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Identification of New Metabolites of Ifosfamide in Rat Urine Using Ion Cluster Technique†

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Metabolism of the anticancer drug ifosfamide was investigated in Sprague–Dawley rats. Along with four known metabolites, namely N^2-dechloroethylifosfamide, N^3-dechloroethylifosfamide, alcoifosfamide and isophosphoramide mustard, four new urinary metabolites were identified utilizing combined techniques of chemical modification/derivatization, capillary gas chromatography/chemical ionization mass spectrometry (ammonia), deuterium-labeling/ion cluster analysis and chemical synthesis. Secondary metabolites of N^2-dechloroethyl and N^3-dechloroethylifosfamide formed by 4-hydroxylation, *i.e.* 4-hydroxy-N^2-dechloroethylifosfamide and 4-hydroxy-N^3-dechloroethylifosfamide, respectively, and their subsequent decomposition product, *N*-dechloroethylisophosphoramide mustard, were identified. Secondary dealkylation pathways of N^2-dechloroethylifosfamide and/or N^3-dechloroethylifosfamide were also demonstrated through characterization of $N^{2,3}$-didechloroethyl ifosfamide. The key active metabolite of ifosfamide, 4-hydroxyifosfamide, was characterized as a cyanohydrin adduct for the first time.

INTRODUCTION

Ifosfamide (IF), a structural isomer of the oxazaphosphorine cyclophosphamide (CP),[1,2] has been found to possess significant activity against human and experimental malignancies such as non-seminomatous testicular cancer, small-cell lung cancer, pediatric solid tumors, non-Hodgkin's and Hodgkin's lymphomas and ovarian cancer.[3-5] Like CP, IF is a prodrug that requires *in vivo* enzymatic activation to be cytotoxic.[1,2,6,7] Hydroxylation mediated by the hepatic cytochrome P450 system at the 4-position of the oxazaphosphorine ring leads to the formation of 4-hydroxyifosfamide (4-OHIF), the most important metabolite for the activity of this drug. This species spontaneously equilibrates with its open-chain tautomer, aldoifosfamide (aldoIF). Further oxidation of these species by alcohol oxidase or aldehyde dehydrogenase gives 4-ketoifosfamide (4-ketoIF) and carboxyifosfamide (carboxyIF), respectively. AldoIF is reduced by aldehyde reductase to alcoifosfamide (alcoIF), and at the same time undergoes spontaneous decomposition to form isophosphoramide mustard (IPM), the purported ultimate intracellular alkylating metabolite[8,9] with the elimination of acrolein, the causative agent for the urotoxicity of oxazaphosphorines.[10] Unlike CP, *N*-dealkylation of IF occurs to a much greater extent, leading to the formation of monoalkylating species, N^2-dechloroethylifosfamide (N2D) and N^3-dechloroethylifosfamide (N3D),[11-15] with the release of the co-product, chloroacetaldehyde (CAA), the metabolite implicated in the neurotoxicity observed in patients receiving IF therapy.[16] Although 4-OHIF and aldoIF are believed to be the most important metabolites of IF, neither of them has been unequivocally identified as a metabolite because of their chemical instability. The metabolic pathway of IF is shown in Scheme 1. The previously unknown metabolism of N2D and N3D is derived from the results reported herein.

Conventional metabolite identification techniques involve isolation and purification of the metabolite from biological excreta, followed by the usual spectroscopic analyses, such as IR, NMR, UV and mass spectrometry (MS), and are tedious and time consuming. Using a 1:1 mixture of stable isotopically labeled and unlabeled drugs, tentative identification of metabolites can rapidly be achieved on the basis of observed ion doublets or ion clusters using electron impact (EI)[17,18] and chemical ionization (CI) MS[19,20] on either crude extracts or partially purified extracts. The mild fragmentation under CIMS conditions, augmented by a high abundance of the labeled ions, greatly simplifies the location of the potential metabolites. Subsequent structural identification of a metabolite can be accomplished by loss or retention of specific isotope labels, derivatization and chemical synthesis. This approach has been successfully applied to a number of drugs, including (+)-propoxyphene,[18] nortriptyline,[17] warfarin[19] and cyclophosphamide.[20] Using stable isotope labeling, chemical modification, ion cluster analysis and GC/CIMS, we

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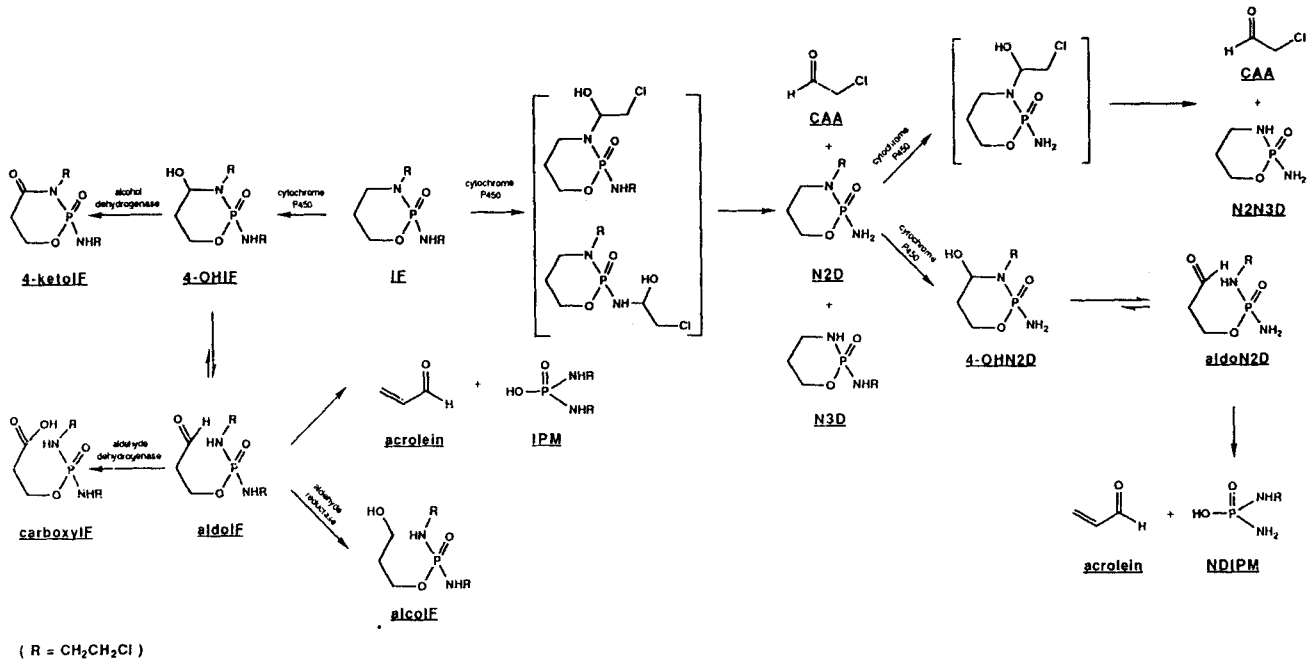


