NOVEL ANTIMITOTIC NUCLEOSIDE DERIVATIVES OF PODOPHYLLOTOXIN AND DMEP

SYNTHESIS AND BIOLOGICAL ACTIVITY OF NOVEL ANTIMITOTIC NUCLEOSIDE DERIVATIVES OF PODOPHYLLOTOXIN AND 4'-DEMETHYLEPIPODOPHYLLOTOXIN

by

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ABSTRACT

The objective of this study was to synthesize and biologically evaluate a series of novel nucleoside derivatives of the naturally occurring lignans, podophyllotoxin and 4'-demethylepipodophyllotoxin. Epipodophyllotoxin derivatives display two types of biological activity: while the naturally occurring compounds are potent inhibitors of microtubule polymerization, two semi-synthetic glycoside derivatives, *viz* VP16-213 (etoposide) and VM26 (teniposide), are specific inhibitors of mammalian DNA topoisomerase II. The latter two compounds have proven to be very useful in the treatment of many different types of cancer. In this study, the glycoside moiety of VP16-213/VM26 was replaced with either a thymidine or a 2'-deoxyadenosine group. The synthetic approach involved protective group chemistry, where functional groups on the nucleosides and DMEP were selectively blocked prior to their condensation in the presence of boron trifluoride etherate catalyst. These compounds are of interest because they involve substitution of the glucoside moiety with a nucleoside residue, whose effect on the biological activity of epipodophyllotoxin derivatives has not been examined. The biological activity of these compounds was assessed by examining their cross-resistance patterns towards a set of Chinese hamster ovary mutants resistant to podophyllotoxin VP16-213/VM26, mitotic index, and by a competition assay. From the cross resistance

assay, all of the thymidine derivatives were found to be considerably less active than the parent podophyllotoxin and 4'-demethylepipodophyllotoxin molecules, while the 2'-deoxyadenosine derivatives were found to be completely inactive. The cross-resistance patterns of the thymidine derivatives suggests that these compounds display podophyllotoxin-like activity *in vivo* and show no VP16-213/VM26-like activity. Treatment of wild type cells with the active thymidine derivatives (compounds 1.2, 2.2, 2.3, and 2.4) increased the mitotic indices approximately ten-fold in a dose-dependent manner, which parallels the results of the dose-response curves in the initial cross-resistance assay. Furthermore, there was a marked increase in the levels of drug required to elevate the mitotic index in second-step mutants resistant to podophyllotoxin, again lending support to the initial results indicating that the cellular target of these compounds is tubulin. Only one compound (1.2), was found to out compete the binding of radiolabelled podophyllotoxin to purified calf brain tubulin. However, due to their low activity relative to podophyllotoxin, most of these compounds were insoluble at concentrations required to out compete the binding of radiolabelled podophyllotoxin. Molecular modelling studies have provided useful insights regarding the structure/activity relationships among the active and inactive nucleoside derivatives showing podophyllotoxin-like antimitotic activity. There appears to be steric limits for substituents attached at the C4 moiety that maintain the antimitotic activities of the parent molecules. The electrostatic potential and hydrophobic properties of these groups also seem to play a role in the degree of activity these compounds show, but remain unclear

at this point. By simple comparison of the three-dimensional structures of these compounds there appears to be a very limited spacial and electrostatic requirement for the bulky glycosidic moiety attached to C4 which is essential for targeting VP16-213 and VM26 to DNA topoisomerase II. Thus, several important structural features which may distinguish the selectivity POD derivatives show for either tubulin or DNA topoisomerase II are described.

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ATP	adenosine triphosphate
t-BDPCS	*tert*-butyldiphenylchlorosilane
t-BDPS	*tert*-butyldiphenylsilyl
BSA	bovine serum albumin
C	carbon
^{13}C	carbon-13 isotope
C4	refers to carbon 4 on the podophyllotoxin molecule
Ca^{2+}	calcium ion
CBZ	carbobenzoxy
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
CHO	Chinese hamster ovary
Ci	Curie
CO_2	carbon dioxide
CR	cross-resistance
d	doublet peak (used in reporting proton nuclear magnetic resonance spectra)
dd	doublet of a doublet peak (used in reporting proton nuclear magnetic resonance spectra)
D_{10}	dose of a compound which reduces the plating efficiency of a cell line to 10% of that observed in the absence of the drug.
DCE	1,2-dichloroethanol
DCI	direct chemical ionization
DEAE	*O*-(diethylaminoethyl)cellulose
DE81	Whatman *O*-(diethylaminoethyl)cellulose filter disc
DMEP	demethylepipodophyllotoxin
DMF	N,N-dimethylformamide
DMSO	dimethyl sulphoxide
DMSO-d_6	deuterated dimethyl sulphoxide
DP	demethylpodophyllotoxin
EA	elemental analysis
EGTA	ethyleneglycol-bis-(ß-aminoethylether)-N,N,N',N'-tetraacetic acid
EOT-3	multiply marked Chinese hamster ovary cell line selected for resistance to emetine (two steps), ouabain, and 6-thioguanine
EP	epipodophyllotoxin

EtOH	ethanol
eq	equivalents
eV	electron volts
FAB^+	positive ion fast atom bombardment
GDP	guanosine diphosphate
GTP	guanosine triphosphate
h	hours
^1H	proton
^3H	tritium (isotope of hydrogen)
HeLa	immortal human fibroblast cells
KCl	potassium chloride
kg	kilograms
l	litre
m	multiplet peak (used in reporting proton nuclear magnetic resonance spectra)
M	molarity (expressed as moles per litre)
MAPs	microtubule associated proteins
mbar	millibars
mCi	milliCuries
α-MEM	alpha-modified minimal essential medium
M_r	relative molecular mass
MeOH	methanol
MeSH	ß-Mercaptoethanol
mg	milligrams
$MgSO_4$	magnesium sulphate
MHz	megahertz
min	minutes
ml	millilitres
mm	millimeters
mmol	millimole
mp	melting point
MS	mass spectra
MT	microtubule
MTs	microtubules
NaOH	sodium hydroxide
Na_2SO_4	sodium sulphate
ng	nanogram
nm	nanometer
nM	nanomolar
NMR	nuclear magnetic resonance
Pd/C	10% palladium on carbon

PEMG	0.1 M PIPES-NaOH, 1 mM EGTA, 1 mM $MgSO_4$, and 0.1 mM GTP, pH 6.6
PIPES	piperazine-*N*,*N*'-bis[2-ethanesulphonic acid]
POD	podophyllotoxin
$Pod^{RI}16$	first-step Chinese hamster ovary mutants selected for resistance to the antimitotic drug, podophyllotoxin
$Pod^{RII}6$	second-step Chinese hamster ovary mutants selected for resistance to the antimitotic drug, podophyllotoxin
ppm	parts per million
q	quadruplet peak (used in reporting proton nuclear magnetic resonance spectra)
rep	reported
RNA	ribonucleic acid
rpm	revolutions per minute
SAR	structure/activity relationship
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	seconds
t	triplet peak (used in reporting proton nuclear magnetic resonance spectra)
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
TOPO II	DNA topoisomerase II
tRNA	transfer ribonucleic acid
UV	ultra violet
Vpm^R5	single-step Chinese hamster ovary mutants selected for resistance to the 4'-demethylepipodophyllotoxin glycosides VP16-213 and VM26
VM26	4'-demethylepipodophyllotoxin thenylidene ß-D-glucoside (teniposide)
VP16-213	4'-demethylepipodophyllotoxinethylidineß-D-glucoside(etoposide)
WT	wild type, proline-requiring Chinese hamster ovary cell line

1.0 INTRODUCTION

1.1 History of Podophyllotoxin

"O, mickle is the powerful grace that lies
In herbs, plants, stones, and their true qualities..."
"Within the infant rind of this small flower
Poison hath residence, and medicine power..."

Shakespeare, "Romeo And Juliet", Act II, scene iii

Podophyllotoxin (POD, Fig. 1.1a) is a natural product obtained from the dried roots of species of *Podophyllum* (family name *Berberidaceae*). *Podophyllum* is derived from two species of plants; *Podophyllum peltatum* Linnaeus (the North American species, commonly known as "May apple" or "American mandrake") and *Podophyllum emodi* Wallach (the Indian species), both of which are herbaceous perennials (Hartwell & Schrecker, 1958). POD and its derivatives belong to the class of compounds known as lignans, which are natural products containing the 2,3-dibenzylbutane skeleton (Fig. 1.2a). *Podophyllum* lignans have also been found in other genera of plants, such as in the roots of the wild chervil (*Anthriscus sylvestris*), the seed oil of *Hernandia ovigera*, needles of the juniper (*Juniperus silicicola*), and from the berries of *Juniperus sabina* (Hartwell &

Schrecker, 1958). In fact, wild chevril and juniper seeds are listed as components of a salve for cancer in the Leech Book of Bald, 900-950 A.D. (Cockayne, 1961), a pre-conquest English medical book. Ironically, this same book recommends the application of a mixture of hound urine and mouse blood for the treatment of warts. The medicinal uses of *Podophyllum* roots were first called to the attention of travellers and colonists in North America by the first nations who were aware of its properties as a cathartic, emetic, anthelmintic, and as a mortal poison (Kelly & Hartwell, 1954).

