CHARACTERIZATION AND REACTIVITY OF AN ULTIMATE CARCINOGEN: THE TAMOXIFEN CARBOCATION

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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For Matthew,

And

Mom & Dad
Characterization and Reactivity of an Ultimate Carcinogen: The Tamoxifen Carbocation

Doctor of Philosophy (2000)

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Abstract

Women treated for breast cancer with tamoxifen (TAM) are at increased risk of developing endometrial cancer. This carcinogenic effect has been attributed to estrogenic stimulation and/or to a genotoxic effect. To examine genotoxicity via the proposed carbocation pathway, a detailed study concerning the solvolysis of α-sulfate/acetoxy-tamoxifen in solvent alone or with added nucleophile was undertaken.

Ground state solvolysis experiments established that the TAM-esters solvolyze via an $S_N 1$ mechanism. A solvent dependency for α-sulfate-TAM hydrolysis was observed by conducting experiments in varying proportions of water: acetonitrile. The plot of the logarithm of the resultant rate constants as a function of the $Y_{Cl}$ parameter is linear with a slope of 0.72. Rates of solvolysis for α-sulfate/acetoxy-TAM were independent of added nucleophile; this is classic evidence for an $S_N 1$ reaction in which the α-sulfate/acetoxy ionizes to a carbocation in the rate-determining step. The formation of four products after hydrolysis also points to a TAM$^+$ intermediate which can isomerize...
between *cis* and *trans* forms, and which can be trapped by water or some other nucleophile, or can undergo intramolecular cyclization followed by deprotonation.

Laser-flash photolysis (LFP) of the TAM-ester produces a transient that decays in an exponential fashion. The carbocation is relatively long-lived, with lifetimes of 40 μs under acidic conditions and 160 μs under basic conditions. HPLC product studies determined the $k_{\text{azide}}: k_w$ selectivity ratio to be $4.4 \times 10^4 \text{ M}^{-1}$, while direct measurement by LFP determined a ratio of $4.45 \times 10^4 \text{ M}^{-1}$. The close agreement of these two distinctly measured ratios provides conclusive evidence that the transient generated by LFP is the exact same carbocation as the one formed in the ground state ionization.

Direct measurement of bimolecular rate constants by LFP shows that TAM+ is quenched by endogenous nucleophiles to varying degrees. At physiological pH, DNA is an effective trapping agent with a $k_{\text{DNA}}: k_w$ ratio of $3.85 \times 10^3 \text{ M}^{-1}$, followed by glutathione; $k_{\text{GSH}}: k_w = 3.3 \times 10^3 \text{ M}^{-1}$, and finally deoxyguanosine (dG); $k_{\text{dG}}: k_w = 70 \text{ M}^{-1}$. The exact nature of the reaction between the TAM+ and DNA is currently under investigation.
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May all the effort and wholesome energy of this project and my studies be a cause for the awakening of all beings.
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Chapter One:

Introduction
Chapter 1: Introduction

1.1 Tamoxifen, the Drug

1.1.1 Introduction

Tamoxifen (TAM, 1) is the most widely used drug in the treatment of breast cancer in the world, with more than 25 years of clinical use.\textsuperscript{1,2} This non-steroidal anti-estrogen of the triphenylethylene type functions by competitively binding to estrogen receptors. Once bound, the antiestrogen receptor complex is either too unstable to translocate into the nucleus of target cells or, if translocated, does not bind properly to the acceptor site of the chromatin to produce an estrogenic response.\textsuperscript{3} The compound administered to patients is the citrate salt of the \textit{trans}-isomer.\textsuperscript{*} The 4-HO-TAM metabolite of this isomer has a higher affinity for estrogen receptors than the \textit{cis} isomer. These receptors are nuclear transcription factors present in normal breast and other tissues as well as in 60 to 70 percent of breast cancers.\textsuperscript{3}

* Cis-trans nomenclature is used to denote the location of the phenyls relative to each other.
In addition to its use as an effective agent in the adjuvant treatment of breast cancer (treatment administered after surgery for breast cancer in an attempt to eradicate the micrometastases), it also reduces the occurrence of contralateral breast cancer in women who have already developed a first tumour. This has led to the current plans to test the efficacy of TAM in preventing primary breast cancer in healthy women with a family history of the disease. One such study, initiated by the National Surgical Adjuvant Breast and Bowel Project (called the Breast Cancer Prevention Trial) recently reported a 45-50% reduction of both invasive and non-invasive breast cancer by TAM. However, a second major chemopreventative trial, headed by the Royal Marsden Hospital, found no significant reduction in breast cancer. While these discrepancies in the drug’s chemopreventative potential clearly need to be addressed, the efficacy of TAM in the adjuvant therapy of breast cancer patients is well established. As such it remains the drug of choice for the treatment of this disease.

1.1.2 Cancer Risk Associated with the Clinical Use of Tamoxifen

Unfortunately, the last decade has seen an emerging deleterious side effect associated with the long-term treatment of breast cancer by TAM. The drug has been found to increase the risk of endometrial cancer in breast cancer patients, as well as in healthy women enrolled in various chemopreventative trials. The increased incidence of endometrial cancers was limited to women 50 years of age and older in the National Surgical Adjuvant Breast and Bowel Project TAM chemopreventative trial. The Early Breast Cancer Trialists’ Collaborative Group found that among the 37,000 women participants in 55 separate trials the incidence of endometrial cancer had approximately
doubled in the trials of 1-2 years and quadrupled in the 5 year trials. Here, the increased risk of developing endometrial cancer was found to be unrelated to age, menopausal status and daily dose, which was generally 20 mg. In an aggregate of three such trials which were conducted in Europe and North America (Stokholm B, Scottish, and NSABP B14), allocation to about five years of tamoxifen was associated with 33 more cases of endometrial cancer (42 during 24,000 woman-years in the TAM group versus 9 during 21,200 woman-years in the control group). A 2-sided p statistical analysis was conducted which found that $2p < 0.00001$. Since $2p > 0.1$ is considered to be insignificant, these results indicate a significant increase in endometrial cancer among women taking the drug. These findings taken together have called into question the wisdom of using TAM prophylactically and underscore the need for a thorough investigation into the mechanism(s) by which the drug exerts this deleterious effect.

1.1.3 Animal Studies with Tamoxifen

In order to address the observed toxicity of TAM in women, several research groups embarked on animal model studies. Williams et al found TAM-induced dose- and time-dependent neoplastic changes in rat liver. Female Sprague-Dawley rats that were given 45.2 mg/kg/day elicited hepatocellular neoplasia sometime between three and six months after administration. At 11.3 mg/kg/day the neoplastic process was evident at 12 months, while at 2.8 mg/kg/day no hepatoproliferative changes were found in the one-year study. Another such study conducted over a two year period found similar results with neoplastic changes occurring at lower doses because of the longer time period. In contrast to rats, mice do not develop hepatic tumours following long-term treatment.
Only minor changes such as fatty change and enlargement of hepatocytes was observed in the Alderley Park mouse at doses of up to 50 mg/kg/day. The reason for this discrepancy is not understood, however, the ongoing elaboration of the mechanism(s) of carcinogenesis will likely shed light into this species-dependent phenomena.

Further study into rat liver toxicity led Hard and coworkers to explore the difference between TAM and toremefine (2), a closely related analogue with a substituted chlorine atom on the β-position of the ethyl side chain.

In the TAM-treated animals, the authors observed the same morphological changes as with the above studies. They also discovered an accumulation of modified nucleosides in the isolated livers of female Sprague-Dawley rats after one year of exposure to the drug. \(^{32}\)P-Postlabeling revealed that in fact TAM produces covalent DNA adducts in the target organs of carcinogenicity. Interestingly, these results were in striking contrast to the toremifene-treated rats, which lacked all signs of
hepatocarcinogenicity. This finding prompted the authors to postulate an alternative to the prevailing hormonal explanation for TAM's observed endometrial carcinogenicity;

"Certainly, the carcinogenic action of TAM cannot be ascribed solely to its hormonal activity because toremifene exerts qualitatively and quantitatively similar hormone-related effects, as it suppresses spontaneous tumor formation in endocrine-regulated organs, specifically mammary and pituitary glands, a likely result of antihormonal effects… Considering the minor difference in molecular structure between the two drugs, the strong hepatocarcinogenicity of TAM contrasting with a lack of hepatocarcinogenicity with toremifene, is striking. The basis for this difference in biological effect is not known, but we suggest that the carcinogenicity of TAM may be due to activation at the ethylene bond region of the molecule, as has been proposed for diethylstilbestrol… Such a hypothesis raises the possibility that toremifene has a lower capacity for generation of genotoxic metabolites because of steric hindrance by the presence of a bulky chlorine atom adjacent to the ethylene bond."

The suggestion that reactive metabolites of TAM may be responsible for the observed genotoxic effect, as established by the 32P-postlabeling assay, was further supported by the work of Mani and Kupfer.13 They found that in human and rat liver microsomal systems in vitro, 14C-TAM was metabolized by NADPH-dependent cytochrome P450 and/or flavin-containing monooxygenase to some intermediate(s) which became covalently bound to microsomal proteins. A considerable number of xenobiotic compounds are known to undergo metabolic activation, resulting in covalent binding to proteins or DNA,14 and it is this covalent binding which results in tissue toxicity and/or carcinogenicity.
1.2 Genotoxicity of Tamoxifen

These initial studies of the early 1990s spawned the current intensive investigation into the mechanism(s) of metabolic activation of TAM and of the chemistry and stereochemistry of DNA adduct formation. At this point DNA damage had only been detected in rat liver and microsomal preparations, however, it certainly raised the question of whether in humans TAM may generate DNA damage within the uterus and that these covalent DNA modifications initiate the development of uterine cancer.

1.2.1 Triphenylethylene Derivatives of Tamoxifen

There are a number of triphenylethylene derivatives of TAM with small structural changes that may also be used in the treatment of breast cancer. The observation that the β-chlorinated analogue of TAM, toremifene, produced neither hepatocarcinogenicity nor DNA adducts in rat was later corroborated by several other groups. Moreover, Davies et al determined that while TAM induced a significant increase in mutation frequency in the liver of lambda/lacI transgenic rats, there was no such detectable increase associated with toremifene.

Interestingly, structural modifications that are further away from the ethylene side chain also show a significant reduction in genotoxicity. Droloxifene (3), which has a 3-hydroxy substituent, has antiestrogenic activity in vitro which is equivalent to or slightly greater than TAM. However, it does not exhibit any detectable hepatocarcinogenesis in rats. Idoxifene (4), whose antiestrogenic potency is comparable to TAM and toremifene, has also been found to be innocuous in rodent studies. This TAM
derivative with a terminal pyrrolidine in place of the dimethylamine and more importantly, a 4-iodo substituent, was found to be much less genotoxic than TAM in human and rat liver cells, even when the α-hydroxy and α-acetoxy derivatives were used in the incubations (a point that will be elaborated on in sections 1.2.3 and 1.2.4).

![Chemical structures](image)

The structural/electronic modifications imposed by the substituents on these three triphenylethylene analogues likely alter the mechanism of biotransformation to a reactive intermediate(s) seen with TAM. However, the initial explanation put forward by Hard et al\textsuperscript{12} that the chlorine substituent of toremifene interferes with metabolic activation via steric hindrance appears to be simplistic in view of the observations outlined for droloxifene and idoxifene.

Currently, three distinct biotransformation pathways resulting in electrophilic species have been proposed to account for DNA adduct formation.
1.2.2 Metabolism of Tamoxifen to an Electrophilic Redox Active $O$-Quinone

As shown in scheme 1.1, the biotransformation pathway begins with the aromatic oxidation of TAM by cytochrome P450 to generate the known metabolite, 4-hydroxy-TAM. Subsequently, a second hydroxylation at the adjacent 3- position can occur, producing the catechol 3,4-dihydroxyTAM. The enzyme responsible for this second hydroxylation would likely be P450. However, in melanocytes or in the cornea, tyrosinase may also be involved. Once formed, the catechol may be readily oxidized to $o$-quinone by a variety of oxidizing enzymes, metal ions, or even molecular oxygen.

Supporting evidence for this pathway is provided by Dehal and Kupfer, who showed a 17-23% reduction in the 4-hydroxy-TAM-mediated alkylation of microsomal proteins when the incubations contained S-adenosyl-L-methionine and endogenous catechol O-methyltransferase. Inhibition of the formation of $o$-quinone from the catechol

**The pKₐ of the side chain ammonium is 8.85 and is therefore protonated at physiological pH. However, the drug is normally depicted in the neutral form.**
effectively reduced protein binding by TAM, suggesting a partial role of catechol in the process. Furthermore, the ocular toxicity that has been reported by some patients receiving high doses of TAM may be caused by tyrosinase-mediated \( \alpha \)-quinone formation in the cornea.\(^{25}\)

There are numerous ways in which the \( \alpha \)-quinone form of TAM could contribute to carcinogenicity. Oxidation of cellular macromolecules occurs via the redox cycling between \( \alpha \)-quinones and their semiquinone radical counterparts mediated by cytochrome P450/P450 reductase.\(^{26}\) Alternatively, the \( \alpha \)-quinone may be reduced directly back to the catechol by NAD(P)H:(Quinone Acceptor) Oxidoreductase (DT-diaphorase).\(^{27,28}\)

Interestingly, elevated levels of DT-diaphorase have been detected in breast tumors.\(^{29}\) Regardless of the enzymes responsible and the extent of oxidation-reduction (i.e. either 1 or 2 electrons), superoxide radicals can be generated quite readily which would set off a cascade of reactions, resulting in the oxidative damage of cellular proteins and more importantly, DNA.\(^{30}\) Reactive oxygen species have been implicated in the
carcinogenicity of TAM through this oxidative damage with the aid of EPR spectroscopy. Moreover, o-quinones of polycyclic aromatic hydrocarbons have been shown to form N7-guanine adducts that result in depurination. Scheme 1.3 illustrates how an amine might react with an o-quinone, modeled after the 1999 publication.

\[ \text{Scheme 1.3} \]

Similarly, the remaining two suggested bioactivation pathways involve the formation of an electrophilic species that binds directly to DNA. The resultant covalent adduct(s) purportedly causes gene mutation resulting in carcinogenesis, as discussed in section 1.2.1.

1.2.3 Metabolism of Tamoxifen to an Electrophilic Quinone Methide

Following the formation of 4-hydroxyTAM, a P450-directed 2-electron oxidation mechanism can form a quinone methide, an electrophile capable of yielding DNA adducts through a Michael-type addition reaction. Another possible route would involve hydroxylation at the α-position by P450 giving 4,α-dihydroxyTAM followed by
dehydration to the quinone methide.\textsuperscript{32} Formation of the 4,α-dihydroxyTAM may also occur by an initial α-hydroxylation followed by aromatic oxidation at the 4-position.

Several different pieces of evidence support the role of TAM quinone methide as the ultimate carcinogen. (1) The 4-hydroxyTAM precursor gave covalent binding to rat or human hepatic microsomal proteins that was 3-5 fold higher than that of TAM, evidence that the metabolite may be a proximate carcinogen.\textsuperscript{33} (2) In vivo hepatic DNA adducts, detected with the aid of $^{32}$P-postlabeling, isolated from mice treated with TAM co-chromatographed with adducts formed in incubations of DNA and 4-hydroxyTAM subjected to chemical or microsomal oxidation.\textsuperscript{34} (3) An 11-fold increase in adduct
formation is observed when the sulfotransferase inhibitor, pentachlorophenol, is co-administered with TAM. This may be the result of a greater availability of 4-hydroxyTAM which has not been depleted through sulfate conjugation. (4) The 3 TAM derivatives droloxifene, idoxifene and toremifene, as well as the D₅-ethyl TAM are all considerably less genotoxic than TAM. All four compounds, especially droloxifene and idoxifene, have substituents that would be expected to interfere with quinone methide formation. However, these observations of reduced genotoxicity could also apply to the α-hydroxylation pathway (see section 1.2.4), especially for toremifene and the D₅-ethyl derivative. Finally, (5) a 15-63-fold increase in the levels of DNA adduct formation is found in rat hepatocytes treated with α-hydroxyTAM as compared to TAM alone at comparable concentrations. Once again, this observation could just as easily apply to the α-hydroxylation pathway.

It is not at all surprising that the last two observations can be applied to either the quinone methide bioactivation pathway or the α-hydroxylation pathway (discussed in the following section). Quinone methides are in fact resonance stabilized carbocations due to the important contribution of the charged aromatic resonance form. Therefore, one would expect that the structural/electronic modifications affecting the stability of the quinone methide-TAM would similarly affect the carbocation-TAM generated from the α-hydroxylation pathway. The important difference between the 2 pathways involves the biotransformation steps leading up to the α-electrophilic species.

TAM-quinone methide is unusually stable due to the influence by the two aryl substituents and the π system of the additional vinyl group. This π-stabilization in addition to steric factors causes a significant reduction in the rate of nucleophilic
trapping. Bolton and coworkers\textsuperscript{41} found the half-life of the TAM-quinone methide under physiological temperature and pH to be 3 hours. Normally quinone methides react very rapidly with glutathione (GSH). However, it was found that the rate of trapping of the TAM quinone methide could be followed by a conventional UV/visible spectrophotometer, resulting in a half-life of 4 minutes in the presence of 50 mM GSH under physiological conditions.\textsuperscript{41} While a small amount of GSH adduct was found, the adduct(s) were found to be unstable and to decompose slowly over time (7 days). It appears that the quinone methide is regenerated, suggesting that the reaction with GSH is a reversible process. Davies and coworkers\textsuperscript{30} found that 4-hydroxytamoxifen causes a stoichiometric time dependent formation of GSSG in the presence of peroxidase. These data imply that DNA damage by quinone methide may in fact be due to oxidative stress within cells through oxidation of reducing equivalents (NAD(P)H, GSH, etc.) rather than alkylation of cellular macromolecules. The importance of 4-hydroxytamoxifen in the overall carcinogenesis pathway has been called into question\textsuperscript{41,42} as the subsequent and final metabolic pathway gains popularity.

1.2.4 Metabolism of Tamoxifen to an Electrophilic Carbocation

1.2.4.1 Metabolic Pathway

The final postulated bioactivation pathway involves the formation of a carbocation as the electrophilic species responsible for protein and/or DNA binding. This pathway begins with the α-oxidation by cytochrome P450 to yield α-hydroxyTAM, which has been identified as a metabolite in rat hepatocytes,\textsuperscript{43} human liver,\textsuperscript{22} and the plasma of patients treated with TAM.\textsuperscript{22}
Prior to generating the reactive carbocation, the α-hydroxyl group requires either protonation or conjugation before it can act as a leaving group. Mounting evidence is pointing towards O-sulfonation by a sulfotransferase enzyme like hydroxysteroid (alcohol) sulfotransferase (STA), although other conjugative leaving groups such as phosphate, glucuronide, or some other ester may be involved. α-Sulfate-TAM is highly reactive towards DNA, forming four diastereomers of dG-N\(^2\)-TAM in several different incubation studies. These diastereomers, which are believed to be epimeric pairs of the \textit{trans} and \textit{cis} forms of TAM, strongly implicate a carbocation intermediate. Scheme 1.6 shows the interconversion between \textit{trans} and \textit{cis} via the carbocation intermediate to account for the formation of stereoisomers.
Numerous studies have come out in the last five years supporting this biotransformation pathway. Once α-hydroxyTAM (and α-hydroxy-N-desmethylTAM) was confirmed to be a metabolite in rats and humans, intensive investigation sought to assess the relative importance on TAM-DNA adduct formation, as well as to determine the mechanism of this process. In one study by Phillips et al., treatment of rat cells with
α-hydroxyTAM resulted in 15-63 fold higher levels of adducts than with comparable concentrations of TAM. Further probing into the importance of the initial α-hydroxylation of TAM led to the substitution of the ethyl protons with deuteriums,\textsuperscript{36,37} the idea being that TAM deuterated at the α-position (as well as the β-position) would be less genotoxic than the non-deuterated compound owing to an isotope effect that would reduce the rate of oxidative metabolism at this position. [D\textsubscript{5}-ethyl]-TAM was in fact found to be significantly less genotoxic than TAM in rats and in human cells.