Scheme 1. Metabolic pathways of ifosfamide incorporating new metabolities found in the present study. Metabolism of N^3-dechloroethylifosfamide is similar to that of N^2-dechloroethylifosfamide and is therefore omitted from the scheme.

report here the identification of four new metabolites of IF.

EXPERIMENTAL

Materials

(±)-IF-d_0 was provided by Drug Synthesis and Chemistry Branch, the National Cancer Institute. (±)-[1′,1′,2′,2′-$^2H_4$]IF, (+)- and (−)-IF, (+)- and (−)-[2′,2′,6,6-$^2H_4$]IF, 4-OHIF, N2D, N3D and IPM were all synthesized in this laboratory (Wang and Chan, unpublished data). All HPLC-grade organic solvents were purchased from EM Science (Gibbstown, NJ, USA). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *N*-trimethylsilylimidazole (TMSI) were purchased from Pierce (Rockford, IL, USA). C_{18} reversed-phase resin was obtained from Analytichem International (Harbor City, CA, USA).

Animal studies

Animal experiments were carried out according to a protocol approved by the Animal Use Review Committee at Ohio State University. Four male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) weighing 250–300 g were used. One animal was given (±)-IF, one given a 1:1 mixture of (±)-IF and (±)-[1′,1′,2′,2′-$^2H_4$]IF, one given a pseudoracemate consisting of (+)-[2′,2′,6,6-$^2H_4$]IF and (−)-IF and one given a pseudoracemate consisting of the opposite composition. Rat chow (Teklad, Indianapolis, IN, USA) and water were given *ad libitum*. The jugular vein of each rat was cannulated under ether anesthesia according to the standard procedures.[20] After cannulation, animals were allowed to recover for 2 h before dosing. IF dissolved in normal saline (1 ml) at a total dose of 40 mg kg^{-1} was injected into the animal through the jugular vein cannula. The cannula was washed three times with 0.3 ml each of 0.9% sodium chloride solution. Urine was collected continuously for 24 h in a container immersed in ice, and immediately stored at −76 °C until analysis.

Extraction and derivatization

Rat urine was divided into two portions and each portion was screened for possible new metabolites, using different methods as described below. For the analysis of dichloromethane (CH_2Cl_2)-extractable metabolites (*e.g.* 4-OHIF and 4-hydroxy-N2D), 50 mg of KCN or K^{13}CN were added to 0.5 ml of urine from each rat. The mixture was allowed to stand at room temperature for 30 min. CH_2Cl_2 (5 ml) was added and the mixture was shaken for 15 min in a horizontal shaker (Eberbath, Ann Arbor, MI, USA). The organic phase was separated after centrifugation and evaporated under a stream of N_2. The residue was derivatized with 35 μl of MSTFA for 60 min at 120 °C. An aliquot (0.5 μl) of the mixture was analyzed by GC/MS.

For the assay of more polar components (*e.g.*, *N*-dechloroethylisophosphoramide mustard (NDIPM) and N^2,N^3-didechloroethylifosfamide (N2N3D)), solid-phase extraction was used. Rat urine (0.5 ml) was loaded on to a disposable Poly-prep column (Bio-Rad, Richmond, CA, USA) containing 400 mg of C_{18} reversed-phase resin, and the resin was washed with 0.5 ml of cold saline followed by centrifugation at 200 *g* for 20 min to remove water. The mini-column was then eluted with 1 ml of methanol, which was collected and evaporated

under a stream of N_2 at room temperature. The residue was derivatized with 35 μl of a mixture of BSTFA and TMSI (5:1) at 120 °C for 60 min. An aliquot (0.5 μl) of the mixture was analyzed by GC/MS.

GC/MS analysis

A Finnigan ITS40 ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) coupled to a Model 3300/3400 gas chromatograph (Varian, Walnut City, CA, USA) was used for the analysis. An A200S autosampler with a capillary splitless injector was purchased from Finnigan MAT. The temperatures of the injection port, transfer line, and source were maintained at 220, 260 and 230 °C, respectively. The analysis was carried out using the CI mode with ammonia as the reagent gas. A DB-5 capillary column (30 m × 0.25 mm I.D.) bonded with a 0.25 μm film thickness of 5% methylsilicone (J&W Scientific, Folsom, CA, USA) was used for the separation. The oven temperature program was 150 °C for 2 min, then increased to 190 °C at 5 °C min^{-1} and to 250 °C at 15 °C min^{-1}, the final temperature being held for 3 min.

Synthesis

Alcoifosfamide (alcoIF). *3-Benzyloxylpropyl* N,N'-*bis(2-chloroethyl)phosphorodiamidate.* Into a cooled (−78 °C) solution of phosphorus oxychloride (1.35 g, 10.0 mmol) in CH_2Cl_2 (20 ml) was added dropwise a solution of *O*-benzylpropane-1,3-diol (1.66 g, 10.0 mmol) and tri ethylamine (1.01 g, 10.0 mmol) in the same solvent (10 ml), while vigorous stirring was maintained. The progress of reaction was followed by TLC. When all the starting material had disappeared (in 10 min), 2-chloroethylamine monohydrochloride (3.32 g, 20.0 mmol) was added to the reaction mixture, followed by a solution of triethylamine (4.04 g, 40.0 mmol) in CH_2Cl_2 (5 ml). The mixture was stirred at room temperature for 1 h. The solvent was removed under reduced pressure and the residue was extracted with acetone. The extract was concentrated to give a pale-yellow oil (4.04 g), which was purified by silica column chromatography (CH_2Cl_2–acetone, 2:1) to give a colorless oil product (3.42 g, 92.7%): R_f 0.47 (CH_2Cl_2–acetone, 1:1); NMR ($CDCl_3$), δ 1.96 (p, J = 6.5 Hz, 2H, $CH_2CH_2CH_2$), 3.02–3.40 (m, 6H, 2 × $NHCH_2$), 3.52–3.66 (m, 6H, 2 × CH_2Cl, CH_2OBn), 4.12 (q, J = 6.5 Hz, 2H, CH_2OP), 7.28–7.42 (m, 5H, PhH).

