Pergamon

Bioorganic & Medicinal Chemistry, Vol. 5, No. 7, pp 1321–1325, 1997

PII: S0968-0896(97)00056-4
0968-0896/97 $17.00 + 0.00

Potential Latent Nitrogen Mustard Derivatives Designed to Target Monoamine Oxidase Rich Cells

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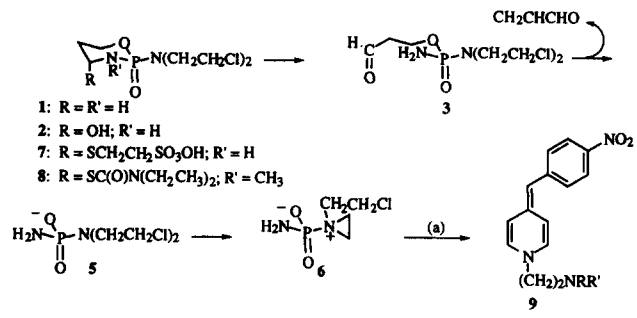
Abstract—The monoamine oxidases A and B (MAO-A and MAO-B) catalyze the α-carbon oxidation of a variety of 4-substituted 1-methyl-1,2,3,6-tetrahydropyridine derivatives to yield the corresponding 2,3-dihydropyridinium species. When the substituent at C-4 of the tetrahydropyridine moiety is a carbamoyloxy functionality, the resulting dihydropyridinium metabolite undergoes spontaneous hydrolytic cleavage to yield 1-methyl-5,6-dihydro-4-pyridone, CO_2, and the corresponding secondary amine. In this paper we summarize our efforts to exploit this metabolic pathway to develop latent nitrogen mustard derivatives related to the oxazaphosphorine antitumor agent cyclophosphamide which may target MAO-A and/or MAO-B rich cells. © 1997 Elsevier Science Ltd.

Introduction

The latent bioalkylating and cytotoxic properties of the oxazaphosphorine antitumor agent cyclophosphamide [2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide (**1**)] are released by a cytochrome P450 catalyzed bioactivation process.[1] The resulting 4-hydroxycyclophosphamide metabolite **2**, via the ring-opened phosphoramidoaldehyde **3**, undergoes a reverse Michael reaction to yield acrolein and the phosphoramide mustard **5**. The aziridinium ion **6** derived from intermediate **5** is thought to mediate the chemotherapeutic properties of the parent drug via the formation of covalent adducts that lead to the cross-linking of DNA.[2] The reaction with 4-nitrobenzylpyridine to yield the purple chromophoric species **9** has been used to monitor the metabolic formation of **5** (Scheme 1).[3]

Efforts to document the molecular mechanism of action of cyclophosphamide and to improve its therapeutic profile have led to the synthesis of a variety of oxazaphosphorine analogues.[4] Other masked phosphoramide mustards also have been investigated[5] including mafosfamide (**7**, Scheme 1), the 2-mercaptoethanesulfonate analogue of 4-hydroxycyclophosphamide[6] which undergoes hydrolytic[7] rather than oxidative bioactivation, and the 3-methylamido-4-thiocarbamoyl derivative **8** which is bioactivated by oxidative *N*-demethylation.[8] The phosphoramide mustard **5** is released in all of these prodrugs.

The concept explored in this study is based on the excellent monoamine oxidase-A (MAO-A)/MAO-B substrate properties of 1,4-disubstituted 1,2,3,6-tetrahydropyridine derivatives including the parkinsonian-inducing neurotoxin MPTP (**10**).[9] The dihydropyridinium intermediate $MPDP^+$ (**11**), formed from MPTP in this reaction, undergoes spontaneous oxidation to the pyridinium species MPP^+ (**12**), the ultimate toxin (Scheme 2).[10] As part of our metabolic studies,[11] we have discovered that dihydropyridinium metabolites generated from tetrahydropyridine substrates bearing a leaving group at C-4 undergo spontaneous hydrolysis.[12] Hydrolysis of the dihydropyridinium metabolites **14** generated from 4-carbamoyloxy-1-methyltetrahydropyridine derivatives (**13**) leads to 1-methyl-5,6-dihydro-4-pyridone **15**, CO_2 and the corresponding secondary amines **16**.[13] These results suggested the possibility of designing tetrahydropyridine derivatives bearing the masked phosphoramide mustard moiety that might be useful as cytotoxic agents that target cells rich in MAO-A/-B. In a previous study we were able to document the potential utility of this approach with an MAO-A bioactivated prodrug designed to release (R)-nordeprenyl [**16**: R = propargyl; R′ = (*R*)-1-methyl-2-phenylethyl].[14] In this paper we describe the synthesis and MAO-A and MAO-B substrate properties of



