2-Methylcinnolinium Herbicides: Effect of 2-Methylcinnolinium-4-(*O*-methyl phosphonate) on Photosynthetic Electron Transport

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This is the first paper on the mode of action of a new class of broadly active foliar herbicides, 2-methylcinnoliniums. One of the most active compounds of this class is 2-methylcinnolinium-4-(*O*-methyl phosphonate) (MCP). The symptoms of phytotoxicity include desiccation, chlorosis, and necrosis, and are very similar to those caused by methylviologen. Both MCP and methylviologen have reversible oxidation–reduction waves as measured by cyclic voltammetry, and both molecules have reduction potentials that are sufficiently positive to compete for electrons with the endogenous electron acceptors of photosystem I (PS I). MCP is a highly active inhibitor of whole-chain electron transport, similar to methylviologen, with an I_{50} of about 25 μM. This compound is not an inhibitor of photosystem II (PS II). However, when PS II is inhibited by diuron, partial-chain electron transport from the artificial electron donor DPIPH2 to NADP is still strongly inhibited (I_{50} = 40 μM) by this molecule. We conclude that MCP acts in the same fashion as methylviologen (Bowyer, J. R.; Camilleri, P. In *Herbicides*; Hutson, D. H., Roberts, T. R., Eds.; Wiley: New York, 1987; pp 105–145), by competing with endogenous acceptors for electrons that would normally flow through the reducing side of PSI to NADP.

INTRODUCTION

Quaternized 4-substituted cinnolines (cinnoliniums) constitute a new class of broadly active foliar herbicides. One of the most active of this class has been 2-methylcinnolinium-4-(*O*-methyl phosphonate) (MCP, Figure 1) (Ponte et al., 1987). Several observations have led to the speculation that MCP acts in the same way as methylviologen (paraquat), i.e., competition with endogenous substances on the acceptor side of photosystem I (PS I) for electrons that are thereby diverted to the reduction of molecular oxygen rather than to the reduction of NADP (Bowyer and Camilleri, 1987). The symptoms of phytotoxicity—desiccation, chlorosis, and necrosis—are very similar to those caused by methylviologen. The mode of action of methylviologen is thought to be mediated by reduction to a radical cation. An analogous reduction can be postulated for MCP. These observations have led us to compare the electrochemical properties of both molecules and to examine the activity of MCP on photosynthetic electron transport in isolated chloroplast membranes.

EXPERIMENTAL PROCEDURES

Membrane Preparations. Chloroplast thylakoid membranes were prepared from 10 g of 12-day-old Alaska peas (*Pisum sativum* L.). Leaves were homogenized in ice-cold grinding buffer (50 mM tricine, pH 7.8; 0.4 M sorbitol; 10 mM KCl; 5 mM $MgCl_2$). The slurry was filtered through 12 layers of cheesecloth, and the brei was centrifuged at 1000*g* for 10 min. The pellet was resuspended in hypotonic medium (50 mM tricine, pH 7.8; 10 mM KCl; 5 mM $MgCl_2$) and centrifuged at 1000*g* for 10 min. The pellet was resuspended in resuspension buffer (50 mM tricine, pH 7.8; 5 mM $MgCl_2$), recentrifuged at 1000*g* for 10 min, and resuspended again in a minimal amount of buffer. Chlorophyll content was determined according to the method of Arnon (1949).

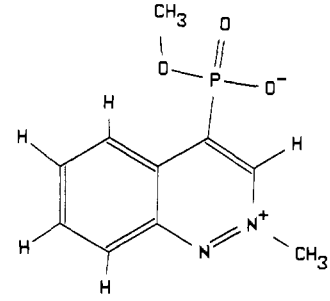

Figure 1. Structure of 2-methylcinnolinium-4-(*O*-methyl phosphonate) (MCP).

Photosynthetic Electron Transport. Photosystem II (PS II) activity was assayed as previously described (Gardner et al., 1985), except the buffer contained 40 μM dichlorophenolindophenol (DPIP). The PS II-mediated reduction of DPIP was measured at 580 nm using a Hewlett-Packard Model 8450A UV–vis spectrophotometer equipped with a spectrophotometer-controlled actinic source.