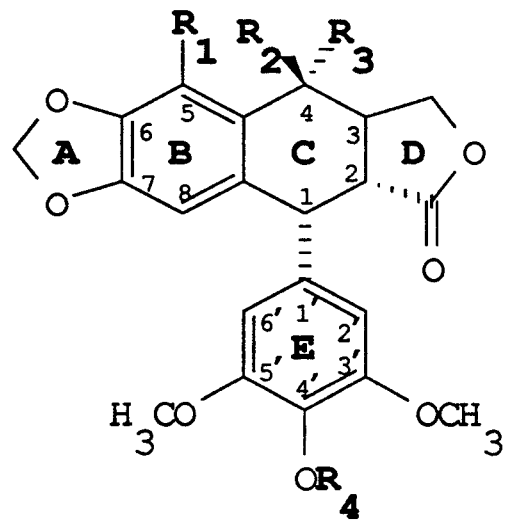
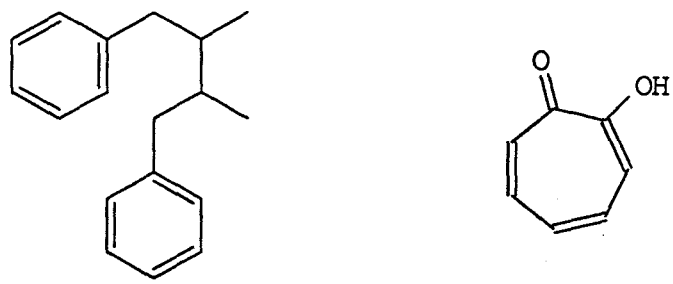


Figure 1.1: Structure of podophyllotoxin and its derivatives:
a) Podophyllotoxin: R_1=H, R_2=H, R_3=OH, R_4=CH3;
b) 4'-Demethylpodophyllotoxin: R_1=H, R_2=H, R_3=OH, R_4=H;
c) Desoxypodophyllotoxin: R_1=H, R_2=H, R_3=H, R_4=CH3;
d) Epipodophyllotoxin: R_1=H, R_2=OH, R_3=H, R_4=CH3;
e) 4'-Demethylepipodophyllotoxin: R_1=H, R_2=OH, R_3=H, R_4=H;
f) α-Peltatin: R_1=OH, R_2=H, R_3=H, R_4=H;
g) ß-Peltatin: R_1=OH, R_2=H, R_3=H, R_4=CH3.

The alcohol soluble portion of *Podophyllum* yields a resin (podophyllin), first separated in 1835 by Dr. John King (see Kelly & Hartwell, 1954; and references therein), which is a complex mixture containing most of the original biological activity found in the root. The chemical composition of podophyllins from different sources varies greatly although the physiological properties appear to be primarily the same (Hartwell & Schrecker, 1958). In 1880, Podwyssotzki separated a crystalline, white substance from the resin of American *Podophyllum*, which he named podophyllotoxin and hypothesized to be responsible for the purgative activity of podophyllin. It should be noted that the Indian species contains a larger amount of POD and a smaller proportion of other POD derivatives than American podophyllin (Hartwell & Schrecker, 1958). The correct structure of POD (Fig. 1.1a) was proposed by Hartwell and Schrecker (1951) who also isolated many other components of podophyllin. With the advent of adsorption chromatography isolation of other lignans such as α-peltatin (Hartwell, 1947; Fig. 1.1f), ß-peltatin (Hartwell & Detty, 1948; Fig. 1.1g), 4'-demethylpodophyllotoxin (DMP; Fig. 1.1b) and picropodophyllotoxin glucoside (Nadkarni *et al.*, 1952) was made possible. Solvent partition and paper chromatography led to the separation of dehydropodophyllotoxin (Kofod & Jϕrgensen, 1954), desoxypodophyllotoxin (Kofod & Jϕrgensen, 1955; Fig. 1.1c), and the glucosides of POD (Stoll *et al.*, 1954a) and DMP (Stoll *et al.*, 1954b).

In the early 1940's successful treatment of *Condylomata acuminata*, a type of venereal wart, was accomplished with the topical application of podophyllin (Kaplan,

1942). King and Sullivan (1946) observed that the antiproliferative effects of podophyllin were similar to those of colchicine. These clinical studies eventually led to intensive chemical and biochemical investigations which elucidated podophyllotoxin as the principal active component of podophyllin (Hartwell and Schrecker, 1958).



a b

Figure 1.2: Basic structural components of two major antimitotic compounds: a) the 2,3-dibenzylbutane skeleton of plant lignans such as POD and DMEP; b) the seven-membered tropolone ring of colchicine.

1.2 Biochemical Properties of Tubulin

Microtubules (MTs) are an integral component of the cellular apparatus responsible for cell division (mitosis) and play critical roles in cell shape, movement, and organelle transport (Darnell *et al.*, 1986; Dustin, 1984; Gupta, 1989b; Olmsted & Borisy, 1973a). MTs are helical assemblies of protein dimers (tubulin) about 250 Å in diameter, composed of two corresponding subunits (α and ß) of approximately 50,000 relative

molecular mass (Dustin, 1984). From most sources MTs embody 13 protofilaments that form a tubular bundle, with each protofilament constructed by the head to tail joining of αß tubulin dimers, thereby yielding a defined polarity (Dustin, 1984). Immunofluorescent *in vivo* growth studies of MTs has been shown to occur from specific cellular sites, termed microtubule organizing centres (MTOCs) (Brinkley, 1985). These structures are visualized as centrosomes and are located in the perinuclear region of interphase cells, whereas in mitotic cells, the MTOCs coincide with the spindle poles (Dustin, 1984; Brinkley, 1985; Gupta, 1989b).

Both α and ß tubulin have been sequenced and shown to consist of about 450 amino acids (Ponstingl *et al.*, 1981; Krauhs *et al.*, 1981). Tubulin has been isolated and sequenced from a multitude of organs and species of eukaryotes, however, no indication of its presence in prokaryotes has been put forth (Dustin, 1984). The α and ß subunits differ slightly in their electrophoretic mobility with isoelectric points of 5.3 and 5.4, respectively (Feit *et al.*, 1977; Berkowitz *et al.*, 1977). The carboxy terminal of α tubulin is very acidic and is thought to play an important role in the binding of microtubule associated proteins (MAPS) or cations, such as Ca^{2+} (Hamel, 1989). No covalent disulfide bonds are known to exist between the two heteromeric subunits although there are likely two intrapolypeptide cysteine bridges (Lee *et al.*, 1973). Tubulin heterodimers contain two molar equivalents of guanine nucleotide (Levi *et al.*, 1974; Stephens *et al.*, 1967; Weisenberg *et al.*, 1968), which is essential for the formation of MTs. Half of the bound nucleotide can be rapidly exchanged with free GDP or GTP, while the other molar

equivalent, believed to be GTP, cannot be removed from the protein without its denaturation (Levi *et al.*, 1974; Stephens *et al.*, 1967; Weisenberg *et al.*, 1968; Hamel, 1989). Polymerization of tubulin is generally associated with hydrolysis of the exchangeably bound GTP to GDP (Hamel & Lin, 1981; Penningroth & Kirschner, 1977), although the precise mechanism linking GTP hydrolysis with tubulin polymerization remains elusive (Stewart *et al.*, 1990). Cross-linking experiments carried out by Hesse *et al.* (1987) have localized the binding site of the exchangeable nucleotide to amino acid residues 155-162 of ß tubulin. However, there is no evidence discriminating the subunit to which non-exchangeable nucleotide binds (Hamel, 1989), albeit amino acid sequence considerations may favour its binding to the α subunit (Sternlicht *et al.*, 1987).

Advanced electrophoretic techniques have identified up to 21 tubulins (7 α and 14 ß subunits), the ratios of which have been shown to vary in different cell types (Dustin, 1984). Multiple copies of the tubulin gene exist in the cell (Hyams, 1982) and all cells of higher organisms examined accommodate a mixture of different tubulin isotypes (Burns & Surridge, 1990 and references therein). In many vertebrates it appears that α and ß tubulins are independently regulated, with changes in α tubulins occurring before birth, while the increase in ß tubulins proceeds throughout development (Dustin, 1984). The α and ß tubulins are both highly conserved, with the most variability being confined to the C-terminal sequences (Burns & Surridge, 1990). Post-transcriptional changes in the tubulin molecule, notably tyrosinolation of the α subunit by tubulin-tyrosine ligase, augment the complexity of the tubulin molecule. Tyrosinolation of tubulin has been

linked with differentiation since this activity decreases during brain growth (Thompson, 1982). The activity of this enzyme peaks during mid-S phase in Chinese hamster ovary (CHO) cells implying a role in cellular regulation (Deanin *et al.*, 1980; Forrest & Klevecz, 1978). Antimitotic drugs such as colchicine and podophyllotoxin inhibit the activity of tubulin-tyrosine ligase, which supports the notion that removal of the terminal tyrosine molecule requires intact MTs (Thompson, 1982). Evidence for post-transcriptional modifications, such as phosphorylation and glycosylation, remains obscure and debatable (Dustin, 1984).