Scheme 1. The proposed bioactivation pathway for oxazacyclophosphorines. (a) Reaction of 4-(4-nitrophenyl)pyridine with 6 at ph 4.6 for 20 min followed by pH adjustment to 10 to generate the chromophoric species 9 (λ_{max} = 540 nm) where R = CH_2CH_2Cl and R′ = $PO(NH_2)O^-$.

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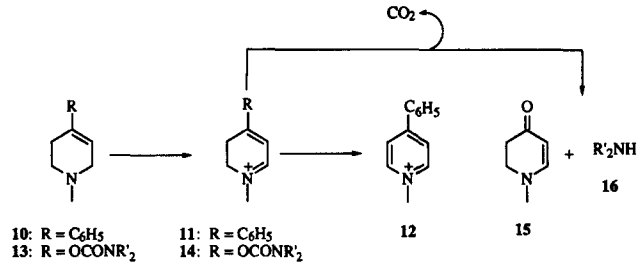
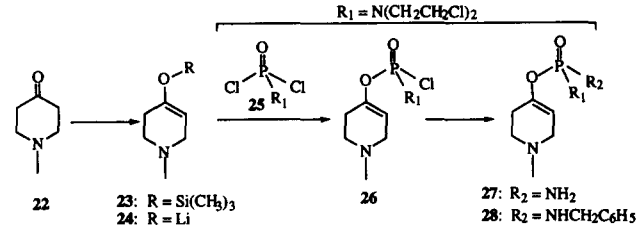


Scheme 2. Pathway for the MAO-catalyzed oxidation of tetrahydropyridine derivatives.



Scheme 4. Synthesis of the tetrahydropyridyl phosphorodiamidates 27 and 28.

1-methyl-1,2,3,6-tetrahydropyridine derivatives bearing bis(2-chloroethyl)carbamoyloxy and bis(2-chloroethyl)-phosphoramoyloxy groups at C-4.

Results and Discussion

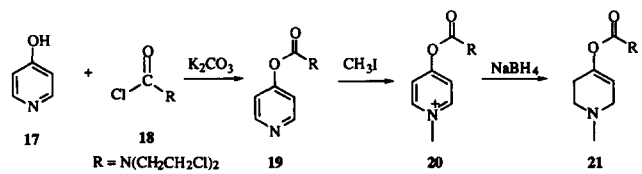
Chemistry

The synthesis of the tetrahydropyridyl carbamate **21** (Scheme 3) was achieved via $NaBH_4$ reduction of the corresponding pyridinium intermediate **20** which in turn was obtained by treatment of the pyridyl carbamate **19** with iodomethane. Compound **19** was prepared from commercially available 4-hydroxypyridine (**17**) and bis(2-chloroethyl)carbamoyl chloride (**18**) by a modification of the previously reported synthesis of related pyridyl carbamates.[13] The oily free base **21** was stored as its stable oxalate salt.

Failure of the condensation reaction between **17** and bis(2-chloroethyl)phosphoroamidic dichloride (**25**) led to an alternative approach for the tetrahydropyridyl phosphorodiamidates **27** and **28** that required the lithium enolate **24**. Compound **24** was obtained in good yield by treatment of the known trimethylsilyl enol ether **23**[15] with methyllithium.[16] Subsequent condensation of **24** and **25** gave the unstable monoamide intermediate **26** which, on treatment with ammonia and benzylamine, gave the desired phosphorodiamidates **27** and **28**, respectively.

