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Anodic Oxidation of Ifosfamide and Cyclophosphamide: A Biomimetic Metabolism Model of the Oxazaphosphorinane Anticancer Drugs

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Abstract—The electrochemical oxidation of anticancer drugs ifosfamide and cyclophosphamide produced in high yield methoxylated analogues of the key hydroxy-metabolites of these oxazaphosphorine prodrugs. The cytotoxicity of these compounds was evaluated, and found to be as high as the hydroxy-metabolite. © 2001 Elsevier Science Ltd. All rights reserved.

Oxazaphosphorinane drugs are alkylating antineoplastic agents used in various cancer chemotherapy regimens for the treatment of sarcoma and cerebral tumors. Ifosfamide (IFM, **1a**) and cyclophosphamide (CPM, **1b**) (Scheme 1) share a similar enzyme-catalyzed activation process necessary for the therapeutic activity.[1] They are non-cytotoxic prodrugs which require a cytochrome P450-mediated hepatic bioactivation step consisting of a ring oxidation. In brief, the enzyme-catalyzed oxidation of **1a**,**b** at the C-4 position produces 4-hydroxyifosfamide (**2a**) or 4-hydroxycyclophosphamide (**2b**) precursors of aldehydes. Subsequent retro-Michael reaction leads to the true alkylating moiety, isophosphoramide mustard (**4a**) or phosphoramide mustard (**4b**) and acrolein (**5**), concomitantly (Scheme 1).

Many attempts of reaching 4-oxidated moieties in vitro were reported,[2–7] especially for cyclophosphamide. Oxidation of cyclophosphamide **1b** at C-4 was achieved by microsomal incubation techniques[7] or by chemical reactions.[2–6] The use of Fenton's reagent ($FeSO_4$/H_2O_2), $KMnO_4$ or ozone with hydrogen peroxide (O_3/H_2O_2) were reported. Since such chemical oxidations are typically unspecific, very poor yields ($<5\%$) of the expected products were obtained. Furthermore, in vivo oxidation of the side chain of the drugs was reported to occur (via putative compounds **3** or **3′**; Scheme 1) without formation of the mustard and producing neurotoxic derivatives.[8]

In this paper, we report a highly efficient electrochemical synthesis of 4-methoxy-ifosfamide (**6**) and 4-methoxy-cyclophosphamide (**7**) (Scheme 2), the cytotoxic activities of which were evaluated in vitro on human carcinoma KB cells.

Results and Discussion

Electro-oxidation of tertiary amides and carbamates are well documented. Extensive work by Shono showed the facile preparation of α-methoxylated derivatives useful for subsequent syntheses. Shono[9] also reported one example of oxidation of amidophosphate to the corresponding α-methoxylated derivative showing a similar electronic effect of phosphonate group compared to classical *N*-acyl deactivating group. We then undertook an electrochemical study in order to prepare equivalents of 4-hydroxy metabolites, and to propose a model for the metabolism of the phosphamide drugs. It is also interesting to examine such anodic oxidation regarding its regiochemistry.

The cyclic voltamogram of (±)ifosfamide (**1a**) running in acetonitrile containing lithium perchlorate as supporting electrolyte on a glassy carbon electrode showed two irreversible anodic signals at, respectively, 1.85 and 2.00 V versus SCE. Under the same conditions, (±) cyclophophamide (**1b**) exhibited a unique signal at 2.00 V versus SCE. In both cases the oxidation corresponds to a two electron process.

This preliminary analytical study showed the possible electrochemical oxidation of phophoramides **1a** and **1b**.