1.3 Mechanism of Action of Podophyllotoxin

The mechanism by which POD inhibits microtubule polymerization has been investigated by a number of laboratories. However, the classic example of a spindle poison has long been regarded as the alkaloid colchicine (Fig. 1.3), which at concentrations as low as 10^{-8} M disrupts microtubule assembly and function producing metaphase arrest in dividing cells (Hartwell & Schrecker, 1958; Jardine, 1980; Dustin, 1984; Gupta, 1989b). Colchicine noncovalently binds tubulin dimer, which contains one high-affinity binding site (Dustin, 1984; Hamel, 1989) and a second, low-affinity site (Ringel & Sternlicht, 1984; Floyd *et al.*, 1989). Association constants for colchicine binding to the high-affinity site range from 1 to 40 μM, depending on the source of tubulin and experimental conditions (Wilson *et al.*, 1974; Bhattacharyya & Wolff, 1974; Sherline *et al.*, 1975; Bhattacharyya & Wolff, 1976; Garland, 1978). The high-affinity

colchicine binding site overlaps with a high-affinity binding site for POD, which produces the same physiological and morphological effects as colchicine (Jardine, 1980). Wilson and Friedkin (1967) first noted that the mechanism of action of POD was very similar to that of colchicine from the observation that POD prevented the binding of radiolabeled colchicine to grasshopper embryo tubulin. Tubulin from several different sources has been used to demonstrate that POD competes with colchicine for the high-affinity colchicine binding site, with inhibition constants (Ki) ranging from 0.3 to 3 μM (Wilson, 1970; Bryan, 1972; Wilson & Meza, 1973; Zweig & Chignell, 1973; Bhattacharyya & Wolff, 1974; Flavin & Slaughter, 1974; Wilson *et al.*, 1974; Bhattacharyya & Wolff, 1975; Wilson, 1975; Pfeffer *et al.*, 1978; Cortese *et al.*, 1977; Hadjian *et al.*, 1977; Kelleher, 1977; McClure & Paulson, 1977; Barnes & Roberson, 1979; Lacey *et al.*, 1987). Although inhibition of colchicine binding to tubulin by POD is competitive, their mechanisms of binding appear to be quite different. The chemical structures of these two molecules (see Figs. 1.1a and 1.3) differ sufficiently enough to suggest that some extent of structural variation may be accommodated at their binding site (Kelleher, 1977). Colchicine binding to tubulin results in positive enthalpy and entropy changes and a relatively large free energy change, which indicates that the binding site may be located in a hydrophobic or nonpolar pocket (Bryan, 1972). Unlike colchicine, POD binds to tubulin in a rapid and reversible manner (Wilson, 1975), and in a less temperature-dependent fashion (Kelleher, 1977). Nevertheless, it has been demonstrated that tropolone (Fig. 1.2b), which is a single, seven membered ring analogue of colchicine (ring C, Fig.

1.3), inhibits the binding of colchicine but not POD to tubulin (Bhattacharyya & Wolff, 1974; Cortese *et al.*, 1977). The binding of colchicine to tubulin has been shown to alter the fluorescence spectrum (Garland, 1978; Lambier & Engelborghs, 1981) and the circular dichroism (CD) spectrum of tubulin (Andreu & Timasheff, 1982b).

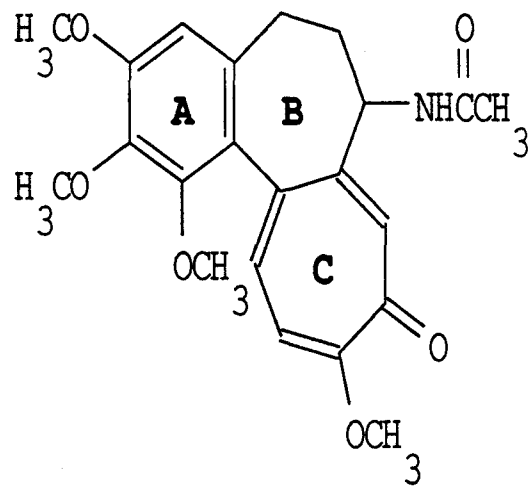


Figure 1.3: Chemical structure of the antimitotic plant alkaloid colchicine.

Furthermore, using CD conformational changes in colchicine were observed upon its binding to tubulin (Detrich *et al.*, 1981). However, no conformational changes in tubulin complexed with POD were detected using CD, fluorescence and absorption spectroscopy

(Andreu & Timasheff, 1982a, 1982b). In contrast, Morgan and Spooner (1983) report conformational changes in tubulin that is complexed with POD, colchicine and vinblastine (Fig. 1.4a) using antitubulin antibodies. Infact, these authors suggest that each drug induces a different conformational change upon binding tubulin since the inhibition curves do not overlap. Granted that the specific interaction between an antibody and its antigenic determinant have been very useful in studying the structure and functions of many proteins, it should be noted that the antibody used in these studies was generated using tubulin dimer, which may not localize the subunit with which these drugs specifically interact. Limited proteolysis experiments suggest that the colchicine binding site is regulated by the C-terminal region of α tubulin (Avila *et al.*, 1987; Mukhopadhyay *et al.*, 1990), while direct photoaffinity labelling studies reveal that the binding sites of POD and colchicine are likely to span the α/ß interface (Wolff *et al.*, 1991). The kinetics of colchicine binding to tubulin has been more extensively studied than those of POD. The binding reaction appears to proceed in two steps; with the initial rapid formation of a transient and reversible low-affinity pre-equilibrium complex followed by conformational changes in tubulin and colchicine leading to the final-state tubulin-colchicine complex (Andreu & Timasheff, 1982b; Garland, 1978; Lambier & Engelborghs, 1981; Skoufias & Wilson, 1992). Tubulin-colchicine and tubulin-POD complexes substoichiometrically inhibit MT polymerization by "capping" the assembly ends of MTs (Olmsted & Borisy, 1973b; Margolis & Wilson, 1977, 1978; Sternlicht and Ringel, 1979; Margolis *et al.*, 1980) as well as inhibiting the flux of subunits

(treadmilling) through MTs (Margolis & Wilson, 1978; Farrell & Wilson, 1984). Interestingly, the tubulin-drug complex alone polymerizes to form nonmicrotubule polymers (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982c, Andreu *et al.*, 1983). Andreu and Timasheff (1982c) propose that bound colchicine contorts the bonding geometry between tubulin molecules in such a manner that incorporation of the tubulin-colchicine complex suppresses MT polymerization by agitating the polymer lattice. This alteration in bonding geometry has been mechanistically explained by a steric hinderance model, where the high-affinity colchicine site is located within or near the tubulin domains that interact with adjacent microtubule protofilaments (Sandoval & Weber, 1979; Palanivelu & Ludueña, 1982; Saltarelli & Pantaloni, 1982). Alternatively, Andreu *et al.* (1983) propose a conformational model where colchicine induces a conformational change in tubulin that results in distortion of interdimer contacts and, hence, prevents polymerization.

Although the primary mechanism of action of POD is its ability to interact with tubulin, it has also been shown to inhibit nucleoside transport in HeLa cells (Loike & Horwitz, 1976). However, these effects are only seen at drug concentrations substantially higher than the levels required to disrupt MT dynamics. Interestingly, this transport is also inhibited by compounds such as picropodophyllotoxin and VP16-213 (Fig. 1.4), which display little or no tubulin binding activity.

1.4 Structural Requirements For Podophyllotoxin-like Antimitotic Activity

The crystal structures of several biologically inactive POD derivatives have been described (Bates & Wood, 1972; Petcher *et al.*, 1973; Yamaguchi *et al.*, 1982), as well as analogues that display antimitotic activity (Yamaguchi *et al.*, 1984; Beard *et al.*, 1987; Sicheri *et al.*, 1991, 1992a, 1992b). From this crystallographic data and solution nuclear magnetic resonance (NMR) studies (Brewer *et al.*, 1979) the three-dimensional structural requirements of POD derivatives that show antimitotic activity has been inferred. Essentially, many inactive and active analogues possess similar conformations in the rigid ABCD ring system (refer to Fig. 1.1), but subtle differences in structure account for the range of potency these compounds display. It should be noted that there are often conflicting reports on the potency of many POD analogues, depending on the method used to assess biological activity.