Enzymology

Repeated UV scans (400–200 nm) of incubation mixtures showed that **21**, **27**, and **28** are stable in the presence of MAO-B. This outcome was not unexpected since poor MAO-B substrate properties have already been observed with tetrahydropyridine derivatives

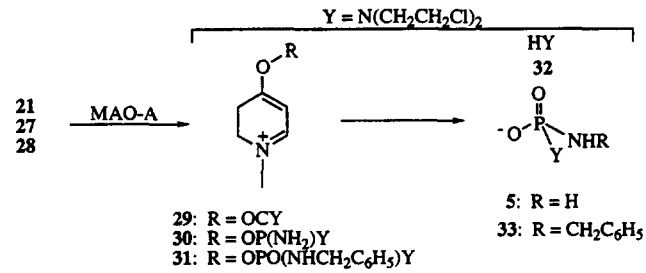


Scheme 3. Synthesis of carbamate mustard **21**.

bearing bulky substituents on C-4.[13] On the other hand, all three compounds proved to be MAO-A substrates as was evidenced by the time-dependent increase in a chromophore with λ_{max} 324 nm corresponding to the expected aminoenone **15**. Plots of the absorbance at 324 nm vs time obtained with 500 μM solutions of the test compounds in the presence of 0.16 μM MAO-A are shown in Figure 1. Of some interest is the observation that introduction of the relatively large, lipophilic benzyl group on one of the phophoroamidate nitrogen atoms increases the rate of oxidation relative to the compound bearing the NH_2 group. This behavior is consistent with the flexibility of the active site of MAO-A[17] and may reflect a lipophilic binding pocket in the active site.

The MAO-A-dependent formation of the aminoenone species from the tetrahydropyridine substrates **21**, **27**, and **28** should coincide with the release of the reactive nitrogen mustards [bis(2-chloroethyl)amine (**32**) and the phosphoramide mustards **5** and **33**] from the hydrolysis of the corresponding dihydropyridinium metabolites **29**, **30**, and **31**, respectively (Scheme 5). The observation of the time and MAO-A-dependent formation of a purple chromophoric species (corresponding to **9**, Scheme 1) when 4-(4-nitro)benzylpyridine was added to the incubation mixtures is consistent with the predicted behavior.

Kinetic studies on the MAO-A catalyzed oxidations of these compounds led in all three cases to linear initial rate plots at substrate concentrations that bracketed the K_m values. The k_{cat} and K_m values were obtained from the corresponding double reciprocal plots, which were also linear. The results are summarized in Table 1. The differences in the substrate properties as measured by



Scheme 5. MAO-A-catalyzed bioactivation of latent nitrogen mustard substrates.

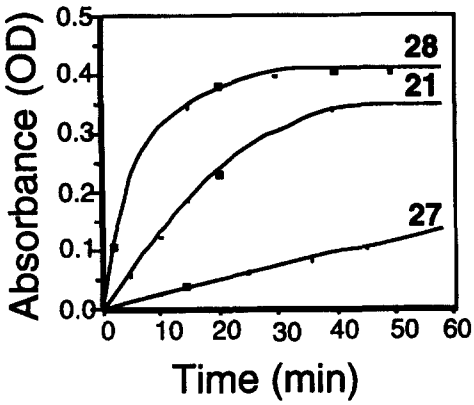


Figure 1. Absorbance vs time plots for the formation of aminoenone 15 from the MAO-A catalyzed bioactivation of the latent nitrogen mustard derivatives 21, 27, and 28.

k_{cat}/K_m reflect the differences in the rates of turnover since the K_m values are quite similar. The *N*-benzyl-bearing phosphorodiamidate derivative **28** is the best substrate with k_{cat}/K_m = 38 min^{-1} mM^{-1}. This value is somewhat better than that reported for the cytochrome P450 catalyzed oxidation of cyclophosphamide (about 10 min^{-1} mM^{-1}).[18]

In summary, these studies demonstrate a potentially novel molecular design that may find utility when attempting to target MAO-rich cells with a cytotoxic agent. Studies currently under consideration will attempt to evaluate the potential selective destruction of MAO-A-rich neurons in the central nervous system of mice treated with **28**, the member of this series with the best MAO-A substrate properties.