NADP reduction was assayed either as whole-chain electron transfer from water to NADP or as a partial reaction in the presence of either diuron or 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), using a DPIPH2/ascorbate couple as the electron donor to NADP (Izawa, 1980; Trebst, 1980). The reaction buffer for these assays consisted of 50 mM bicine (pH 8.0), 100 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 2.5 mM NH_4Cl, and 1 μM gramicidin. The buffer for the diuron- and DBMIB-insensitive reactions also contained 0.2 mM DPIP. For whole-chain electron transport the reaction buffer contained 10 μg/mL chlorophyll, 200 μg/mL ferredoxin, and 0.2 mM NADP. The partial-chain assays also contained 1 mM ascorbate and either 1 μM diuron or 0.1 μM DBMIB. Activity was measured as change in absorbance at 340 nm. This wavelength is characteristic of reduced NADP and is readily followed as a function of time using the Hewlett-Packard 8450A UV–vis spectrophotometer. Activity in Figure 2 and 3 is expressed relative to a solvent control with no MCP. Each experiment was carried out at least three times, and representative examples are shown in the figures.

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Electrochemical Measurements. One millimolar solutions of MCP were prepared in phosphate buffer (0.1 M, pH 6, 7, and 8). The solutions were deaerated by nitrogen sparging for 10 min and repetitively and cyclically scanned through the potential range 0 to −1000 mV, with scan rates in the range 20–50 mV/s, employing an oil-based carbon paste working electrode, a silver/silver chloride reference electrode, and a platinum auxiliary electrode attached to a cyclic voltammeter (BioAnalytical Systems CV-5). Change in current flow with change in potential was followed on an x–y recorder (Bausch and Lomb Series 100) attached to the voltammeter. There was no attempt at temperature control of the solutions, although all were at normal laboratory temperature (20–25 °C).

The calibration of the electrodes was checked using a 10 μM solution of methylviologen in phosphate buffer (0.1 M, pH 8). The half-wave reduction potential of a freshly prepared electrode was usually found to be −650 mV (vs Ag/AgCl).

Chemical Synthesis. *4-(p-Toluenesulfonyl)cinnoline.* Sodium *p*-toluenesulfinate (1.90 g, 0.01 mol) was added in one portion to a stirred solution of 4-chlorocinnoline (1.64 g, 0.01 mol) in dry dimethylformamide (25 mL) under nitrogen at 0 °C. After 1.5 h, the reaction was allowed to warm to room temperature and stirred overnight. The resulting solution was poured into water (150 mL) and filtered. The solids were dried and triturated with ether to yield to yellow product (2.33 g, 82%): mp 166–168 °C; MS (EI, solid probe) *m/z* (% base peak) 284 (M^+, 25), 155 (1), 129 (15), 101 (100), 91 (25), 75 (40), 65 (30). Elemental analysis calcd for $C_{15}H_{12}N_2O_2S$: C, 63.4; H, 4.2; N, 9.9. Found: C, 63.1; H, 4.1; N, 9.9.