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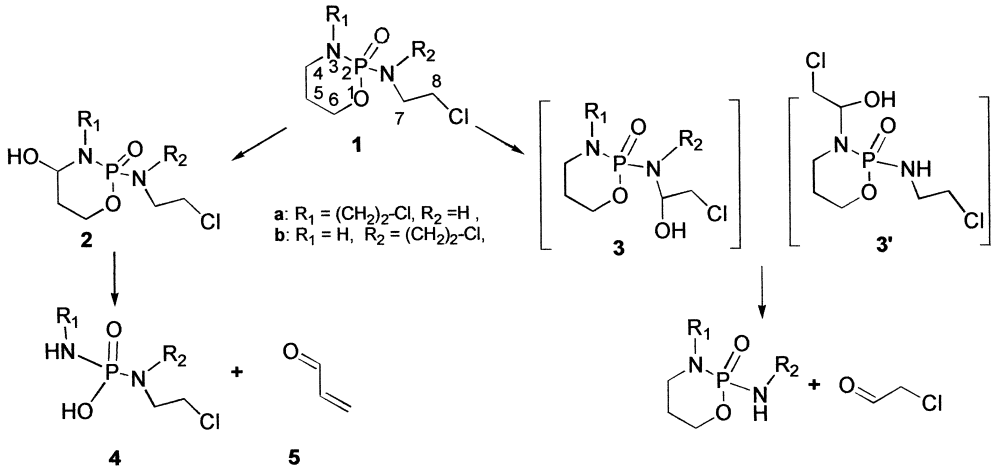
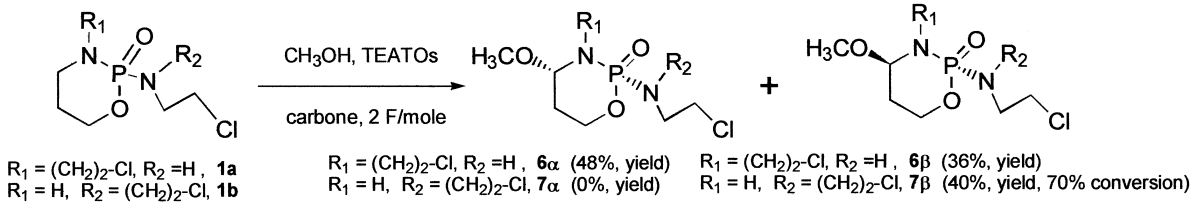


Scheme 1.



Scheme 2.

The electrolysis of **1a** was thus undertaken galvanostically in methanol and in the presence of Et_4NOTs as supporting electrolyte. This electrochemical reaction leads specifically within 90 min (2.2 F/mol) to the α-methoxylated product **6** isolated in 84% yield, which is extremely high compared to the values found in the literature.[2–6]

Chromatographic and NMR analyses of the reaction product revealed the presence of two components **6α** and **6β** in a 60:40 ratio, easily separated by flash chromatography on silica gel. These products were proved to be epimeric by means of NMR analysis. Both stereomers carry the methoxy group in the axial position. This means that the phosphoryl moiety (P=O) is in an equatorial position for one diastereoisomer and in an axial position for the other one as confirmed by examination of IR spectra (**6α**: $\nu_{P=O}$ 1257 cm^{-1}; **6β**: $\nu_{P=O}$ 1237 cm^{-1}).

Cyclophosphamide (**1b**) was also oxidized through a similar electrochemical process. Since the oxidation potential is high (ca. 2 V) overoxidation products were observed and it was proved more convenient to stop the electrolysis after consumption of 60% of starting material. In this case the 4-OMe derivative **7** was isolated as a unique compound in 40% yield (about 30% of starting material was recovered) with the methoxy group and the P=O moiety in axial positions (**7β**: $\nu_{P=O}$ 1240 cm^{-1}).

The formation of a diastereomeric mixture in the case of ifosfamide could not be the result of epimerisation at phosphorus as had been postulated by different authors.[4,10] This epimerisation should occur in acidic medium and our experimental conditions were essentially neutral ones. It is noteworthy that, in contrast, cyclophosphamide was oxidized to a unique derivative. We suggest that the different behaviour of **1a** and **1b** was the result of an unfavourable $A^{1,2}$ allylic interaction which only occurs in the iminium ion derived from ifosfamide as illustrated in Fig. 1. This assumption was supported by the chemical stability of methoxylated compounds: there is no interconversion between **6α** and **6β**.

It is interesting to note the regioselectivity of the reaction which occurs exclusively at the C-4 position, whereas the reaction is theoretically possible at the two other positions α to nitrogen. It seems that the endocyclic position is more sensitive to anodic oxidation since it occurred with both compounds **1a** and **1b** (where the endocyclic nitrogen is secondary or tertiary). Furthermore, this result highly correlates with the in vivo metabolic behaviour of these drugs.

