Halogenation Enhances the Photosystem II Inhibitory Activity of 4-Hydroxypyridines: Structure-Activity Relationships and Their Mode of Action

Tadao Asami[a], Masatoshi Baba[b], Hiroyuki Koike[c], Yorinao Inoue[a], and Shigeo Yoshida[a]

[a] The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-01, Japan

[b] Agricultural Chemicals Research Department, Nissan Chemical Ind., Ltd., Tsuboi 722-1, Funabashi, Chiba 274, Japan

[c] Department of Life Science, Faculty of Science, Himeji Institute of Technology, Harima Science Garden City, Hyogo 678-12, Japan

Z. Naturforsch. **48c**, 152–158 (1993); received October 30, 1992

4-Pyridone, 4-Hydroxypyridine, Photosynthetic Electron Transport, Inhibition, Halogenation

A range of 4-hydroxypyridines were synthesized and their activity as PET inhibitors were investigated with regard to their structural resemblance to plastoquinone in photosynthetic electron transport (PET). The activity of these compounds was markedly enhanced upon modifying their structures: introduction of halogens into both the 3- and 5-positions of the pyridine ring and additional substitution at the α-position of the side chain at 6-position were effective among others in enhancing the activity. Insertion of a phenyl ring into the side chain at 6-position of the pyridine ring also increased the activity. Substituents on the phenyl ring greatly affected the activity: when substituted with an appropriate functional group, the compounds became 10- to 100-fold more active. The mode of action of both halogenated and non-halogenated 4-hydroxypyridines were investigated by means of thermoluminescence measurements and cross resistance examination against atrazine-resistant thylakoids having mutation in D1 protein. It was inferred that upon halogenation, 4-hydroxypyridines changed their mode of action from plastoquinone-pool inhibitors to phenol-type inhibitors.

Introduction

In the design of inhibitors acting at an enzymatic level, one important feature is the structural resemblance between inhibitors and substrates. For example, in mitochondrial electron transport system, piericidines and their analogs bearing a close structural resemblance to ubiquinone, act as potent inhibitors [1]. In photosynthetic electron transport system, on the other hand, the plastoquinone site is the main target of various photosynthesis inhibiting herbicides. Their mode of inhibition is a consequence of displacement of plastoquinone from its binding site on the protein subunits of photosystem II [2]. In view of this, we may reasonably expect that chemicals structurally resembling plastoquinone should inhibit photosynthetic electron flow at the plastoquinone binding site. In fact, dibromothymoquinone [3] and tetra-halogenated 4-hydroxypyridine [4], both structurally analogous to plastoquinone, are known as potent inhibitors. 4-Pyrones and 4-hydroxypyridines are also known as structurally related inhibitors, but their activity level are low. It has been also reported that 4-hydroxypyridines enhance their activity upon halogenation both on the pyridine ring and α-position of the side chain at 6-position, while 4-pyrones do not [5].

It is believed that lipophilicity of the compounds plays an important role in enhancing the PET inhibitory activity [6–9]. It has further been suggested that the phenyl group in the lipophilic side chain may have interactions with some amino acid residues surrounding the binding site of the D1 protein [10]. A convenient synthetic route for 4-hydroxypyridines allows us to prepare many types of compounds having various substituents on the pyridine ring and to examine the effect of halogenation, change of lipophilicity, and insertion of phenyl group into the molecule. We report here the structure-activity relationships of halogenated 4-hydroxypyridine and the possible reason for the activity enhancement upon halogenation. We further analyzed comparatively the features of inhibition of the Hill reaction by various compounds in atrazine-resistant and -susceptible

Reprint requests to T. Asami.

Verlag der Zeitschrift für Naturforschung, D-W-7400 Tübingen
0939–5075/93/0300–0152 $ 01.30/0

thylakoids from *Brassica napus* by employing thermoluminescence measurements, and confirmed that upon halogenation, 4-hydroxypyridines change their mode of binding to the D 1 protein. It was also suggested that this change occurs in accordance with the change of their predominant tautomeric forms. Both change of UV-spectrum upon halogenation and chemical analysis of non- and dibromo-4-hydroxypyridines support this suggestion.