Methyl 2-Methylcinnolinium-4-(O-methyl phosphonate) Inner Salt. Sodium hydride (0.19 g, 3.8 mmol of a 50% oil dispersion) was twice washed with dry hexane and suspended in dry THF (2 mL). Dimethyl phosphite (0.43 g, 3.8 mmol) in dry THF (0.8 mL) was added dropwise with stirring under nitrogen at 0 °C over 10 min. The resulting mixture was transferred by canula under nitrogen pressure to a stirred suspension of 4-toluenesulfonylcinnoline (1.00 g, 3.5 mmol) in THF (4.2 mL at −50 °C). The reaction mixture was stirred at this temperature for 1 h, at −20 °C for 6 h, and at room temperature overnight. It was then diluted with 3 volumes of water and extracted with ethyl acetate (3 × 15 mL). The combined organic extracts were washed with water (10 mL) and saturated NaCl solution (10 mL), dried (anhydrous Na_2SO_4), and evaporated. Dimethyl cinnoline-4-phosphonate (0.83 g) was obtained as an unstable green oil: NMR ($CDCl_3$) 3.86 (d, J = 12 Hz, 6 H), 7.6 (m, 2 H), 8.3 (m, 2 H), 9.58 (d, J = 9 Hz, 1 H) ppm downfield from TMS. The product was dissolved in methanol and heated at reflux for 8 h. The reaction mixture was evaporated under reduced pressure and the residue triturated with acetone to yield the desired cinnoliniumphosphonate as a green solid (0.16 g, 19%). Recrystallization from methanol–ether gave a light gray solid: mp 174–176 °C (decomp.); NMR (CD_3OD) 3.61 (d, J = 12 Hz, 3 H), 4.88 (s, 3 H), 8.33 (m, 2 H), 8.60 (d, J = 7.9 Hz, 1 H), 9.02 (d, J = 7.5 Hz, 1 H), 9.62 (d, J = 8.6 Hz) ppm downfield from TMS; MS (electron impact, solid probe) *m/z* (% base peak) 238 (M^+, 2), 224 (12), 207 (3), 145 (100), 130 (11), 110 (19), 102 (31), 101 (16), 101 (15), 90 (15), 75 (19). Elemental analysis calcd for $C_{10}H_{11}N_2O_3P$: C, 50.43; H, 4.66; N, 11.76. Found: C, 50.20; H, 4.67; N, 11.58. NMR nuclear Overhauser effect experiments confirmed the methylation of the N-2 nitrogen. Thus, irradiation of the *N*-methyl signal leads to enhancement of the resonance for the C-3 proton. Conversely, irradiation of the C-3 proton resonance leads to enhancement of the *N*-methyl proton signal.

The DBMIB was synthesized by K. H. Pilgram as previously described (Trebst et al., 1970).

RESULTS AND DISCUSSION

As has been previously discussed (Trebst, 1974), photosynthetic electron transport involves three major membrane-bound proteinaceous components: PS II, the cytochrome *b/f* complex, and PS I. In the complete pathway, the initial electron donor is water, and the final electron acceptor is NADP. Inhibition of whole-chain electron transport by MCP is shown in Figure 2. For comparison,

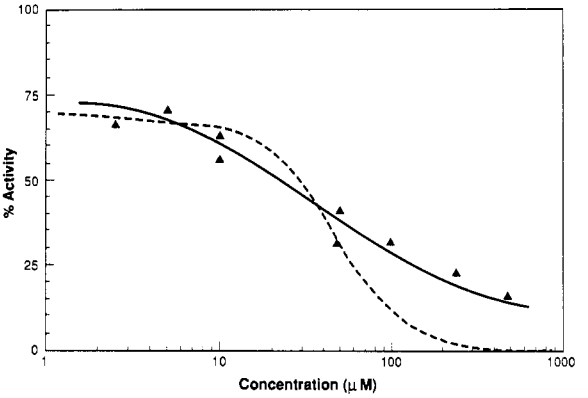

Figure 2. Inhibition of photosynthetic electron transport from water to NADP. Solid curve is for MCP. Dashed curve is for methylviologen.

activity for methylviologen is indicated by the dashed line. It is clear that MCP is a highly active inhibitor of whole-chain electron transport, similar to methylviologen, with an I_{50} of about 25 μM. A careful examination of Figure 2 indicates that the dose–response curve for MCP is not exactly like that of methylviologen. Whereas both compounds have similar I_{50} values, at higher concentrations methylviologen caused complete inhibition but MCP did not. This phenomenon was not always observed, and in some experiments MCP completely inhibited whole-chain NADP reduction at 100 μM.