4-Methoxy derivatives were checked in vitro against carcinoma KB cells line and the results were reported in

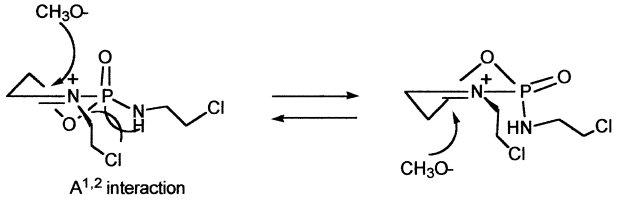


Figure 1.

Table 1. It can be seen that these derivatives can be considered as pre-activated analogues of oxazaphosphorinane drugs since they exhibit moderate but significantly higher cytotoxicity compared to the unoxidised drugs ($IC_{50} > 200\,\mu M$). Indeed, this oxidation level allows the liberation of the phosphoramide mustard without the cytochrome mediated ring oxidation. The cytotoxicity of these derivatives can be compared with the data found in the literature for 4-hydroxy-IFM (**2a**) and 4-hydroxy-CPM (**2b**) ($IC_{50} = 10$–$20\,\mu M$)[3] which are the first metabolites of those oxazaphosphorine drugs.

Table 1. Cytotoxicity[11] of 4-methoxy derivatives

Compounds	**6α**	**6β**	**7α**
IC_{50} (μM)	20	22	16

Studies currently in progress will attempt to evaluate the potentials of oxidation of the other positions α to the nitrogen on the side chain of these drugs (C7 and C9) and the possibility to manage further oxidations of the α-methoxylated products **6** and **7**.

General Procedure for Anodic Oxidation

A solution of ifosfamide **1a** (100 mg, 0.383 mmol) or **1b** (100 mg, 0.383 mmol) and Et_4NOTs (200 mg, 0.66 mmol) in methanol (7 mL) was introduced into an undivided cell equipped with two graphite rod electrodes (5 mm, diameter) and cooled with ice water. The anodic oxidation was followed by checking the consumption of the starting material with thin-layer chromatography. After 2.2 F/mol of electricity was passed through the solution at a constant current ($i_{ox} = 15$ mA), lithium carbonate (65 mg, 1 mmol) was added and the solvent was removed in vacuo. The oily residue was chromatographed on silica gel (CH_2Cl_2/CH_3OH: 98/2) to afford 4-methoxy-ifosfamide (**6α**) and (**6β**) in, respectively, 48 and 36% yield, or 4-methoxy-cyclophosphamide (**7β**) in 40% yield when the starting material was **1b.**

6α (53 mg, 48% yield): $R_f = 0.65$ [CH_2Cl_2/CH_3OH (95:5)]. IR (film) ν cm^{-1}: 3289; 2943 (C–H); 1460 (P–N); 1257 (P=O); 1114–1067 (P–O–C). ^1H NMR ($CDCl_3$) δ (ppm): 1.90 (1H, m, $^4J_{H-P} = 15$ Hz, $H_{5\ eq}$), 2.25 (1H, m, $^3J_{H-H} = 3$ Hz, $^4J_{H-P} = 3$ Hz, $^2J_{H-Hgem} = 9$ Hz, $^3J_{H-H} = 3$ Hz, $^3J_{H-H} = 6$ Hz, $H_{5\ ax}$), 3.10–3.40 (4H, m, $H_{7,7'}$ and $H_{9,9'}$), 3.30 (3H, s, MeO), 3.40–3.60 (4H, m, $H_{8,8'}$ and $H_{10,10'}$), 4.10–4.20 (1H, m, $H_{6\ eq}$), 4.40 (1H, m, $^3J_{H-H} = 3$ Hz, $^3J_{H-P} = 2$ Hz, $^2J_{H-Hgem} = 9$ Hz, $^3J_{H-H} = 3$ Hz, $^3J_{H-H} = 6$ Hz, $H_{6\ ax}$), 4.50 (1H, dt, $^3J_{H-P} = 22$ Hz, $^3J_{H-H} = 3$ Hz, H_4). ^{13}C NMR ($CDCl_3$) δ (ppm) 29.5 (C_5); 42.8 (C_7); 44.2 (C_8); 46.5 (C_{10}); 49.8 (C_9); 55.9 (MeO); 62.5 (C_6); 90.8 (C_4). ^{31}P NMR ($CDCl_3$): 9.8 ppm. SM (IC) $m/z = 308$ (310, 312) $[M + NH_4]^+$, 291 (293, 295) $[M + H]^+$.