1.2.4.2 Supporting Evidence from the Triphenylethylene Derivatives

The observed reduction in genotoxicity by TAM analogues, especially for droloxifene\textsuperscript{17} and toremifene\textsuperscript{12,15-17} lends further support to this biotransformation pathway. For reasons that are not entirely clear, droloxifene (3-hydroxyTAM) has a completely different metabolic profile than TAM. It has been found to undergo very little phase I (oxidative) metabolism, being metabolized predominantly by the phase II (conjugative) pathway by direct glucuronidation of the 3-hydroxy group.\textsuperscript{48} Given that it does not form adducts with DNA,\textsuperscript{17} the implication is that α-oxidation in a necessary step in the genotoxicity process. Toremifene exhibits only a very low level of hepatic DNA adducts and is found to be non-carcinogenic in rats. It is not difficult to see how the β-chloro substituent would interfere with hydroxylation at the adjacent carbon. It may well inhibit P450-mediated hydroxylation at the α-position, possibly through steric hindrance, and it would certainly destabilize the carbocation through induction. While the evidence stated so far unequivocally supports the importance of α-hydroxylation, there is no way of distinguishing between the relative importance of subsequent 4-hydroxylation to form
the quinone methide and the α-sulfonation to generate the carbocation. The following evidence favours the latter process.

1.2.4.3 Importance of O-Sulfonation

O-Sulfonation is a Phase II conjugative metabolic reaction that functions to render xenobiotic compounds more water soluble, and hence, facilitates their excretion. In rare cases as with safrole, estragole, and 7,12-dimethylbenz[a]anthracene, the formation of an ester results in bioactivation to a reactive electrophile. While in principle any hydroxy group on TAM is subject to sulfonation, it does not necessarily result in its conversion to a reactive species. The major metabolite of TAM, 4-hydroxyTAM, may well be sulfonated. However, the sulfate in this case is not expected to leave as the resultant carbocation on the phenyl is highly unstable and is therefore unlikely to be formed. In fact, sulfonation here would be expected to inactivate the proximate carcinogen (section 1.2.4). On the other hand, sulfonation at the α-position on the ethyl side chain can be seen as a precursor to the generation of a stabilized, but nevertheless highly electrophilic, carbocation.

In one study using rat hepatocytes, a direct correlation was found between the level of DNA adduct formation of TAM and α-hydroxyTAM and the concentration of magnesium sulfate. Between 0 and 10 μM MgSO₄, the DNA adduct level increased 10-fold with both compounds. Furthermore, the levels of DNA adducts reduced to approximately one-fifth when the two compounds were incubated with dehydroisoandrosterone-3-sulfate (DHEAS; 0.1 mM, a sulfotransferase inhibitor). Benzo[a]pyrene, known to form DNA adducts following a biotransformation pathway
that does not involve sulfonation, was not significantly affected by sulfate concentration or by addition of DHEAS. Three other studies sought to examine the difference between the extent of DNA adduct formation and type of sulfotransferase used. The amount of TAM adducts formed by human hydroxy sulfotransferase SULT2A1 (hHST) was anywhere from 3-20 times less than that formed by an equivalent amount of rat hydroxysteroid (alcohol) sulfotransferase. This result suggests that the risk of genotoxicity in human liver is much lower than in rat liver, which correlates with clinical and animal studies. However, there is a strong possibility that a different sulfotransferase enzyme found in the human uterus is responsible for esterification of α-hydroxyTAM.

1.2.4.4 Characterization of the DNA-TAM Adducts

The final and perhaps most compelling piece of evidence comes from TAM-DNA adduct isolation and characterization studies. α-Sulfate-TAM and α-acetoxyTAM, a model for the sulfate, were reacted directly with DNA. P-Postlabeling identified the products of the in vitro incubations to be identical to the TAM-DNA adducts isolated from the liver of rats treated with drug. Following incubation, the DNA was digested and the products isolated as the nucleosides (note that dR refers to deoxyribose).
Characterization by ultraviolet, mass and proton magnetic resonance spectroscopy revealed three different adducts (2 of which are epimeric pairs) which account for >90% of the reaction products. Of these, the 2 major products were identified as the *trans* and *cis* forms of the TAM-deoxyguanosine adduct (5 and 6, respectively) in which the α-position of TAM is linked to the exocyclic amino group of guanine. Dasaradhi and
Shibutani managed to separate these products further via HPLC into pairs of epimers resulting from the α-carbon stereocenter. In another study, the minor product was found to be TAM-deoxyadenosine (7), where linkage was through the N6-amino group of adenine. In another study that isolated and characterized the adducts taken directly from rat liver, only 2 major adducts were identified. One was the trans-TAM-dG adduct as with previous studies. The other, and more significant, adduct was the N-desmethylTAM-dG (8) counterpart, presumably derived from the major metabolite N-desmethylTAM. Not surprisingly, this metabolite was not present in any of the previous studies. The previous incubations only involved α-esterTAM and DNA, and none of the metabolic enzymes found in vivo. Taken together, these observations (the increased genotoxicity of α-hydroxyTAM, the reduced genotoxicity of various TAM analogues, the importance of O-sulfonation, and the characterization of the major TAM-DNA adducts) point to a significant contribution of the carbocation pathway to the genotoxicity of TAM. However, the importance of this genotoxic mechanism to the development of uterine cancer has yet to be established.
1.3 Chemistry of the Carbocation

A carbocation is generally considered to be any species containing a trivalent carbon atom with only six electrons in its valence shell, which consequently carries a charge of +1. George Olah and coworkers found evidence for another type of intermediate in which there is a positive charge at a carbon atom, but in which the carbon atom bears a formal covalency of five rather than three.\(^5\) The simplest example is the methanoniurn ion CH\(_3^+\). Olah proposed that the name "carbonium ion" be henceforth reserved for pentacoordinated positive ions and that the trivalent positive carbon (R\(_3\)C\(^+\)) be called "carbenium ions". He then proposed that the term "carbocation" encompass both types. IUPAC has accepted these definitions.\(^5\) For R\(_3\)C\(^+\), the carbon bearing the positive charge is bonded to three other atoms, and as predicted by the valence-shell electron-pair repulsion (VSEPR) model, ideally the three bonds are coplanar and form angles of 120°. According to the molecular orbital model, the electron deficient carbon of a carbocation uses sp\(^2\) hybrid orbitals to form sigma bonds to the three attached groups. The unhybridized 2p orbital lies perpendicular to the sigma bond framework and contains no electrons. Carbocations are excellent Lewis acids and are readily trapped by nucleophiles to generate an sp\(^3\) carbon-centered product.\(^\text{58}\)

1.3.1 Direct Observation of the Carbocation

In the past, evidence for SN\(_1\)-derived carbocations was obtained indirectly, primarily through the study of reaction kinetics and trapping processes to measure selectivities, i.e., ratios of rate constants.\(^\text{58a}\) By using the competition kinetics method, a
cationic intermediate is allowed to partition between two nucleophiles to produce different trapped products.\textsuperscript{59} Absolute rate constants can then be deduced from such selectivities by assuming that one of the competing nucleophiles, typically azide ion N\textsubscript{3}⁻, reacts at the diffusion limit.\textsuperscript{60}

However, direct observation of the carbocation intermediate is expected to provide more useful information about the S\textsubscript{N}1 reaction mechanism. While certain types of compounds have long been known to produce observable concentrations of the positive ions relatively easily, the studies have been limited. For example, triarylmethyl carbocations have been easily generated and observed; the corresponding halides ionize readily in nonnucleophilic solvents such as sulfur trioxide, and the corresponding alcohols yield solutions of the ions in concentrated sulfuric acid. Application of the freezing point depression technique established that each mole of trityl alcohol yields 4 moles of ions in sulfuric acid,\textsuperscript{61} presumably by the following equation:

\[
\text{Ph}^-\text{COH} + 2\text{H}_2\text{SO}_4 \rightarrow \text{Ph}^+ + \text{H}_3\text{O}^+ + 2\text{HSO}_4^- \\
\text{Eq 1.1}
\]

This cryoscopic method is applicable only to a select number of compounds\textsuperscript{62} (e.g. triarylmethyl systems, some diarylmethyl and allylic ions, and aryl acylium ions with an ortho substituent). Moreover, side reactions frustrate most attempts to generate carbocations in sulfuric acid.
1.3.1.1 Superacid Generation of Carbocations

Development of the superacid (or stable ion) system has permitted the preparation of stable solutions of carbocations of many structural types at low temperature. The solvents normally used consist of the strong Lewis acid antimony pentfluoride with or without added protic acid. The ready availability of solutions of many types of carbocations have made possible spectroscopic observations of a greatly expanded variety of structures. Proton and $^{13}$C-nuclear magnetic resonance, as well as infrared and Raman spectroscopy are commonly employed. Such investigations have furnished detailed information about the structure of the cations, but studies of reactivities have been generally limited to internal rearrangements and fragmentations where they occur. The experimental conditions required for such studies are very different from those encountered in the usual \( S_N1 \) reaction and the strongly acidic character of the solvent precludes the addition of potential nucleophiles. Thus superacid studies are unable to address the fundamental questions relating to the reactivity of the carbocation intermediate towards a nucleophile, either in the form of solvent such as water or some externally added species.

1.3.1.2 Flash Photolysis Generation of Carbocations

The technique of flash photolysis (FP) allows for the direct observation of a short-lived species such as a carbocation. The experimental conditions often closely resemble those of the thermal reaction where the same species has been proposed as a reactive intermediate. In addition to establishing the very existence of the carbocation, the technique also provides direct information about the kinetics of its subsequent reaction.
In FP, a short pulse of light is used to produce the reactive species. Thus, any species that can be generated photochemically can in theory be studied by this method. As the intermediate is usually detected and studied by absorption spectroscopy, it must have a UV-visible spectrum with a fairly high extinction coefficient at the wavelength of maximal absorbance ($\lambda_{\text{max}}$). The transient must also survive as long as the pulse of light used for its generation, hence the natural evolution from conventional flash to nanosecond and then to picosecond laser flash photolysis (nsLFP and psLFP) to study shorter lived intermediates. Once these conditions are met, the lifetime of a cation in a solvent with which it reacts can be easily determined by taking the reciprocal of the first-order rate constant ($k_s$) for decay in that solvent. Likewise, the absolute rate constant for trapping by a nucleophile ($k_{\text{Nu-}}$) can also be measured.

1.3.1.3 Is the Transient a Carbocation?

With any flash photolysis experiment, it is of crucial importance to unambiguously assign the transient being observed, in this case to the carbocation. There are a number of criteria that can be employed in making such an assignment:

(1) **Spectrum.** A UV-visible spectrum can be easily generated using flash photolysis by measuring the absorbance of the transient across a spectrum of monitoring wavelengths. A match of this spectrum to one obtained conventionally under “stable ion” conditions provides excellent evidence that the transient spectrum does correspond to the cation.

(2) **Rate Constant Ratios.** This method combines the competition kinetics method by product analysis with the flash photolysis-derived rate constants. The ground
state selectivity ratio, \( k_{\text{Nu}^-} : k_{\text{solvent}} \), can be determined via quantification of the trapped products resulting from varying concentrations of nucleophile. Strong evidence that the transient is in fact the proposed cation is provided if this value agrees with the ratio of the two corresponding rate constants measured directly by flash photolysis.

\[
\begin{align*}
\text{RX} & \xrightarrow{k_{\text{i}}{{\text{on}}}_{or \text{ hu}}} \text{R}^+ \\
& \quad \downarrow \quad +\text{SOH} \rightarrow \text{ROS} \\
& \quad \downarrow \quad +\text{Nu}^- : k_{\text{Nu}^-} \rightarrow \text{RNu}
\end{align*}
\]

(3) **Quenching Behaviour.** The question sometimes arises as to whether the transient is a cation, a radical, or some excited-state species. By conducting the studies mentioned in (2), one observes that an increasing concentration of nucleophile is met with a corresponding increase in the rate of first order decay of the transient. This is typical behaviour of a carbocation. Moreover, a transient that is not affected by oxygen concentrations is unlikely to be a radical or a triplet excited state as \( \text{O}_2 \) is an effective quencher of these two species and unreactive towards cations.
1.4 Purpose of This Research Project

The main objective of this study was to determine whether the TAM carbocation (9) was a viable intermediate for the observed carcinogenicity of the parent drug, Tamoxifen (1). α-Sulfate-TAM (10), and more often, α-acetoxy-TAM (11), were used as precursors to 9. Initial studies used the sulfate derivative because of its postulated role as an intermediate along the biotransformation pathway, shown in scheme 1.5. However, its short half-life (~10 s in 100% aqueous conditions) made LFP studies very difficult and ground state solvolysis studies in highly aqueous solvents impossible. Consequently, the α-acetate derivative was substituted as a model for the α-sulfate precursor, and has proven to be very useful in all of the experimental work. Both derivatives have been shown to produce the same carbocation intermediate.
Trapping of the TAM carbocation (9), either by solvent or added nucleophile, to products was followed by nsLFP, high performance liquid chromatography (HPLC), and UV-visible spectroscopy. These studies sought to characterize 9, determine its lifetime under varying solvent conditions, and its reactivity towards a number of different nucleophiles, including those of biological relevance. An unexpected finding led us to explore the tendency of the TAM carbocation to rearrange to form a cyclic compound in addition to being trapped. Another interesting result arose from the discrepancy in the reactivity of the tamoxifen derived transient towards deoxyguanosine (dG) alone versus its reactivity towards dG in DNA. These topics, among others, are covered in the subsequent chapters of this thesis. It is my sincere wish that this initial work into the elucidation of the tamoxifen carbocation will serve as a useful contribution to the overall study of the mechanism of carcinogenesis by this most important drug.
Chapter Two:

Experimental
Chapter 2: Experimental

2.1 Synthesis

2.1.1 General

Unless otherwise stated, all chemicals used in the syntheses were purchased from the Sigma-Aldrich Chemical Company.

$^1$H-NMR, $^{13}$C-NMR, and mass spectral analyses confirmed the identity of the tamoxifen derivatives. $^1$H NMR spectra were obtained on either one of the Gemini 200, 300 or 400 MHz spectrometers in the deuterated solvent indicated. The chemical shifts are reported with multiplicities, where s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. Low and high resolution mass spectra were obtained on a Micromass 70-250S (double focusing) instrument.

2.1.2 α-Hydroxytamoxifen

The first two steps of the multistep synthesis were modeled after a 1947 US patent.45 4-Hydroxybenzophenone (61.5 g, 0.3 moles) was dissolved in 950 ml dry ether. This was added dropwise to 615 ml of a 2 M solution of benzylmagnesium chloride (1.23 moles) also in ether, and allowed to reflux for a minimum of one hour. The resultant solution was brown in colour with a yellow-green precipitate. After the reaction mixture was allowed to cool to room temperature, it was added to 700 g of ice with 200 ml of water containing 100 g of ammonium chloride. From this slurry, the ether layer was collected, dried over anhydrous magnesium sulfate, and filtered. The ether was removed by rotary evaporator, leaving a pale yellow solid that was recrystallized from $p$-xylene.
The melting point of the triaryl-substituted ethanol (12: 67% yield) was found to be 134 °C (literature m.p. = 137 – 139 °C).

$^1$H-NMR (300 MHz, DMSO-$d_6$): $\delta = 3.5$ (s, 2H, -CH$_2$-); 5.5 (s, 1H, ethanol-OH); 6.6-7.4 (m, 14H, ArH); 9.2 (s, 1H, phenol-OH).

Approximately half of the product from the first step was used for the subsequent synthetic step. Compound 12 (30g, 0.103 moles) was suspended in 420 ml carbon tetrachloride. To this solution, bromine (16.5 g, 0.103 moles in 82.5 ml CCl$_4$) was added dropwise very slowly with vigorous stirring. The bright orange colour of molecular bromine quickly disappeared as it entered the solution and reacted to form the thick suspension of off-white product, which was collected by vacuum filtration. This procedure was repeated a second time on the remaining lot, and the resultant brominated products (13) were combined and then recrystallized from methanol (38 g, 53% yield). The product likely consisted of both $E$- and $Z$-isomers. However, the 200 MHz spectrum is not sufficient to resolve these two isomers.

$^1$H-NMR (300 MHz, d-6 DMSO): $\delta = 6.4$ (d, 2H, $J = 8.8$ Hz, ArH o- to O); 6.7 (d, 2H, $J = 8.7$ Hz, ArH m- to O); 7.2-7.4 (m, 10H, ArH).
The final two steps of the synthesis were modeled after a 1985 publication by Foster and coworkers. Once again, half of the product from this step was used in the following alcohol alkylation step. To a stirred solution of (E, Z)-1-bromo-1,2-diphenyl-2-(4-hydroxyphenyl)ethene (13: 19 g, 0.054 moles) in dry HCONMe₂ (225 ml) under argon at room temperature was added sodium hydride (9.5 g, 0.396 moles), and the mixture heated to 60 °C. N,N-Dimethylaminoethyl chloride hydrochloride (19 g, 0.133 moles) was then added portionwise during 20 – 30 minutes. The thick opaque pale yellow mixture, which was kept stirring at 60 °C for another 15 minutes, gradually turned a pale greenish-yellow. Once cooled to room temperature, this mixture was poured into ice water (650 ml). The O-alkylated product (14) was extracted into ether (3 x 150 ml). The ether layers were combined and washed with water (2 x 150 ml) and dried over anhydrous magnesium sulfate. The ether was removed using the rotary evaporator. The dimethylamino-ethylated product was recrystallized from hexanes and gave a yield of 28% (6.3 g). Characterization revealed the product (14) to be very pure, with both E- and Z-isomers likely present once again.
$^1$H-NMR (300 MHz, CDCl$_3$): $\delta = 2.27$ (s, 6H, NMe$_2$); 2.61 (t, 2H, $J = 6$ Hz, CH$_2$N); 3.91 (t, 2H, $J = 6$ Hz, CH$_2$O); 6.59 (d, 2H, $J = 9$ Hz, ArH o- to O); 6.83 (d, 2H, $J = 9$ Hz, ArH m- to O); 7.0-7.4 (m, 10H, ArH).

In the final step of the synthesis of $\alpha$-hydroxytamoxifen (15), all 6.3 g (14.9 mmoles) of alkylated product (14) was dissolved in dry tetrahydrofuran (105 ml). The solution was brought to $-78$ °C using an acetone/dry ice bath, and was kept under argon. To this was added 8.4 ml of a 2.5 M solution of $n$-butyllithium in hexanes (21 mmoles). The mixture was allowed to stir for 5 minutes before 5.25 ml acetaldehyde (87.5 mmoles) was added. The mixture was allowed to attain 0 °C during 20 minutes, then quenched with H$_2$O (35 ml), and partitioned between Et$_2$O (350 ml) and H$_2$O (350 ml). The ether layer was collected and washed with H$_2$O (250 ml), dried (over MgSO$_4$), concentrated, and the resulting residue was subjected to column chromatography on silica gel (Aldrich
130-270 mesh, 60 Å) using the following solvent mixtures in sequence: hexane; hexane:ether (1:1); hexane:ether (1:1) + 1% triethylamine; hexane:ether (1:1) + 2% triethylamine; hexane:ether:triethylamine (1:1:1); and finally [hexane:ether (1:1), 20% triethylamine]:methanol [1:1]. The E-isomer eluted first with a 33% yield followed by the Z-isomer with a 22% yield.

Characterization for the trans-isomer:

$^1$H-NMR (400 MHz, CDCl$_3$): $\delta = 1.19$ (d, 3H, $J = 6.6$ Hz, CH$_3$CH(OH)); 2.33 (s, 6H, NMe$_2$); 2.71 (t, 2H, $J = 5.8$ Hz, OCH$_2$CH$_2$N); 3.94 (t, 2H, $J = 5.8$ Hz, OCH$_2$CH$_2$N); 4.81 (q, 1H, CH$_3$CH(OH)); 6.57 (d, 2H, $J = 8.7$ Hz, ArH o- to O); 6.78 (d, 2H, $J = 8.7$ Hz, ArH m- to O); 7.1-7.4 (m, 10H, ArH).