The coplanarity of the methylenedioxybenzene ring system (rings A and B) with carbon atoms 1 and 4 (ring C), combined with the geometric demands of the trans-lactone ring (ring D) constructs the rigid backbone of the POD molecule. In contrast, the biologically inactive picropodophyllotoxin molecule, which has a cis-fused lactone ring, is considerably less rigid and adopts limiting conformations when compared with its active counterpart. Gensler and Gatsonis (1966) suggest that the low level of activity sometimes observed for picropodophyllotoxin may be attributable to the small amount of POD generated by equilibrium. However, analysis of POD and several of its analogues using nuclear Overhauser enhancement spectroscopy (NOESY) indicate that

picropodophyllotoxin exists in two conformations (Brewer *et al.*, 1979). In one conformation the E ring is quasi-equatorial to the ABCD ring system and rotates about the C1-C1' bond. These studies revealed that the C and D rings of the POD derivatives are involved in their interactions with tubulin, and these interactions were shown to be sensitive to the configuration, size, and hydrophilic character of substituents at the C4 position. Structural alterations that do not reduce the activity of POD include demethylation at the C4' position, removal of the hydroxyl group at the C4 position, and addition of a hydroxy or methoxy group at the C5 position (Kelleher, 1977; 1978; Flavin & Slaughter, 1974; Loike et al, 1978). Gupta and Chenchaiah (1987) synthesized a number of ester derivatives of the C4 hydroxyl group of 4'-demethylepipodophyllotoxin (DMEP, Fig. 1.1e), all of which showed POD-like activity. Structure/activity studies of these derivatives indicate that the placement of a bulky substituent in epi configuration at the C4 position does not interfere with the POD-like activities of the compounds. Earlier studies show that the hydroxy group at C4 of deoxypodophyllotoxin is not essential for its POD-like activity (Kelleher, 1977; Loike *et al.*, 1978; Gupta, 1983a). Furthermore, some substitutions at C4 markedly enhance the POD-like activity of the compound, such as the p-hydroxybenzyl and the dichlorobenzyl esters of DMEP, both of which contain aromatic rings with electron donating groups. It has also been shown from several studies that the presence of a free C4' hydroxyl group does not significantly enhance nor diminish the POD-like activity of the compound (Kelleher, 1977; Loike *et al.*, 1978; Gupta & Singh, 1984; Chenchaiah & Gupta; 1987). In contrast with its lack

of effect on POD-like activity, the free hydroxy group at C4' remarkably magnifies the activity of DNA topoisomerase II targeting glycoside derivatives of DMEP (Gupta *et al.*, 1987).

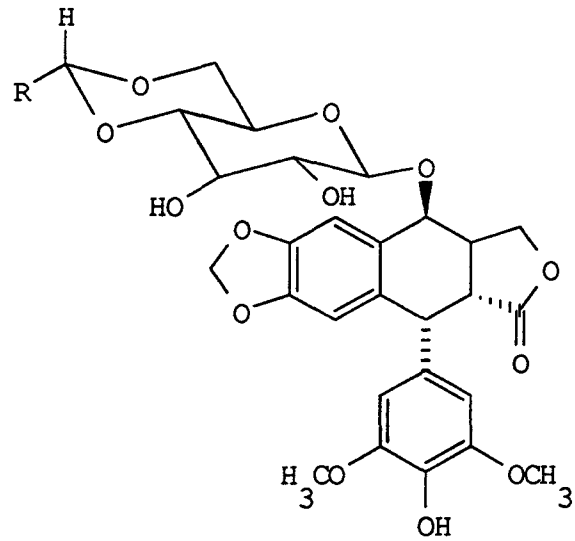
1.5 Biochemical Properties of Non-Antimitotic Podophyllotoxin Analogues

In analogy with the cardiac glycosides (particularly *Digitalis* glycosides), where the pharmacological properties of these natural compounds increase when glycosylated, von Wartburg and coworkers aimed to develop a compound with high cytostatic potential and low toxicity. With the intention of improving the therapeutic value of POD derivatives, they undertook a programme of semi-synthetic chemical modifications of POD and related lignans (reviewed in Stähelin and von Wartburg, 1989). Up to 1965 all *Podophyllum* compounds were known to exhibit their antiproliferative effects by blocking cells in mitosis. However, semi-synthetic glycoside derivatives of EP, such as 4'-demethylepipodophyllotoxin ethylidene-ß-D-glucopyranoside (VP16-213, clinically known as etoposide, Fig. 1.4a) and 4'-demethylepipodophyllotoxin thenylidene-ß-D-glucopyranoside, (VM26, clinically known as teniposide, Fig. 1.4b) were found to arrest cells in late S phase or G2 phase of the cell cycle (Stähelin, 1970; Stähelin & von Wartburg, 1991). These compounds show no significant MT-inhibitory activity, are inactive in competitively displacing [^{3}H]colchicine or [^{3}H]POD binding to tubulin, and cause extensive DNA strand breaks in treated cells (Loike *et al.*, 1974; Lokie and Horwitz, 1976a, 1976b; Lokie *et al.*, 1978). The DNA fragmentation effects of these

compounds were shown to arise from inhibition of the nuclear enzyme DNA topoisomerase II (TOPO II), which functions in decatenating supercoiled DNA prior to transcription (Long & Minocha, 1983; Minocha & Long, 1984; Ross *et al.*, 1984). TOPO II is a homologous dimer which unwinds supercoiled DNA by transiently breaking then resealing double-stranded DNA to facilitate passage of a separate strand through the gap (Wang, 1985). During this process a reaction intermediate, known as the "cleavable complex", is formed with the enzyme subunits covalently bound to the 5' termini of the break site via a phosphotyrosyl linkage. This cleavable complex facilitates the passage of a second double helical strand of DNA through the break site with the coupled hydrolysis of ATP (Wang, 1985; Gupta & Ross, 1989). VP16-213 and VM26 (Fig. 1.4) stimulate site-specific DNA cleavage by interfering with the breakage-reunion reaction of TOPO II, presumably by stabilizing a cleavable complex between DNA and the enzyme (Liu *et al.*, 1983; Liu, 1989; Ross, 1985; D'Arpa & Liu, 1989).

Previous structure-activity relationship (SAR) studies on DMEP derivatives demonstrated the requirement of four structural features for VP16/VM26-like activity (Gupta and Singh, 1984; Loike and Horwitz, 1976b; Long *et al.*, 1984; Stähelin, 1972). These are: i) the presence of a hydroxyl group at 4'-carbon; ii) an epi configuration at the C4 position, iii) the attachment of a glycosidic moiety to the C4 hydroxyl group and iv) condensation of an aldehyde or ketone with the C4 sugar group. To examine the requirement of the C4' hydroxyl group in the activity of such compounds, Gupta *et al.*

(1987) compared the activity of a series of acetal and ketal glucosides of EP and DMEP. Results of these studies established that a free C4'-OH was not necessary for



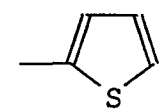
Compound	R Group
A) VP16.213	—CH_3
B) VM26	

Figure 1.4: Template structures of the DNA topoisomerase poisons, A) VP16-213 (etoposide) and, B) VM26 (teniposide).

VP16/VM26-like activity, although its presence had an enhancing effect on this type of activity (Gupta *et al.*, 1987). In order to examine the effect of substituting the glucose moiety with other sugars on the activity of DMEP derivatives, Pamidi *et al.* (1991) synthesized a number of acetal and ketal derivatives of DMEP-galactose and assessed their biological activities using the cross-resistance (CR) assay (described below) and with regard to DNA-strand breakage. The results of these studies showed that the

galactopyranoside derivatives of DMEP generally display a similar type of biological activity as the corresponding glucopyranoside compounds. However, the altered stereochemistry of the glycoside moiety in the galactoside series seems to affect the interaction of the acetal or ketal substituent R groups with the drug's presumptive cellular receptor. In the glucoside derivatives (*viz* VP16-213 and VM26) potency increases with size and hydrophobicity of the R group, whereas in the galactoside derivatives the R group does not appear to affect the drugs' potency (Pamidi *et al.*, 1991).

1.6 Clinical Uses of Podophyllotoxin And Its Derivatives

Species specific warts are known to afflict several animals, and in man, are classified according to morphology, distribution and epidemiology (von Krogh, 1981). *Condylomata acuminata* exemplify macromorphologically typical warts of anogenital areas. In 1946, King and Sullivan, described the cytological changes in normal human and rabbit skin, as well as in *Condylomata acuminata* following topical application of podophyllin or colchicine. They found that the histological and clinical consequences of *Condylomata acuminata* after application of colchicine were more intense and of shorter duration than those treated with podophyllin. By the late 1940's Sullivan and collaborators (Sullivan, 1949; Sullivan & Blanchard, 1947; Sullivan *et al.*, 1948) postulated that the active component of podophyllin responsible for damaging cells was POD. Traditional *Condylomata acuminata* therapy has since involved the topical application of dilute podophyllin, and of the components which comprise podophyllin

resin, POD has been shown to be the most effective in combating this affliction (von Krogh, 1981). An alternative treatment to podophyllin is the nucleoside analogue, 5-fluorouracil, which inhibits cellular DNA synthesis (von Krogh, 1981).

