electron flow. In this case the optimized inhibition of AP (**16**) was surpassing the effect of DCMU.

It is noteworthy that APs (**5–7**) with the methylidene structure ($R^2 = -H$) was totally inactive. This point is one of the most significant structural difference between APs and ACs which exhibited very high activity with the methylidene structure [3]. Furthermore another basic difference between those two series was recognized in the location of lipophilic moieties in their molecules. This means that a lipophilic *N*-substituent is necessary for the active APs, while the active ACs carry a polar group as the corresponding part. Thus, a curious problem concerning the binding site(s) of those new inhibitors occurred again from the structural consideration, although their modes of action had not been examined in detail.

As described above, the vicinity of the conjugated enamine system of APs, ACs and cyanoacrylates should be a key part to generate the photosynthetic electron transport inhibition. Consequently an accurate evaluation of their inhibition manners depending on the enamine structures should be required for the further investigation of binding studies. For the analysis on the binding mode of D1 protein inhibitors, the thermoluminescence measurement [9] is one of the most effective to make evidences at the *intra*-protein level.

Thermoluminescence method

A number of inhibitors which interrupt the electron flow at photosystem II (PS II) has been reported and the mode of action has been studied extensively. These PS II inhibitors were classified into two major groups, an urea/triazine type and a phenol type by their chemical structures and inhibitory patterns [10]. Both types of inhibitors inhibit the electron flow between Q_A and Q_B by binding at the plastoquinone (Q_B) domain resulting in replacement of the quinone with inhibitors. Since an inhibitor is replaceable with another inhibitor of different class, the presence of a common binding domain on PS II is suggested in the receptor of those inhibitors. There is a clear correlation between the binding sites and the structure of these two groups. The urea/triazine type inhibitors have common structural features of lipophilic part(s) in association with the azo-functionalities [$-C(=X)-N<$] [11]. Although ureas and triazines were classified into one group of D1 protein inhibitors, significant gaps of activity were often observed by the use of triazine-resistant D1 proteins which were obtained from mutant plants [12].

The redox state of Q_A and Q_B in PS II can be monitored by thermoluminescence (TL) measurements, and the TL glow curve shows a main band at around 30 °C (B-band) which is attributed to recombination between S_2 (and/or S_3) and Q_B^-. Inhibitors cause a lower temperature shift of the TL-band due to recombination between S_2 and Q_A^- (Q-band) [13–15]. The peak temperature of the Q-band is dependent on the treatment of a particular inhibitor class [9]. From the peak emission temperature of Q-band, the orthodox inhibitors are classified into three groups; ureas, triazines and phenols in agreement with differences indicated by the mutant experiments [13]. Thus, the TL glow curve measurement is a powerful tool for grouping new inhibitors.

Table II shows the TL glow peaks under the presence of various inhibitors. The peak temperature of Q-band in the presence of DCMU, atrazine and ioxynil were +7, +1, and −7 °C, respectively. The peaks of TL glow curves by AC (+8 °C) and cyano-

Table II. Thermoluminescence glow peaks under the presence of various inhibitors.

Compounds	Concentration [μM]	T_{max} [°C]
DCMU	10	+7
Propanil	10	+6
Bromacil	10	+8
Buthidazole	10	+6
Atrazine	6	+1
Ioxynil	100	−7
Bromopyridinol*	10	−6
Cyanoacrylate	10	+6
AC	10	+8
AP	10	+18
Control	–	+38

* Abbreviated name of 2-(α-bromooctyl)-3,5-dibromo-2-methylpyridine-4-ol; see ref. [16].

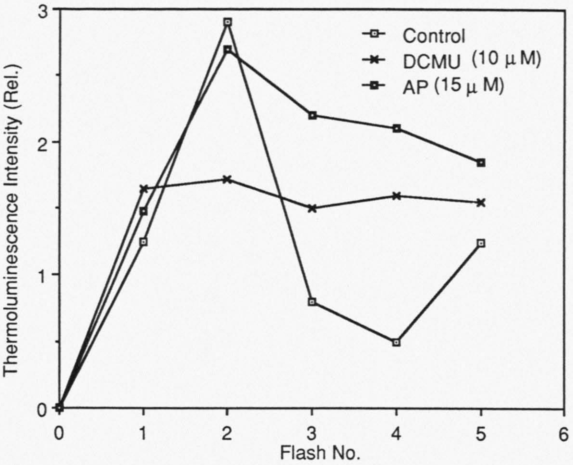


Fig. 3. Oscillation patterns of the intensity of thermoluminescence with flash times under the normal condition and under the presence of DCMU and AP.

acrylates (+6 °C) were very similar to that of DCMU, therefore those new inhibitors should be classified into the DCMU-type inhibitor. On the other hand, the glow peak of AP (+20 °C) was found to be intermediate between B-band and DCMU-induced Q-band. There have been no report on the inhibitor which shows the emission peak at such a high temperature. This suggests that AP binds to the D1 protein with a different manner from those of other inhibitors.