If a compound is active on electron transport from water to NADP, then the effect of the compound on electron transport can be measured through photosystem II (water to DPIP) or through photosystem I (DPIP/ascorbate to NADP in the presence of diuron). The use of specific inhibitors can reveal additional information about the site of action of an active compound. Since diuron blocks electron transport between Q_A and Q_B (Moreland and Hill, 1962), transport from DPIP/ascorbate to NADP in the presence of diuron really measures transport from plastoquinone through PS I. DBMIB inhibits electron flow at the next step, i.e., from plastoquinone to the cytochrome *b/f* complex (Boehme et al., 1971). Thus, NADP reduction by DPIP/ascorbate in the presence of DBMIB is measured from the cytochrome *b/f* complex through PS I. Therefore, if MCP inhibits NADP reduction by DPIP/ascorbate in the presence of diuron, but not in the presence of DBMIB, then the action of MCP could be attributed to a specific interaction with the plastoquinone pool. MCP is not an inhibitor of photosystem II. Even at concentrations as high 500 μM, it does not inhibit PS II-specific reduction of DPIP. However, when PS II is inhibited by diuron, partial-chain electron transport from the artificial electron donor DPIPH2 to NADP is still strongly inhibited by MCP (Figure 3). The I_{50} for this reaction is about 40 μM, roughly the same as that for whole-chain electron transport. Similar results were seen in the presence of DBMIB, which blocks electron transport at the oxidation site of plastoquinone (data not shown). Therefore, MCP inhibits electron flow from the *b/f* complex through PS I. We conclude that this phosphonate cinnolinium acts in the same fashion as methylviologen, by inhibiting electron flow through PS I.

If methylcinnoliniums have a mechanism of action similar to that of methylviologen, they would be expected to intercept electrons at some point along the electron-transfer pathway between photoexcited P700 and $NADP^+$ and then to autoxidize, producing superoxide as a phytotoxic product (Bowyer and Camilleri, 1987; Farrington et al., 1973). Thus, from a thermodynamic standpoint,

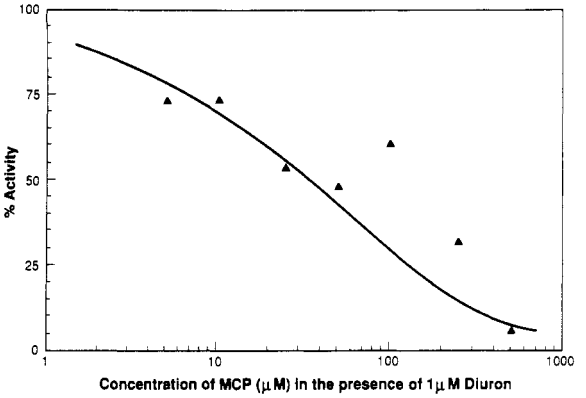

Figure 3. Inhibition of photosynthetic electron transport from DPIPH2 to NADP.

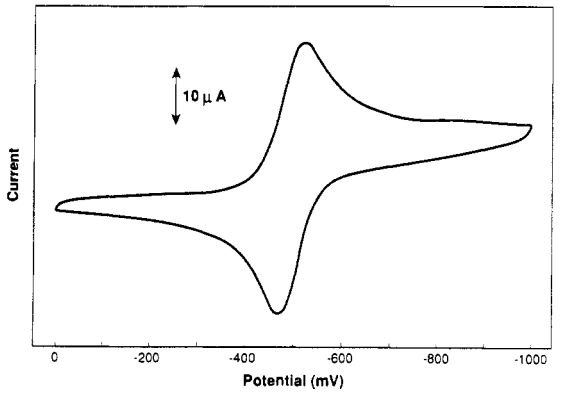

Figure 4. Cyclic voltammogram of a 1 mM solution of 2-methylcinnolinium-4-(*O*-methyl phosphonate) (MCP) in pH 8 phosphate buffer at room temperature. Ag/AgCl is the reference electrode. The scan rate is 50 mV/s.