The semi-synthetic glycoside derivatives of DMEP, VP16-213 (Fig 1.3a) and VM26 (Fig. 1.4b), have been established as potent chemotherapeutic drugs used in the treatment of acute leukemias and certain solid tumors (Rozencweiz *et al.*, 1977; Issell *et al.*, 1984; Gupta & Ross, 1989). Currently, VP16-213 is the only DMEP-glycoside commercially available, although VM26 has been utilized in a number of clinical trials. These drugs are generally administered intravenously and their toxicities consist primarily of noncumulative myelosuppression (Gupta & Ross, 1989). Side effects, which include alopecia (hair loss), nausea, vomiting and oral mucositis, are all mild in comparison to other commonly used chemotherapeutic agents (Gupta & Ross, 1989). VP16-213 has emerged as one of the most active agents in the antineoplastic armamentarium and has recently been moved into many front-line chemotherapy regimens used in the treatment of lymphomas, small cell carcinoma of the lung, and testicular carcinoma (Gupta & Ross, 1989). For example, O'Dwyer *et al.* (1985) report objective response rates of 25 to 40% with VP16-213 in the treatment of both Hodgkin's and non-Hodgkins lymphomas. When used as a single agent in the treatment of small cell lung carcinoma, an average response rate of 38% was reported (Schmoll, 1982). Other tumors in which VP16-213 has been found to be active include Kaposi's sarcoma (Laubenstein *et al.*, 1984), gestational trophoblastic choriocarcinoma (Newlands & Bagshawe, 1980), and ovarian carcinoma

(Hillcoat *et al.*, 1985). Although VP16-213 is more widely studied than VM26, both show similar clinical antitumour efficacy and are thus very important agents in the treatment of human cancers. It is of interest to note that the glycoside derivatives of DMEP (which do not target tubulin) are used as potent anticancer drugs, whereas other microtubule poisons, such as vinblastine (Fig. 1.5A) and taxol (Fig. 1.5C), have become very important drugs in many chemotherapeutic armamentaria. Because these drugs have either distinct cellular targets (viz VP16-213 and VM26) or possess unique binding sites on tubulin, there are likely myriads of cellular processes unique to each type of cancer which are favourably inhibited by select compounds. Also, since these drugs have different rates of metabolism they display a wide range therapeutic properties. For example, in a study on the actions of *vinca* alkaloids on HeLa cells, both vinblastine and vincristine were found to concentrate in the cells (approximately 150 to 500 times in the incubation medium) with large differences between the two alkaloids (Lengsfeld *et al.*, 1982). Vinblastine is efficiently released whereas vincristine is retained much longer, which explains the greater toxicity of vincristine in chemotherapy (Lengsfeld *et al.*, 1982). These are just a few examples of the problems that are encountered when moving a potential anticancer drug from the laboratory into clinical trials.

1.7 Other Microtubule Inhibitory Compounds

Apart from colchicine and POD there is an extensive and ever growing list of compounds, most of which are natural products, that interact with tubulin. Infact, this list

has been subdivided into over 20 distinct chemical classes (Wilson, 1986). The chemical structures depicting several of the most common inhibitors are shown in figure 1.5, which include vinblastine (Fig. 1.5A), steganacin (Fig. 1.5B), taxol (Fig. 1.5C), griseofulvin (Fig. 1.5D), maytansine (Fig. 1.5E), and nocodazole (Fig. 1.5F).

The *Vinca* alkaloids, such as vinblastine, vincristine and vindesine, are widely used in cancer chemotherapy in combination with other MT inhibitory drugs (Gupta, 1989b). Although the cellular action of *vinca* alkaloids is very similar to colchicine, they bind to tubulin at a two unique sites (Dustin, 1984). The interaction of *Vinca* alkaloids with tubulin results in the formation of crystalline structures (Bensch & Malawista, 1969; Bensch *et al.*, 1969; Marantz *et al.*, 1969). There are two postulated binding sites for the *Vinca* alkaloids on tubulin; one high affinity site and one low affinity site. Like the colchicine binding site, the high affinity binding site for vinblastine decays rapidly but is stabilized by colchicine. Likewise, the colchicine binding site is stabilized by vinblastine (Wilson *et al.*, 1974; Dustin, 1984). Contrary to colchicine, the kinetics of vinblastine binding is similar to POD: rapid, reversible, and temperature independent (Bhattacharyya & Wolff, 1976). Maytansine, a macrolide similar in structure to the rifamycins, inhibits the action of vinblastine while having no effect on colchicine binding (Dustin, 1984). In contrast with colchicine and nocodazole, which stimulate the hydrolysis of GTP by tubulin, maytansine and vinblastine inhibit this activity (Lin *et al.*, 1981). Steganacin is a lactone derivative extracted from *Steganotaenia araliacea* Höchst and inhibits MT polymerization at micromolar concentrations (Dustin, 1984). This

compound competes with colchicine and POD binding and likely binds tubulin at the same or an overlapping site (Wang *et al.*, 1977; Schiff *et al.*, 1978).

Taxol is perhaps one of the most important cancer chemotherapeutic agents in recent history, and the discovery of its mechanism of action has opened new fields in MT research. Isolated from bark of the Pacific Yew tree, *Taxus brevifolia*, this compound inhibits the growth of HeLa cells by stabilizing the formation of MTs and increasing their stability, making it impossible for the cell to disassemble its MTs and form the mitotic spindle (Schiff *et al.*, 1979; Schiff & Horwitz, 1980). Taxol does not interfere with the binding of POD and colchicine (Kumar, 1981) and binds to both free tubulin and assembled microtubules (Parness & Horwitz, 1981) in a reversible manner (Manfredi *et al.*, 1982). Because of its unique mechanism of action taxol is being used more as a tool for studying the properties of tubulin and MTs (Dustin, 1984).

Other MT-inhibitory drugs include the antibiotic griseofulvin (Fig. 1.5D), which is isolated from *Penicillium griseofulvum*, and the benzimidazole derivatives, such as nocodazole (Fig. 1.5F). Clinically, the benzimidazoles are used as fungistatics and anthelminthics, while nocodazole has been shown to be an effective antineoplastic agent (Atassi & Tagnon, 1975). Nocodazole competes with radiolabeled colchicine binding to tubulin (Hamel, 1989). However, from cross-resistance studies using Chinese hamster ovary cell mutants resistant to both POD and nocodazole, Gupta (1986) has demonstrated that this class of antimitotic compounds possess a different mechanism of action than most other classes of MT inhibitors.

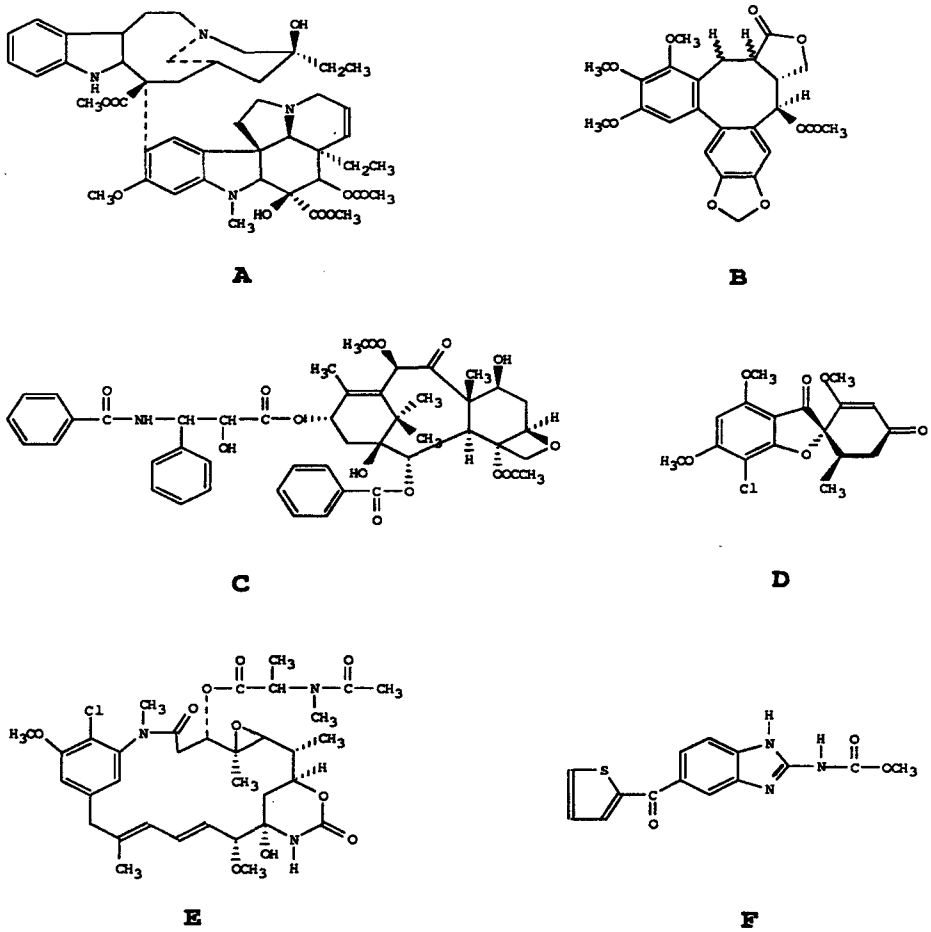


Figure 1.5: Chemical structures of several common microtubule inhibitory drugs. **A**, vinblastine; **B**, steganacin; **C**, taxol; **D**, griseofulvin; **E**, maytansine; and **F**, nocodazole.

