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TRANSFORMATIONS OF MPTP BY CERULOPLASMIN AND PEROXIDASE: COMPARISON WITH VINCA ALKALOID BIOTRANSFORMATIONS

ANIMESH GOSWAMI, MICHAEL W. DUFFEL, JOHN P. ROSAZZA,*

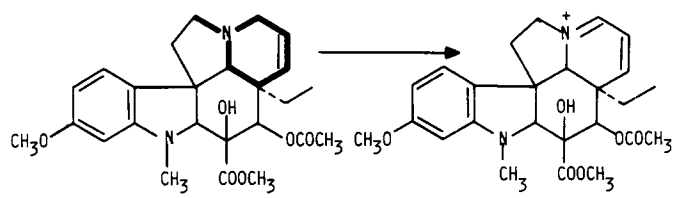
Division of Medicinal Chemistry and Natural Products, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242

WIESLAW GESSNER, and ARNOLD BROSSI

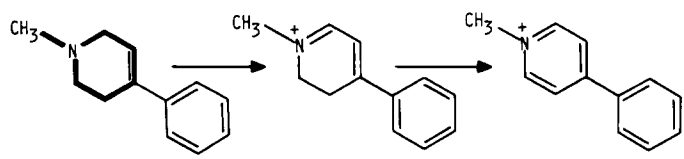
Medicinal Chemistry Section, Laboratory of Chemistry, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Vindoline [**1**] is a major alkaloid of the Vinca plant, also known as *Catharanthus roseus*. The structure of this toxic compound is found essentially intact as ½ of the so-called dimeric alkaloids vinblastine and vincristine, both of which have been used in the clinical treatment of cancer for nearly three decades. In spite of their wide-spread use, surprisingly little is known of the possible role of metabolism in mechanism(s) of action and/or dose limiting neurotoxicities (1) of these alkaloids. We have shown that vindoline and other Vinca alkaloids undergo metabolic oxidation by a variety of enzymes including copper oxidases (ceruloplasmin, laccases) (2), peroxidase (3), cytochrome P-450 (4), and unknown enzymes of a bacterium (5). With these systems vindoline undergoes initial one-electron oxidation (3) leading to the formation of a reactive iminium intermediate [**2**] (6) that could be trapped and fully characterized.

The structure of vindoline embodies an unsaturated tertiary allylic-amine moiety similar to that contained in the structure of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [**3**]. In mammals MPTP is sequentially transformed by the enzyme monoamine oxidase B into 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) [**4**], and 1-methyl-4-phenyl pyridinium (MPP^+) [**5**] (7-14). The formation of these metabolites has been linked to the occurrence of neurological dysfunction with symptoms similar to those observed with Parkinson's disease (8, 9, 15-20). Interestingly, the portion of the MPTP structure prone to metabolic activation is the tertiary allyl-amine moiety contained within the unsaturated piperidine ring. The structure and metabolic similarities between MPTP



1 vindoline **2** vindoline-iminium



3 MPTP **4** $MPDP^+$ **5** MPP^+

and vindoline led us to explore the potential for MPTP to undergo oxidations by the copper oxidases and peroxidase.

MPTP, $MPDP^+$, and MPP^+ were prepared as described (21,22). Rates of oxidation of MPTP by human serum ceruloplasmin were determined by measuring the uptake of molecular oxygen with a Clark oxygen electrode (2). MPTP and $MPDP^+$ were not oxidized unless chlorpromazine was present in enzyme reaction mixtures. Thus, MPTP is actually a "pseudosubstrate" for the copper oxidase (2). With ceruloplasmin oxygen was consumed at the rate of 1.58 μmoles/min/liter. $MPDP^+$ was also a pseudosubstrate with ceruloplasmin and was oxidized at a rate of 1.77 μmoles of oxygen/min/liter. The Vinca alkaloids are also "pseudosubstrates" for ceruloplasmin (2), and under identical reaction conditions, vindoline and the Iboga alkaloid catharanthine were oxidized at rates of 6.13 and 24.54 μmole of oxygen/min/liter, respectively. Iboga alkaloids, like catharanthine and cleavamine (23), are more readily oxidized than MPTP or $MPDP^+$. Reasons for differences in observed reaction rates among MPTP, vindoline, and Iboga alkaloids are unclear.