MS (EI, 70 eV): 387 (M$^+$, 7.2%); 72 (I$^-$, -O-CH$_2$CH$_2$N(CH$_3$)$_2$, 36%); 58 (I$^+$, -CH$_2$N(CH$_3$)$_2$, 100%).

Characterization for the cis-isomer:

$^1$H-NMR (400 MHz, CDCl$_3$) for: $\delta = 1.19$ (d, 3H, $J = 7$ Hz, CH$_3$CH(OH)); 2.16 (s, 6H, NMe$_2$); 2.50 (t, 2H, $J = 6$ Hz, OCH$_2$CH$_2$N), 2.93 (br s, 1H, OH); 4.19 (t, 2H, $J = 6$ Hz, OCH$_2$CH$_2$N); 4.88 (q, 1H, $J = 7$ Hz, CH$_3$CH(OH)); 6.8-7.2 (m, 14H, ArH).

MS (EI, 70 eV): 387 (M$^+$, 7.2%); 72 (I$^-$, -O-CH$_2$CH$_2$N(CH$_3$)$_2$, 36%); 58 (I$^+$, -CH$_2$N(CH$_3$)$_2$, 100%).
2.1.3 α–Sulfatetamoxifen

The α–sulfate derivative of tamoxifen (10) was synthesized as its pyridinium salt in a facile one step process. To pure trans-α–hydroxytamoxifen (400 mg, 1.03 mmoles) dissolved in dry pyridine (10 ml) under argon was added SO3•pyridine (834 mg, 5.24 mmoles). After stirring for one hour, ether (20 ml) was added and the product was allowed to precipitate out. Once the off-white solid had settled to the bottom of the round-bottom flask, the remaining liquid was decanted off. This step was repeated two more times. Residual solvent was removed by connecting the round-bottom flask to a vacuum pump for 16 hours.

1H-NMR (300 MHz, d-6 DMSO): δ = 1.1 (d, 3H, J = 5.1 Hz, CH3CH(OSO3)); 2.8 (s, 6H, NMe2); 3.4 (br, 2H, OCH2CH2N); 4.1 (br, 2H, OCH2CH2N); 5.1 (q, 1H, unresolved, CH3CH(OSO3)); 6.6 (d, 2H, J = 8.6 Hz, ArH o- to O); 6.8 (d, 2H, J = 8.6 Hz, ArH m- to O); 7.1-7.4 (m, 10H, ArH).
2.1.4 \(\alpha\)-Acetoxytamoxifen

\(\alpha\)-Acetoxytamoxifen (11) was prepared as follows.\(^5\) To a solution of \(trans\)-\(\alpha\)-hydroxytamoxifen (0.523 g, 1.29 mmoles) in anhydrous pyridine (10 ml) was added acetic anhydride (0.5 ml, 5 mmoles). Stirring was continued under argon for 16 hr. The mixture was diluted with ether (50 ml) and water (50 ml) to remove pyridine. The ether layer was collected and placed on the rotary evaporator, leaving behind an off-white solid. For some experiments this was used as is; for others the material was purified by column chromatography.

\(^1\)H-NMR (300 MHz, CDCl\(_3\)): \(\delta = 1.28\) (d, 3H, \(J = 6.6\) Hz, \(CH_3CH(OAc)\)); 1.9 (s, 3H, \(OC(O)CH_3\)); 2.28 (s, 6H, NMe\(_2\)); 2.63 (t, 2H, \(J = 5.8\) Hz, \(OCH_2CH_2N\)); 3.90 (t, 2H, \(J = 5.8\) Hz, \(OCH_2CH_2N\)); 5.77 (q, 1H, \(J = 6.6\) Hz, \(CH_3CH(OSO_3^-)\)); 6.55 (d, 2H, \(J = 8.85\) Hz, ArH o- to O); 6.79 (d, 2H, \(J = 8.85\) Hz, ArH m- to O); 7.1-7.4 (m, 10H, ArH).
2.1.5 α-Azido-Tamoxifen

First α-sulfatetamoxifen was synthesized using the same procedure as above, using 65 mg (0.16 mmoles) of trans (or cis)-α-hydroxytamoxifen and 135 mg (0.83 mmoles) of SO₃-pyridine. The product was washed 3 times with ether and decanted off. The round bottom flask was placed on the vacuum pump for 1 hour. Immediately following this, 50 ml of a pH 7 buffered sodium azide solution (10 mM phosphate (1:1) + 10 (and 100) mM NaN₃) was added and allowed to stir for one hour. In order to extract the product (16), enough aqueous NaOH was added dropwise to bring the solution to pH ~10 -12. As the pH increased, the solution gradually turned cloudy due to deprotonation of the ammonium ion, rendering the product less water soluble. This neutral species was then extracted into CH₂Cl₂, dried over MgSO₄, filtered and placed on the rotary evaporator, and finally on the vacuum pump to remove any trace of solvent. Attempts to
separate the $E$- and $Z$-isomers by column chromatography failed, so identification had to be carried out on the mixture. The $E$: $Z$ ratio of the azide-adduct isomers depended on which isomer of the starting material was used.

$^1$H-NMR (400 MHz, CDCl$_3$): δ = 1.02 ($E$) and 1.04 ($Z$) (d, 3H, $J = 6.8$ Hz, CH$_3$CHN$_3$); 2.26 ($E$) and 2.34 ($Z$) (s, 6H, NMe$_2$); 2.62 ($E$) and 2.74 ($Z$) (t, 2H, $J = 5.7$ Hz, OCH$_2$CH$_2$N); 3.90 ($E$) and 4.08 ($Z$) (t, 2H, $J = 5.7$ Hz, OCH$_2$CH$_2$N); 4.7 ($E$) and 4.8 ($Z$) (q, 1H, $J = 6.8$ Hz, CH$_3$CHN$_3$); 6.47 ($E$) and 6.49 ($Z$) (d, 2H, ArH o- to O); 6.74 ($E$) and 6.76 ($Z$) (d, 2H, ArH m- to O); 7.1-7.4 (m, 10H, ArH).

MS (EI, 70 eV): 412 (M$^+$, 7%); 370 (M$^+$-N$_3$, 36%); 72 (I$^+$, -CH$_2$CH$_2$N(CH$_3$)$_2$, 42%); 58 (I$^+$, -CH$_2$N(CH$_3$)$_2$, 100%). HRMS calculated for C$_{26}$H$_{28}$N$_4$O 412.2263 [M$^+$], found 412.2269.
2.1.6 Cyclic-Tamoxifen (Indene)

Cis-α-hydroxytamoxifen (50 mg) was dissolved in 1 M HCl (20 ml) and stirred overnight. The following morning, TLC (8: 11: 1; hexane: ether: triethylamine) on a small amount of extract from the solution revealed a new spot with a greater RF value than either of the cis- or trans-α-hydroxytamoxifens. Furthermore, the spot was a visible yellow colour under normal lighting and fluoresced to give a deep bluish-purple colour under an ultraviolet lamp. The workup consisted of neutralizing the cyclized tamoxifen product (17) with aqueous NaOH (to pH ~10-12), extracting into ether (50 ml), and washing the ether layer with water (3 x 50 ml). The ether layer was dried over MgSO₄, filtered and placed on the rotary evaporator, as usual. Column chromatography, unfortunately failed to provide any separation whatsoever, so that identification had to be carried out on the mixture.

¹H-NMR (400 MHz, CDCl₃): δ = 1.32(major) and 1.32 (minor) (d, 3H, J = 7.5 Hz, -CH₃); 2.38 (major and minor) (s, 6H, NMe₂); 2.79 (major) and 2.78 (minor) (t, 2H, J = 5.6 Hz, OCH₂CH₂N); 4.025 (major) and 4.04 (minor) (q, 1H, J = 7.6 Hz, -H); 4.15 (major) and 4.12 (minor) (t, 2H, J = 5.6 Hz, OCH₂CH₂N); 6.87 (major) (d of d, 1 H, J = 2.2 and 8.2 Hz, H₄) (5H of indene ring of Z-In); 6.95 (minor) (d, 2H, J = 8.2 Hz, H₄) 7.8 (d, 1H, J = 2.2 Hz, H₅) 7.16 – 7.14 (multiplet).

MS (EI, 70 eV): 369 (M⁺, 5%); 105 (I⁺, C₆H₃OCH₂, 6%); 91 (I⁺, C₆H₃O, 14%); 72 (I⁺, -CH₂CH₂N(CH₃)₂, 46%); 58 (I⁺, -CH₂N(CH₃)₂, 100%). HRMS calculated for C₂₆H₂₇NO 369.2092 [M⁺], found 369.2097.
2.1.7 14-Nucleotide Oligomer

The 14-nucleotide long strand of deoxyribonucleic acid (DNA, 5' ATATTAGCTAATAT 3') was synthesized using standard autosynthetic techniques with the "Applied Biosystems 392 DNA/RNA synthesizer" supplied by Professor A. MacMillan with the help of Steve Chaulk. Solvents and reagents were purchased from Perkin and ABI.

The DNA synthesis was relatively straightforward. The reactive 3' phosphorous group of one nucleoside was coupled to the 5' hydroxyl of a second nucleoside. The former monomer was delivered in solution while the latter was immobilized on a solid support (10 μM T column). Three other chemical reactions followed the formation of the internucleotide linkage to prepare for the subsequent coupling step. Therefore, the synthesis cycle was conducted by adding one nucleotide at a time. Once the desired chain length (with the desired sequence) was met, the crude DNA (oligonucleotide) had
to be cleaved from the support and deprotected. The synthesis cycle is depicted in figure 2.1.

Figure 2.1. The oligonucleotide synthesis cycle.
The first step of the synthesis cycle involved freeing the 5'-hydroxyl by treating the derivatized solid support (in this instance with a thymidine) with trichloroacetic acid (TCA). The acid readily cleaves the dimethoxytrityl (DMT) protecting group. Coupling proceeded by the simultaneous addition of the phosphoramidite nucleoside monomer and tetrazole, a weak acid, to the reaction column. Protonation of the nitrogen of the phosphoramidite makes the phosphorous susceptible to nucleophilic attack. Under these conditions, coupling to the 3' hydroxyl of the supported monomer was complete within thirty seconds. Unreacted chains will contain a free 5'-hydroxyl, therefore capping by acetylation (acetic anhydride and 1-methylimidazole) terminates any chains that did not undergo addition.

Finally, the internucleotide linkage is oxidized from the phosphite to the more stable phosphotriester. Iodine was used as the oxidizing agent and water as the oxygen donor.

After oxidation, the DMT group was removed with TCA, and the cycle was repeated until completion of the chain elongation. The oligonucleotide was cleaved from the support by a one-hour treatment with concentrated ammonium hydroxide. This treatment also serves to remove the cyanoethyl phosphate protecting groups. Furthermore, the protecting groups on the exocyclic amines on bases deoxyadenosine (dA), deoxyguanosine (dG), and deoxycytidine (dC) were removed by treating the crude DNA solution in ammonium hydroxide at 55 °C for a minimum of 8 hours. Each step of the process was expected to generate a 95% yield, therefore the overall yield was expected to be $0.95^{14} \approx 50\%$. 
The oligomer was purified by filtering through a syringe-mounted oligonucleotide purification cartridge (OPC, Applied Biosystems part # 400771). Further purification involved running the sample on a polyacrylamide gel electrophoresis (PAGE) setup. The percent yield was determined by taking the absorbance of a 1.25 µl aliquot of the DNA sample in 1.5 ml water at its λ_max of 260 nm. The absorbance was divided by the calculated extinction coefficient of \((A = 15.4 \times 6 + G = 11.7 \times 1 + T = 8.8 \times 6 + C = 7.3 \times 1) \times 164.2 \times 10^{-6} \text{ pmol}^{-1}\) in order to determine the concentration in the cuvette (pathlength was 1 cm). From this value a total amount of 2.9 µmol DNA was determined, which works out to a 29% yield ((2.9µmol /10µmol) x 100).
2.2 Experimental Techniques

2.2.1 High Performance Liquid Chromatography (HPLC)

Rates of solvolysis and product analysis were performed at room temperature (~22°C) with a Waters 600E HPLC, a Waters 486 Tunable Absorbance detector set at either 285 nm or 260 nm, and a Waters 746 Data Module, using a μ-Bondapak C\textsubscript{18} reversed phase column or a Waters Symmetry C\textsubscript{18} μm column of dimensions 4.6 mm x 150 mm. Most experiments were carried out with the latter column, using one of two gradients, shown in tables 2.1 and 2.2. The gradient shown in table 2.1 was used for experiments with the α-hydroxytamoxifens as starting material. Retention times were 4.6 min (E-HO-TAM), 5.9 min (Z-HO-TAM), 14.2 min (Z-Indine), and 15.4 min (E-Indine). The gradient shown in table 2.2 was used for experiments starting with α-acetoxy-TAM. Retention times were 11.3 min (E-HO-TAM), 12.1 min (Z-HO-TAM), 13.7 min (E-OC(O)CH\textsubscript{3}), 14.8 min (Z-Indine), and 15.3 min (E-Indine). Quenching experiments using various nucleophiles (chapter 5) were conducted using isocratic eluting solvent (70% CH\textsubscript{3}CN, 30% buffer – usually 1 mM HClO\textsubscript{4}) at 3 ml/min, λ = 280 nm. Retention times for the alcohols were 12.0 min and 13.5 min for E- and Z-isomers respectively.

For most of the experiments, solutions contained \(1 \times 10^{-4}\) M of the substrate, with 60 μl being directly injected into the HPLC. Quantitative measurements were based on comparison of peak areas of authentic samples. The tamoxifen indenes share an isosbestic point with the alcohols at 260 nm, and so the assumption was made that equivalent areas of the indenes to the alcohols represented the same concentration. This
is supported be the fact that total peak areas remained constant throughout the conversion of alcohols to indenes.

Table 2.1. Gradient program used for elution on HPLC for experiments using α-hydroxytamoxifen, where a represents the rate of change of the solvent conditions; * = isocratic, 1 = immediate step, 6 = linear gradient.

<table>
<thead>
<tr>
<th>Time, minutes</th>
<th>Flow Rate, ml/min</th>
<th>% Buffer</th>
<th>% CH₃CN</th>
<th>Curvea</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1</td>
<td>60</td>
<td>40</td>
<td>*</td>
</tr>
<tr>
<td>2.0</td>
<td>1</td>
<td>60</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>8.0</td>
<td>1</td>
<td>15</td>
<td>85</td>
<td>6</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>15</td>
<td>85</td>
<td>*</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>60</td>
<td>40</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2.2. Gradient program used for elution on HPLC for experiments using α-acetoxytamoxifen, where \( a \) represents the rate of change of the solvent conditions; * = isocratic, 1 = immediate step, 6 = linear gradient.

<table>
<thead>
<tr>
<th>Time, minutes</th>
<th>Flow Rate, ml/min</th>
<th>% Buffer</th>
<th>% CH₃CN</th>
<th>Gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1</td>
<td>75</td>
<td>25</td>
<td>*</td>
</tr>
<tr>
<td>1.0</td>
<td>1</td>
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<td>25</td>
<td>1</td>
</tr>
<tr>
<td>14.0</td>
<td>1</td>
<td>15</td>
<td>85</td>
<td>6</td>
</tr>
<tr>
<td>18.5</td>
<td>1</td>
<td>15</td>
<td>85</td>
<td>*</td>
</tr>
<tr>
<td>23</td>
<td>2</td>
<td>75</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>0.5</td>
<td>75</td>
<td>25</td>
<td>*</td>
</tr>
</tbody>
</table>

2.2.2 Laser-Flash Photolysis

The tamoxifen carbocation was generated and monitored using a nanosecond laser flash photolysis device, depicted in figure 2.2, at room temperature (22 °C). The carbocation precursors were photolyzed using a Lumenics Excimer 510 Laser with a 20 nanosecond pulse width. The actinic flash, typically ~150 ±30 mJ per pulse, was set to 248 nm (KrF* emission) for most experiments and to 308 nm (XeCl* emission) for all experiments involving deoxyguanosine or DNA as the added nucleophile. The laser was equipped with a UV detector to monitor the transients. A Tetronics SCD-1000 digitizer connected to a Tetronics DX 386 computer digitized the signals from the detector. The
raw data (changes in voltage with time) on the digitizer was further processed by the computer to generate a graph showing changes in optical density versus time. The computer program called Grafit™ was used to conduct fits to the traces in order to determine rate constants, and Excel was used to determine ΔOD values at given time intervals along the trace decay.

Figure 2.2. Schematic diagram of laser-flash photolysis apparatus.
HPLC grade solvents were used for LFP experiments. Stock solutions of the TAM-carbocation precursor (either \( \alpha \)-acetate or \( \alpha \)-sulfate) were prepared in CH\(_3\)CN. A small and consistent (for the particular experiment) amount of this stock solution was injected either directly into the cuvette (\( \alpha \)-sulfate), or into a 25 ml volumetric flask (\( \alpha \)-acetate) of the appropriate solvent (with or without added nucleophile) to bring the optical density of this final solution to between 0.5 and 2.0 \( \text{cm}^{-1} \). The final concentration of the precursor ranged from \( \sim 1.5 \times 10^{-5} \text{ M} \) (for 248 nm actinic flash) and \( 7.5 \times 10^{-5} \text{ M} - 1 \times 10^{-4} \text{ M} \) (for 308 nm actinic flash; depending on solvent). A laser quality four-sided transparent quartz cuvette of dimension 4 x 1 x 1 cm, supplied by Hellma Canada Limited, was used for all experiments.

### 2.2.2.1 LFP Experiments

Transient spectra were recorded using the \( \alpha \)-acetoxytamoxifen precursor in 0.0025 M acetate buffer (1:1), 40\% CH\(_3\)CN. Formation of the transient was investigated over the wavelength range of 330 – 580 nm at 5 nm increments. The absorbance was taken at a specific time interval of the decay trace at various times after the laser pulse for each detector wavelength. The average optical density over the time period of the interval at each delay time after the pulse were plotted against wavelength to generate the absorption spectra.

All rate constants and absorbance measurements were taken as the average of at least four kinetic runs or absorbance measurements. Solutions of substrate alone in buffer were flashed up to three times each, whereas solutions with added nucleophile were flashed only once each.
Rate constants were determined from the rate of decay (k_{obs}) at 450 nm (the \( \lambda_{\text{max}} \) for the tamoxifen carbocation). Plots of \( k_{\text{obs}} \) versus the concentration of the added nucleophile were linear (except with DNA), the slopes of which gave the second order rate constants for quenching by the nucleophile (\( k_{\text{N-U}} \)).

Rate constants for quenching of the transient were also determined at varying pH. The solutions were: 1 x 10^{-3} M HClO_4; 0.0025 M acetate, 11.5:1 acid, 1:1, 11.5:1 base; 0.0025 M phosphate, 11.5:1 acid, 4:1 acid, 1:1, 4:1 base, 11.5:1 base; 0.0025 M tris 11.5:1 acid, 1:1, 11.5:1 base; 0.0025 M carbonate, 11.5:1 acid, 1:1, 11.5:1 base; and 1 x 10^{-3} NaOH. These solutions were either 100% aqueous, or contained 20% or 40% CH_3CN, both of which were either under constant ionic strength (with added NaClO_4) or not. Buffer dilution plots were also constructed with the rate constant taken as the y-intercept value to eliminate buffer catalysis.

2.2.3 Ultraviolet-Visible Spectrophotometry

All UV-visible spectra were obtained from solutions in quartz cuvettes of 1cm path length at room temperature (22 °C) on a Hewlett Packard 8452 A Diode Array spectrophotometer. This same instrument was sometimes used to measure reaction kinetics at a fixed wavelength from a series of spectra. Alternatively, kinetics were measured on a Varian Cary 2200 spectrophotometer.
2.2.4 Melting Curve

Melting curves for various solutions of the 14-mer oligonucleotide were obtained by monitoring the UV absorbance at 260 nm in the temperature range 5° to 70 °C at a heating – cooling rate of 0.5 °C/minute. All solutions contained a thin layer of silicon oil in addition to being capped in order to prevent evaporation.