AlcoIF. To a solution of benzyloxypropyl phosphorodiamidate (0.70 g, 1.90 mmol) was added freshly prepared hexane-washed 10% Pd/C (0.50 g) and the mixture was hydrogenated at room temperature. The reaction was monitored by TLC. When the starting material had disappeared (2 h), the catalyst was removed by filtration. Concentration of the filtrate under reduced pressure afforded the crude product (0.62 g), which was purified by column chromatography (CH_2Cl_2–acetone, 1:1) to give alcoifosfamide, a colorless oil (0.38 g, 68.4%): R_f 0.44 (CH_2Cl_2–acetone, 1:3); NMR ($CDCl_3$), δ 1.76 (bs, 1H, OH), 1.85 (p, J = 5.6 Hz, 2H, $CH_2CH_2CH_2$), 3.08–3.40 (m, 6H, 2 × $NHCH_2$), 3.64 (t, J = 5.6 Hz, 4H, 2 × CH_2Cl), 3.75 (t, J = 5.6 Hz, 2H, CH_2OH), 4.10–4.24 (m, 2H, CH_2OP). The assigned structure was consistent with GC/MS analysis.

4-Hydroxy N^3-dechloroethylifosfamide (4-OHN3D). The synthesis of 4-OHN3D was conducted according to the procedure of Peter *et al.*[21] for the synthesis of 4-hydroperoxyifosfamide. N3D (0.10 g, 0.50 mmol) was dissolved in aqueous acetone (1:1, 10 ml). After addition of 30% H_2O_2 (1 ml), the solution was ozonized at 0 °C for 2 h. Acetone was removed under reduced pressure at room temperature. The isolation of the product was found to be difficult and the resulting mixture was used directly as the source of 4-OHN3D. The chemical identity was confirmed by GC/MS analysis. To a culture tube containing 100 μl of the resulting mixture was added 1 M $Na_2S_2O_3$ solution (100 μl) followed by 1 M KCN (250 μl). The mixture was allowed to stand at room temperature for 30 min. CH_2Cl_2 (5 ml) was added to extract the cyanohydrin adduct of 4-OHN3D. The extract was evaporated under a stream of N_2 and the residue derivatized with MSTFA (40 μl) at 120 °C for 1 h. An aliquot was subjected to GC/MS analysis. A major peak at m/z 422 (no Cl) was observed, which is consistent with the assigned structure of the tri(trimethylsilyl)dechlorinated cyanohydrin adduct of 4-hydroxy N3D.

***N*-Dechloroethyl isophosphoramide mustard (NDIPM).** *Benzyl* N-*diphenylmethyl*-N'-*2-chloroethylphosphorodiamidate.* To 20 ml of a cooled (−78 °C) solution of phosphorus oxychloride (1.53 g, 10.0 mmol) in CH_2Cl_2 were added 10 ml of benzyl alcohol (1.08 g, 10.0 mmol) and triethylamine (1.01 g, 10.0 mmol) in CH_2Cl_2. The mixture was stirred for 15 min, then 5 ml of diphenylmethylamine (1.83 g, 10.0 mmol) and triethylamine (1.01 g, 10.0 mmol) in CH_2Cl_2 were added. After vigorous stirring for 2 h at ambient temperature, 2-chloroethylamine monohydrochloride (1.16 g, 10.0 mmol) was added to the reaction mixture, followed by 5 ml of triethylamine (2.02 g, 20.0 mmol) in CH_2Cl_2. The stirring was maintained overnight. The reaction mixture was washed with 4 × 20 ml of water to remove triethylamine chloride. The CH_2Cl_2 layer was dried over anhydrous Na_2SO_4 and the filtrate concentrated under reduced pressure to afford a thick oil (4.52 g), which was purified by column chromatography (CH_2Cl_2–acetone 20:1) to give the diamidate as a colorless oil (3.24 g, 78.3%): R_f 0.28 (CH_2Cl_2–acetone, 20:1); NMR ($CDCl_3$), δ 2.75–2.95 (m, 1H, NH), 3.00–3.18 (m, 2H, $NHCH_2$), 3.30–3.46 (m, 3H, NH, CH_2Cl), 4.75–5.07 (m, 2H, CH_2O), 5.42–5.54 (m, 1H, *CH*NH).

N-(*2-Chloroethyl*) *phosphorodiamidic acid* (*NDIPM*). The resultant diamidate (0.83 g, 2.0 mmol) in MeOH (40 ml) was hydrogenated under normal pressure at room temperature with 10% Pd/C (0.40 g) and $Pd(OH)_2/C$ (0.40 g) as catalysts. The reaction was followed by TLC. When the starting material had disappeared (1 h), the catalysts were removed by filtration. The solvent in the filtrate was removed under vacuum. To the residue was added diethyl ether (5 ml), resulting in the formation of crystalline material, which was collected by filtration. The solid was washed with ethanol

to afford the expected product (0.25 g, 78.9%, m.p. 103–105 °C). The chemical identity was confirmed by GC/MS analysis.

N^2,N^3-Didechloroethylifosfamide (N2N3D). To a stirred solution of phosphorus oxychloride (1.53 g, 10.0 mmol) in Et_2O (40 ml) at −78 °C was added a mixture of 3-aminopropan-1-ol (0.75 g, 10.0 mmol) and triethylamine (2.02 g, 20.0 mmol). The stirring was maintained for 2 h at ambient temperature. The precipitated triethylamine hydrochloride was removed by filtration and the filtrate was used directly in the subsequent step. Ammonia was bubbled into the vigorously stirred solution for 1 h at room temperature. The precipitated ammonium chloride was removed by filtration and, after concentration of the filtrate, it gave N2N3D as a white solid (0.48 g, 35.3%): m.p. 105–108 °C; R_f 0.18 (acetone); NMR (acetone-d_6), δ 1.54–1.86 (m, 2H, C-5-H), 3.14–3.62 (m, 5H, NH, NH_2,C-4-H), 4.08–4.30 (m, 2H, C-6-H); The chemical identity was further confirmed by GC/MS analysis. The MSTFA-derivatized sample gave a peak with ion at m/z 353, consistent with the assigned structure, N,N,N'-tri-(trimethylsilyl)N2N3D.