Materials and Methods

PS II inhibition in thylakoids

Spinach (*Spinacia oleracea* L.) thylakoids prepared by usual methods [11] were stored in liquid nitrogen. Photosynthetic electron transport activity was measured at pH 7.0 in 2 ml of a medium (50 mM HEPES, 10 mM NaCl, 20 mM methylamine) containing 50 μM DCIP (2,6-dichlorophenolindophenol) and 0.5 μg/ml of chlorophyll. Photoreduction of DCIP was measured spectrophotometrically at 600 nm, after dilution of the thylakoid suspension with 0.4 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$ and 40 mM tricine (pH 7.8). The PS II inhibitory activities of the compounds were expressed in terms of pI_{50} values, the negative logarithms of the concentration (M) of the compounds for 50% inhibition of electron transport. Experiments were repeated at least three times, and the average values were reported. Thylakoids from both wild type and atrazine-resistant *Brassica napus* were isolated in the same manner and were used for in assays as described above for spinach thylakoids.

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, and then dark-adapted for 5 min at room temperature. Thermoluminescence was measured as described in [12]. The dark adapted thylakoids were illuminated with xenon flashes, rapidly cooled to −196 °C, then luminescence emission was recorded during heating at a rate of 0.8 °C/sec.

Results

Structure-activity relationships

All the compounds examined in this study are classified into two groups based on the position of the longest side chain on the pyridine ring: Type I compounds have the long side chain adjacent to the nitrogen atom, while Type II compounds have the long side chain adjacent to the 4-hydroxyl attaching carbon atom. Fig. 1 shows the effect of 3,5-dihalogenation of the pyridine ring of Type I compounds on the activity. Compounds **1**–**4** have a non-brominated side chain, and none of them exhibited the activity. However, 3,5-dibromination of their pyridine rings enhanced the activity as indicated in Fig. 1. This activity enhancement was observed particularly for Type I compounds that carried a phenyl ring in their side chains (**5**–**8**). While 3,5-dibrominated compounds having a straight alkyl chain, but no phenyl ring did not show any marked increase in activity upon the dibromination [5]. The activity enhancement by side chain modification indicates that lipophilicity and/or existence of phenyl ring in the side chain are the important requirement for the expression of PET inhibiting activity by such compounds as demonstrated in the modification of compound **5.** The activity of **5** was low ($pI_{50} = 4.4$), but compounds **9, 10,** 4-*n*-butoxyphenyl- (**12**), 4-phenylphenyl- (**13**), 2,4-dichlorophenyl- (**14**), 4-phenoxyphenyl- (**15**) and 3,4-methylenedioxyphenyl- (**16**) derivatives were 10- to 100-fold more active than compound **5.** Compound **17,** in which the pyridine ring was substituted with iodine atoms, showed the highest activity among the 3,5-dihalogeno-4-hydroxypyridines examined.

As shown in Table I, the activity of 3,5-dibromo-4-hydroxypyridines was increased by bromination of the α-position of the side chain at 6-position (**5** → **18, 12** → **23, 15** → **24, 13** → **25**). One exception was compound **14,** bromination of which caused no distinct change in the activity (**22**). Di-iodinated compound **26** also showed the activity as high as DCMU. Elongation of the side chain of compound **18** clearly enhanced the activity (**19**–**21**). This activity enhancement is probably due to increase in lipophilicity of the side chain as has been suggested for other PET inhibitors [6–9].

In Fig. 2, the effect of halogenation of 4-hydroxypyridines possessing a long side chain at