Two factors were found to be crucial for the proper annealing of the oligomer. The first was to raise the temperature of the solution containing the oligomer to ~ 70 °C to ensure exclusive formation of the monomer without hairpins, followed by a very slow and gradual decrease in the temperature of the solution to the starting temperature of 5 °C (this was done overnight). The second factor was the salt concentration. The greater the salt concentration, the higher the melting temperature. Therefore the initial conditions of no added salt (4 mM phosphate 1:1, μ = 8 mM) were modified to solutions of 40 mM, and finally 80 mM NaClO₄. Another complicating factor arose from the formation of condensation on the surface of the cuvettes when going from the cold extreme of the temperature range to the warm one, causing the artifact of a rapid absorbance increase in the lower temperature range when absorbance is normally steady and low. A stream of gaseous nitrogen was directed at the cuvettes in the holder to prevent condensation from forming.

All melting curve experiments were performed on a temperature regulated iso – hyperbaric spectrometer (TRIHBS) provided by Professor R.B. MacGreggor from the Faculty of Pharmacy at the University of Toronto. The instrument is controlled by an AT&T 6300+ personal computer with a numerical coprocessor, and a medium resolution graphics board. The computer was operated under Asyst version 3.0 (Asyst technologies,
Inc., Rochester, NY). Transfer of analog signals to and from the computer was through a Data Translation, Inc. (Marlboro, MA) Model 2801A 12 bit analogue – to digital/ digital – to - analogue converter with 16 analogue inputs and 2 analogue outputs.

2.2.5 pH Measurements

pH was measured for all solutions using a Corning pH meter 130 at room temperature (22 °C). The pH meter was calibrated using the pHs of 4 and 7, or 7 and 10, depending on the solution to be measured. pH readings were accurate to ± 0.02 pH units.
Chapter Three:

Ground State Solvolysis Studies
Chapter 3: Ground State Solvolysis Studies

3.1 Introduction

As was mentioned in chapter 1, TAM is known to cause endometrial cancer in a small percentage of the women who take the drug,\textsuperscript{6,7} and is also a hepatocarcinogen in rats.\textsuperscript{9,10,12} Furthermore, TAM-DNA adducts have been isolated and characterized both in animal model studies\textsuperscript{17,52,65} as well as in women.\textsuperscript{70-74} Of all the metabolic pathways that have been proposed to account for carcinogenesis by TAM, the pathway shown in schemes 1.5 and 1.6 has been gaining favour over the last couple of years (refer to section 1.2.4 for a review). α-Hydroxytamoxifen is a metabolite known to undergo O-sulfonation.\textsuperscript{43-46} This may well represent a bioactivation pathway that effectively produces the TAM carbocation precursor.

While it has been established that the exocyclic amine of guanine traps TAM at the α-position to generate adducts, the exact mechanism of this reaction is unknown. Does α-sulfate TAM undergo $S_N1$ or $S_N2$ substitution? If the mechanism is found to be unimolecular, what is the nature/reactivity of the TAM carbocation intermediate? Chapters 3, 4, and 5 all attempt to answer these questions in the hope that the final pieces will be added to the metabolic puzzle.
Several studies of the ground state solvolysis reaction have been carried out to answer some of these questions and are the subject of this chapter. The TAM carbocation cannot be observed directly with these studies. However, the mechanism of solvolysis can be determined, and the presence of the carbocation inferred. Initial studies sought to examine the effect of solvent polarity on the rates of solvolysis (section 3.2.1). Rates were also determined with added nucleophile at varying concentrations (section 3.2.2). Product analysis was also performed to confirm the mechanism of solvolysis (and substitution by added nucleophiles; sections 3.3 and 3.4). Finally, an extensive mathematical analysis was conducted to determine the rate constants of the reactions involved in the acid-catalyzed isomerization of the TAM-alcohols (section 3.5).
Once derivatized with either a hydroxy or sulfate, the α-position of TAM represents a stereocenter. Experiments using pure enantiomeric starting material could in theory be followed using plane-polarized light, in order to determine the mechanism of substitution. SN2 attack at the chiral center would result in inversion of configuration. Alternatively, racemization, resulting in loss of optical activity, would imply an SN1 mechanism. These experiments were not conducted, as optically pure α-sulfate-TAM was not available. Nevertheless, product analysis was carried out following hydrolysis, and confirmation of the structure of these products was made. The resulting cis-trans isomers of α-hydroxy-TAM that were observed (section 3.3) can only be explained by the TAM carbocation intermediate. Another product of hydrolysis (indenes; also present as a pair of “cis-trans” isomers) was also discovered quite unexpectedly, which again, could only result from the carbocation intermediate.
3.2 Rates of Hydrolysis

Ground state solvolysis studies on α-sulfate- and α-acetoxytamoxifen were conducted on the HPLC. Rate constants for the disappearance of the substrate were measured by repeat injections of solutions containing ~50 – 100 μM of either α-sulfate-or α-acetoxy-TAM, depending on the particular experiment. The area of the substrate peak on the chromatogram was measured as a function of time. Figure 3.1 shows three chromatograms to illustrate the gradual decrease in α-acetoxy-TAM (where retention time = 5 min). A typical experiment is shown in figure 3.2.

![Chromatogram](image)

Figure 3.1. Three chromatograms from time points t = 1 min. (A); t = 32.7 min. (B); and t = 163 min. (C) illustrating the gradual decrease in substrate (where retention time = 5 min) as it solvolyzes over time.
Figure 3.2. Area of $\alpha$-acetoxy-TAM peak as a function of time in 0.0025 M phosphate buffer (2:1 base, pH 7.00), at 37° C.

Ground state solvolysis experiments were conducted under varying H$_2$O/CH$_3$CN proportions, with or without added nucleophile, and at varying temperatures. In all cases, the $\alpha$-sulfate-$\alpha$-acetate-TAM disappeared in an exponential function. First-order rate constants were obtained by fitting the data points to the exponential equation 3.1. The offset is within experimental error of zero, however a value must be input into the fitting program in order to conduct a proper fit.

$$[A] = [A]_0 \cdot e^{-kt} + \text{offset}$$  
Eq 3.1
The half-life \( t_{\frac{1}{2}} \) can be easily calculated from the first-order rate constant by the equation:

\[
    t_{\frac{1}{2}} = \ln(2)/k \tag{Eq 3.2}
\]

3.2.1 Acetonitrile: Water Experiments

Ground state solvolysis experiments were performed on the HPLC using \( \alpha \)-sulfate-TAM in varying proportions of water: acetonitrile. Rate constants were found to decrease with increasing acetonitrile content. A plot of the logarithm of the rate constants versus the \( Y_{Cl} \) parameter is given in figure 3.3.\(^75\)

<table>
<thead>
<tr>
<th>Volume Percent Water</th>
<th>( k_{(solv)}, \text{s}^{-1} )</th>
<th>( Y_{Cl} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>66.7</td>
<td>0.0095</td>
<td>2.21</td>
</tr>
<tr>
<td>60</td>
<td>0.0063</td>
<td>1.92</td>
</tr>
<tr>
<td>50</td>
<td>0.0029</td>
<td>1.45</td>
</tr>
<tr>
<td>35</td>
<td>0.00093</td>
<td>0.79</td>
</tr>
<tr>
<td>20</td>
<td>0.00025</td>
<td>Not available</td>
</tr>
</tbody>
</table>
Figure 3.3. Plot of log $k_{\text{solv}}$ versus the solvent ionization parameter $Y_{\text{Cl}}$.

The 'Y' parameter is based on the rate constants for the solvolysis of tert-butyl chloride, known to occur via the $S_N1$ mechanism. The parameter provides a measure of the polarity of the mixed solvent systems as seen in $S_N1$ solvolysis. Other compounds undergoing $S_N1$ solvolysis are expected to follow the Y parameter in a linear fashion with a reasonable slope ($m \approx 1$). The fact that the four data points obtained for $\alpha$-sulfate-TAM do plot linearly against $Y_{\text{Cl}}$ with the slope of the correlation equal to 0.72 suggests that disappearance of the substrate is occurring in an $S_N1$ fashion. The implication is that $\alpha$-sulfate-TAM undergoes solvolysis to produce a carbocation intermediate.
It should also be noted that extrapolation of the line in figure 3.3 to 100% water ($Y_{Cl} = 3.49$) produces the estimate $k_{(solv)} = 0.08 \text{ s}^{-1}$ for the rate constant in water, indicating a half-life of the sulfate of 8.7 seconds at room temperature. Numerous solvolysis experiments were later conducted using $\alpha$-acetoxy-TAM as the substrate, and the corresponding rate constants were found to be much slower. In the example illustrated in figure 3.4, the rate of solvolysis ($k_{(solv)}$) was 0.0078 min$^{-1}$, with a calculated half-life ($t_{1/2}$) of $\sim$ 90 minutes in 100% water at room temperature. These different rates of solvolysis are due to the leaving group, $\text{OSO}_3^2$ versus $\text{OC}(\text{O})\text{CH}_3^-$. 

**Figure 3.4.** Area of $\alpha$-acetoxy-TAM peak as a function of time in 0.0025 M phosphate buffer (2:1 base, pH 7.00), at 22°C.
As a result of this slower rate of solvolysis, and consequent ease of handling, α-acetoxy-TAM was used as a model for α-sulfate-TAM in most of the experiments that followed. The acetoxy derivative has proven to be a suitable substrate as it generates the same tamoxifen carbocation as the sulfate does (as indicated by the transient spectra in section 4.3), albeit more slowly under ground state conditions.

3.2.2 Effect of Adding Nucleophile to the Rate of Solvolysis

Ground state solvolysis experiments of α-acetoxy/sulfate-TAM were also conducted in the presence of added nucleophiles, such as azide ion (N₃⁻) and 2′-deoxyguanosine (dG, 18).

![Diagram of 2'-Deoxyguanosine (18)](image)

In one such experiment, the sulfate was solvolyzed in 40% acetonitrile containing 0 mM, 0.1 mM, 0.2 mM, and 0.4 mM NaN₃. As the concentration of NaN₃ increased, the
two peaks on the HPLC chromatograms normally observed as products became replaced by two new peaks with considerably longer retention times. Figure 3.5 shows a chromatogram of α-sulfate-TAM in 0.1 mM NaN₃ left overnight. Even though it was evident that reaction with N₃⁻ was taking place, the rate of disappearance of the sulfate remained unaffected, as shown in Table 3.2.

**Table 3.2.** First-order rate constants for the disappearance of α-sulfate-TAM in aqueous acetonitrile (60: 40; H₂O: CH₃CN) as a function of azide concentration, at 22°C.

<table>
<thead>
<tr>
<th>Azide Concentration, mM</th>
<th>k_{solv.} s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0063</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0068</td>
</tr>
<tr>
<td>0.2</td>
<td>0.0063</td>
</tr>
<tr>
<td>0.3</td>
<td>0.0062</td>
</tr>
<tr>
<td>0.4</td>
<td>0.0064</td>
</tr>
</tbody>
</table>

**Figure 3.5.** Chromatogram of α-sulfate-TAM in 0.1 mM NaN₃ left overnight, with the two azide-adduct peaks of retention times 12.29 and 14.08 min.
Similar behaviour was observed for $\alpha$-acetoxy-TAM in 100% aqueous phosphate buffer (0.0025 mM; 2:1 base, pH = 7.00) at 37° C. Rates of solvolysis and emergence of dG-TAM adducts were determined at 0 mM, 4.48 mM, and 9.79 mM dG with respective rate constants of $4.23 \times 10^{-4}$ s$^{-1}$, $4.97 \times 10^{-4}$ s$^{-1}$, and $4.67 \times 10^{-4}$ s$^{-1}$ ($t_{1/2} \equiv 23 - 27$ min). Not surprisingly, rates were elevated due to the higher temperature. However, no significant difference was observed with increasing dG concentration.

The behaviour shown in the presence of the nucleophile is classic evidence for an $S_N1$ reaction in which the $\alpha$-acetoxy/sulfate ionizes to a carbocation in the rate-determining step; the carbocation subsequently reacts with water to form alcohols, and with the added nucleophile to form the appropriate adducts. The rate constant for the decay of the starting material is unaffected since the conjugation step does not depend on the concentration of the nucleophile.
3.3 Products of Hydrolysis: Alcohols

As the peak in the HPLC for the α-sulfate/acetoxy-TAM decreased, it became replaced by two new peaks, with shorter retention times than the starting material, in a ratio of ~ 2.2:1 (see figure 3.1). The ratio of these two products was found to be independent of the configuration of the starting ester and was also independent of the extent of conversion. Disruption of this ratio was observed only with experiments involving high azide concentrations.

Initially, the products were believed to be Z-HO-TAM, 15 (for Z-ester as the substrate), and the allylic rearrangement alcohol, 19. However, one would expect three different products with three separate peaks on the HPLC chromatogram, as 2 isomers would likely result from the allylic rearrangement. Furthermore, solvolysis of α-sulfate-TAM followed on the UV-visible spectrophotometer over the time period required to observe loss of starting material on the HPLC was found to produce negligible change in the spectra. The rearranged alcohols, with the double bond conjugated to only one of the aryls, rather than to all three aryls, are expected to have a sufficiently different spectra from the substrate that solvolysis would be detectable by a gradual change in the spectra. However, the E- and Z-HO-TAMs differ only marginally in their structure from the starting ester, so their formation would be consistent with this observation.
Clearly, it was important to synthesize authentic samples of $E$- and $Z$-HO-TAM and to inject them on the HPLC to confirm our suspicion that they represented the hydrolysis products. Authentic samples were synthesized and characterized (section 2.1.2) and, when injected on the HPLC, gave the exact same retention times as our two products of hydrolysis. Retention times of the $E$- and $Z$-HO-TAMs varied depending on the eluting solvent. However, in all cases the $E$-alcohol eluted first, and accounted for the 2.2 proportion of the ratio, i.e. $(E):(Z); 2.2: 1$. Peak area ratios easily translate into molar ratios (as determined by quantification by authentic samples) due to virtually identical
spectra of the two isomers, with the same extinction coefficient at 260 nm ($\varepsilon \approx 9000$). Therefore, the ratio of 2.2:1 represents moles of $E$-HO-TAM to $Z$-HO-TAM.

Isomerization to the two alcohols can be explained by noting that the carbocation is best viewed as an $\alpha$-vinyl substituted diarylmethyl cation. As a result, there is little double bond character in the central C-C bond and significant double bond character at the $\alpha$-carbon instead (see AM1 calculation results in appendix 1). This allows for rotation about the central C-C bond of the TAM carbocation intermediate, which upon being trapped by water at the $\alpha$-position, generates the two isomers observed. The very fact that these two isomers are formed with the same ratio from either configuration of the starting material provides very compelling evidence for $S_N1$ solvolysis.

![Scheme 3.2]
3.4 Products of Hydrolysis: Indenes

3.4.1 From α-Acetoxytamoxifen

Another interesting consequence of the quantification of the alcohol products by authentic samples was the discovery that these two products did not account for 100% of hydrolysis products. Typically, after the TAM ester hydrolyzed, 64% of product was $E$-HO-TAM and 29% $Z$-HO-TAM. Some unknown product(s) with a total yield of 7% accounted for the rest.

Initially, it was suspected that the other products must be the rearranged alcohols (19). AM1 calculations showed mainly single bond character about the central two carbons. Furthermore, steric congestion forces the vinyl group to rotate 60-70 degrees out of the plane of the $C^+$ center. This conformation suggests that the $C^+$ center alpha to the two aryl groups would be a likely site for attack by a nucleophile. In searching for these “rearranged alcohols”, two minor peaks were noted in the HPLC chromatogram eluting at considerably longer retention times than the $E/Z$-HO-TAMs. By modifying the HPLC conditions to those shown in table 2.2 and using the Waters Symmetry C$_{18}$ μm column, five discrete peaks could be clearly seen, and three of them clearly identified. Retention times were 11.3 min ($E$-HO-TAM), 12.1 min ($Z$-HO-TAM), 13.7 min ($E$-C(O)O-TAM), 14.8 min (?), and 15.3 min (?). Figure 3.6 shows a chromatogram where the TAM ester has completely hydrolyzed, generating four products.
Figure 3.6. Chromatogram of α-acetoxy-TAM left to hydrolyze overnight.

Hydrolysis of α-acetoxy-TAM was also followed by recording the absorption spectra as a function of time, over times much longer than what was required for loss of starting material. In one such experiment, 100 µM α-acetoxy-TAM in 0.0025 M phosphate buffer (2:1 base, pH 7.00) was scanned every five minutes for 4.5 hours. In another, 200 µM α-acetoxy-TAM in 0.0025 M phosphate buffer (10:1 base, pH 8.00), was scanned every 10 minutes overnight. In both experiments, the UV-visible spectrum was found to change over time, which further indicated that product(s) other than the E/Z-HO-TAM was being generated. Authentic samples of the tamoxifen alcohols generate a spectrum that is virtually identical to the α-acetoxy-TAM. Most notable was the gradual increase in absorbance at ~310 nm. The identity of this product was not known, however, increase in conjugation was suspected due to the rise in absorbance at
higher wavelengths. The rearranged alcohol products (19) would not be expected to display such behaviour as conjugation is decreased in these structures.

3.4.2 From E/Z-α-Hydroxytamoxifen

Experiments under acidic conditions were conducted in order to follow the rate of cis to trans isomerization of the α-HO-TAMs. Leaving group ability can be expressed in terms of the pKₐ of the conjugate acid, where it increases with increasing acidity (decreasing pKₐ). OH⁻ is a very poor leaving group as the pKₐ for H₂O is 15.74. In acid, the alcohol becomes protonated and its leaving ability is remarkably enhanced. The pKₐ for the conjugate acid H₃O⁺ is -1.7. Thus, under acidic aqueous conditions, the two HO-TAMs were found to isomerize readily between E and Z forms, as followed by repeat injections on the HPLC.

3.4.2.1 UV-Visible Spectra

The reactions of E-HO-TAM and Z-HO-TAM in acidic aqueous solutions were first seen in absorption spectra recorded as a function of time. Solutions included E-HO-TAM in 55% H₂SO₄; in 1 M HClO₄; in 0.1 M HClO₄ and 40% acetonitrile; and Z-HO-TAM in 0.1 M HClO₄ and 40% acetonitrile. Acetonitrile was found to be necessary because the product(s) formed precipitated out of 100% aqueous solution, turning the final solution cloudy. This effect was attenuated with added CH₃CN. As illustrated in figure 3.7, these show the slow growth of a new peak with λₘₐₓ at 310 nm. This increase follows good single exponential kinetics with reasonable isosbestic points at 260 and 270 nm. This is similar to what was observed when α-acetoxy-TAM was used as the
substrate (in both neutral and slightly basic solutions), and suggests a first-order conversion of the starting material to product(s).

Figure 3.7. Spectra obtained with $2 \times 10^{-4}$ M $E$-HO-TAM in 0.1 M HCl (22 °C). Spectra were recorded every 20 minutes. The absorbance increase at 310 nm fits to a single exponential with a rate constant of $1.1 \times 10^{-4}$ s$^{-1}$.

3.4.2.2 HPLC Experiments

HPLC chromatograms recorded on the solutions containing 40% CH$_3$CN showed that the behavior is more complicated than a simple first-order conversion. Thus $E$-HO-TAM was found to convert to an approximate 1:1 ratio with its Z-isomer Z-HO-TAM. The same interconversion to an ~1:1 $E$: $Z$ ratio is obtained with Z-HO-TAM as the starting alcohol. At the same time as the isomerization, the two peaks with longer retention times begin to appear. This process was found to be considerably slower, and appeared to match the spectral changes in the UV-visible spectrum. Ultimately, the two
HO-TAMs completely disappear, and the chromatogram contains only the two peaks for the new products.