Another characteristic effect of AP in the TL measurement was observed in the band height after the second flash, which was higher than that of the first flash whereas the other types of inhibitors gave a constant band height for every flash (Fig. 3). The height of the B-band which arises from the recombination between S_2/S_3 and Q_B^- in the absence of inhibitors shows a quadruple oscillation with a maximum at 2nd flash and a minimum at 4th flash. It should be noted that the concentration of the inhibitors (15 μM) tested in Fig. 3 was sufficient for complete inhibition of electron flow (see also Table I). Thus, the anomalous behavior of AP in the TL measurement is not due to insufficient blocking of the electron flow.

The oscillation of Q-band induced by atrazine, AC and ioxynil showed practically the same pattern as that of DCMU although the emission temperature is different with each other. The difference in the peak temperature might reflect the interaction between Q_A^- and inhibitors. Thus, as far as the results of TL measurements, APs should be classified as totally new type inhibitors in the binding manner at the D1 protein.

Conclusion

Our present study indicated that the compounds holding a conjugated enamine system should be an effective functionality to inhibit the photosynthetic electron transport. In spite of the similarity in the chemical nature of the enamine systems, there are obvious differences in their inhibition mode due to variation of the substitutional pattern around the system. This fact leads us to the assumption that a conjugated enamine system presumably has a potent affinity to peptide bond(s) and/or residue(s) of amino acid(s) in the D1 protein. According to this idea, those inhibitors described in this paper might be useful as good probes to investigate a niche of herbicides in the D1 protein because of easiness in their structural modification.

Further characterization for the unique binding site of AP is now under way using various TL techniques and mutant chloroplasts.

Acknowledgements

A part of this research was supported by Nissan Science Foundation. The authors gratefully acknowledge Dr. J. N. Phillips (CSIRO, Australia) for his kind supply of cyanoacrylates.

[1] J. N. Phillips and J. L. Huppatz, Agric. Biol. Chem. **48,** 51–54 (1984).

[2] J. N. Phillips and J. L. Huppatz, Agric. Biol. Chem. **48,** 55–59 (1984).

[3] T. Asami, N. Takahashi, and S. Yoshida, Z. Naturforsch. **41c,** 751–757 (1986).

[4] T. Asami, N. Takahashi, and S. Yoshida, Agric. Biol. Chem. **51,** 2775–2780 (1987).

[5] C. J. Arntzen and C. L. Ditto, Biochim. Biophys. Acta **449,** 259–279 (1976).

[6] T. Ichikawa, Y. Inoue, and K. Shibata, Biochim. Biophys. Acta **408,** 228–239 (1975).

[7] J. N. Phillips and J. N. Huppatz, Z. Naturforsch. **39c,** 335–337 (1984).

[8] J. L. Huppatz and J. N. Phillips, Z. Naturforsch. **42c,** 674–678 (1987).

[9] I. Vass and S. Demeter, Biochim. Biophys. Acta **682,** 496–499 (1982).

[10] W. Oettemeier and A. Trebst, The Oxygen Evolving System in Photosynthesis (Y. Inoue *et al.,* eds.), pp. 411–420, Academic Press, Tokyo 1983.

[11] A. Trebst and W. Draber, Advances in Pesticide Science (H. Geissbühler, ed.), pp. 353–369, Pergamon Press, Oxford 1982.

[12] C. Fedtke, Biochemistry and Physiology of Herbicide Action, pp. 51–60, Springer Verlag, Berlin 1982.

[13] M. Droppa, G. Horvath, I. Vass, and S. Demeter, Biochim. Biophys. Acta **638,** 210–216 (1981).

[14] A. W. Rutherford, A. R. Crofts, and Y. Inoue, Biochim. Biophys. Acta **682,** 457–465 (1982).

[15] S. Demeter and I. Vass, Biochim. Biophys. Acta **764,** 24–32 (1984).

[16] T. Asami, S. Yoshida, and N. Takahashi, Agric. Biol. Chem. **50,** 469–474 (1986).