1.8 Cross-Resistance Assay Using Cultured Cells

The observation that non-specific DNA strand breaks were produced by antimitotic POD derivatives at concentrations 100- to 10,000-fold above their levels of cellular toxicity underscored the need for a specific assay that clearly distinguishes between the two types of activities of POD derivatives, while at the same time indicating which of the activities is responsible for cellular toxicity. The isolation of stable, single-step mutants (designated as Pod^{RI} mutants), which are from two- to five-fold resistant to POD, has been achieved in cultured human (HeLa) and mouse L cells (Gupta, 1981b), mouse tetracarcinoma (OC15) and Syrian Hamster (BHK21) cells (Gupta & Hodgson, 1981), and CHO cells (Gupta, 1981a). More resistant mutants have been isolated after a single-step selection on the Pod^{RI} mutants in the presence of higher concentrations of the drug (designated as Pod^{RII} mutants, Gupta, 1981a). These mutants display proportionately increased cross-resistance towards POD derivatives possessing antimitotic activity (Gupta, 1983a; Gupta & Singh, 1984; Gupta & Chenchaiah, 1987). However, the $Pod^{RII}6$ mutants became hypersensitive to other antimitotic compounds such as colchicine, colcemid and steganacin (Gupta *et al.*, 1982; Gupta, 1983a). These results strongly support earlier biochemical studies which show the binding site for POD is different from other antimitotic compounds. Furthermore, the Pod^{RI} and Pod^{RII} mutants do not show any CR towards the POD derivatives VP16-213 and VM26, again reinforcing the inference that the mechanism of action of VP16-213 and VM26 differs from that of the other POD derivatives (Gupta, 1983; Gupta & Singh, 1984; Gupta, 1989b). The second-step mutants

were shown to possess two altered cellular proteins, designated P1 (~60 kDa) and P2 (~70 kDa) (Gupta & Gupta, 1984; Gupta *et al.*, 1985). These proteins coprecipitate with MTs and are homologues of chaperonin and heat-shock proteins (Gupta & Gupta, 1984; Gupta *et al.*, 1985; Picketts *et al.*, 1989). Because P1 is localized within mitochondria, which associates with MTs in interphase cells (Gupta *et al.*, 1985; Gupta, 1989b), it is likely that these proteins play an important role in the cellular resistance to various MT inhibitory drugs. Moreover, Gupta (1990) has proposed an attractive model for the involvement of these chaperonin proteins in the *in vivo* assembly of MTs. For the purpose of simplification this thesis will only utilize these mutants for their ability select POD-like antimitotic compounds.

In 1983, Gupta isolated the first mutants resistant to VP16-213 and VM26 in cultured CHO cells (denoted as Vpm^R mutants), after a single-step selection (Gupta, 1983b). Like the Pod^R lines, these mutants are stable after prolonged growth in non-selective medium (Gupta, 1989b). The Vpm^R mutants show increased resistance to several TOPO II inhibitory drugs, but remain sensitive to POD derivatives which display antimitotic activity (Gupta, 1983b). The mechanism of resistance of the Vpm^R mutants does not involve altered cellular uptake of the drug (Glisson *et al.*, 1986a, 1986b), however, the precise genetic lesion and its role in resistance toward this class of drugs is not clear at present (Gupta & Ross, 1989). Cross-resistance studies with Pod^R and Vpm^R mutants provides a sensitive assay for determining which type of activity POD derivatives display. The mutually exclusive and complimentary CR pattern coupled with

the fact that different cellular components are altered in mutants resistant to either the antimitotic drugs or the TOPO II poisons, provides strong evidence that the two types of activities exhibited by POD derivatives are unrelated and involve different cellular targets. Work carried out with these mutants in our laboratory has provided valuable information regarding many different properties of podophyllotoxin derivatives. These include: (i) mechanisms of action of the drugs; (ii) the mechanisms by which cellular resistance to these drugs develops and examination of cross-resistance patterns of mutants towards other compounds; (iii) development of a simple cross-resistance assay for screening/identifying such compounds; (iv) demonstration of genotoxic activity of certain drugs in mammalian systems and lack of such an effect in prokaryote cells (e.g. TOPO II poisons); (v) structure/activity relationships; and (vi) the rational design of new compounds possessing these types of activity.

1.9 Nucleoside Analogues

Nucleoside analogues represent a useful class of antimetabolites for the treatment of acute leukemia and some solid tumors. Many pyrimidine nucleoside analogues have become useful in the clinical setting in recent years, one of the most active being cytosine arabanoside (Rossi, 1979). Interestingly, many nucleoside analogues are inferior to their base analogues (*viz* 5-fluorouracil, 6-mercaptopurine, 6-thioguanine). However, some of the most potent nucleoside analogues (*viz* toyocamycin, tubercidin, 8-azaadenosine, and 2-fluoroadenosine) exhibit a broad spectrum of biological activities, such as antibacterial,

antiparasitic, antineoplastic, and antiviral (Gupta, 1989a). Many of these compounds are incorporated into RNA and DNA and eventually lead to pleiotropic effects such as inhibition of protein and nucleic acid biosynthesis, mitochondrial respiration, polyamine biosynthesis, and nuclear and tRNA methylation (Gupta, 1989a).

1.10 Research Objectives Of The Current Investigation

The design of effective anticancer drugs is often a long and arduous task with few rewards and many disappointments. The field of cancer research is abundant in attempts at deciphering the mechanisms of action of an endless array of potent natural products, semi-synthetic derivatives, and novel synthetic compounds. For over 10 years the laboratory of Dr. R.S. Gupta has been working at understanding the mechanisms of action of several classes of biologically active and medically important compounds, which range from nucleoside analogues (Gupta, 1989a) and protein synthesis inhibitors (Gupta, 1989c) to several antimitotic compounds (Gupta, 1989b). In particular, this laboratory has focused on determining the structure/activity relationships among POD and its derivatives, using the sensitive mutant screening assay described above as well as other genetic and biochemical methods. The unique relationship between POD derivatives which inhibit the polymerization of tubulin and those which poison DNA topoisomerase II has fascinated a plethora of scientists ranging from chemists to geneticists. Generally, the aim of most researchers is to better understand these phenomena with the hope of designing more effective drugs to combat cancer. In 1987, Gupta and Chenchaiah synthesized a

number of glycoside derivatives of DMEP which showed TOPO II inhibitory activity (Gupta *et al.*, 1987) as well as a series of ester derivatives of DMEP which displayed antimitotic activity (Gupta & Chenchaiah, 1987). From these studies it was inferred that the glycoside moiety is required for TOPO II inhibitory activity, such as that seen with VP16-213 and VM26. Interestingly, some of the DMEP-ester derivatives exhibited potencies that rivalled the parent POD molecule using the CHO mutant assay (Gupta & Chenchaiah, 1987). In the past, all compounds which displayed VP16/VM26-like activities have been glucoside derivatives of EP or DMEP. However, Pamidi *et al.* (1991) recently showed that galactoside derivatives of DMEP show the same type of activity as the corresponding glucoside derivatives. Other groups have recently reported that substitution of the glucoside moiety with aromatic substituents has led to compounds which inhibit both tubulin and TOPO II (Lee *et al.*, 1990; Wang *et al.*, 1990; Chang *et al.*, 1991). However, the effects these drugs have on tubulin is only detectable at very high concentrations.

To follow the flow of logic from these earlier investigations we have synthesized a series of nucleoside derivatives of POD and DMEP to further investigate the requirement of the glucoside moiety in targeting to TOPO II. Because many nucleoside analogues are biologically active compounds used in the treatment of cancer and many other diseases (Osswald, 1978; Rossi, 1979; Gupta, 1989a), it is of interest to combine the structural features of more than one group of biologically active molecules. Certain nucleosides actually enhance the chemotherapeutic action of antineoplastic agents when

combined (Osswald, 1978). In this study, the glycoside moiety was substituted with a nucleoside (either thymidine or 2'-deoxyadenosine) which contains a five carbon sugar as opposed to the six carbon sugar present in VP16-213 and VM26. Since the pyrimidine and purine bases are able to directly interact with DNA and RNA, the resultant compound may display nucleoside analogue-like activity, or even interact better with the TOPO II/DNA cleavable complex which is responsible for DNA strand breakage in cells treated with VP16-213 and VM26. These derivatives may even retain their POD-like antimitotic activity or display any combination of the aforementioned activities. Nevertheless, whatever activities are observed with these compounds it is hoped that important structure/activity relationships will emerge from this study.