Figure 3.8. HPLC chromatograms obtained with $2 \times 10^{-4}$ M $E$-HO-TAM in 0.1 M HCl, monitoring $\lambda = 260$ nm, $22^\circ$ C. Solutions were injected at 0.8 (A), 23.8 (B), 95.3(C), and 164.3(D) minutes after preparation of the solution.
3.4.3 Identification of the Indenes

The unknown products were identified by working on a larger scale with 50 mg Z-HO-TAM in 1 M HCl (section 2.1.6). The reaction was given sufficient time so that none of the α-HO-TAMs remained. The reaction was followed by TLC, which revealed a new spot with a greater RF value than either of the α-HO-TAMs. This spot was a visible yellow color under normal lighting, and fluoresced to give a deep bluish-purple color under an ultraviolet lamp. When the TLC indicated that none of the α-HO-TAM remained, a small extract of the solution was injected on the HPLC. The chromatogram was similar to that obtained at long times on the smaller scales (section 3.4.2.2). Just as before, the peak at shorter retention time had twice the area as that of longer retention time.

The products were then extracted into dichloromethane after the solution was neutralized. All attempts at separating the two products via column chromatography failed, so that identification had to be carried out on the mixture. The mass spectrum showed a molecular ion at m/z 369 MS, with high resolution MS providing a molecular formula of C_{26}H_{27}NO. This formula indicates loss of a molecule of water from the α-HO-TAM. Based on the suspicion that these products are derived from the cis and trans forms of the tamoxifen carbocation (these products are generated under the same conditions as the α-HO-TAMs are produced and isomerized), we suspected our unknown products to be the isomeric indenes Z-In and E-In. E/Z terminology is used for the indenes to indicate their relation to starting alcohols/esters. As illustrated in scheme 3.3, these products arise from intramolecular cyclization of the tamoxifen carbocation.
Characterization by $^1$H-NMR is consistent with this interpretation, and shows the major product to be Z-In (the product of shorter retention time), which results from cyclization into the alkoxy-substituted aromatic ring. Quantification of Z-In relative to E-In was based on the aromatic protons next to the alkoxy group, which give discrete peaks on the NMR spectrum. In the Z-In, these protons are non-equivalent, whereas in E-In the protons are equivalent.
In figure 3.9, the proton in Z-In labeled $H_a$ appears as a doublet of doublets at 6.87 ppm, coupling to $H_b$ ($J = 2.2$ Hz) and $H_c$ ($J = 8.2$ Hz). The equivalent protons $H_d$ of $E$-In appear at 6.95 ppm as a doublet ($J = 8.6$ Hz). The integration of $\delta$ 6.87: $\delta$ 6.95 is $\sim 1:1$. Since two protons provide the latter doublet, the ratio of Z-In: E-In is $\sim 2:1$. Therefore, Z-In represents the major product eluting at the earlier retention time in the HPLC experiments.
3.5 Analysis

Once our known products were confirmed to be the $Z$- and $E$-indenones, we subjected our HPLC derived cis- trans isomerization and $\alpha$-acetate-TAM solvolysis experiments to an extensive kinetic analysis in order to obtain the rates of the individual contributing reactions. Prior to conducting the kinetic analysis, it was necessary to make the assumption that the four products, the 2 alcohols and the 2 indenes, had the same sensitivity in the HPLC.

3.5.1 Quantitative Analysis

The HPLC chromatograms for the cis- trans isomerization experiments showed a total of four peaks, now positively identified as the $E$-HO-TAM, $Z$-HO-TAM, $Z$-In, and $E$-In. with respective retention times of 11.3 min., 12.1 min., 14.8 min., and 15.3 min. The spectra of conversion of the $\alpha$-HO-TAM to indenes shown in figure 3.7 indicate an isosbestic point at 260 nm. By operating the HPLC at that wavelength, we were able to make the assumption that relative peak areas were in fact relative molar amounts. This was verified for $E$-HO-TAM and $Z$-HO-TAM by injecting known amounts of authentic alcohols. Furthermore, the sum of the areas of the four peaks was found to remain constant throughout the reaction to within 3%.

Based on the assumption that all four species have the same HPLC sensitivity at 260 nm, figures 3.10A and 3.10C were constructed. These plot the fractions of each of the four species as a function of time in 0.02 HCl, 0.08M NaCl (22 °C) starting with $Z$-HO-TAM (figure 3.10A) and $E$-HO-TAM (figure 3.10C). The two alcohols are seen to interconvert to an equilibrium position where the $[E$-HO-TAM]: $[Z$-HO-TAM] ratio is
slightly greater than unity regardless of the starting alcohol. Superimposed on this is the conversion of the alcohols to the indenes. This is a slower process that yields a constant [Z-In]: [E-In] ratio that is also independent of the starting alcohol.

Figure 3.10. HPLC analysis of solutions of Z-HO-TAM (A and B) and E-HO-TAM (C and D) in 0.02 M HCl, 0.08 M NaCl (22 °C). Legend for Figs. A, C: (■) – [Z-HO-TAM] : [Total], (□) – [E-HO-TAM] : [Total], (○) – [Z-In] : [Total], (●) – [E-In] : [Total], where [Total] = [Z-HO-TAM] + [E-HO-TAM] + [Z-In] + [E-In]. Legend for Figs. B, D: (△) – [Z-HO-TAM]/([E-HO-TAM] + [Z-HO-TAM]), (▲) – [E-HO-TAM]/([E-HO-TAM] + [Z-HO-TAM]), (□) – ([E-HO-TAM] + [Z-HO-TAM]) : [Total]. The points are experimental. The lines are based upon fitting the data to a double exponential or single exponential (see table 3.2).
The results of the kinetic analysis of the data in figures 3.10A and 3.9C are provided in table 3.3. Satisfactory fits are obtained when the data for the E- and Z-HO-TAMS are fit to a double exponential equation, and the data for the increases in the indenes are fit to a single exponential. Not surprisingly, the rate constants of the latter are the same as those associated with the slower process of the double exponential fits. The same rate constants are obtained whether starting with Z-HO-TAM or E-HO-TAM within experimental uncertainty. Also noteworthy is the observation that the slower rate constant from the HPLC data is, within experimental error, the same as the single rate constant obtained from the exponential rise in the UV spectra, under the same experimental conditions (i.e. acid concentration). This can be explained by the fact that the interconversion of the two alcohols is invisible in the UV due to their identical spectra. Therefore, only the irreversible process resulting in formation of the indene products is observed via a spectral change.
Table 3.3. Kinetic analyses of HPLC chromatograms of figure 10 obtained with Z-HO-TAM and E-HO-TAM in 0.02 M HCl, 0.08 M NaCl at 22 °C.

<table>
<thead>
<tr>
<th>Constant</th>
<th>Start with Z-HO-TAM</th>
<th>Start with E-HO-TAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double exponential decay of starting alcohol</td>
<td>$k_1 \ 2.57 \times 10^4$ s$^{-1}$</td>
<td>$k_1 \ 2.33 \times 10^4$ s$^{-1}$</td>
</tr>
<tr>
<td>Double exponential rise and fall of isomer</td>
<td>$k_1 \ 2.61 \times 10^4$ s$^{-1}$</td>
<td>$k_1 \ 2.40 \times 10^4$ s$^{-1}$</td>
</tr>
<tr>
<td>Single exponential rise of major indene product</td>
<td>$1.88 \times 10^5$ s$^{-1}$</td>
<td>$2.11 \times 10^5$ s$^{-1}$</td>
</tr>
<tr>
<td>Single exponential rise of minor indene product</td>
<td>$1.87 \times 10^5$ s$^{-1}$</td>
<td>$2.07 \times 10^5$ s$^{-1}$</td>
</tr>
<tr>
<td>Ratio of major indene to minor indene</td>
<td>$1.86 \pm 0.05$</td>
<td>$1.83 \pm 0.04$</td>
</tr>
<tr>
<td>Single exponential approach to equilibrium - $k_{iso}$</td>
<td>$2.37 \times 10^4$ s$^{-1}$</td>
<td>$2.13 \times 10^4$ s$^{-1}$</td>
</tr>
<tr>
<td>$[E-HO-TAM]/([E-HO-TAM] + [Z-HO-TAM])$</td>
<td>$[E-HO-TAM]/([E-HO-TAM] + [Z-HO-TAM])$ at equilibrium</td>
<td></td>
</tr>
<tr>
<td>$[E-HO-TAM]/([E-HO-TAM] + [Z-HO-TAM]) + Z-HO-TAM]$:total - $k_{cyc}$</td>
<td>$1.89 \times 10^5$ s$^{-1}$</td>
<td>$2.12 \times 10^5$ s$^{-1}$</td>
</tr>
</tbody>
</table>

$^a$Fit to $A_1 \cdot \exp(-k_1t) + A_2 \cdot \exp(-k_2t)$ with $A_1 + A_2 = 1$. (Forced to begin at one and end at zero). $^b$Fit to $A_1 \cdot \{\exp(-k_1t) - \exp(-k_2t)\}$. (Forced to begin and end at zero). $^c$Fit to $(-A_1) \cdot \exp(-kt) + A_1$. (Forced to begin at zero). $^d$Averaged over all the points. $^e$Fit to eq 12. (Forced to begin at zero when Z-HO-TAM was starting material and to begin at one when E-HO-TAM was starting material. $^f(k2:(k1+k2))$ of eq 12. Fit to eq 13. (Forced to begin at one and end at zero).
3.5.2 Kinetic Analysis

Scheme 3.4 illustrates the proposed kinetic model. Note that there are two intermediate carbocations $E$-TAM$^+$ and $Z$-TAM$^+$ in equilibrium. Each reacts with water to form the corresponding alcohol, or cyclizes to the indene, the competing irreversible reaction. Associated with these reactions are four first order rate constants $k_w(E)$, $k_{cyc}(E)$, $k_w(Z)$, and $k_{cyc}(Z)$. The alcohols form the corresponding carbocations via the acid-catalyzed reaction with second order rate constants defined as $k_{H}(E)[H^+]$ and $k_{H}(Z)[H^+]$. 

Scheme 3.4
When solvolysis of the TAM ester (acetate or sulfate) is carried out in solutions of very low acid concentration, the reaction is under kinetic control. The same TAM$^+$ intermediates are formed, however, the resultant alcohol products are stable as they do not become protonated to generate the water leaving group (and as has been noted, OH$^-$ is a very poor leaving group). An important observation from such experiments is that regardless of whether the starting ester is (E) or (Z), the alcohols are produced in the same 2.2:1 ratio in favour of $E$-HO-TAM. The implication is that $E$-TAM$^+$ and $Z$-TAM$^+$ achieve equilibrium within their lifetime. Laser flash photolysis experiments (see chapter 4) have shown the lifetime of the equilibrating cations to be 40 $\mu$s in aqueous acid. Thus, the (E) – (Z) carbocation interconversion must be very rapid.

The following four equations can be written for the fraction of each product obtained for the solvolysis reactions under kinetic control. Numerical values of these fractions were provided from TAM ester solvolysis experiments conducted in very dilute acid solution (where the side chain amine is protonated). It can be noted that the ratio [Z-In] : [E-In] is 1.75 ± 0.2, the same as the value obtained (within experimental error) when they are formed from the alcohol starting material in more acidic solutions.

\[
Fr(E - HO - TAM) = \frac{k_w(E)}{k_w(E) + k_w(Z)K^+ + k_{cyc}(Z)K^+ + k_{cy}(E)} = 0.640 \pm 0.005 \quad \text{Eq 3.3}
\]

\[
Fr(Z - HO - TAM) = \frac{k_w(Z)K^+}{k_w(E) + k_w(Z)K^+ + k_{cyc}(Z)K^+ + k_{cy}(E)} = 0.290 \pm 0.003 \quad \text{Eq 3.4}
\]

\[
Fr(E - In) = \frac{k_{cy}(E)}{k_w(E) + k_w(Z)K^+ + k_{cyc}(Z)K^+ + k_{cy}(E)} = 0.025 \pm 0.002 \quad \text{Eq 3.5}
\]
\[ Fr(Z - In) = \left( \frac{k_{\text{cyc}}(Z)K^*}{k_w(E) + k_w(Z)K^* + k_{\text{cyc}}(Z)K^* + k_{\text{cyc}}(E)} \right) = 0.045 \pm 0.002 \]  

Eq 3.6

To analyze the cis-trans isomerization experiments with one of the alcohols as starting material in high acid concentration, we calculate the steady state concentration of the two carbocations assuming them to be in equilibrium. In order to derive the three differential equations 3.7 - 3.9, another simplification is made. The two indene isomers are treated as one by setting \([\text{In}] = [E\text{-In}] + [Z\text{-In}]\) and \(Fr(\text{In}) = (Fr(E\text{-In}) + Fr(Z\text{-In}))\).

\[
\frac{d[E - HO - TAM]}{dt} = k_1[Z - HO - TAM] - (k_1 + k_2)[E - HO - TAM] \tag{Eq 3.7}
\]

\[
\frac{d[Z - HO - TAM]}{dt} = k_1[E - HO - TAM] - (k_2 + k_4)[Z - HO - TAM] \tag{Eq 3.8}
\]

\[
\frac{d[\text{In}]}{dt} = k_3[E - HO - TAM] + k_4[Z - HO - TAM] \tag{Eq 3.9}
\]

where

\[
k_1 = Fr(Z\text{-HO-TAM}) \cdot k_w(E)[H^+] \tag{Eq 3.10}
\]

\[
k_2 = Fr(E\text{-HO-TAM}) \cdot k_w(Z)[H^+] \tag{Eq 3.11}
\]

\[
k_3 = Fr(\text{In}) \cdot k_w(E)[H^+] \tag{Eq 3.12}
\]

\[
k_4 = Fr(\text{In}) \cdot k_w(Z)[H^+] \tag{Eq 3.13}
\]
The simplified model shown in scheme 3.5 illustrates how E-HO-TAM and Z-HO-TAM interconvert with rate constants $k_1$ and $k_2$, and react irreversibly to form the indenes with rate constants $k_3$ and $k_4$. It is evident from our HPLC data (Fig. 3.9) that the alcohols equilibrate approximately 10 times faster than they cyclize to form the indenes. Therefore, the approximation $k_1, k_2 \gg k_3, k_4$ is made. This results in equations 3.14 and 3.15 representing the rapid kinetic phase where alcohols equilibrate and the slow kinetic phase where they react irreversibly to form indenes, respectively.

\[ \frac{[E-HO-TAM]}{[E-HO-TAM]+[Z-HO-TAM]} = Ae^{-k_{(isom)}t} + \left( \frac{k_2}{k_1+k_2} \right) \]  

Eq 3.14

where $k_{(isom)} = (k_1+k_2)$, and $A = k_1:(k_1+k_2)$ or $-k_2:(k_1+k_2)$ for the cases where E-HO-TAM and Z-HO-TAM are the starting materials, respectively.

\[ \frac{[E-HO-TAM]+[Z-HO-TAM]}{[E-HO-TAM]+[Z-HO-TAM]+[In]} = e^{-k_{(cyc)}t} \]  

Eq 3.15

where $k_{(cyc)} = (k_2k_3+k_1k_4)/(k_1+k_2)$
The resultant values $k_{(isom)}$ and $k_{(cyc)}$ calculated from these two single exponential equations correspond nicely to the rate constants determined from the standard fits to the HPLC data, as shown in table 3.2.

In order to determine the second-order rate constants $k_{H(E)}$ and $k_{H(Z)}$, HPLC experiments were conducted at different acid concentrations. Both $k_{(isom)}$ and $k_{(cyc)}$ were found to be proportional to acid concentration. Equations 3.16 and 3.18 give the average rate constants by dividing by $[H^+]$. For the isomerization process, the following relations apply:

\[
\left( \frac{k_{(isom)}}{[H^+]} \right) = \frac{k_1 + k_2}{[H^+]} = (1.16 \pm 0.06) \times 10^{-2} M^{-1}s^{-1} \quad \text{Eq 3.16}
\]

\[
= Fr(Z-HO-TAM) \cdot k_{H(E)} + Fr(E-HO-TAM) \cdot k_{H(Z)}
\]

Assuming equilibrium is established between the two alcohols, the fit to equation 3.14 also provides the fraction of $E$-HO-TAM.

\[
\left( \frac{k_2}{k_1 + k_2} \right) = 0.535 = \left( \frac{[E-HO-TAM]_{eq}}{[E-HO-TAM]_{eq} + [Z-HO-TAM]_{eq}} \right) \quad \text{Eq 3.17}
\]

\[
= \left( \frac{Fr(E-HO-TAM) \cdot k_{H(Z)}}{Fr(Z-HO-TAM) \cdot k_{H}(E) + Fr(E-HO-TAM) \cdot k_{H}(Z)} \right)
\]
The second-order rate constants can then be determined by inputting the values of $\text{Fr}(Z\text{-HO-TAM})$ and $\text{Fr}(E\text{-HO-TAM})$ and subjecting the equation to some algebraic manipulation:

$$k_H(E) = 2.14 \times 10^{-2} \text{ M}^{-1}\text{s}^{-1}$$

$$k_H(Z) = 8.42 \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$$

We can now explain the apparent discrepancy between the $E\text{-HO-TAM}: Z\text{-HO-TAM}$ ratios from kinetically-controlled experiments (TAM ester hydrolysis in low acid, where it is $\sim2.2:1$) and from the cis-trans isomerization experiments (in high acid, where it is $\sim1:1$). $E\text{-HO-TAM}$ forms from the equilibrating TAM carbocations with a slightly greater than 2:1 preference, but it also undergoes acid-catalyzed ionization over 2-fold faster. As a result, the ratio of the two alcohols at equilibrium conditions, where all reactions are reversible, is close to unity.

Finally, for the cyclization process,

$$\left( \frac{k_{(\text{cyc})}}{[H^+]} \right) = (1.01 \pm 0.04) \times 10^{-3} \text{ M}^{-1}\text{s}^{-1} = \text{Fr(In)} \left( k_H(E) \left( \frac{k_2}{k_1 + k_2} \right) + k_H(Z) \left( \frac{k_1}{k_1 + k_2} \right) \right)$$  \[\text{Eq 3.18}\]

where the last term is derived by substituting $k_{(\text{cyc})}$ from equation 3.15, and $k_3$ and $k_4$ from equations 3.12 and 3.13. $\text{Fr(In)}$ can be calculated by inputting all the numerical values which are known from the isomerization kinetics. Substitution gives $\text{Fr(In)} = 0.066$. This is in excellent agreement with the value of 0.070 obtained from the sum of $\text{Fr}(E\text{-In})$ and $\text{Fr}(Z\text{-In})$ from the HPLC product analysis in the acetate solvolysis.
3.6 Summary

These experiments show indirectly that the tamoxifen ester undergoes $S_N1$ solvolysis via the carbocation intermediate. In addition to being trapped by water to form the alcohol isomers, the equilibrating TAM carbocations also undergo intramolecular cyclization to form indene products. HPLC product analysis under kinetic control has shown these products to represent 7% of the fate of the TAM cation. Laser-flash photolysis experiments have determined the rate of decay of the cation under acidic aqueous conditions to be $2.5 \times 10^4 \text{ s}^{-1}$ (see chapter 4), therefore the rate constant for cyclization must be $1.8 \times 10^3 \text{ s}^{-1}$. This reaction represents an irreversible process that results in removal of a molecule of water, forming products that are stable under aqueous acid conditions.

The difference in the $E$-HO-TAM: $Z$-HO-TAM ratios seen in the TAM ester hydrolysis experiments (~2.2: 1) as compared to the cis-trans alcohol isomerization experiments (~1: 1) can be explained as follows. Under equilibrium conditions (high acid-concentration), both the rates of $E$-HO-TAM formation from the cation and the reverse reaction, $H^+$-catalyzed ionization to the cation, occur 2-fold faster than with the corresponding $Z$-HO-TAM. Under equilibrium conditions, the consequence is that the ratio of the two alcohols approaches unity. Under kinetic conditions, the alcohols are relatively stable once formed (in the time it takes the TAM ester to hydrolyze completely), and are therefore present in the ratios that reflect the relative rates of $k_w$ (or the equilibrium position for the 2 cations).