RESULTS

AlcoIF

The GC/MS trace of the derivatized urinary CH_2Cl_2 extract obtained from a rat given (±)-IF is shown in Fig. 1(A). The ion chromatograms with specific ions are shown in Fig. 1(B)–(D). As shown, an ion at m/z 387 with a retention time of 14.30 min was observed [Fig. 1(B)] and this ion was absent in the blank urine [Fig. 1(B), inset]. The mass spectrum for this component shows a strong ion at m/z 387 and a small ion at m/z 389, the abundance of which is consistent with one chlorine atom. Based on this information, the structure of trimethylsilyldehydrochlorinated alcoIF was tentatively assigned. When the urine sample obtained from a rat treated with a 1 : 1 mixture of (±)-IF and (±)-[1',1',2',2'-$^2H_4$]IF was analyzed, an additional set of ions at m/z 391/393 at the same retention time was seen (Fig. 2). The mass difference between the corresponding ions in these two sets of ions was 4 u, indicating that these metabolites were derived from the labeled and non-labeled parent drugs. Similar results were found from urine samples obtained from rats treated with pseudoracemates (+)-[2',2',6,6-$^2H_4$]IF–(−)-IF or (−)-[2',2',6,6-$^2H_4$]IF–(+)-IF. Definitive identification of alcoIF as a metabolite of IF in the rat was accomplished from the analysis of the authentic compound. When the synthetic alcoIF was derivatized under identical conditions and the derivative analyzed by GC/MS, it gave an ion set at m/z 387/389 representing MH^+ and the chlorine isotope peak of the assigned trimethylsilyldechlorinated alcoIF, respectively, approximately in the expected ratio and at the same retention time (data not shown).

4-OHIF

4-OHIF has been postulated as the primary metabolite of IF;[5] however, direct demonstration of its existence has not appeared in the literature. Because its presence in urine was anticipated, authentic 4-OHIF was first synthesized as described previously[11] to facilitate its identification. Then this synthetic compound was characterized using a methodology similar to that of Fenselau *et al.*[22] for the identification of 4-hydroxycyclophosphamide (4-OHCP) as a metabolite of CP. When the authentic 4-OHIF was treated with KCN followed by extraction and derivatization with MSTFA, it gave a single component with a retention time of 16.0

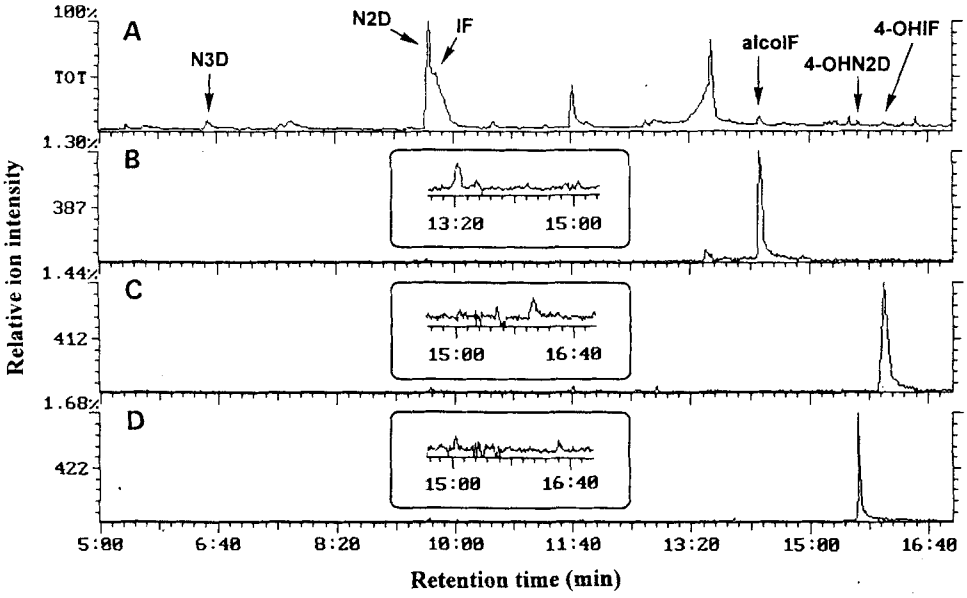


Figure 1. Total and selected-ion GC/CIMS chromatograms of the derivatized CH_2Cl_2 extract from the urine of a rat receiving racemic ifosfamide. (A) Total ion; (B) selected-ion m/z 387 for alcoifosfamide; (C) selected-ion m/z 412 for 4-hydroxyifosfamide; (D) selected-ion m/z 422 for 4-hydroxy-N^2-dechloroethylifosfamide or 4-hydroxy-N^3-dechloroethylifosfamide.

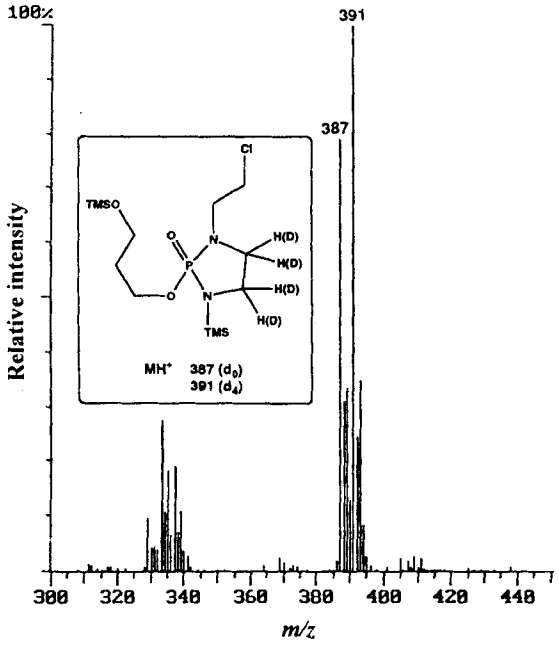


Figure 2. Ion cluster mass spectrum of derivatives of alcoifosfamide detected in the urine of a rat receiving a 1 : 1 mixture of (±)-ifosfamide and (±)-[1′,1′,2′,2′-$^2H_4$]ifosfamide.