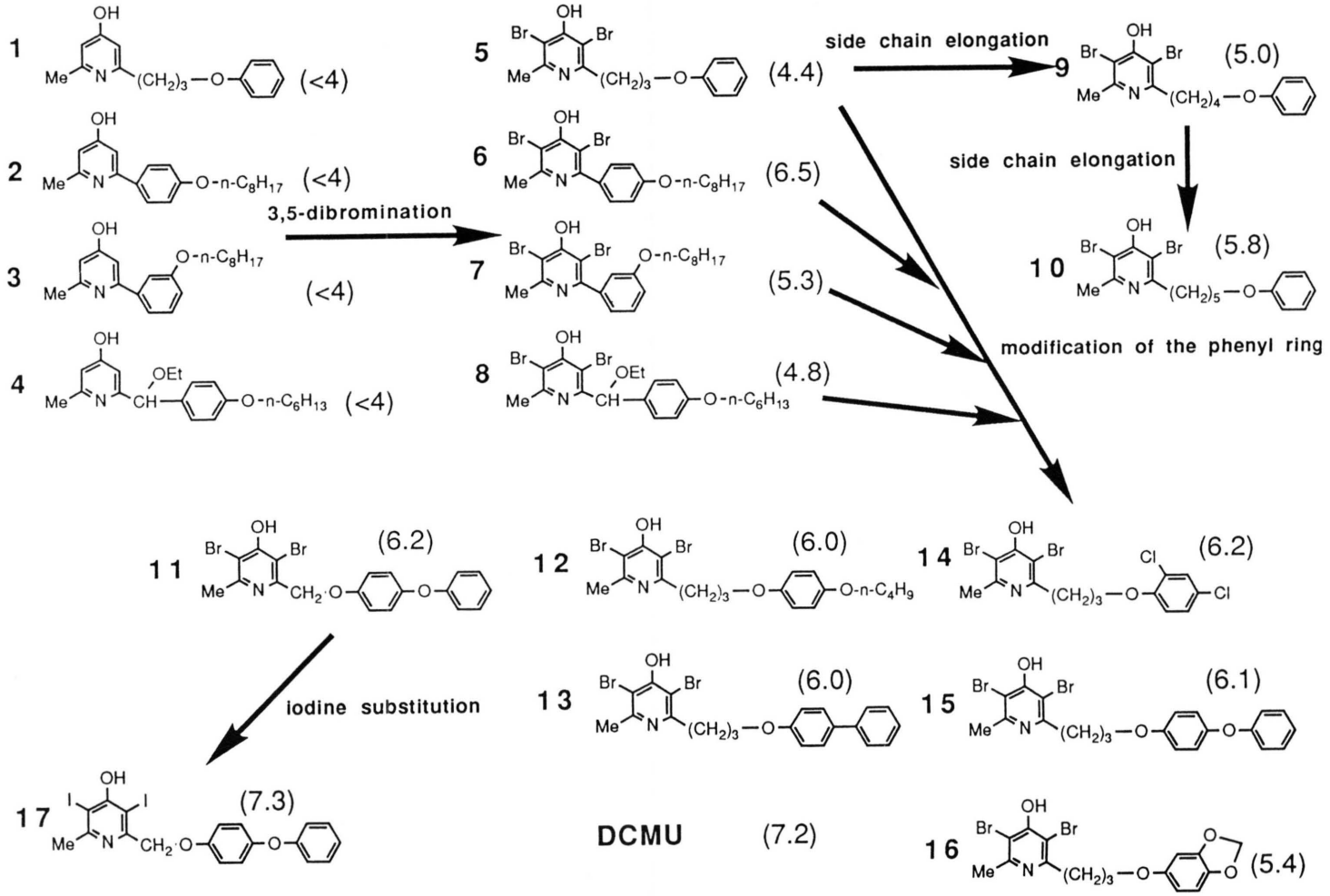
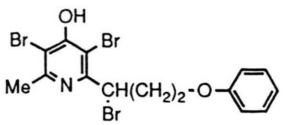
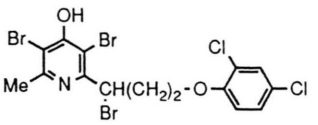
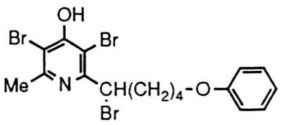
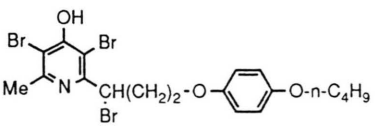
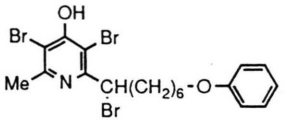
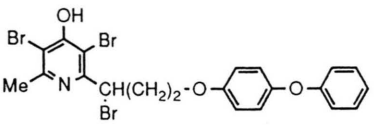
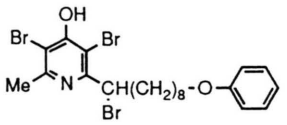
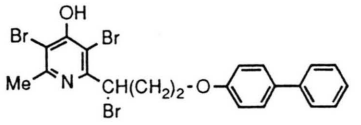
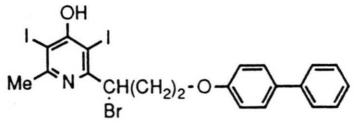


Fig. 1. Effect of 3,5-dihalogenation of 6-substituted 4-hydroxypyridines (Type I) on activity enhancement.