Due to the fact that the indenes are produced at neutral pH during the solvolysis of the TAM esters, observation of these indenes could be used as an indicator for the
formation of the TAM carbocation (via the mechanism shown in schemes 1.5 and 1.6) in cellular systems, and even in vivo. The indenes have sufficiently different UV-visible spectra and retention times on reversed-phase HPLC from the TAM-ester substrate and TAM alcohols, that their detection is quite favourable. Their formation in cells and in vivo would provide unambiguous proof of the formation of the tamoxifen carbocation, and the $S_N$1 precursor to this cation.
Chapter Four:

Direct Observation of the Tamoxifen Carbocation
Chapter 4: Direct Observation of the Tamoxifen Carbocation

4.1 Introduction

Once it became evident from our ground state solvolysis experiments that substitution of the sulfate of α-SO₄²⁻-TAM occurs via S₉1 (chapter 3), we set out to characterize the carbocation intermediate. Direct observation of this short-lived intermediate was made possible by using the technique of nanosecond laser flash photolysis (LFP). LFP allows one to establish the existence of a particular intermediate and to acquire direct information about the kinetics of its further reactions. Comparison of our LFP results with those obtained under ground state conditions (chapter 3) were easily made as the experimental conditions were virtually identical.

Preliminary flashing of TAM-sulfate (and TAM-acetate) confirmed it to be a suitable substrate for LFP studies. The three general requirements were met for this technique to be successful for the study of the cationic intermediate.⁶⁴ (1) The TAM-ester readily undergoes a photochemical reaction to generate the carbocation. (2) The transient generated upon flashing was ‘observable’ by an absorbance change in the UV-visible spectrum. (3) The transient lifetime was easily detected in the microsecond timeframe of the LFP set-up. The initial experiments of transient detection, characterization, and its identification as the tamoxifen-carbocation (TAM⁺) are the subject of this chapter, while the following chapter elaborates further on the reactivity of the allylic cation towards a number of biologically relevant nucleophiles.

Once again, experiments began with the α-sulfate-TAM, and were later conducted with the α-acetoxy-TAM. Both substrates generated the identical transient,
however most experiments utilized α-acetoxy-TAM as the precursor due to its ease of handling and greater stability in the ground state in aqueous solutions.
4.2 Photochemical Generation of the Transient

LFP of a solution of the TAM-ester in either 100% aqueous buffer or a mixture of water and acetonitrile produced a single decaying transient. Substrate concentration varied between 15-110 μM, depending on the solvent system and the wavelength of the actinic flash, either 248 or 308 nm. The transient, believed to correspond to the formation of the TAM$^+$ intermediate (and later confirmed as such, section 4.5), was observed as a very sharp rise followed by a slower decay. Figure 4.1 shows a representative trace. The formation of the transient species occurred within the 20 ns laser pulse, while the decay occurred on the order of microseconds. It can be noted that there was a small residual absorbance upon completion of the decay. The nature of the species responsible has not been identified.

Figure 4.1. A typical transient observed upon LFP of 25 μM α-acetoxy-TAM in 100% aqueous phosphate buffer, pH 5.9, monitoring at 450 nm.
The rate constant for the decay of the intermediate formed can be accurately determined by fitting the decay portion of the curve to the first-order exponential decay equation:

\[ A_t = (A_0 - A_\infty)e^{-k_{\text{obs}}t} + A_\infty \]  

Eq 4.1

where \( A_0 \) and \( A_\infty \) are the absorbances immediately after the flash (maximal absorbance) and at infinity respectively, for the trace. Excellent fits were always obtained. The rate of decay of the transient corresponds to the rate of trapping of the intermediate by water or some other nucleophilic species.
4.3 Spectrum of the Transient

Transient spectra were obtained from both of the tamoxifen ester precursors, α-sulfate-TAM and α-acetoxy-TAM. Formation of the transient was investigated over a wavelength range of 330 – 550 nm, at 5 – 10 nm increments depending on the wavelength region. The absorbance of the intermediate at various times after the laser pulse was determined for each increment. These absorbances were plotted against wavelength in order to obtain the spectrum of the transient. Figure 4.2 shows the spectrum corresponding to the α-acetoxy-TAM precursor. This plot was found to be identical to the one generated from the α-sulfate-TAM precursor. The spectrum is a simple one with a single broad $\lambda_{\text{max}}$ between 450 – 460 nm. The spectrum is similar to that of 4-MeOC$_6$H$_4$PhCH+, whose $\lambda_{\text{max}}$ is 455 nm. As a result, the LFP kinetic experiments were conducted at a monitoring wavelength of 450 nm. As is seen with the transient decay trace, there is a residual absorbance in the spectrum.
Figure 4.2. Transient spectrum for the intermediate derived from α-acetoxy-TAM in acetate buffer (0.0025 M, 1: 1), 40% CH$_3$CN.

Ideally, the tamoxifen carbocation could be produced under 'stable ion' conditions, where the cation could then be characterized by NMR. A match of its UV-visible spectrum with the one shown in figure 4.2 would provide excellent evidence that the transient spectrum does in fact correspond to the carbocation. Unfortunately, attempts to observe the tamoxifen carbocation under super-acid conditions have been unsuccessful due to cyclization and subsequent destruction of the α-HO-TAM substrate. This complication may well be averted by conducting the experiment at very low temperatures, and is currently under investigation. Nevertheless, there is compelling
evidence that the transient is the tamoxifen carbocation represented in scheme 3.2. The kinetic evidence for this is discussed in sections 4.4 and 4.5. Some of the other possibilities that can be ruled out are discussed below.

One of the possibilities is a radical cation derived by one electron oxidation of the triarylethylene chromophore. Such a photoionization is known to occur with laser spectroscopy in some cases. However, this can be ruled out since the transient spectrum is not correct for this type of radical cation. Majima and coworkers obtained the spectra for the radical-cations of a number of stilbene derivatives. In all cases the $\lambda_{\text{max}}$ is red-shifted compared to the TAM transient spectrum. More importantly, the radical-cation $\lambda_{\text{max}}$ peak is very narrow and sharp. Furthermore, there is no transient generated upon LFP of $\alpha$-HO-TAM. This rules out the radical-cation, as it would be generated regardless of the substituent at the $\alpha$-position.

![Scheme 4.1](image)

The other possible cation that could form arises by photoprotonation of the C=C bond of the substrate. This reaction has been observed with 1-phenyl-1-$p$-methoxyphenylethane. There are two good reasons to dismiss formation of this cation. (1) Protonation of this type is not normally observed in water, but rather in more acidic
solutions such as 1,1,1-trifluoroethanol. (2) More importantly, no transient is formed when flashing the hydrolysis product, α-hydroxy-TAM substrate. In other words, a transient is only formed by the acetate or sulfate esters. Just as with the formation of a radical-cation, photoprotonation is expected to occur equally with the ester or alcohol substrate.

Scheme 4.2

It can also be noted that the transient is not quenched by oxygen. This rules out triplet excited states and free-radicals.
4.4 Rate-pH Profile of the Transient

LFP experiments were carried out with α-acetoxyl-TAM as the precursor in various buffer solutions. The ionic strength was maintained at 0.1 M by adding appropriate amounts of sodium perchlorate. Rate constants ($k_{obs}$) were determined at various measured pHs, and a graph of log $k_{obs}$ versus pH was generated. Buffer dilution plots were conducted for all of the solutions, and $k_{obs}$ was extrapolated to zero buffer concentration to eliminate any potential rate acceleration due to buffer catalysis. The buffers did have a small effect, but this was not investigated in detail. Figure 4.3 illustrates a rate-pH profile with the open triangles representing actual data points, and the curve representing a fit of the data to equation 4.2.

$$k_{obs} = \frac{k_w^2[H^+] + k_w^-K_a + k_{OH}^-K_a[OH^-]}{[H^+] + K_a}$$  \[Eq 4.2\]
Figure 4.3. Rate-pH profile for transient generated upon LFP of α-acetoxy-TAM in various buffer solutions, I = 0.1 M, monitoring at 450 nm.

The graph has four distinct regions, which on the basis of scheme 4.3, can be explained as follows. Region (1) arises from the tamoxifen dication reacting with water. In region (2) the acid-base equilibrium has shifted to the monocation, but the reaction is still occurring by the more reactive dication, while region (3) arises from the monocation reacting with water. Finally, region (4) arises from the monocation reacting with the strongly nucleophilic hydroxide anion, OH⁻.
The pK$_a$ of the tamoxifen carbocation can be easily calculated by taking the negative logarithm of K$_a$ from equation 4.2. Under the experimental conditions of 100% aqueous and constant ionic strength of $\mu = 0.1$ M, pK$_a$ was determined to be 7.9. This is an order of magnitude lower than the parent drug's pK$_a$ of 8.85. Clearly, the positive charge of the allylic cation has the effect of lowering the pK$_a$ of the amine. In addition to an inductive effect, another possible explanation stems from a resonance contributor of the carbocation where the oxygen bears the positive charge, illustrated in scheme 4.4. As such, the oxygen is no longer able to stabilize the proton through a hydrogen-bonded five-membered ring that may well contribute to its stabilization in the parent compound.
The other two important values that can be obtained from the rate-pH profile relate to $k_w^{-2}$ and $k_w^+$, from regions 1 and 3 respectively of figure 4.3. Limiting lifetimes $(1/k_{obs})$ are 40 μs in region 1 where the side chain amine is protonated, and 160 μs in region 3 where the amine is a free base. These carbocations are indeed relatively long-lived, at least on the scale of other SN1 intermediates. For example, a simple tertiary carbocation has a lifetime of under 1 ps,$^{80}$ while the diphenylmethylcation (Ph$_2$CH$^+$) has a lifetime of 700 ps.$^{81}$ Substitution of a methoxy group on the para position of one of the phenyIs (i.e. 4-MeOC$_6$H$_4$PhCH$^+$) increases the lifetime to 500 ns.$^{76}$ Nevertheless, this is still much shorter than the lifetime of the tamoxifen carbocation.
4.4.1 Solvent and Ionic-Strength Dependence

Several rate-pH profile experiments were conducted with both TAM-ester substrates, where solvent and ionic strength ($\mu$) were varied. In all cases, the plots were similar; however, the $pK_a$ did vary by a small margin. The results are as follows:

- 100% Aqueous, $\mu = 0.1$ M; $pK_a = 7.9$
- 100% Aqueous, no constant $\mu$; $pK_a = 7.3$
- 80% H$_2$O, 20% CH$_3$CN, no constant $\mu$; $pK_a = 7.0$
- 60% H$_2$O, 40% CH$_3$CN, $\mu = 0.1$ M; $pK_a = 7.7$
- 60% H$_2$O, 40% CH$_3$CN, no constant $\mu$; $pK_a = 6.6$

The two 'trends' that appear in this data are: (1) the $pK_a$ of the protonated amine decreases as the solvent decreases in polarity and (2) the $pK_a$ decreases in the absence of added salt. Both of these trends are best explained by the fact that the ionization is occurring between a charged acid (protonated amine) and a neutral conjugate base (free amine). A polar solvent with added salt would be expected to stabilize the acid and hence increase the $pK_a$. This explanation is purely hypothetical, and these 'trends' were not explored further, as the determination of the $pK_a$ of the tamoxifen carbocation in 100% aqueous conditions at constant ionic-strength (and comparison with the $pK_a$ of the parent drug) was the main objective in this study.
4.5 Azide Quenching Studies

4.5.1 Introduction

As was discussed in the introduction (section 1.3.1.3), the LFP-generated transient can be identified as the carbocation by determining rate constant ratios. This method combines the competition kinetics method by product analysis with flash photolysis-derived rate constants. The ground state selectivity ratio, \( k_{\text{Nu}} : k_{\text{solvent}} \), can be determined via the quantification of the trapped products resulting from varying concentrations of nucleophile. Strong evidence that the transient is in fact the tamoxifen carbocation is then provided if this ground-state-derived value agrees with the ratio of the two corresponding rate constants measured directly by flash photolysis.

\[
\text{R-OSO}_3^- \xrightarrow{k_{\text{ion}}} \text{R}^+ \xrightarrow{k_w} \text{Z-alcohol + E-alcohol} \quad \xrightarrow{k_{\text{az}[N_3^-]}} \text{Z-azole} + \text{E-azole}
\]

(Ar: N3)

Azide ion is a very effective trap for reactive carbocations, reacting with these species with rate constants that are either at the diffusion limit, or at least approaching the diffusion limit, i.e., approaching \(5-10 \times 10^9 \text{ M}^{-1}\text{s}^{-1}\). The bimolecular rate constants do become slower when the cation becomes highly stabilized. From the previous section, we have seen that the tamoxifen carbocation has a water rate constant of \(6.25 \times 10^3 \text{ s}^{-1}\) to
2.5 \times 10^4 \text{ s}^{-1}, \text{ depending on the protonation state of the side chain amine. Experiences with other cations suggests that at these rates, we are approaching the point where the behaviour is changing.}^{64} \text{ The reaction with azide ion should still be very fast, although perhaps not quite at the diffusion limit.}

4.5.2 Laser-Flash Photolysis Studies

In order to determine the exact rate constant for trapping of the tamoxifen carbocation by the azide ion, the following LFP experiment was conducted. A series of solutions were prepared containing 60% aqueous carbonate buffer (0.0025 M; 11.5: 1 acid, pH 9.8) and 40% acetonitrile with an overall concentration of 0, 0.2, 0.4, 0.6, 0.8, and 1 mM NaN₃. To these solutions was added 50 \mu M \alpha\text{-sulfate-TAM immediately prior to flashing. The transient generated by LFP was quenched by sodium azide, as the first-order rate constant for its decay was found to increase in a linear fashion with azide concentration. Table 4.1 lists the first order rate constants measured for each of the six solutions. Each rate constant is taken as the average of 5 – 7 laser-flash shots (one shot per fresh solution). The bimolecular rate constant for azide is determined by taking the slope of the linear plot of } k_{obs} \text{ versus } [N_3^-], \text{ shown in figure 4.4. For this experiment, } k_w = 7.83 \times 10^3 \text{ s}^{-1} \text{ and } k_{azide} = 3.48 \times 10^8 \text{ M}^{-1}\text{s}^{-1}. \text{ Therefore, azide ion is an effective quencher of the tamoxifen transient, reacting with a rate constant about an order of magnitude below diffusion control.}
Table 4.1. Average rate constant (s\(^{-1}\)) as a function of azide concentration (mM) in 60% aqueous carbonate buffer (0.0025 M; 11.5: 1 acid, pH 9.8) and 40% CH\(_3\)CN. Data points are plotted in figure 4.4.

<table>
<thead>
<tr>
<th>Concentration of NaN(_3) (mM)</th>
<th>Average Rate Constant (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(7.83 \times 10^4 \pm 2.74 \times 10^2)</td>
</tr>
<tr>
<td>0.2</td>
<td>(8.17 \times 10^4 \pm 3.67 \times 10^3)</td>
</tr>
<tr>
<td>0.4</td>
<td>(1.56 \times 10^5 \pm 5.23 \times 10^3)</td>
</tr>
<tr>
<td>0.6</td>
<td>(2.30 \times 10^5 \pm 7.72 \times 10^3)</td>
</tr>
<tr>
<td>0.8</td>
<td>(2.94 \times 10^5 \pm 1.54 \times 10^4)</td>
</tr>
<tr>
<td>1</td>
<td>(3.56 \times 10^5 \pm 2.72 \times 10^4)</td>
</tr>
</tbody>
</table>

Figure 4.4. Plot of \(k_{\text{obs}}\) versus [NaN\(_3\)] for the transient generated upon LFP of \(\alpha\)-sulfate-TAM. Conditions: 50 \(\mu\)M substrate in 60% carbonate buffer (0.0025 M; 11.5: 1 acid), 40% CH\(_3\)CN, monitoring at 450 nm.
Most important is the flash photolysis ratio, \( k_{\text{azide}}: k_w \) of \( 4.45 \times 10^4 \text{ M}^{-1} \), calculated using the two directly measured rate constants. This value must now be compared to the ratio determined by conducting an HPLC product analysis for the ground state solvolysis under the exact same experimental conditions.

### 4.5.3 HPLC-Ground State Solvolysis Studies

\( \alpha \)-Sulfate-TAM (100 \( \mu \text{M} \)) was solvolyzed in 60% aqueous carbonate buffer (0.0025 M; 11.5:1 acid, pH 9.8) and 40% acetonitrile with 0, 0.1, 0.2, and 0.4 mM NaN\(_3\). Concentrations of azide ion were kept lower than for the LFP experiment so that all four products would be present in a measurable amount. The rate of solvolysis of the sulfate was unaffected by the increasing concentrations of azide. However, two new peaks at considerably longer retention times (12.5 min. and 14 min.) replaced the alcohols (6.6 min. and 7.2 min.) with increasing azide concentration. Synthesis and characterization of authentic products (section 2.1.5) later confirmed these two new peaks as the cis-trans azide-adduct isomers (16). The HPLC retention times are different from those of chapter 3 because this experiment was conducted under isocratic conditions (85% CH\(_3\)CN, 15% 1mM HClO\(_4\), at a flow rate of 2 ml/min).

Product analysis and determination of the \( k_{\text{azide}}: k_w \) ratio was done by quantifying the yield of alcohols at the varying azide concentrations in the following way. The fraction of alcohol products of the total solvolysis/azide-adduct products can be expressed as the ratio of the corresponding rates of trapping.

\[
Fr(ROH) = \frac{[ROH]}{[ROH] + [RN_3^-]} = \frac{ROH_{\text{area}}(\text{with} N_3^-)}{ROH_{\text{area}}(\text{no} N_3^-)} = \frac{k_w}{k_w + k_{\text{az}}[N_3^-]}
\]

**Eq 4.4**
Then by taking the reciprocal of the last two terms:

\[
\frac{ROH_{area\ (noN_3^-)}}{ROH_{area\ (withN_3^-)}} = 1 + \frac{k_{az}[N_3^-]}{k_w}
\]  

Eq 4.5

By subjecting equation 4.5 to some minor algebraic manipulation, equation 4.6 is derived, which allows us to calculate the \( k_{az} : k_w \) ratio easily from the alcohol peak areas alone.

\[
\frac{\left( \frac{ROH_{area\ (noN_3^-)}}{ROH_{area\ (withN_3^-)}} - 1 \right)}{[N_3^-]} = \frac{k_{az}}{k_w}
\]  

Eq 4.6

Alcohol peak areas were taken as the average of three separate injections of the same solution, for each of the azide concentrations. The results are summarized in table 4.2.
Table 4.2. Total α-HO-TAM area as a function of azide concentration in 60% aqueous carbonate buffer (0.0025 M; 11.5: 1 acid, pH 9.8) and 40% CH₃CN. Calculated corresponding kₐzide: kₚ ratios appear in the right column.

<table>
<thead>
<tr>
<th>[N₃⁻] (M)</th>
<th>Total ROH_area</th>
<th>1ˢᵗ Term of Eq 4.6 (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>41224</td>
<td></td>
</tr>
<tr>
<td>1 x 10⁻⁴</td>
<td>8282</td>
<td>3.98 x 10⁴</td>
</tr>
<tr>
<td>2 x 10⁻⁴</td>
<td>4218</td>
<td>4.39 x 10⁴</td>
</tr>
<tr>
<td>4 x 10⁻⁴</td>
<td>2067</td>
<td>4.74 x 10⁴</td>
</tr>
</tbody>
</table>

Average = 4.4 x 10⁴ M⁻¹

The average value of 4.4 x 10⁴ M⁻¹ for the kₐzide: kₚ ratio is in excellent agreement with the directly measured ratio of 4.45 x 10⁴ M⁻¹ from our LFP study. This is proof positive that the laser-flash photolysis transient is the same carbocation as the one formed in the ground state ionization, as shown in equation 4.3.
4.6 Summary

The main objective of the studies discussed in this chapter was to identify the LFP transient as the tamoxifen carbocation illustrated in scheme 3.2. Initial work using α-sulfate-TAM (and later α-acetoxy-TAM) was encouraging as the TAM ester substrates were found to produce an observable transient in the micro-millisecond time scale. The traces decayed in a first-order fashion, with corresponding rates easily obtained from equation 4.1. The transient spectrum, generated by measuring ΔOD (at a given time interval) as a function of monitoring wavelength, was relatively simple with a λ_{max} at ~450 nm. Furthermore, the spectrum was characteristic of a carbocation and not the possible radical cation. Unfortunately, the LFP-derived spectrum has not been compared to the ‘stable-ion’-derived spectrum, as characterization of the tamoxifen carbocation by NMR has yet to be performed.