Experimental

Chemistry

All chemicals were reagent or HPLC grade. Proton NMR spectra were recorded on a Bruker WP 270 MHz spectrophotometer. Chemical shifts are reported in ppm relative to tetramethylsilane (TMS) and spin multiplicities are given as s (singlet), d (doublet), t (triplet), or m (multiplet). Enzyme kinetic studies were performed on a Beckman Model DU-50 spectrophotometer. Melting points were determined using a Thomas–Hoover melting point apparatus and are uncorrected. Microanalyses, performed by Atlantic Microlab, Inc., Norcross, GA, were within 0.4% of theoretical values calculated for C, H, N. The aminoenone **15**,[19] the bis(2-chloroethyl)carbamoyl chloride (**18**),[20] 1-methyl-4-trimethylsiloxy-1,2,3,6-tetrahydropyridine (**23**)[14] and the bis(2-chloroethyl)phosphoramidic dichloride (**25**)[21] were synthesized as described previously. The newly synthesized phosphorodiamidates are racemic mixtures.

Oxalate salt of 1-methyl-1,2,3,6-tetrahydro-4-pyridyl 4-bis(2-chloroethyl)carbamate (21). The carbamoyl chloride **18** (2.04 g, 10 mmol) in DMF (5 mL) was added dropwise at room temperature with stirring to a solution of 4-hydroxypyridine (**17**, 0.95 g, 10 mmol) in DMF (20 mL) containing K_2CO_3 (1.66 g, 12 mmol). The mixture then was heated at 80 °C for 4 h. The resulting mixture was filtered and the DMF was removed by vacuum distillation. The residue was chromatographed (25 g silica gel, CH_2Cl_2) to give the crude pyridyl carbamate **19** which was treated with iodomethane (7.05 g, 0.05 mol) in 50 mL THF at room temperature with stirring. After two days the solvent and excess iodomethane were removed under vacuum to give methiodide **20** (2.6 g, 64.4%) as an oil: ^{1}H NMR ($CDCl_3$) δ 9.33 (d, 2H, C2 and C6), 8.40 (d, 2H, C3 and C5), 4.78 (s, 3H, N-CH_3), 4.13–4.35 (m, 8H, CH_2CH_2Cl). Since this intermediate could not be obtained in crystalline form, it was used in the next step without further characterization.

$NaBH_4$ (0.374 g, 10.0 mmol) was added in portions to a stirred solution of the above pyridinium compound **20** (2.50 g, 6.2 mmol) in methanol (50 mL) at 0 °C. After stirring for an additional 15 min, the methanol was removed under vacuum and a solution of the residue in 20 mL cold water was extracted with ethyl acetate to provide 1.7 g (98%) of oily free base **21**: ^{1}H NMR ($CDCl_3$) δ 5.41 (unresolved m, 1H, C5), 3.68 (m, 10H, C6 and CH_2CH_2Cl), 3.04 (unresolved m, 2H, C2), 2.66 (t, 2H, C3), 2.35 (s, 3H, N-CH_3). The solid oxalate salt was prepared by the dropwise addition of a solution of oxalic acid (0.363 g, 41.0 mmol) in diethyl ether (10 mL) to a solution of **21** (0.75 g, 2.7 mmol) in diethyl ether (30 mL) at room temperature with stirring: mp 124–125 °C; ^{1}H NMR (D_2O) δ 5.58 (unresolved m, 1H, C5), 3.69–3.79 (m, 10 H, C6 and CH_2CH_2Cl), 3.40 (m, 2H, C2), 2.98 (s, 3H, N-CH_3), 2.57 (unresolved m, 2H, C3). Anal. calcd for $C_{13}H_{20}Cl_2N_2O_6 \cdot 0.25\ H_2O$: C, 41.45; H, 5.16; N, 7.46%. Found C, 41.43; H, 5.29; N, 7.33%.