Table I. Effect of α-Bromination of Side Chain at 6-Position on Activity Enhancement (Type I)

		pI_{50}			pI_{50}
18		5.4 (+1.0)	22		5.9 (-0.3)
19		6.6 (+0.8)	23		6.6 (+0.6)
20		6.7	24		6.9 (+0.8)
21		6.8	25		7.3 (+1.3)
	DCMU	7.2	26		7.2

The degree of the activity enhancement is expressed in ( ).

3-position is depicted. Although Type II compounds do not carry two halogens on their pyridine rings, halogenation of these compounds at 5-position of the pyridine ring enhanced the activity (**27** → **30** and **31**, **28** → **32**, **33** and **34**, **29** → **35** and **36**, **37** → **38**). The order of effectiveness in activity enhancement is iodine > bromine > chlorine. Side chain elongation at 3-position enhanced the activity, probably due to the increase in lipophilicity. Insertion of a phenyl group into the long side chain was appreciably more effective (**38**) as compared with the effect on the compounds possessing an alkyl straight chain. We note that these Type II compounds are totally different from Type I compounds in the structural requirement for expression of the activity: Type I compounds require 3,5-dihalogens of the pyridine ring for activity expression whereas Type II compounds require only 5-halogen of the pyridine ring. When the 3-halogen of Type I compounds was substituted with a methyl group, inhibitory activity was drastically lost, as demonstrated by compounds **33** and 3-bromo-2-decyl-4-hydroxy-5,6-dimethylpyridine [5]: the structural difference between the two compounds is only the positions of the methyl and *n*-decyl groups, however their activity is greatly different by a factor of about 1000.

3-Alkoxy-4-hydroxypyridines of Type II acquired higher activity by bromination at the 5-position, and side chain elongation was also effective for activity enhancement. This result suggests that for Type II compounds the lipophilicity of the 3-position is the most important factor while the methyl group at 6-position plays no role in the activity expression.

Mode of action

The mode of action of non-halogenated 4-hydroxypyridines has already been investigated, and they are known to act as plastoquinone-pool