Nevertheless, two other methods of transient characterization have been completed. The first of these involved measuring rate-pH profile of the intermediate. Four distinct regions were observed that easily fit our model shown in scheme 4.2. The rates of decay were 2.5 x 10^4 s^{-1} (in the acidic region where the side chain amine is protonated) and 6.25 x 10^3 s^{-1} (in the basic region where the side chain amine is a free base), with respective lifetimes of 40 μs and 160 μs. This represents a relatively long-lived cation on the time scale of other SN1 intermediates. The measured pK_a of 7.9 for the intermediate is an order of magnitude lower than that of the parent drug, providing very compelling evidence that the transient is a carbocation. Clearly, the positive charge is exerting an influence on the ability of the amine to donate its electrons to a proton. However the exact mechanism of this influence is not understood. Presumably, the
cation must be distributed in such a manner as to affect the side chain amine, which originates ten atoms away.

Finally, there is conclusive evidence that the transient generated by laser flash photolysis is the exact same carbocation as the one formed in the ground state ionization, illustrated in equation 4.3. HPLC product studies determined the $k_{\text{azide}} : k_w$ ratio to be $4.4 \times 10^4 \text{ M}^{-1}$, while direct measurement of the rates by LFP yielded a $k_{\text{azide}} : k_w$ ratio of $4.45 \times 10^4 \text{ M}^{-1}$. The close agreement of these two distinctly measured ratios is seen as proof positive for the above assertion.
Chapter Five:

Quenching of the Tamoxifen Carbocation by Endogenous Nucleophiles
Chapter 5: Quenching of the Tamoxifen Carbocation by Endogenous Nucleophiles

5.1 Introduction

Working on the basis that the LFP-generated transient is in fact the same tamoxifen carbocation generated during ground state solvolysis, a series of experiments were then conducted to investigate the nature of trapping by various biologically relevant nucleophiles. As was discussed in section 1.2.4.4, tamoxifen-DNA adducts have been isolated and characterized from animal studies,\textsuperscript{38,54,55} and more recently from the leukocytes and endometrium of women being treated with the drug.\textsuperscript{71,72,82} Characterization of these TAM-DNA adducts indicate the major adduct as the N\textsuperscript{2}-deoxyguanosine (dG)-TAM product (present as diastereomers), with some minor N\textsuperscript{6}-deoxyadenosine (dA)-TAM adduct formation.\textsuperscript{47,56} Nevertheless, the nature of the reaction between these nucleotides (namely dG) and TAM remains poorly understood.

The objective of the work discussed in this chapter is to measure the bimolecular rate constant for a number important endogenous nucleophiles (glutathione, deoxyguanosine, DNA, and a 14-nucleotide oligomer as a DNA model). These quenching studies also allow for the determination of the selectivity ratios (k\textsubscript{nu}: k\textsubscript{w}), and were conducted over a range of pHs to examine the effect of the protonation state of both TAM and the nucleophile on quenching. Ground state solvolysis experiments were also conducted for deoxyguanosine, due to the important role of this nucleophile in the overall mechanism of carcinogenesis. Finally, comparisons between the various nucleophiles (namely between deoxyguanosine and DNA) are made in order to consider the behaviour of the tamoxifen carbocation in a true biological medium.
5.2 Glutathione

\( \gamma \)-Glutamylcysteinylglycine, more commonly referred to as glutathione (GSH, 20), is a ubiquitous endogenous tripeptide with an unusual \( \gamma \)-amide bond between glutamic acid and cysteine. GSH participates in a variety of detoxification, transport and metabolic processes.\(^8\) Conjugation of GSH to many chemicals renders them more water soluble, a crucial step in their excretion.\(^4\) In fact, a number of chemopreventative strategies have been developed to increase levels of both GSH and glutathione-S-transferase (GST, the enzyme responsible for catalysis of GSH conjugation).\(^5\) The \( pK_a \) of the sulfhydryl moiety has been determined experimentally as 8.89,\(^6\) however other literature values vary somewhat (for example, the \( pK_a \) is listed as 9.12 in Lange's Handbook of Chemistry\(^7\)).

![Glutathione (19)](image)

5.2.1 Laser-Flash Photolysis Studies

Trapping of the tamoxifen carbocation by GSH was investigated by LFP in much the same way that the azide quenching studies were conducted. A series of solutions were prepared containing phosphate buffer (0.1 M; 3:1 base; \( \mu = 0.1M \)) with 0 mM, 0.5
mM, 0.75 mM, 1.0 mM, and 1.5 mM GSH. To these was added 17 μM α-acetoxy-TAM (final concentration) immediately prior to flashing. All solutions containing GSH were bubbled under argon to prevent oxidation to the disulfide. Furthermore, following the LFP experiment, pHs were determined for each solution to ensure consistency. For example, in one set of experiments the measured pHs were 7.32 (0 mM GSH), 7.30 (0.5 mM GSH), 7.29 (0.75 mM GSH), 7.28 (1.0 mM GSH), and 7.25 (1.5 mM GSH). While there was a trend towards lower pH with increasing concentrations of GSH, the variation was slight, due to the high buffer concentration, and therefore regarded as insignificant.

Figure 5.1 shows the quenching plot in 100% aqueous phosphate buffer at pH 7.3. The bimolecular rate constant, $k_{GSH}$, is $9.76 \times 10^6$ M$^{-1}$s$^{-1}$, as determined by measuring the slope of the linear fit. Since $k_w = 2.81 \times 10^4$ s$^{-1}$, the $k_{GSH} : k_w$ selectivity ratio was calculated to be $3.35 \times 10^2$ M$^{-1}$. This value is approximately two orders of magnitude lower than that for the azide anion (in carbonate solution of pH 9.8). Nevertheless, GSH still represents an effective trapping agent of the tamoxifen carbocation in the absence of GST at physiological pH.
Quenching plots were then obtained at varying pH in a fairly narrow range between 6.8 and 7.8. Attempts to extend the pH range into the basic region were unsuccessful. α-Acetoxy-TAM is poorly soluble in a 100% aqueous solution of 50 mM carbonate buffer (1: 1, pH 9.7) due to deprotonation of the side chain amine. Although 17 μM substrate is sufficient for a good transient signal in carbonate buffer of pH 9.7, approximately 80% of the glutathione is present as the anion (GS⁻). This species absorbs the 248 nm actinic flash, obscuring the transient signal. The laser pulse was then set to 308 nm to prevent absorption by GS⁻. Unfortunately, a higher concentration of substrate is required to absorb the actinic flash, which is not possible in basic solution. Carbonate
solutions containing ten times the substrate concentration were opaque. At lower pH, it would have been necessary to go to much higher glutathione concentrations. It may have been possible to conduct these experiments in high proportions of CH$_3$CN, however it was felt that this would represent a significant deviation from physiological conditions. Moreover, bubbling argon gas into solutions containing GSH would alter the proportions of CH$_3$CN: H$_2$O. Therefore, the range of pH was achieved over one log unit using phosphate buffer in 100% aqueous conditions. Figure 5.2 illustrates that in the physiological pH range, glutathione quenching is first order in hydroxide concentration with the rate approaching zero in the absence of hydroxide.

\[\begin{align*}
  k(GSH) & \text{ (M$^{-1}$s$^{-1}$)} \\
  0 & 6e^{-08} 1.2e^{-07} 1.8e^{-07} 2.4e^{-07} \\
  0 & 2e+06 4e+06 6e+06 8e+06 1e+07 1.2e+07 1.4e+07
\end{align*}\]

**Figure 5.2.** Rate versus hydroxide concentration for trapping of the tamoxifen carbocation by glutathione.
Figure 5.3. Log rate versus pH for trapping of the tamoxifen carbocation by glutathione.

The resultant plot of the logarithm of the observed rate (indicated as log $k_{GSH}$) versus pH is shown in figure 5.3. The closed triangles represent the actual data points, while the curve represents a fit of the data to equation 5.1. This equation is derived based on exclusive trapping of the tamoxifen dication by the anion form of glutathione. Note that at pH 6.8 less than 1% of total glutathione exists as GS$^-$, while at pH 7.8, ~7.5% of the GSH$_{total}$ is in the form of GS$^-$.

$$k_{obs} = \frac{k_{GS} - K_a(GSH)}{[H^+] + K_a(GSH)} \quad \text{Eq 5.1}$$
The data points fit to the linear portion of the curve where \([H^+] > K_a\) (GSH) or [GSH] > [GS⁻]. The pKₐ for GSH was inputted as a fixed value of 8.89. The determined bimolecular rate constant for the anion, \(k_{GS^-}\), is \(3 \times 10^8 \text{ M}^{-1}\text{s}^{-1}\). It is also noteworthy that the last data point of the series falls below the curve. This deviation is likely due to the deprotonation of the ammonium group of the tamoxifen carbocation (pKₐ = 7.9, see section 4.4). As the pH of the solution approaches the tamoxifen carbocation pKₐ, one begins to observe the reaction between GS⁻ and the more stable tamoxifen monocation, resulting in a slightly reduced overall rate of quenching.
5.3 Deoxyguanosine

Deoxyguanosine (dG, 18) is a deoxyribonucleoside of a purine derivative where the nitrogenous base is linked to C1' of deoxyribose. Nucleosides differ from nucleotides in that they lack the 5' phosphate functionality necessary in the formation of the phosphate ester linkage to a second sugar residue to from the sugar-phosphate backbone of DNA.88

![Deoxyguanosine structure](image)

Close examination of the structure of dG reveals a number of potential nucleophilic sites. In fact, positions 1, 7, 8, the exocyclic amine at 2, and the exocyclic oxygen at 6, have all been shown to be targets for adduct formation.89 While it is not fully understood why certain alkylating agents react at specific sites, properties such as stability and polarizability of the electrophile, positioning within (or on the surface of) the DNA double helix, as well as the stability of the adduct have all been implicated. It appears that stabilized carbocations such as those found with the diol-epoxides of various polycyclic aromatic hydrocarbons, tend to react at the exocyclic amine.89 It may therefore be expected that the tamoxifen carbocation would react at the same site with
The importance of dG as a nucleophile towards tamoxifen and the structure of the resulting adduct has been discussed at some length in section 1.2.4.4. The following two sections examine the reactivity of dG towards the tamoxifen carbocation in an effort to complete this picture.

5.3.1 Laser-Flash Photolysis Studies

Trapping of the tamoxifen carbocation by dG was investigated by LFP in the same way that the azide quenching studies were conducted. A series of buffered solutions were prepared containing varying concentrations of dG (0 – 15 mM). Acetate, phosphate, and dG-buffered with NaOH solutions all contained 20% CH₃CN for improved solubility of both substrate and dG. To these was added 110 µM α-acetoxy-TAM (final concentration) immediately prior to flashing. Following the LFP experiments, pHs were measured and recorded for each solution.

As with the azide and GSH experiments, addition of dG increases the rate of decay with plots linear in dG concentration at constant pH. Figure 5.4 shows the $k_{obs}$ versus $[dG]$ quenching plot in dG-buffered with NaOH, pH 9.54 in 20% CH₃CN. The bimolecular rate constant $k_{dG}$ is $8.16 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, as determined by measuring the slope of the linear fit. Since $k_w = 1.26 \times 10^4 \text{ s}^{-1}$, the $k_{dG}:k_w$ selectivity ratio in this solution was calculated to be $6.48 \times 10^2 \text{ M}^{-1}$. 
Figure 5.4. Plot of $k_{obs}$ versus [dG] for the transient generated upon LFP of ω-acetoxy-TAM. Conditions: 110 μM substrate in 80% aqueous dG-NaOH buffer and 20% CH$_3$CN, pH 9.5, monitoring at 450 nm.

Quenching plots were obtained at varying pH in the much broader range of 3.9 – 11.3, and their corresponding $k_{dG}$s determined. Log $k_{dG}$ values were plotted against pH as shown in figure 5.5. Open triangles represent data points, while the curve is derived by fitting the data to the following equation where (R+2) represents the $K_a$ for the tamoxifen carbocation and $K_a$(dG) is the $pK_a$ for ionization of $N_1$H of dG:

$$k_{obs} = \frac{k_0^2(dG)[H^+] + k_0^2(dG^-)K_a(dG)[H^+] + k^+ (dG^-)K_a(dG)K_a(R+2)}{([H^+] + K_a(R+2))([H^+] + K_a(dG))}$$

\[\text{Eq 5.2}\]
This equation is based on scheme 5.1, and a model where both the tamoxifen dication and monocation can react with both the neutral form of dG and the anion.

\[ \text{Scheme 5.1} \]
Figure 5.5. Log rate versus pH for trapping of the tamoxifen carbocation by deoxyguanosine. Conditions: 110 μM α-acetoxy-TAM in 80% aqueous buffer and 20% CH₃CN, monitoring at 450 nm.

In contrast to the rate-pH profile in water alone (section 4.4, figure 4.3), rate constants increase with increasing pH, starting around pH 7. This is explained by a complicated kinetic model involving two reactive forms of the tamoxifen carbocation with two reactive forms of dG, as shown in scheme 5.1.

The three marked portions of the curve of figure 5.5 correspond to 1) the tamoxifen dication reacting with dG, 2) the tamoxifen dication reacting with dG⁻, and 3) the tamoxifen monocation reacting with dG⁻. The fourth possible reaction, the tamoxifen monocation reacting with dG is not important. This is ruled out since it requires that the monocation reacts with neutral dG faster than the dication. It is apparent from the data
that the anion form of dG is a much more effective nucleophile than its neutral form. This is supported by the values determined from the fit to equation 5.1, where:

\[
\begin{align*}
    k_{dG} \text{ (reacting with } T\text{A}M^2\text{)} &= 1.00 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \\
    k_{dG^-} \text{ (reacting with } T\text{A}M^2\text{)} &= 1.85 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \\
    k_{dG^-} \text{ (reacting with } T\text{A}M^+\text{)} &= 1.32 \times 10^7 \text{ M}^{-1}\text{s}^{-1}
\end{align*}
\]

A comparison between the rate of reaction of the anion form versus the neutral form of dG towards the tamoxifen dication shows a ~2000-fold increase in reactivity. This is consistent with previous work on the bis(\textit{para}-methoxyphenyl)methyl cation (2(4-MeOC$_6$H$_4$)CH$^+$),\textsuperscript{90} which determined the selectivity ratios of $k_{dG}: k_w$ to be 2 M$^{-1}$ and $k_{dG^-}: k_w$ to be $1.4 \times 10^3$ M$^{-1}$, resulting in a 700-fold increase in reactivity by dG$^-$ as compared to neutral dG.

The final objective of this work was to compare and analyze the two rate constants found for the reaction of the tamoxifen carbocation with water and dG, that is, determine the $k_{dG}: k_w$ selectivity ratios. Figure 5.6 illustrates the two plots under consideration. The top curve represents log $k_{dG}$ versus pH (taken from figure 5.5), while the bottom curve represents log $k_w$ versus pH. Both plots were obtained in 20% CH$_3$CN.
Figure 5.6. Log rate versus pH for trapping of the tamoxifen carbocation by deoxyguanosine (top curve) and water alone (bottom curve). Conditions: 110 μM α-acetoxy-TAM in 80% aqueous buffer and 20% CH₃CN, monitoring at 450 nm.

By superimposing these two plots, the ratio of $k_{dG}$: $k_w$ can be determined, as shown in figure 5.7.

Figure 5.7. Log ($k_{dG}$: $k_w$) versus pH for trapping of the tamoxifen carbocation.
These data clearly demonstrate a trend towards increasing log \((k_{dG}: k_w)\) with increasing pH. In other words, as the solution becomes more basic, dG becomes a more effective nucleophile relative to water, due to the formation of dG⁻.

5.3.2 HPLC-Ground State Solvolysis Studies

Selectivity ratios were then determined by conducting a product analysis on ground state solvolysis studies. The principle is similar to the azide quenching experiments discussed in section 4.5, where the ground state selectivity ratio, \(k_{dG}: k_w\), can be determined via the quantification of the solvolysis products resulting from varying concentrations of dG. Scheme 5.2 illustrates the partitioning of the tamoxifen carbocation between water to produce the \(E-\) and \(Z\)-alcohols (present as \(R\) and \(S\) isomers at the \(\alpha\)-carbon that cannot be resolved by HPLC) and dG to produce \(E-\) and \(Z\)-dG-TAM adducts (also present as \(R\) and \(S\) isomers at the \(\alpha\)-carbon that can be resolved by HPLC).

![Scheme 5.2](image_url)
α-Acetoxy-TAM (100 μM) was solvolyzed in 80% aqueous buffer and 20% CH₃CN with 0 – 8 mM dG. Note that dG concentrations are higher than those for azide because of its weaker nucleophilicity. The rate of solvolysis was unaffected by increasing concentrations of dG. Two new peaks of shorter retention times (8.4 min and 9.7 min as compared to 12.0 min for E-HO-TAM and 13.5 min for Z-HO-TAM) appeared with increasing concentrations of dG. The HPLC protocol (section 2.2.1) was modeled after Dasaradhi and Shibutani,⁴⁷ who characterized the TAM-dG adduct peaks resulting from incubations of α-acetoxy-TAM with DNA followed by digestion.

Product analysis and determination of the k₄₆: k₆ ratio was done in the same way as with the azide experiments (section 4.5.3). Solvolysis experiments were conducted at varying pH and their corresponding k₄₆: k₆ ratios determined, as with their counterpart LFP experiments. Once again, a comparison was made between the LFP-generated selectivities and the HPLC-generated ones. Figure 5.7 contains the logarithm of all these ratios plotted against pH. The open squares represent LFP data (taken from figure 5.5) and the black diamonds represent the new HPLC-derived data.
Both sets of data show the same trend, where dG behaves as a better nucleophile relative to water with increasing pH. However, the selectivities from the product analysis are slightly lower than those measured by laser-flash. It is important to note that the plot is logarithmic in $k_{dG}$: $k_w$; therefore a difference of 0.5 log units represents an approximate three-fold increase in the LFP-selectivities relative to the HPLC-selectivities.

The exact reason for this discrepancy is not known and was not explored further. However, a possible explanation can be proposed based on a 1981 publication by Dipple and coworkers. They found that dG reacts with the paramethoxybenzyl cation to
generate an unstable O6 adduct with a half-life of ~ 20 minutes. In the event that a similar adduct is being formed with the tamoxifen carbocation, one would not expect to find it via product analysis on the HPLC as the solutions are left to incubate overnight. However, trapping of the cation by dG to form an unstable adduct will increase the apparent \( k_{dG} \), as measured by laser-flash, resulting in an elevated \( k_{dG}: k_w \) selectivity ratio. Scheme 5.3 illustrates the partitioning of the TAM carbocation between dG to form two products (one of them unstable) and water to form the alcohols, modeled after Dipple’s work. \(^ {91} \)
5.4 Deoxyribonucleic Acid

Following the dG studies, quenching of the tamoxifen carbocation by deoxyribonucleic acid (DNA) was explored. Nucleic acids are, with few exceptions, linear polymers of nucleotides whose phosphates bridge the 3' and 5' positions of successive sugar residues. While DNA's base composition varies widely between different organisms, among mammals guanine (G) + cytosine (C) ranges from 39 to 46%. Experiments were conducted using salmon testes DNA whose G content accounts for 20.6% of all bases. The most important structural feature of B-DNA (native form of DNA) is that it consists of two polynucleotide strands that wind about a common axis with a right-handed twist to form a ~20-A-diameter double helix. The two strands are antiparallel with complementary bases occupying the core of the helix and sugar-phosphate chains are coiled about its periphery, thereby minimizing repulsions between charged phosphate groups. The planes of the bases are nearly perpendicular to the helix axis. Each base is hydrogen-bonded to a base on the opposite strand to form a planar base pair. Scheme 5.4 illustrates such a pairing between guanine and cytosine.