6β (40 mg, 36% yield): $R_f = 0.50$ [CH_2Cl_2/CH_3OH (95:5)]. IR (film) ν cm^{-1}: 3289; 3237 large (NH); 2932 (C–H); 1437 (P–N); 1237 (P=O); 1114–1067 (P–O–C). ^1H NMR ($CDCl_3$) δ (ppm): 2.00 (2H, m, $H_{5\ eq}$ and $H_{5\ ax}$), 3.10–3.45 (4H, m, $H_{7,7'}$ and $H_{9,9'}$), 3.35 (3H, s, MeO), 3.40–3.60 (4H, m, $H_{8,8'}$ and $H_{10,10'}$), 4.00–4.10 (1H, m, $H_{6\ eq}$), 4.40–4.50 (1H, dt, $^3J_{H-P} = 21$ Hz, $^3J_{H-H} = 3$ Hz, H_4), 4.60 (1H, m, $H_{6\ ax}$). ^{13}C NMR ($CDCl_3$) δ (ppm): 28.8 (C_5); 42.6 (C_7); 43.4 (C_8); 45.6 (C_{10}); 48.2 (C_9); 55.4 (MeO); 62.0 (C_6); 89.4 (C_4). ^{31}P NMR ($CDCl_3$): 10.2 ppm. SM (IC) $m/z = 308$ (310, 312) $[M + NH_4]^+$, 291 (293, 295) $[M + H]^+$.

7β (44 mg, 40%): $R_f = 0.50$ [CH_2Cl_2/CH_3OH (95:5)]. IR (film) ν cm^{-1}: 3216 large (NH); 2980–2900 (C–H); 1459 (P–N); 1240 (P=O); 1116–1055 (P–O–C). ^1H NMR ($CDCl_3$) δ (ppm) 1.90 (1H, d, $^2J_{H-H} = 11$ Hz, $H_{5\ eq}$), 2.05 (1H, m, $^3J_{H-H} = 11$ Hz, $^2J_{H-Hgem} = 11$ Hz, $^3J_{H-H} = 3$ Hz, $^3J_{H-H} = 2$ Hz, $H_{5\ ax}$), 3.30–3.40 (5H, m, MeO and $H_{9,9'}$), 3.40–3.50 (2H, m, $H_{7,7'}$), 3.60 (4H, m, $H_{8,8'}$ and $H_{10,10'}$), 3.80 (1H, t, NH), 4.10–4.20 (1H, dddd, $^3J_{H-H} = 3$ Hz, $^2J_{H-Hgem} = 12$ Hz, $^3J_{H-H} = 3$ Hz, $^3J_{P-H} = 26$ Hz, $H_{6\ eq}$), 4.60 (1H, m, $^3J_{H-P} = 26$ Hz, $^3J_{H-H} = 3$ Hz, H_4), 4.80 (1H, dddd, $^3J_{H-H} = 11$ Hz, $^2J_{H-Hgem} = 11$ Hz, $^3J_{H-H} = 3$ Hz, $^3J_{P-H} = 2.5$ Hz, $H_{6\ ax}$). ^{13}C NMR ($CDCl_3$) δ (ppm): 30.3 (C_5); 42.3 (C_8, C_{10}); 48,4 (C_7, C_9); 54.1 (MeO); 63.0 (C_6); 84.0 (C_4). ^{31}P NMR ($CDCl_3$): 9.8 ppm. SM (IC) $m/z = 308$ (310, 312) $[M + NH_4]^+$, 291 (293, 295) $[M + H]^+$.

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