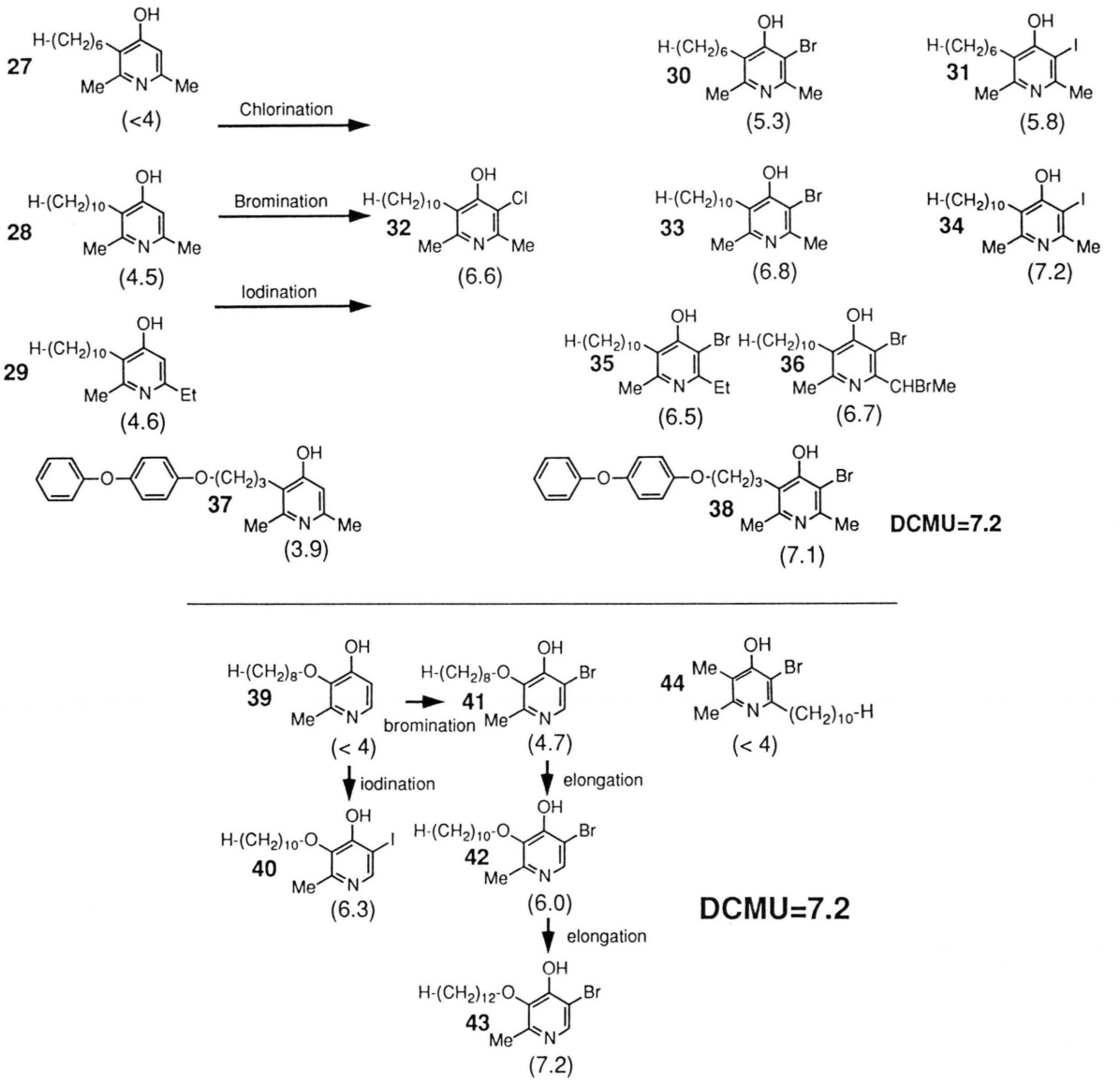


Fig. 2. Effect of halogenation of 4-hydroxypyridines possessing a long side chain at 3-position (Type II).

inhibitors [15]. PET inhibitory activity of 4-hydroxypyridines was enhanced by halogenation regardless of the position of the long side chain. This led us to assume that the mode of action of 4-hydroxypyridines may be altered upon halogenation.

Table II lists the peak temperatures of thermoluminescence glow curves of spinach thylakoids in the presence of various inhibitors. From the peak temperature of thermoluminescence Q-band, the orthodox inhibitors are known to be classified into three groups: upon treatment with ureas, triazines and phenols the thermoluminescence B-band is converted to the Q-band due to interruption of electron transport from Q_A to Q_B, and the peak position of the resulted Q-band is located at different temperatures corresponding to the types of the inhibitors, +8, −2, and −9 °C with DCMU, atrazine and ioxynil, respectively [13]. Notably, the glow peak of the thylakoids treated with non-brominated 4-hydroxypyridines was found at

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

Compound	Type	Concentration [μM]	TL grow peek temperature [°C]
21	I	10	−9
24	I	10	−6
35	II	10	3
36	II	10	4
4-Hydroxypyridine*		1000	25
Ioxynil		100	−9
DCMU		10	8
Atrazine		10	−2
Control		–	38

* Abbreviated name of 2,3-dimethyl-6-*n*-octyl-4-hydroxypyridine.

around +25 °C, an intermediate peak temperature between the B-band (control) and the DCMU-induced Q-band. These compounds are the exceptions among 4-hydroxypyridines, exhibiting the emission peak at such a high temperature as reported for AP series compounds [14]. This suggests that the mode of action of non-brominated 4-hydroxypyridines differs from that of other inhibitors, in good accordance with the results of our previous study [15]. Type I-4-hydroxypyridines (**21** and **24**) exhibited the glow peak at −9 and −6 °C, respectively, suggesting that compounds **21** and **24** are classified as phenol-type inhibitors. On the other hand, Type II-4-hydroxypyridines (**35** and **36**) exhibited the glow peak at +3 and +4 °C, respectively, suggesting that these two compounds are rather classified as urea-atrazine type inhibitors than phenol-type inhibitors.