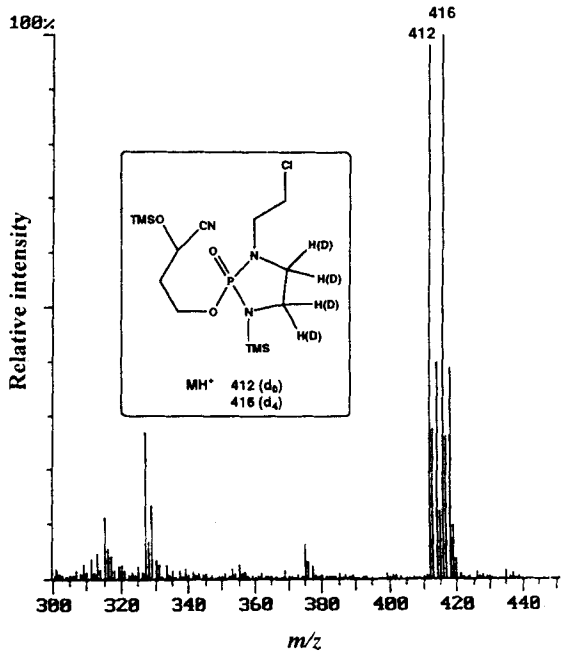


Figure 3. Ion cluster mass spectrum of the silylated cyanohydrin of 4-hydroxyifosfamide from the urine extract of a rat given a 1 : 1 mixture of (±)-ifosfafmide and (±)-[1′,1′,2′,2′-$^2H_4$]ifosfamide.

min with ions at *m/z* 412/414 on GC/MS analysis. The mass spectral data were consistent with the formation of a cyclic trimethylsilylated derivative similar to that of 4-OHCP.[22,23] Once authentic 4-OHIF had been characterized by GC/MS, its possible presence in the urine sample from a rat treated with (±)-IF was examined using the analytical approach similar to that for the authentic compound. After KCN treatment, extraction and derivatization, a set of ions at *m/z* 412/414 was detected at the same retention time as the authentic compound under identical conditions [Fig. 1(C)]. No ion at *m/z* 412 was detected at that retention time in blank rat urine [Fig. 1(C), inset]. To ascertain that the observed component was indeed derived from KCN trapping, the experiment was repeated with K^{13}CN. The resultant product gave a component at 16.0 min, but with a mass unit shifted from 412/414 to 413/415 as expected. Ion cluster analysis was also conducted using samples from a rat treated with a 1 : 1 mixture of (±)-IF and (±)-[1′,1′,2′,2′-$^2H_4$]IF. The ammonia CI mass spectrum for the derivatized extract from the urine is shown in Fig. 3. A set of ion clusters were seen at *m/z* 412/414 and *m/z* 416/418, consistent with 4-OHIF generated from these precursors. Similar results were obtained in other samples from rats treated with a 1 : 1 mixture of (+)-[2′,2′,6,6-$^2H_4$]IF and (−)-IF and a 1 : 1 mixture of (−)-[2′,2′,6,6-$^2H_4$]IF and (+)-IF. Thus, 4-OHIF as a metabolite of IF was unequivocally established.

4-OHN2D and 4-OHN3D

Since N2D and N3D have been previously found to be two of the major metabolites of IF, sequential 4-hydroxylation of these metabolites might be possible. Therefore, urine was screened for the possible presence of 4-OHN2D and 4-OHN3D, using an experimental approach similar to that for the identification of 4-OHIF. When a sample obtained from a rat treated with (±)-IF was processed through KCN treatment followed by extraction, silylation and GC/MS analysis, a component was eluted at a retention time of 14.0 min with an ion at *m/z* 422 [Fig. 1(D)], which was absent in blank rat urine [Fig. 1(D), inset]. The mass spectrum showed no chlorine present in the molecule. The structure of cyclic trimethylsilyldechlorinated derivative of N2D and/or N3D was proposed. Replacement of KCN by K^{13}CN resulted in a single mass unit shift from *m/z* 422 to 423 in the component of interest. These data indicated that this component contained an aldehydic group similar to that of 4-OHIF. When a 1 : 1 mixture of (+)-[2′,2′,6,6-$^2H_4$]IF and (−)-IF was given to a rat, the urine was subjected to treatment with KCN as before. As expected, the mass spectrum of the silylated extract gave ion doublets at *m/z* 422 and 424 at the same retention time as before, indicating that this component was a metabolite derived from the parent drug (Fig. 4). A similar result was obtained in the urine sample from a rat treated with a 1 : 1 mixture of (−)-[2′,2′,6,6-$^2H_4$]IF and (+)-IF (data not shown).

The final identity of this metabolite was confirmed by GC/MS analysis of the synthetic 4-OHN3D. The authentic sample was treated with KCN followed by silylation as before. GC/MS analysis revealed that the trimethylsilylated dehydrochlorinated cyanohydrin adduct of 4-OHN3D eluted at a retention time identical with and with a mass spectrum virtually identical with those observed for the component in the urinary extracts, thus confirming the structure as 4-OHN3D (data not shown). However, both 4-OHN2D and 4-OHN3D would lead to the same derivative (stereochemistry not implied) following this same treatment (Scheme 2, R = H). It was therefore not possible to distinguish between the two structural isomers on the