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Sources of Chemicals and Reagents

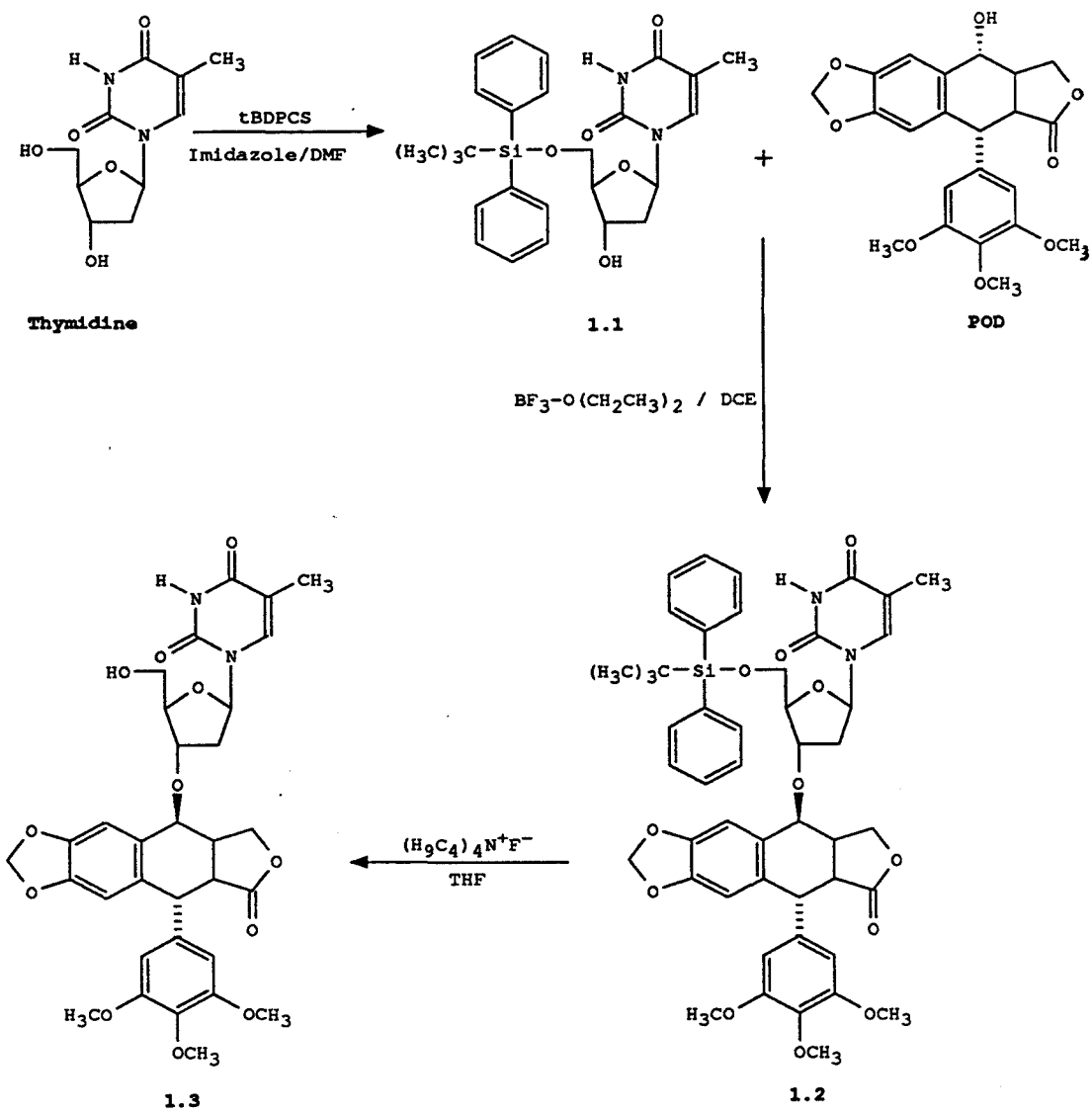
Adenosine triphosphate, bovine serum albumin (BSA), 2'-deoxyadenosine, guanosine triphosphate, hydrogen bromide (gas), imidazole, 10% palladium on carbon, *Podophyllum* resin, pyridine, sodium dodecyl sulphate, *tert*-butyldiphenylsilyl chloride, and thymidine were purchased from Sigma Chemical Co., St. Louis, MO. Barium carbonate, 1,2-dichloroethane, magnesium sulphate, ß-mercaptoethanol, piperazine-N,N'-bis[2-ethane, sulphonic acid] (PIPES), potassium chloride, sodium hydroxide, and sodium sulphate were purchased from BDH Laboratories, Toronto, ON. Alumina basic (Brockman activity 1), boron trifluoride etherate, carbobenzoxy chloride, celite, and tetra-n-butylammonium fluoride were purchased from Aldrich Chemical Co. (Milwaukee, WI). [^{3}H]Podophyllotoxin, labelled in the 4' position (specific activity, 3.4 Ci/mmol), was a gift obtained from Dr. Martin Flavin, National Heart And Lung Institute (Bethesda, MD). Acrylamide, ammonium persulphate, bis-acrylamide, and TEMED were purchased from BRL (Bethesda, MD). Silica gel (35-70 micron particles) was purchased from Toronto Research Laboratories, Downsview, ON. Alpha-minimum essential medium, fetal calf serum, and tryptone phosphate broth were purchased from Gibco Canada, Inc. (Burlington, ON).