Atrazine-resistant weeds provide another information about the mode of binding of various PET inhibitors. By comparing the R/S ratio [ratio of I_{50} (resistant type) divided by I_{50} (susceptible type)] of various compounds, we can classify them into several groups [16–18]. Generally, a mutation giving rise to atrazine-resistance results in an increased sensitivity to phenol-type herbicides to exhibit an R/S value lower than one, whereas the inhibitory activity of urea-type remains mostly unchanged showing an R/S value nearly the same or slightly higher. In Table III, pI_{50} values for PET inhibition are compared for thylakoids from susceptible and atrazine-resistant *Brassica napus*. DCMU and ioxynil exhibited R/S values of 3.2 and 0.4, respectively. Notably, both Type I and Type II compounds showed R/S values lower than one, indicative of phenol-type inhibition, while the R/S value of non-halogenated 4-hydroxypyridine remained unaffected, suggesting that the mutation in the D 1 protein does not change the mode of binding of this compound. Based on these results we classify both Type I and Type II compounds to phenol-

Table III. Inhibition of the Hill reaction by various compounds in triazine-resistant (R) and -susceptible (S) chloroplasts from *Brassica napus*.

Compound	Type	pI_{50} (S)	pI_{50} (R)	R/S
21	I	6.8	7.7	0.13
24	I	7.2	7.7	0.32
33	II	6.9	7.5	0.25
35	II	7.0	7.5	0.32
36	II	6.6	7.2	0.25
4-Hydroxypyridine*		4.0	3.9	1.1
DCMU		7.1	6.6	3.2
Ioxynil		6.5	6.9	0.4

* Abbreviated name of 2,3-dimethyl-6-*n*-octyl-4-hydroxypyridine.

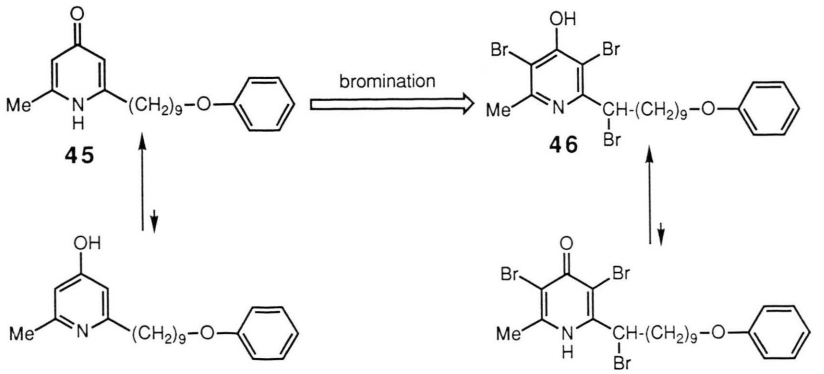


Fig. 3. Change of predominant tautomeric form of 4-hydroxypyridines upon halogenation.

type inhibitors. As a consequence of this classification, we infer that 4-hydroxypyridines change their mode of action upon halogenation.

Discussion

Trebst *et al.* [19] were the first to report tetrahalogenated-4-hydroxypyridines as PET inhibitors. They classified them as phenol-type inhibitors based on their analyses of their mode of action advocating same essential features of this type of inhibitors. The structural difference between their derivatives and ours is the existence of lipophilic side chains on the heteroring. Comparison of these two sets of results led us to conclude that 4-hydroxypyridines change their mode of action upon halogenation, and this change gives rise to the activity enhancement. Generally, when the hydroxyl group of phenol-type inhibitors is methylated by diazomethane treatment, they cannot act any more as a phenol, and lose the inhibitory activity. The O-methylated bromo-4-hydroxypyridines also lose their high inhibitory activity upon methylation and act merely as plastoquinone-pool inhibitors. The idea of change in mode of action is supported by the change in the UV-absorption spectrum and color reaction by $FeCl_3$ spray ($FeCl_3$ is a good spray reagent for identifying the phenol hydroxyl group). When the hydroxyl group of non-brominated 4-hydroxypyridines is methylated by diazomethane treatment, a large change in UV absorption spectrum takes place. This is probably because the heteroring of non-brominated compounds is in tautomeric equilibrium with preferential population of keto-form (**45**), but upon methylation the equilibrium reverses toward enol-form (**46**) to lock all the molecules in the pyridine form, the requirement for phenol-type inhibitors. In contrast, diazomethane treatment of dibrominated 4-hydroxypyridines did not induce any discernible change in UV-absorption spectrum. This indicates that the heteroring of these dibrominated 4-hydroxypyridines were in the enol-form both before and after methylation. These interpretations account well for the fact that brominated 4-hydroxypyridines can react with $FeCl_3$ to undergo color reaction while non-brominated 4-hydroxypyridines do not, and also explain well the observation in this study that the PET inhibitory activity of 4-hydroxypyridines was markedly enhanced by bromination: unless brominated, the compounds could not act as phenol-type inhibitors due to the preferential keto-form structure of their non-brominated heteroring.