Human liver MAO B is irreversibly inactivated during MPTP oxidations (12). We considered the possibility that iminium products like $MPDP^+$ and MPP^+ formed during the reaction could similarly inhibit the copper oxidase. This hypothesis was tested by preincubating a mixture of enzyme, chlorpromazine, and either MPTP, $MPDP^+$, or MPP^+ for 7.5-8.75 min before adding catharanthine hydrochloride to the reaction mixture. Under these conditions rates of oxidation of catharanthine were essentially identical to those observed without MPTP, $MPDP^+$, or MPP^+. Therefore, these compounds do not inactivate human serum ceruloplasmin.

The identification of metabolites formed by ceruloplasmin reaction mixtures was accomplished by preparative incubation and by analyzing the reaction mixture by tlc, hplc, and uv spectral measurements. No reaction was observed in any of the control incubations. In the complete reaction mixture, yields of metabolites increased gradually during 27 h of incubation with the major portion of MPTP remaining unreacted. Tlc and co-tlc demonstrated the formation of $MPDP^+$ and MPP^+ and a trace of MPTP-*N*-oxide. Semiquantitative tlc estimates indicated that $MPDP^+$ and MPP^+ were each formed in 10-15% yield.

Spectral analysis of reaction mixtures revealed the presence of uv absorption bands at 237, 274, 293, and 343 nm only in complete reaction mixtures. Absorption maxima for MPTP, $MPDP^+$, and MPP^+ occur at 243, 343, and 293 nm, respectively (21). At 343 nm ($\epsilon = 19,200$) (21) and 293 nm ($\epsilon = 25,296$), the uv absorption maxima of $MPDP^+$ and MPP^+, respectively, are well separated from all other absorption bands. These physical property differences were used to advantage in determining the amounts of $MPDP^+$ and MPP^+ formed. The observed absorbances of fiftyfold diluted reaction mixtures were 0.3853 at 293 nm and 0.1551 at 343 nm, indicating yields of 15% for both $MPDP^+$ and MPP^+. Spectral analysis of MPP^+ at 293 nm is complicated by the presence of chlorpromazine, which absorbs moderately at this wavelength ($\epsilon = 2,147$). However, the contribution of chlorpromazine to reaction mixture absorbances at 293 nm was readily eliminated by using chlorpromazine-containing blanks.

Vindoline is also oxidized by a peroxidase/hydrogen peroxide enzyme system to yield the same reactive iminium derivative produced by the copper oxidases (3). Thus, the potential for MPTP to be oxidized by the same peroxidase/hydrogen peroxide was examined. Analyses of this reaction revealed the formation of a single polar

product which was also observed in the control flask containing only hydrogen peroxide and MPTP. The result suggested that MPTP was undergoing chemical transformation to MPTP-*N*-oxide.

Tlc comparison of the enzyme incubation mixture with an authentic sample of MPTP-*N*-oxide (21) and the reaction product formed when MPTP was oxidized by *m*-chloroperbenzoic acid in CH_2Cl_2 indicated that the polar product was the *N*-oxide derivative. To avoid complications in this reaction caused by the presence of realtively large amounts of H_2O_2 at any instant, the reaction was conducted with glucose and glucose oxidase as an in situ H_2O_2 generating system. Again, the only product observed in the incubation mixture was MPTP-*N*-oxide. Thus, unlike vindoline, MPTP was not converted into iminium intermediates like $MPDP^+$ and later MPP^+ by peroxidase.