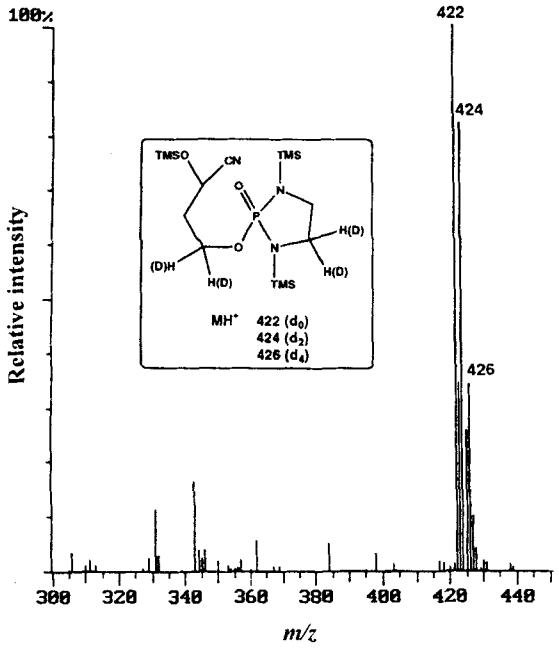


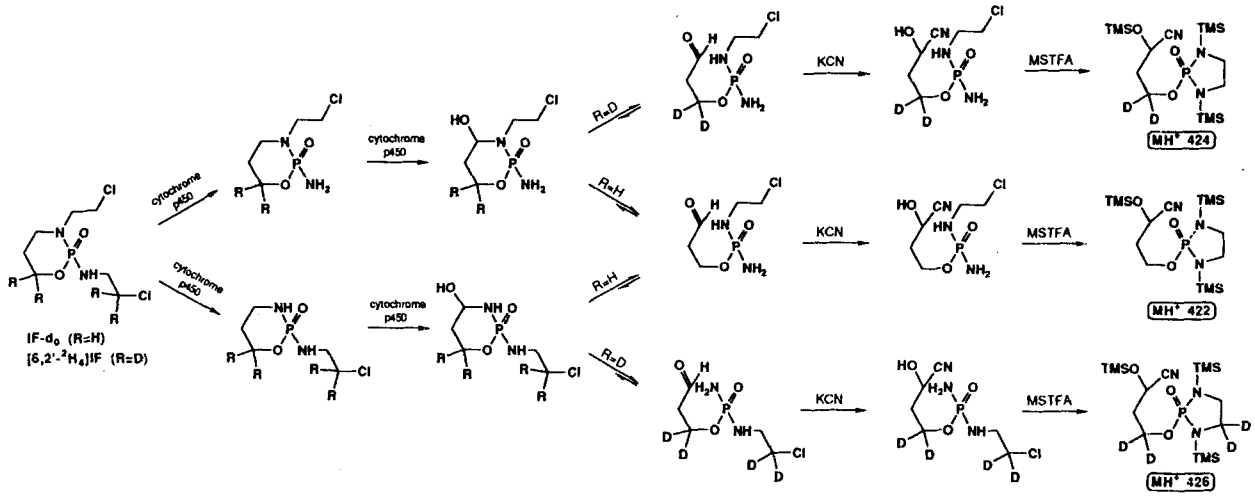
Figure 4. Ion cluster mass spectrum of the silylated cyanohydrin of 4-hydroxy-N^2-dechloroethylifosfamide or 4-hydroxy-N^3-dechloroethylifosfamide from the urine extract of a rat given a 1 : 1 mixture of (+)-IF and (−)-[2′,2′,6,6-$^2H_4$]ifosfamide.

basis of the authentic sample. Hence the remaining task was to determine the exact location of the chloroethyl side-chain.

Differentiation between the two structures could be accomplished by specific deuterium labeling as shown in Scheme 2 (R = D). Following administration of [2′,2′,6,6-$^2H_4$]IF, the metabolite N2D and subsequent 4-OHN2D would retain only two deuterium atoms. The silylated cyanohydrin adduct for the latter would generate the expected cyclic structure with only two deuterium atoms (*m/z* 424). On the other hand, N^3-dealkylation would give rise to N3D and subsequent 4-OHN3D with retention of all four deuterium atoms. The silylated cyanohydrin adduct under the GC/MS conditions would generate the expected cyclic structure with retention of four deuterium atoms (*m/z* 426). After careful examination of the ion cluster in the mass spectrum obtained from the urine of a rat treated with a 1 : 1 mixture of IF and [2′,2′,6,6-$^2H_4$]IF, an ion at *m/z* 426 was discerned. However, an ion at *m/z* 424 was also found but with higher abundance (Fig. 4). Therefore, it was concluded that both primary metabolites N2D and N3D were further hydroxylated by cytochrome P450 mixed-function oxygenases to form the secondary metabolites 4-OHN2D and 4-OHN3D, respectively, with the preponderance of the former. This contention was further investigated by giving a rat a 1 : 1 mixture of (±)-IF and (±)-[1′,1′,2′,2′-$^2H_4$]IF. GC/MS analysis of the urinary extract revealed the presence of a trace amount of the tetradeuterated analog (representing 4-OHN3D, data not shown). These data confirmed that 4-OHN2D, in comparison with 4-OHN3D, was the major secondary metabolite of IF.

NDIPM

The identification of 4-OHN2D and 4-OHN3D prompted the search for their decomposition product/metabolite, NDIPM. A urine sample obtained from a rat treated with (±)-IF was extracted by C_{18} reversed-phase resin and the residue was derivatized with silylating agents (BSTFA-TMSI, 5 : 1). The derivatized sample was subjected to GC/MS analysis. A component with *m/z* 339 was eluted at a retention time of 6.2 min [Fig. 5(B)], which was absent from the blank rat urine sample following the same treatment [Fig. 5(B), inset]. The mass unit at *m/z 339* is consistent with the protonated molecular ion of trimethylsilylated dechlorinated NDIPM. Further confirmation was obtained from the analysis of a urine sample obtained from a rat injected with a 1 : 1 mixture of (±)-IF and (±)-[1′,1′,2′,2′-$^2H_4$]IF. GC/MS analysis showed a major component with an intensive ion at *m/z* 339 and a weak ion at *m/z* 343 (Fig. 6). The ion at *m/z* 339 represented the NDIPM derived from the metabolite N2D, and the ion at *m/z* 343 derived from N3D-d_4. The relative abundance of these two peaks was consistent with the finding that 4-



Scheme 2. Differentiation of metabolic pathways to 4-hydroxy-N^2-dechloroethylifosfamide from those to 4-hydroxy-N^3-dechloroethylifosfamide, two secondary metabolites of IF, *via* specific deuterium labeling and GC/CIMS.