![Scheme 5.4](image-url)
The bimolecular rate of trapping of the transient generated upon LFP of α-acetoxy-TAM by dG alone at physiological pH was found to be $8.59 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, corresponding to a $k_{dG} : k_w$ selectivity ratio of $\sim 70 \text{ M}^{-1}$. The HPLC-derived selectivity ratio is $\sim 30 \text{ M}^{-1}$ at the same pH. This value is quite low considering the crucial role played by dG trapping of the tamoxifen carbocation in the proposed carcinogenesis pathway. Quenching studies using DNA sought to investigate the effectiveness of dG as a trapping agent within DNA compared to nucleoside alone, as this represents the form encountered by TAM under physiological conditions.

5.4.1 Laser-Flash Photolysis Studies

Trapping of the tamoxifen carbocation by purified DNA was investigated by LFP in the same way that the azide quenching studies were conducted. A series of phosphate-buffered solutions were prepared containing varying concentrations of guanosine in DNA (0 - 1.055 mM). The concentration of G in DNA was determined by measuring the absorbance of the DNA stock solution at 260 nm and dividing this value by the extinction coefficient ($\varepsilon_{260} = 6000 \text{ M}^{-1}\text{cm}^{-1}$) in order to determine the total base concentration. This value was divided by 4.85 to calculate the concentration of G alone (recall that in salmon testes DNA; 20.6% guanosine). To these solutions was added 100 μM α-acetoxy-TAM (final concentration) immediately prior to flashing. Following the LFP experiments, pHs were measured and recorded for each solution.

Unlike all previous quenching experiments, addition of DNA increases the rate of decay of the TAM carbocation to a point. That is to say, DNA quenching plots exhibit saturation kinetics rather than the typical linear correlations previously seen. Figure 5.9
shows the $k_{obs}$ versus guanosine concentration in DNA quenching plot in 4 mM phosphate buffer (1:1), pH 6.7, where closed triangles represent the actual data points and curves represent two different fits to the data. A linear fit to the first four values yields a $k_{DNA}$ of $1.15 \times 10^8$ M$^{-1}$s$^{-1}$. Since $k_w = 2.99 \times 10^4$ s$^{-1}$, the $k_{DNA}: k_w$ selectivity ratio was calculated to be $3.85 \times 10^3$ M$^{-1}$. A more appropriate fit for all of the data points is to the following equation for saturation kinetics where $k_1$ represents the plateau rate and $K_b$ the binding constant:

$$k_{obs} = \frac{k_w + k_1K_b(G \text{ in DNA})}{1 + K_b(G \text{ in DNA})}$$

Eq 5.3

This is illustrated in scheme 5.5, where the tamoxifen dication may be trapped by water to yield the $\alpha$-HO-TAMs (E/Z) or by DNA to yield "products". Tamoxifen is shown to bind to DNA prior to quenching, consistent with experimental data. A simple interpretation of the "products" resulting from DNA quenching is that they are TAM-DNA adducts. However, recent evidence from ground state solvolysis experiments points to a mixture of products containing both TAM-DNA adducts, as well as a large amount of $\alpha$-HO-TAMs, suggesting that $k_1$ may contain several terms.
Figure 5.9. Plot of $k_{obs}$ versus [G] in DNA for the transient generated upon LFP of $\alpha$-acetoxy-TAM. Conditions: 100 $\mu$M substrate in 100% aqueous phosphate buffer (4 mM, $1:1$, pH 6.7), monitoring at 450 nm.
A second quenching plot for DNA was obtained with added salt (0.01 M NaClO₄), shown in Figure 5.10. While the data points show curvature, saturation has not been reached at the higher concentrations. A linear fit to the first seven values yields a $k_{DNA}$ of $8.71 \times 10^7$ M\(^{-1}\)s\(^{-1}\). Since $k_w = 3.19 \times 10^4$ s\(^{-1}\), the $k_{DNA}: k_w$ selectivity ratio was calculated to be $2.73 \times 10^3$ M\(^{-1}\). Quenching plots were not conducted at varying pH as the DNA helical structure is susceptible to its protonation state.

![Figure 5.10](image)

**Figure 5.10.** Plot of $k_{obs}$ versus [G] in DNA for the transient generated upon LFP of α-acetoxy-TAM. Conditions: 100 μM substrate 100% aqueous phosphate buffer (4 mM, 1:1, 0.01 M NaClO₄, pH 6.7), monitoring at 450 nm.
5.4.2 HPLC-Ground State Solvolysis Studies

Unlike previous ground state experiments, incubation solutions containing DNA cannot be injected directly onto the HPLC. Following complete solvolysis of α-acetoxy-TAM in DNA of varying concentrations, the solutions must be subjected to an extensive digestion process in order to break the DNA down to its nucleoside components (completely lacking the phosphate moiety). The solution of nucleosides (adenosine, guanosine, cytidine, and thymidine), α-HO-TAMs, and TAM-nucleoside adducts can then be injected and measured on the HPLC. Quantitative analysis of the DNA-adducts is not a trivial process and is under continued investigation at the time of writing this thesis.
5.4 14-Nucleotide Oligomer

A 14-nucleotide-long oligomer with a self-complementary sequence of 5' ATATTAGCTAATAT 3' was synthesized (section 2.1.7) in order to model DNA. Prior to conducting any LFP or ground-state solvolysis experiments, it was important to confirm that the oligomer does in fact exist as a duplex under the usual experimental conditions. To this end, melting curves were obtained (section 2.2.4) to establish the temperature at which the duplex melts to the constituent monomers. Initially, melting curves were conducted in 4 mM phosphate buffer (1: 1) with no added salt. However, the melting temperature was found to be too low (~30° C). Addition of 80 mM NaClO₄ raised the melting temperature to 36° C. A typical melting curve is shown in figure 5.11. At low temperature the oligomer exists as a duplex (formed by the H-bonding between two self-complementary strands), and as such has a lower absorbance at 260 nm. At higher temperature the oligomer exists as the free monomer with a higher overall absorbance at this wavelength. The inflection point represents the melting temperature. LFP and ground state solvolysis experiments are currently under way, and it is important to be careful to conduct these experiments at room temperature (or lower) and with added salt to ensure the duplex formation.
Figure 5.11. Melting curve of 14-nucleotide oligomer. Conditions: 3.15 μM oligonucleotide in 100% aqueous phosphate buffer (4 mM, 1: 1, 0.08 M NaClO₄, pH 6.7), monitoring at 260 nm.
5.5 Summary

Quenching studies of the tamoxifen carbocation have been conducted with a number of different biologically relevant nucleophiles. Comparisons can now be made between the various nucleophiles in order to consider the behaviour of the cation in a true biological medium. The first nucleophile selected for these studies was glutathione due to its role as an electrophile-scavenger. GSH conjugation to many xenobiotics renders them more water soluble, a necessary step in their excretion.\cite{84,85} Trapping by GSH usually occurs in conjunction with GST; however GSH itself is an effective nucleophile.\cite{84} In fact, at physiological pH the bimolecular rate constant, $k_{GSH}$, was found to be $9.76 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ with a $k_{GSH}: k_w$ selectivity ratio of $3.35 \times 10^2 \text{ M}^{-1}$. Also noteworthy is the fact that at physiological pH, only the anion form of glutathione, GS\textsuperscript{−}, is responsible for trapping. This is illustrated in figure 5.2 where glutathione trapping is first order in hydroxide and approaches zero in the complete absence of hydroxide. An important feature of glutathione-S-transferase is that at physiological pH (where ~3% of the total glutathione is present as the anion), it provides the anion form, GS\textsuperscript{−}, within the active site to catalyze GSH-conjugation to the (xenobiotic) substrate.\cite{85}

Trapping of xenobiotics by deoxyguanosine, unlike GSH, represents an activation pathway (towards mutagenesis and carcinogenesis) rather than a detoxifying one.\cite{84,85} Recent reports have found that dG-TAM adducts have high miscoding potential which cause mutations and initiate endometrial cancer.\cite{94,95} Not surprisingly, quenching studies reveal that dG is less effective a nucleophile towards the tamoxifen carbocation than glutathione. Both the neutral and the anion forms of dG react, with $dG + TAM^{2+} = 1.00 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $dG^- + TAM^{2+} = 1.85 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, and $dG^- + TAM^+ = 1.32 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, as
determined by fitting the quenching data to equation 5.2. At physiological pH, where ~1% of the total dG is present as the anion, $k_{dG}$ is more than an order of magnitude slower than $k_{GSH}$, with a selectivity ratio of $\sim 70 \text{ M}^{-1}$ (as determined by LFP) and $\sim 30 \text{ M}^{-1}$ (as determined by HPLC-ground state solvolysis). This discrepancy between the two experimentally determined selectivities is explained by the possible trapping at O6 (in addition to N2) to yield an unstable dG-TAM adduct.

Very little dG-TAM adduct has been found in biological systems (in the endometrial samples of women; 0.9 to 13.1 cis- + trans-TAM adducts/10$^8$ nucleotides). While very few adducts are needed to cause mutagenesis and carcinogenesis, the selectivity ratio for dG is surprisingly low at physiological pH. Quenching studies using DNA sought to investigate the effectiveness of dG as a trapping agent within DNA as compared to nucleoside alone, as this represents the form encountered by TAM under physiological conditions. Unlike all previous nucleophiles, the quenching plot for DNA reacting with tamoxifen cation shows saturation kinetics. In considering the linear portion of the curves (the first few points), $k_{DNA}$ was found to be $1.15 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ at pH 6.7 (it may well be higher at physiological pH), resulting in a $k_{DNA}: k_{W}$ selectivity ratio of $3.85 \times 10^3 \text{ M}^{-1}$. This represents a $\sim 130$-fold increase in selectivity for DNA as compared to dG alone at the same pH. Figure 5.12 illustrates this dramatic difference in quenching by these two nucleophiles.
Figure 5.12. Plot of $k_{\text{obs}}$ versus [dG] alone ( ) and in DNA (●) for the transient generated upon LFP of α-acetoxy-TAM. Conditions: 100 μM substrate 100% aqueous phosphate buffer (4 mM, 1:1, pH 6.7), monitoring at 450 nm.

The precise reason for DNA's improved nucleophilicity towards the tamoxifen carbocation is not fully understood, and is currently under investigation. There are a number of possible explanations: (1) Other nucleotides in addition to dG may contribute to trapping of the allylic cation. There is evidence for the formation of a TAM-deoxyadenosine adduct, although it was found to represent only 7% of the total adducts formed. 56 (2) DNA may be quenching the tamoxifen carbocation to form unstable adduct(s), resulting in an elevated selectivity ratio. This effect is seen with deoxyguanosine, which showed higher $k_{\text{dG}}$: $k_w$ from LFP than with ground-state
solvolysis studies. DNA quenching experiments need to be conducted in the ground-state to address this issue. (3) DNA may catalyze quenching of the cation by water. Again, the selectivity ratio determined by HPLC would be lower than LFP, as quenching by H_{2}O yields the alcohol products, not adducts. (4) The conformation of double-stranded DNA may influence the reactivity of dG towards the tamoxifen carbocation. There are two possible contributing factors: (i) A higher effective molarity of DNA due to pre-reaction binding by way of TAM intercalation. This effect is modeled after enzyme catalysis, where reaction rates are increased because of substrate-enzyme binding.^{96a} The entropy loss occurs prior to covalent adduct formation. (ii) General base catalysis due to hydrogen-bonding of guanine to cytosine in DNA, as illustrated in scheme 5.4. The exocyclic nitrogen is involved in H-bonding to the oxygen of cytosine. This effect is also modeled after enzyme catalysis, where protons are often abstracted from the nucleophilic site during attack.^{96b} An example of this is serine protease, where imidazole abstracts the proton from the alcohol functionality as the oxygen attacks the carbonyl carbon of an amide linkage. It is tempting here to quote the k_{dG} bimolecular rate constant however, the ionizing proton of dG is at N1, not the exocyclic amine at C2.

In all likelihood, no single one of these factors is responsible for elevating k_{DNA} relative to k_{dG}. Clearly, ground state solvolysis work is needed to help clarify the DNA's reactivity towards the carbocation of tamoxifen. The 14-nucleotide oligomer will also be investigated as a model for the DNA double-helix.

A final observation is that salt concentration appears to influence the rate of trapping by DNA. At pH 6.7, the k_{DNA}: k_{w} selectivity ratio decreased from 3.85 x 10^{3}
M$^{-1}$ to 2.73 x 10$^3$ M$^{-1}$, when 10 mM NaClO$_4$ was added. Salt has been shown to be important for the duplex formation of the oligomer (section 5.5). However, the much longer DNA strand forms the double-helix readily at room temperature in 4 mM phosphate. The reason for this observed decrease in bimolecular rate constants with added salt is not known. Perhaps added salt helps to neutralize the sugar-phosphate backbone thereby reducing the electrostatic interaction between the phosphates and nucleotides, resulting in diminished negative charge at the exocyclic amine at C2. Alternatively, TAM may be associating with the sugar-phosphate backbone of DNA rather than intercalating. In this case, salt (Na$^+$) would displace tamoxifen (monocation or dication), thereby reducing the effect the phosphates may be exerting on the rate of quenching. Free phosphate may accelerate quenching of the cation by general base catalysis.
References


49. Toxicol. Text


70. Han, X; Liehr, J.G. Cancer Res. 1992, 52, 1360.
79. TAM information website by Zeneca Inc.; www.ndist.com/cgi/oncology/tamox.htm


86. Communication with Prof. R.A. McClelland.


90. Unpublished data.


Cancer Res. 1996, 56, 1475.

White, I.N.H.; Smith, L.L. Carcinogenesis 1998, 19, 1061-1069.) not useful


1996, 17, 1149-1152.
Appendix One

A Molecular Mechanics and AM1 Semiempirical Study of the Tamoxifen Carbocation

1. Introduction

The energy of the geometry-optimized structures of the two cis- and trans-isomers of the tamoxifen carbocation was determined using the molecular mechanics MM+ and semiempirical AM1 methods, provided by the software package HYPERCHEM™. Tamoxifen is too large a molecule to be studied extensively by ab initio methods in the time allotted to conduct these studies. Alternatively, the more economical MM+ and AM1 methods can be employed to examine bond lengths, torsion angles, and conformational energies with great success. In addition, electron distribution and atomic charges can be approximated using semiempirical calculations. This is particularly useful in determining the distribution of positive charge in the tamoxifen carbocation.

Four different structures (a pair of isomers, each represented by two resonance forms) were inputted for molecular mechanics geometry optimization. Due to the large size of tamoxifen, the para-substituent on the aromatic ring was replaced by a methoxy-substituent. These four structures (A1-A4, shown below) were initially drawn as carbon-oxygen skeletons in HYPERCHEM™, followed by the option “model-build and add hydrogens”. The appropriate hydrogen was then deleted from each of structure to
perform the MM+ geometry optimization. This structure was then inputted into AM1 with the following parameters:

- Total charge: 1
- Spin multiplicity: 1
- Convergence limit: 0.05
- Iteration limit: 50
2. **Results**

The energy minima and gradients from geometry optimization of each of the four structures are reported, as are the Mulliken atomic charges, bond lengths and torsion angles obtained from AM1 calculations. Note the variation in numbering of the atoms.

**Structure A1**

![Structure A1](image)

**Table A1.** The energy minima, gradient, and relevant net atomic charges, bond lengths, and torsional angles for input A1.

<table>
<thead>
<tr>
<th>Atomic Charge (elementary charge)</th>
<th>Bond Length (Å)</th>
<th>Torsion Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>At Convergence:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E = 4863.2$ KJ mol$^{-1}$</td>
<td>1 = +0.3079</td>
<td>17-1 = 1.447</td>
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<tr>
<td>$G = 0.010$</td>
<td>2 = -0.1621</td>
<td>1-2 = 1.461</td>
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<tr>
<td></td>
<td>3 = -0.02136</td>
<td>2-3 = 1.354</td>
</tr>
<tr>
<td></td>
<td>4 = -0.2285</td>
<td>2-5 = 1.470</td>
</tr>
<tr>
<td></td>
<td>1-11 = 1.408</td>
<td>4-3-2-1 = 3.2</td>
</tr>
<tr>
<td></td>
<td>3-4 = 1.470</td>
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Table A2. The energy minima, gradient, and relevant net atomic charges, bond lengths, and torsional angles for input A2.

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</thead>
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<tr>
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<td>17-1 = 1.447</td>
<td>17-1-2-3 = 56.2</td>
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<tr>
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<td>1-11 = 1.408</td>
<td>4-3-2-1 = 3.2</td>
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<td>3-4 = 1.470</td>
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Structure A3

Table A3. The energy minima, gradient, and relevant net atomic charges, bond lengths, and torsional angles for input A3.

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<tr>
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<td>11-1 = 1.424</td>
<td>11-1-2-3 = 35.5</td>
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<td>G = 0.008</td>
<td>2 = -0.1457</td>
<td>1-2 = 1.450</td>
<td>11-1-2-16 = -144.7</td>
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<tr>
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<td>3 = +0.005551</td>
<td>2-3 = 1.364</td>
<td>5-1-2-16 = 36.0</td>
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<tr>
<td></td>
<td>4 = -0.2338</td>
<td>2-16 = 1.468</td>
<td>5-1-2-3 = -143.8</td>
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<td>1-5 = 1.440</td>
<td>4-3-2-1 = 179.6</td>
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<td></td>
<td>3-4 = 1.468</td>
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</tr>
</tbody>
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Structure A4

Table A4. The energy minima, gradient, and relevant net atomic charges, bond lengths, and torsional angles for input A4.

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<tr>
<th>At Convergence:</th>
<th>Atomic Charge (elementary charge)</th>
<th>Bond Length (Å)</th>
<th>Torsion Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E = 4862.5 \text{ KJ mol}^{-1}$</td>
<td>1 = +0.3140</td>
<td>6-1 = 1.415</td>
<td>6-1-2-3 = -60.2</td>
</tr>
<tr>
<td>$G = 0.009$</td>
<td>2 = -0.1539</td>
<td>1-2 = 1.463</td>
<td>6-1-2-5 = 120.6</td>
</tr>
<tr>
<td></td>
<td>3 = -0.04177</td>
<td>2-3 = 1.353</td>
<td>7-1-2-5 = -58.6</td>
</tr>
<tr>
<td></td>
<td>4 = -0.2271</td>
<td>2-5 = 1.469</td>
<td>7-1-2-3 = 120.6</td>
</tr>
<tr>
<td></td>
<td>1-7 = 1.437</td>
<td>1-2-3-4 = -2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-4 = 1.470</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. Discussion

The two resonance forms of the \textit{trans}-isomer, A1 and A2, converged to the same final structure. The most important features of this structure are as follows. 1) The C1-C2 bond length is 1.46 Å, while the C2-C3 bond length is 1.35 Å, indicating single and double bond characters respectively. 2) C1 has a Mulliken atomic charge of +0.31, whereas the other atoms listed all have (small) negative charges, indicating that the carbocation charge is centered at the benzylic carbon. Interestingly, the C1-C11 bond length (1.41 Å) is also somewhat shorter than the standard single bond, suggesting stabilization of the C1 charge, most likely by the methoxy substituent. 3) The ethylene is out of the plane of the carbocation and its two adjacent aryl substituents, with a torsion angle of \(-56^\circ\), indicating that the pi-electrons of the double bond are not contributing to the stabilization of the carbocation.

The two resonance forms of the \textit{cis}-isomer, A3 and A4, did not converge to the same final structure. A4 converged to a structure much like that of A1/A2. The only minor difference relates to the torsion angle of the ethylene relative to the carbocation and its two adjacent aryl substituents, found to be \(-60^\circ\). A3 converged to a structure very similar to the previous three with a smaller ethylene torsion angle of \(-36^\circ\). More importantly, the C4-methyl carbon is now pointing away from the methoxyphenyl rather than towards it. It is not clear why the A3 converged to such a final structure, or why it should be different from A4. Given the (very small) energy difference between the two, perhaps A4 is a better depiction of the \textit{cis}-carbocation. Nevertheless, the most interesting features mentioned above correspond to all four converged structures.