Oxalate salt of 1-methyl-1,2,3,6-tetrahydro-4-pyridyl *N,N*-bis(2-chloroethyl)phosphorodiamidate (27). A 1.4 M solution of methyllithium in diethyl ether (11.0 mL, 15.4 mmol) was added dropwise at room temperature to a solution of freshly distilled **23** (2.60 g, 14 mmol) in 20 mL THF with stirring under nitrogen. After 4.5 h the reaction mixture containing the lithium enolate **24** was transferred under nitrogen pressure via a cannula to a dropping funnel from which it was added dropwise at 0 °C to a solution of phosphoramidic dichloride (**25**, 4.71 g, 182 mmol) in 15 mL THF. After stirring at 0 °C for an additional 2 h, NH_3 was bubbled through the reaction mixture containing intermediate phosphoroamidic

Table 1. Kinetic parameters for the MAO-A catalyzed oxidation of latent nitrogen mustards

Compd	k_{cat} (min^{-1})	K_m (mM)	K_{cat}/K_m ($min^{-1}mM^{-1}$)
21	28	1.2	23
27	12	1.4	9
28	42	1.1	38

chloride **26** at a moderate rate for 15 min. The resulting suspension was stirred at room temperature overnight, filtered, and the filtrate concentrated on a rotary evaporator to dryness. The residue was chromatographed [30 g silica gel, $CHCl_3$:CH_3OH (6:1, v/v)] to give 3.0 g of **27** as a thick oil (66.8%): ^1H NMR ($CDCl_3$) δ 5.45 (unresolved m, 1H, C5), 3.42–3.66 (m, 10 H, C6 and CH_2CH_2Cl), 3.05 (s, 2H, NH_2), 2.98 (t, 2H, C2), 2.61 (t, 2H, C3), 2.35 (s, 3H, NCH_3). The hygroscopic oxalate salt was prepared in CH_3CN and recrystallized from methanol/ether: ^1H NMR (D_2O) δ 5.50 (unresolved m, 1H, C5), 3.71 (m, 6H, C6 and CH_2CH_2Cl), 3.49 (m, 6H, C2 and CH_2CH_2Cl), 2.95 (s, 3H, N-CH_3), 2.59 (unresolved m, 2H, C3). Anal. calcd for $C_{12}H_{22}Cl_2N_3O_6P{\cdot}H_2O$: C, 33.94; H, 5.69; N, 9.90%. Found C, 33.99; H, 5.69; N, 9.89%.

Oxalate salt of 1-methyl-1,2,3,6-tetrahydro-4-pyridyl-*N*-benzyl-*N'*,*N'*-bis(2-chloroethyl)phosphorodiamidate (28). In a similar way a 1.4 M solution of methyllithium in diethyl ether (45.4 mL, 63.5 mmol) was added dropwise to a solution of the silyl enol ether **23** (10.70 g, 55.7 mmol) in 140 mL THF at room temperature with stirring under nitrogen. After 4 h the reaction mixture was transferred under pressure via a cannula to a dropping funnel from which it was added to a solution of the phosphoramidic dichloride **25** (19.42 g, 75.1 mmol) in 140 mL THF dropwise at 0 °C. Following 2 h at 0 °C, benzylamine (15.46 g, 0.144 mol) was added dropwise to the solution of **26** and the resulting suspension was stirred overnight at room temperature. The solid benzylamine hydrochloride that formed was removed by filtration and the filtrate was evaporated to dryness. Chromatography of the residue [150 g silica gel, $CHCl_3$/CH_3OH (6:1, v/v)] gave 13.10 g (37.7%) of **28** as an oil: ^1H NMR ($CDCl_3$) δ 7.27–7.37 (m, 5H, PhH), 5.56 (unresolved m, 1H, C5), 4.11–4.21 (m, 2 H, $PhCH_2$), 3.31–3.62 (m, 14H, CH_2CH_2Cl, C2, C3 and C6), 2.84 (s, 3H, N-CH_3). The solid oxalate was prepared in CH_3CN and was recrystallized from EtOAc/CH_3CN: mp 133–134 °C; ^1H NMR (CD_3OD) δ 7.17–7.28 (m, 5H, PhH), 5.39 (unresolved m, 1H, C5), 3.99–4.05 (dd, 2H, $PhCH_2$) 3.28–3.58 (m, 12H, C6, C2 and CH_2CH_2Cl), 2.86 (s, 4.5H, N-CH_3 + 0.5 CH_3CN), 2.52 (unresolved m, 2H, C3). Anal. calcd for $C_{19}H_{18}Cl_2N_3O_6P{\cdot}0.25\ CH_3CN$: C, 46.29; H, 5.73; N, 9.45%. Found C, 46.13; H, 6.11; N, 9.41%.