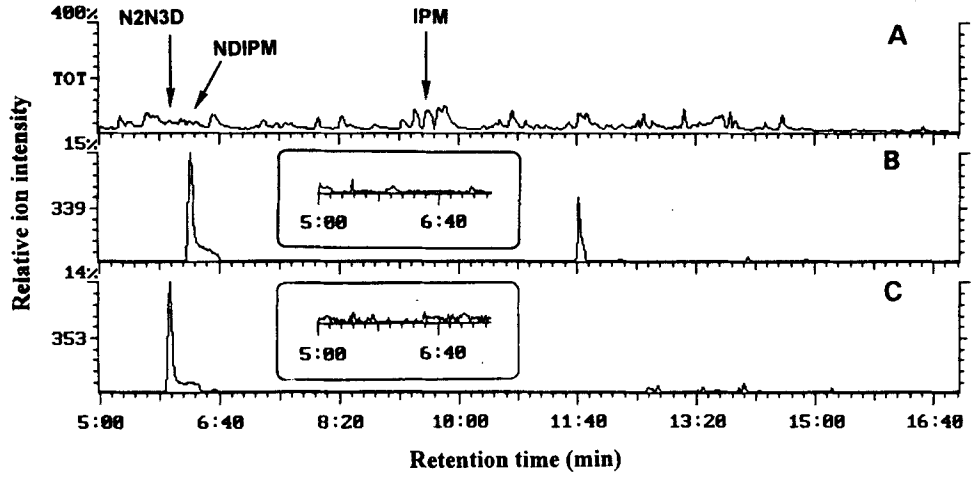


Figure 5. Total and selected-ion GC/CIMS traces of the derivatized solid-phase extract from the urine of a rat receiving racemic ifosfamide. (A) Total ion; (B) selected-ion *m/z* 339 for *N*-dechloroethyliphosphoramide mustard; (C) selected-ion *m/z* 339 for N^2, N^3-didechloroethylifosfamide.

OHN2D, not 4-OHN3D, was the major secondary hydroxylated metabolite. Samples from rats treated with a 1:1 mixture of (+)-[2′,2′,6,6-$^2H_4$]IF and (−)-IF or (−)-[2′,2′,6,6-$^2H_4$]IF and (+)-IF were processed and analyzed in the same manner. An ion at *m/z* 339 was observed as the major peak in this case, representing NDIPM derived from N2D and from N2D-d_2. A small peak at *m/z* 341 represented the existence of deuterated NDIPM (NDIPM-d_2), which was derived from N3D-d_4 (data not shown). The final identification was furnished by the synthetic authentic NDIPM. Under similar treatment, the authentic sample produced a major component at the identical retention time with a nearly identical mass spectrum.

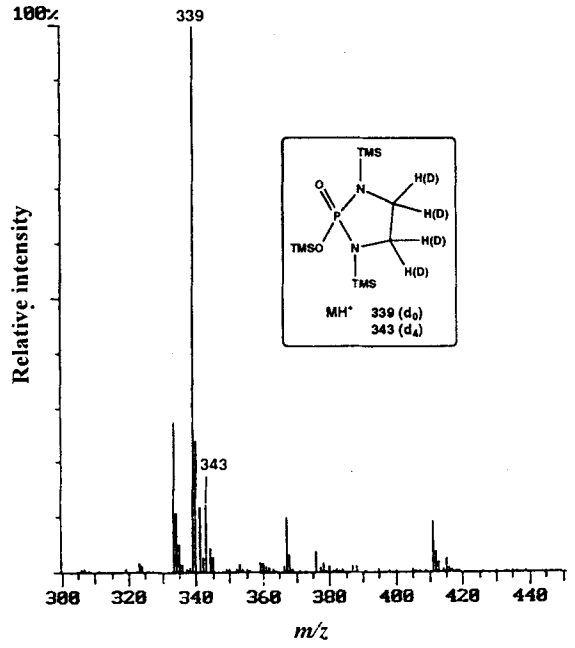


Figure 6. Mass spectrum of the silylated *N*-dechloroethyliphosphoramide mustard from the urine extract of a rat given a 1:1 mixture of (±)-IF and (±)-[1′,1′,2′,2′-$^2H_4$]ifosfamide.

N2N3D

The observation of the secondary hydroxylation pathway also suggested that sequential dechloroethylation might exist. Therefore, the existence of N2N3D was examined in the urine samples from all rats. If such a metabolite existed, the silylated metabolite would exhibit an ion at *m/z* 353 under the present GC/MS conditions. While there was no such ion found in the blank urine [Fig. 5(C), inset], a component with an ion at *m/z* 353 was indeed observed in the sample urine from rats treated either with (±)-IF alone [Fig. 5(C)] or with a 1:1 mixture of (±)-IF and (±)-[1′,1′,2′,2′-$^2H_4$]IF (data not shown). No ion cluster was expected to be found in the latter case because of the total loss of deuterium atoms. Since the four deuterium atoms in the molecule of (±)-[1′,1′,2′,2′-$^2H_4$]IF were all in the N-2 side-chain, they were totally lost during the transformation to N2N3D. However, with [2′,2′,6,6-$^2H_4$]IF, since two of the four deuterium atoms were on the oxazaphosphorine ring, they should be retained in the molecule of N2N3D. Indeed, an ion doublet at *m/z* 353/355 was found in the mass spectrum of a derivatized urine extract from rat treated with 1:1 mixture of (+)-[2′,2′,6,6-$^2H_4$]IF and (−)-IF or (−)-[2′,2′,6,6-$^2H_4$]IF and (+)-IF (Fig. 7). The final confirmation was achieved by the chemical synthesis of N2N3D. The tri(trimethylsilyl) derivative of N2N3D was eluted at a retention time identical with that of the metabolite, with an almost identical mass spectrum.