active methylcinnoliniums should have reduction potentials more positive than approximately −0.6 V for large quantities of the cinnolinyl radicals to be formed by electron transfer from early intermediates on the reducing side of PS I. (Bowyer and Camilleri, 1987; Farrington et al., 1973). On the other hand, if cinnoliniums have reduction potentials substantially higher than that of the O_2/O_2^- couple (about −0.25 V) (Bowyer and Camilleri, 1973; Farrington et al., 1973), only relatively small amounts of phytotoxic O_2^- should be formed. Thus, effective cinnolinium herbicides are expected to have reduction potentials in the −0.6 to −0.2 V range, a range within which the reduction potentials of most other PS I herbicides fall (Bowyer and Camilleri, 1987; Ashton and Crafts, 1981). Methylviologen has a reduction potential of −0.452 V.

To examine further the similarities between MCP and methylviologen, we have measured the reduction potential of MCP using cyclic voltammetry. The cyclic voltammogram for MCP appears in Figure 4. The half-wave reduction potential was calculated by taking the mean value of the anodic and cathodic peak potentials. At pH 8 a half-wave reduction potential of −0.268 V (vs the normal hydrogen electrode) was determined. This reduction potential is slightly more positive than that of most PS I herbicides, including paraquat.

At pH 8 reasonable reversibility was shown, and thus the number n of transferred electrons was calculated from the formula

$$E_p(\text{oxid}) - E_p(\text{red}) = 59\ \text{mV}/n$$

where E_p(oxid) and E_p(red) are the anodic and cathodic peak potentials, respectively. The peak separation of approximately 53 mV indicates that the reduction of MCP is a one-electron process. The dependence of reduction potential upon pH ($\Delta E/\Delta$pH = 23 mV/pH unit) indicates that protons are involved in the reduction process, but the experimental data are not comprehensive enough to permit unambiguous assignment of the number of protons involved. At pH 8 the cyclic voltammogram indicates that the radical species produced by reduction of MCP has a half-life of about 70 s. At pH 5 the reduction appears to be completely irreversible in a matter of 10 s. The activity of MCP as a photosystem I herbicide may be compromised by a combination of this short half-life and its relatively high reduction potential. In particular, MCP may be reduced before reaching the photosystem I reaction center in the thylakoid membrane. In support of this hypothesis it has been observed that an incompletely characterized reaction of MCP with hydroxide ion mediates the production of radicals in pH 8–10 solutions. Although the free radical has not been identified, its existence has been clearly demonstrated by both NMR and EPR spectroscopy (D. A. Kleier, R. E. Taylor, and C. C. Chen, unpublished results). Paraquat does not generate free radicals under the mildly basic conditions that resulted in the production of radicals of MCP. The one-electron reduction of MCP by hydroxide ion may be analogous to the reduction of anthraquinone by hydroxide, although the latter reaction has only been observed in aprotic media (Roberts et al., 1985).

Reduction of MCP before the PS I reaction center is reached may be the first step toward detoxification. Alternatively, it could be the first chemical step in a second mode of action that does not involve the PS I reaction center. It is interesting to observe the production of substantial quantities of hydrogen peroxide when basic solutions of MCP are sparged with oxygen. Thus, toxic oxygen species may be formed without the mediation of PS I in those portions of a treated plant where molecular oxygen is present and where pH values are at or above 8. pH values near or above 8 are realized in many parts of a plant including the sieve tube elements which carry phloem sap and in the chloroplast stroma near the thylakoid membrane under conditions of illumination. In this connection, it should be noted, however, that phytotoxicity of MCP appears to be exhibited in the dark only after prolonged exposure to high rates.

The identification of the intrinsic action of MCP provides a basis for the biochemical evaluation of a broader series of cinnoline and cinnolinium analogues. As has been stressed many times before, high intrinsic activity is a necessary but not sufficient property for agronomically relevant herbicidal activity. Examination of biochemical activity along with a consideration of electrochemical data should allow us to determine whether intrinsic activity is rate-limiting for whole plant phytotoxicity or whether chemical features that might alter penetration, transport, or metabolism of the molecule should be emphasized.

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