Enzyme substrate studies

The isolation and purification of MAO-A from human placenta and MAO-B from beef liver were carried out using the procedures reported by Salach[22] with the following modifications. The phospholipase A used in our preparation was obtained commercially (Sigma, St Louis, MO) rather than from the crude venom. We did not subject the MAO-A preparation to the sephadex purification or the MAO-B preparation to the glucose gradient purification step. In both cases, however, we obtained highly active preparations. The specific activity of MAO-A (17 nmol mL^{-1}) was established with kynuramine as the substrate at 30 °C (k_{cat} = 146 min^{-1}).[12b] The specific activity of MAO-B (10 nmol mL^{-1}) was established with MPTP as the substrate at 30 °C (k_{cat} = 204 min^{-1}) as reported earlier.[12b] The MAO-B preparation was found to be stable when stored at −15 °C. The MAO-A preparation was less stable and its specific activity had to be estimated on a bimonthly basis. Because of its viscosity, the MAO-A preparation was diluted with 3 vols of phosphate buffer just prior to analysis.

Solutions of the oxalate salts of the carbamate **21** and the two phosphorodiamidates **27** and **28** in phosphate buffer (pH = 7.4, 0.5 mM, final volume 500 μL) in a 1 mL quartz cuvette were treated with 20 μL of the MAO-A preparation (final concentration 0.16 μM) or 5 μL of the MAO-B preparation (final concentration 0.10 μM) and the cuvette was placed in a Beckman model DU-50 spectrophotometer maintained at 37 °C. The substrate properties were evaluated qualitatively by obtaining a series of scans (450–250 nm) vs time over a 1-h period for each compound.

Kinetic studies (with MAO-A only) were carried out using a Beckman DU-50 spectrophotometer. Solutions of the test compounds (final volume 500 μL, final substrate concentrations 500–8000 μM) in 100 μM sodium phosphate (pH = 7.4) were incubated in the presence of 0.16 μM MAO-A. The rates of oxidation were obtained by monitoring the increment in absorbance of the aminoenone product **15** at 324 nm (ε 15,300) over a 30–120-s time period. The k_{cat} and K_m values were calculated from double reciprocal plots.

Colorimetric assay to estimate release of mustard-derived aziridinium species[3]

The MAO-A (0.16 μM) dependent generation of bis(2-chloroethyl)amine (**32**) and the phosphoramide mustards **5** and **33** from the three promustards **21**, **27**, and **28** (1 mL of 500 μM solutions) in phosphate buffer (pH 7.4) at 37 °C was examined after a 30-min incubation period by transferring the incubation mixtures to screw-capped test tubes which contained 1.5 mL of 0.2 M acetate buffer (pH 4.6). To each solution was added 0.4 mL of aqueous 5% 4-(4-nitrobenzyl)pyridine. The test tubes were placed in a boiling water bath for 20 min and then were allowed to cool to room temperature. These mixtures were then treated with 4 mL of ethyl acetate:acetone (5:2, v/v) followed by 1.5 mL of 2.5 N aqueous NaOH. The test tubes were shaken 20 times and centrifuged for 2 min (6000 rpm). The formation of a purple chromophore with λ_{max} 540 nm was observed with all three incubations. Control experiments, conducted under identical conditions only in the absence of MAO-A, did not produce the purple solutions.

Acknowledgements

This study was supported by the National Institute of Neurological Disorders and Stroke (NS 28792) and the Harvey W. Peters Center for the Study of Parkinson's Disease.

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(*Received in U.S.A.* 9 *January* 1997; *accepted* 14 *February* 1997)