DISCUSSION

The versatility of deuterium labeling and the ion cluster technique in metabolite identification was demonstrated in this study. Labile, difficult-to-isolate metabolites and those present in very small amounts could be readily identified by applying a properly labeled drug after suitable chemical modification. In addition to the unchanged IF and its known metabolites (IPM, N2D

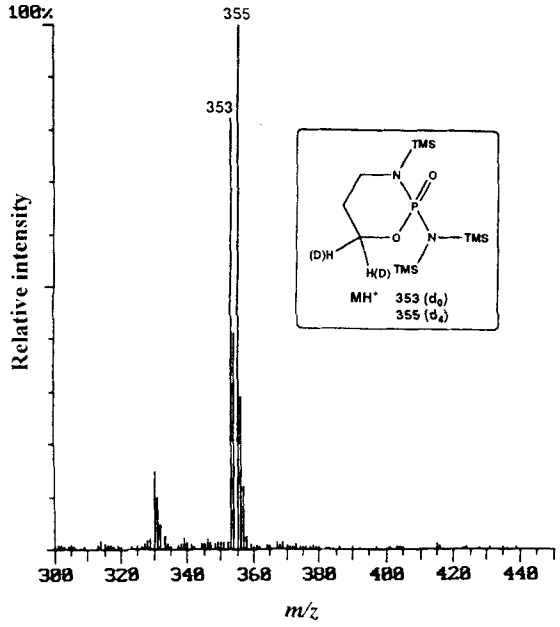


Figure 7. *Ion cluster* mass spectrum of the silylated N^2,N^3-didechloroethylifosfamide from the urine extract of a rat given a 1:1 mixture of (+)-IF and (−)-[2′,2′,6,6-$^2H_4$]ifosfamide.

and N3D), four new metabolites (4-OHN2D, 4-OHN3D, NDIPM and N2N3D) in the urine of rats injected with IF were identified. AlcoIF has been detected in the blood of mice and in the urine of dogs.[9] A recent report described the detection of alcoIF in human urine.[24] However, this is the first report of the presence of alcoIF in rat urine.

The activation of IF to 4-OHIF has been postulated to be parallel to the pathway of CP, since the metabolic successors (*e.g.* IPM, carboxyIF and 4-ketoIF) of 4-OHIF and aldoIF have already been identified as metabolites of IF. However, the definite existence of such metabolites has yet to be proven. Manz *et al.*,[25] using ion-pair extraction and fast atom bombardment MS, identified and quantitated the conjugates of activated CP and IF with mesna (sodium mercaptoethyl sulfonate) in rat and human urine. They suggested that these conjugates derived from the respective 4-hydroxy metabolites (*e.g.* 4-OHCP and 4-OHIF) by displacement of the hydroxy group with mesna. In a separate study, Conners *et al.*[26] identified 4-ethyloxyifosfamide as the trapped 4-OHIF in an *in vivo* rat liver microsomal metabolism experiment using MS. However, all these conjugates could also have been derived from addition of mesna or ethanol across the double bond between N-3 and C-4 of a possible imino intermediate.[22] The identification of the cyanohydrin adduct of aldoIF from the urine of rats treated with IF reported here provided more consistent and direct proof for the identification of 4-OHIF and aldoIF as metabolites of IF. Nevertheless, the position of the postulated equilibrium between 4-OHIF and aldoIF could not be ascertained. In an experiment investigating the stereoselective metabolism of IF in mice, Blaschke and Widey[27] found that 4-ketoIF was formed almost entirely from (−)-IF, thus refuting the previously suggested rapid equilibrium between 4-OHIF and aldoIF. While 4-OHIF is a chiral molecule, ring opening to aldoIF results in the loss of chirality because of the existence of two identical 2-chloroethylamino groups attached to the phosphorus atom. Thus, a rapid tautomeric equilibrium between 4-OHIF and aldoIF would result in racemization, which would made the subsequent stereoselective oxidation to 4-ketoIF impossible. The observed highly stereoselective oxidation to 4-ketoIF would indicate that the subsequent oxidation occurred at a rate faster than the ring closure from the aldo tautomer.

A fluorimetric method[28,29] has been widely used for the quantitation of 4-OHIF and this method was based on the reaction of the released acrolein from 4-OHIF with *m*-aminophenol to form fluoresent 7-hydroxyquinoline. Obviously, this method is proven here to be non-specific since acrolein was also formed from other 4-hydroxy metabolites such as 4-OHN2D and 4-OHN3D.

Under GC/MS conditions, many of the derivatized synthetic metabolites undergo thermal cyclization (intramolecular alkylation) with elimination of HCl. Obviously, under the same conditions, urinary metabolites underwent similar cyclizations. Hence, structures of the intact metabolites were not obtained and these intact structures may be otainable through the direct insertion probe technique. However, we do not think that the cyclization is of metabolic origin since cyclized metabolites of CP or IF have not been demonstrated by MS or other techniques, although we have not completely ruled out the *in vivo* cylization. In our previous work with PM, CP and IF using derivatization and GC/MS, we have detected low levels of uncyclized TMS derivatives, thus supporting the likelihood of cycliztion of this type of derivatives under thermal condition (Chan and Watson, unpublished data).

Gilard *et al.*[24] used ^{31}P NMR to study the urinary metabolite excretion of IF in patients receiving IF therapy. 4-OHIF and IPM were not detected because of chemical instability of the metabolites and intrinsic low sensitivity of the method; however, two degradation products of N2N3D but not N2N3D itself were detected. These two species could be artifacts formed spontaneously during urine collection or sample processing and analysis, since 15–24 h were generally required for the recording of signals.

The contribution of secondary metabolism of these metabolites to the antitumor activity of IF is not expected to be significant. Chemically, NDIPM is a monofuctional alkylating agent, which, in general, is unlikely to possess significant anticancer activity. In our preliminary cytotoxicity study, it showed weak activity against L1210 cells (Wang and Chan, unpublished data).

Although the etiology of the neurotoxicity observed in patients receiving IF has not been well established, evidence from electroencephalograms supports a metabolic or toxic etiology.[30,31] Concomitant to the dealkylation pathways of IF, the presumed toxicophore CAA could also be derived from further metabolism of the two most abundant metabolites, N2D and N3D. CAA generated from hepatic metabolism may be transported to the CNS site; alternatively, CAA could be generated on-site in the CNS, because it has been reported that the CNS also possesses a certain cytochrome P450

activity.[32] Further investigation to elucidate the mechanism of toxicity is necessary.

In conclusion, our results showed that IF metabolism (Scheme 1) is more complex than that of its congener, CP. In addition to the common activation pathway (4-hydroxylation), IF undergoes extensive side-chain oxidation, leading to the formation of dealkylation metabolites (ND and N3D) along with CAA. Further, these dealkylated metabolites can be sequentially hydroxylated and/or dealkylated. The enzymes and isoenzymes involved in these reactions have not yet been fully characterized.[33,34]

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