This study indicates that MPTP is a "pseudosubstrate" for the copper oxidase ceruloplasmin, and that both $MPDP^+$ and MPP^+ are produced with this enzyme system. Copper oxidase catalyzed transformation of MPTP to an iminium intermediate is analogous to previous results obtained with vindoline. Further studies examining the potential for Vinca alkaloids to undergo oxidation by monoamine oxidase B are underway. Preliminary examination of mitochondrial fractions isolated from rat liver indicates that vindoline is not metabolized to isolable iminium derivatives by this subcellular fraction.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Samples of MPTP, $MPDP^+$, MPP^+, and MPTP-*N*-oxide were prepared as described (21,22). Tlc was performed on 0.25-mm thick Si gel GF254 (E. Merck) plates prepared as needed with a Quickfit Industries spreader. Plates were air dried and oven activated at 100° for 30 min prior to use. A mixture of $CHCl_3$-MeOH (3:2) was used as solvent system to develop the tlc plates. After development the spots were visualized by uv and spraying with Dragendorff's reagent. Semiquantitative tlc estimates of reaction yields were accomplished by visually comparing spot sizes and intensities (fluorescence quenching at 254 nm and fluorescence at 365 nm) of $MPDP^+$ and MPP^+ produced in reactions to those of standard amounts of these compounds (0.23-2.3 μg). With this chromatographic system the Rf values were MPTP (0.46), $MPDP^+$ (0.25), MPP^+ (0.05), and MPTP-*N*-oxide (0.15). Hplc was performed using a reversed phase C-18 (Altech, 0.625×25 cm) column and acetonitrile 0.1 M HOAc (pH 5.6 with triethylamine) (86.5:13.5) with a Waters Associates ALC/GPC 202 instrument equipped with a Waters M6000A pump, a U6K Universal injector, and a 254/280 nm differential uv detector. Normal operating conditions were flow rate of 2 ml/min at a back pressure of 500 psi. Retention volumes for MPTP and MPP^+ were 42 ml and 48 ml, respectively. $MPDP^+$ has the same retention volume as MPP^+ in this system, and it was necessary to rely on tlc and uv spectral differences to detect its presence in the reaction mixture.

Uv spectra were recorded with a Hewlett Packard 8250A spectrophotometer in pH 5.5, 0.2 M sodium acetate buffer. Spectral absorbances of MPTP, $MPDP^+$, and MPP^+ were confirmed as reported earlier (21,22).

RATE OF OXYGEN CONSUMPTION BY CLARK ELECTRODE.—Incubations were conducted as described (2) at 37° in 3.60 ml of 0.2 M acetate buffer pH 5.5 in a stirred, jacketed reaction chamber containing 10 μl (10.7 units) of human serum ceruloplasmin (Sigma, C4770, Type X), chlorpromazine hydrochloride (10.34 mg, 8.42×10^{-3} M), MPTP hydrochloride (0.94 mg, 1.25×10^{-3} M), or $MPDP^+$ bromide (0.99 mg, 1.09×10^{-3} M) as substrate. Full scale recorder deflection was calculated as 0.71 μmole of oxygen.

PREPARATIVE SCALE INCUBATION WITH HUMAN SERUM CERULOPLASMIN.—Incubations were conducted as described (2) in 50-ml Delong flasks, each containing 5 ml of 0.2 M acetate buffer of pH 5.5, 3 mg of MPTP.HCl (in 0.1 ml MeOH), 3.1 mg of chlorpromazine hydrochloride (in 0.1 ml MeOH), and 50 μl (58 units) of ceruloplasmin. Flasks were incubated with shaking at 250 rpm at 37°, and aqueous reaction mixtures were examined directly by tlc and hplc without extraction after various time periods. No reactions were observed in any of the control incubations lacking either ceruloplasmin or chlorpromazine.

INCUBATION WITH HORSERADISH PEROXIDASE.—Methods for the incubation of MPTP with horseradish peroxidase were as described (3). A typical reaction mixture contained 1 mg (431 Purpurogallin units) of horseradish peroxidase (Sigma, P8375, Type VI), 3 mg of

MPTP.HCl, and 1.7 mM H_2O_2 in a total volume of 10 ml of 0.1 M phosphate buffer, pH 6.8 in 50-ml Delong flask. Control incubations consisted of H_2O_2 plus MPTP in buffer. Incubations were conducted by shaking at 250 rpm at 37°. Samples were withdrawn hourly for 24 h and analyzed directly by tlc. The enzyme reaction was also conducted as before except that H_2O_2 was omitted, and 100 mg of glucose and 10 µl (13.2 units) of glucose oxidase (Sigma, Type V) was added as an in situ source of H_2O_2.

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