If there are other candidates for new PET inhibitor possessing a heteroring carrying a hydroxyl group in their molecules, halogenation of the heteroring and/or the substituent(s) may yield fruitful results. For the mode of binding of a phenol-type inhibitor to D 1 protein, there remain many black boxes to be investigated, we will be able to utilize those type of compounds for the study of inhibitor-protein binding.

Acknowledgements

The authors greatly acknowledge Mr. Ichinose for his skilful technical supports in the experiments.

[1] S. Yoshida, Y. Nagao, A. Watanabe, and N. Takahashi, Agric. Biol. Chem. **44,** 2921–2926 (1990).
[2] W. Draber, K. Tietjen, J. F. Kluth, and A. Trebst, Angew. Chem., Int. Ed. Engl. **30,** 1621–1633 (1990).
[3] A. Trebst, Methods Enzymol. **69,** 675–715 (1980).
[4] A. Trebst, B. Depka, S. M. Ridley, and A. F. Hawkins, Z. Naturforsch. **40c,** 391–399 (1985).
[5] T. Asami, S. Yoshida, and N. Takahashi, Agric. Biol. Chem. **50,** 469–474 (1986).
[6] K. Mitsutake, H. Iwamura, R. Shimizu, and T. Fujita, J. Agric. Food Chem. **34,** 725–732 (1986).
[7] K. Yoneyama, M. Konnai, T. Takematsu, H. Iwamura, T. Asami, N. Takahashi, and S. Yoshida, Agric. Biol. Chem. **53,** 1953–1959 (1989).
[8] Y. Shigematsu, Fumihiko Sato, and Y. Yamada, Pest. Biochem. Physiol. **35,** 33–41 (1989).
[9] J. N. Phillips and J. L. Huppatz, Z. Naturforsch. **39c,** 335–337 (1984).
[10] M. Kishida, T. Mabuchi, T. Asami, S. Yoshida, and N. Takahashi, Abstracts. Molecular Function of Chloroplast Functions, IXth International Congress on Photosynthesis Satellite Symposium, Omiya, PII-8.
[11] S. Katoh, Method in Photosynthesis Research (S. Katoh, S. Miyaji, and Y. Murata, eds.), pp. 251–253, Kyoritsu Syuppan Inc., Tokyo 1981.
[12] T. Asami, H. Koike, Y. Inoue, N. Takahashi, and S. Yoshida, Z. Naturforsch. **43c,** 857–861 (1988).
[13] M. Droppa, G. Horvath, I. Vass, and S. Demeter, Biochim. Biophys. Acta **638,** 210–216 (1981).
[14] T. Asami, H. Koike, Y. Inoue, N. Takahashi, and S. Yoshida, Z. Naturforsch. **43c,** 857–861 (1988).
[15] M. Kawamura, S. Yoshida, N. Takahashi, and Y. Fujita, Plant and Cell Physiol. **21,** 745–752 (1980).
[16] K. Pfister and C. J. Arntzen, Z. Naturforsch. **34c,** 996–1009 (1979).
[17] W. Oettmeier, K. Masson, C. Fedtke, J. Konze, and R. R. Schmidt, Pest. Biochem. Physiol. **18,** 357–367 (1982).
[18] A. Thiel and P. Böger, Pest. Biochem. Physiol. **22,** 232–241 (1984).
[19] A. Trebst, W. Donner, and W. Draber, Z. Naturforsch. **39c,** 405–411 (1984).