2.2 Methods

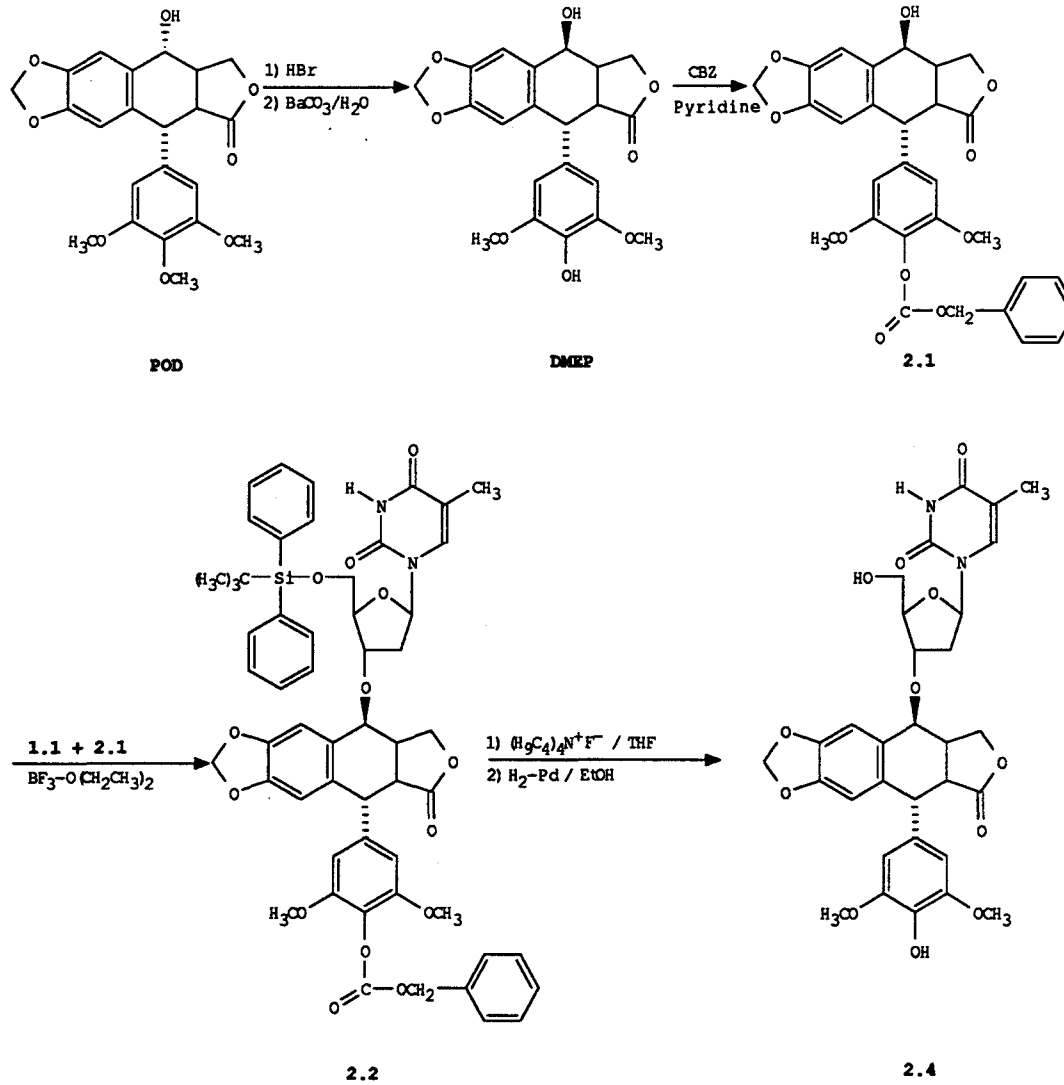
2.2.1 Synthetic Chemistry

The schemes employed for the synthesis of nucleoside derivatives of DMEP and EP using thymidine and 2'-deoxyadenosine are illustrated in schemes 2.1, 2.2, and 2.3. The synthetic scheme involves protection of the primary (i.e. 5') hydroxyl group of thymidine and 2'-deoxyadenosine using *tert*-butyldiphenylsilyl chloride to form compounds 1.1 and 3.1, respectively. Because of its preferential silylation of primary over secondary hydroxyl groups and the stability of tert-butyldiphenyl ethers in the presence of acid and under conditions of hydrogenolysis (Hanessian & Lavallee, 1975), the *tert*-butyldiphenylsilyl (*t*BDPS) group was utilized in the synthesis of the nucleoside derivatives. The silylated thymidine and 2'-deoxyadenosine derivatives can be directly conjugated via an ether linkage at their 2' carbon with the hydroxyl group at carbon 4 of POD and DMEP. This reaction is catalyzed by the Lewis acid, boron trifluoride etherate (BF_3-OEt_2), causing epimerization at the C4 position and leading to the formation of epipodophyllotoxin derivatives (Kuhn & von Wartburg, 1968; Kuhn *et al.*, 1969). Because of its scarcity in nature DMEP was synthesized from POD by selective cleavage of the 4' methoxy group with HBr gas, followed by hydrolysis and epimerization of the 1-bromo intermediate with aqueous barium carbonate (Kuhn *et al.*, 1969). Condensation of the protected nucleoside with POD or DMEP (protected as a benzyloxycarbonyl derivative, CBZ) was carried out using the stereoselective method developed by Kuhn and von Wartburg (1968 & 1969) for the preparation of 1-epi glycoside derivatives, such as

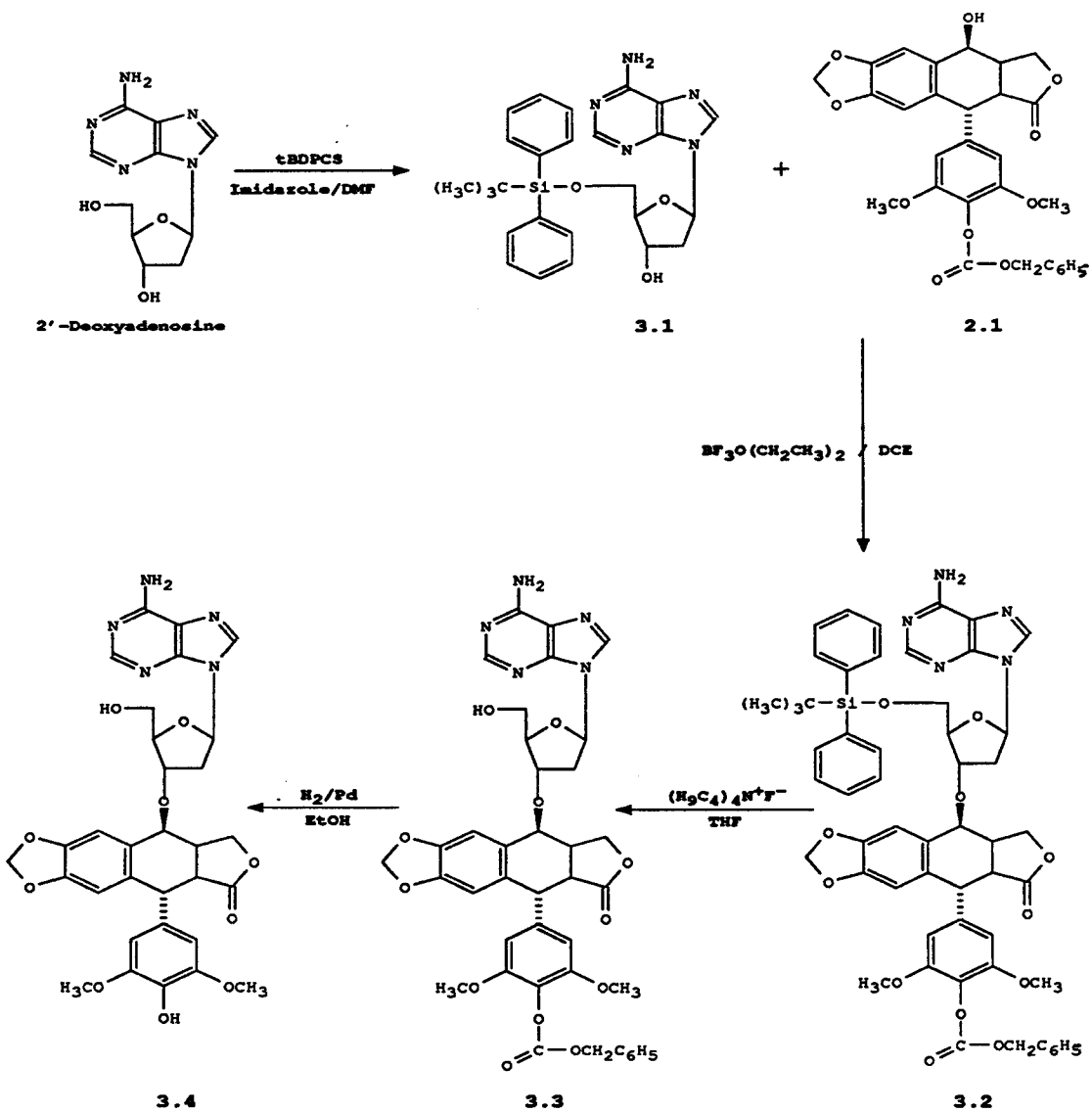
the anticancer drugs VP16.213 and VM26 (see Stähelin & von Wartburg, 1989). Selective deprotection of the *t*BDPS and CBZ groups was afforded with tetrabutyl-n-ammonium fluoride and catalytic hydrogenation, respectively. The synthetic steps illustrating the 2'-deoxyadenosine derivatives of DMEP, shown in scheme 2.3, represent the intended goal of this series of experiments. Unfortunately, only compounds 3.2 and 3.3 could be purified, and while laborious attempts were made at conjugating the silyl protected 2'-deoxyadenosine to POD, there was no progress in this phase of the synthetic chemistry.



Scheme 2.1: Synthetic routes illustrating the podophyllotoxin series of thymidine derivatives.



Scheme 2.2: Synthetic route illustrating the 4'-demethylepipodophyllotoxin series of thymidine derivatives.



Scheme 2.3: Synthetic route illustrating the 4'-demethylepipodophyllotoxin series of 2'-deoxyadenosine derivatives.

2.2.2 Instrumentation

Both proton (^1H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on Varian EM 390, Bruker AC 200 and Bruker AM 500 nuclear magnetic resonance spectrometers. All samples were dissolved in either deuterated chloroform ($CDCl_3$) or deuterated dimethyl sulfoxide (DMSO-d_6). Chemical-shift assignments are given in parts per million (ppm) relative to tetramethylsilane (TMS) and the following abbreviations (s=singlet, d=doublet, dd=doublet of doublets, t=triplet, q=quartet and m=multiplet) are used in reporting the ^1H NMR spectra. See the appendix for ^1H and ^{13}C NMR spectra for compounds 1.3 and 2.4 obtained on the Bruker AM 500 NMR spectrometer.

Mass spectra (MS) were recorded on a VG ZAB-E double focusing mass spectrometer using either direct chemical ionization (DCI) with ammonium ion (NH_3^+) or positive ion fast atom bombardment (FAB^+). In the DCI experiments the sample temperature was 200 °C, sample pressure was 3 x 10^{-5} mbar and ion energy was 70 eV. For the FAB^+ experiments sample temperature was 25 °C and sample pressure was 5 x 10^{-6} mbar. Resolution for all MS experiments was 1000.

Thin layer chromatography (TLC) was performed using Polygram Sil G/UV_{254} (Macherey-Nagel) and spots were visualized under a UV lamp at 254 nm and after exposure to iodine vapour.

Melting points (mp) were obtained using a Gallenkemp melting point apparatus and are uncorrected.

2.2.3 Purification of Podophyllotoxin (1.0)

Podophyllotoxin (1.0) was purified from the resin of *Podophyllum peltatin* as described by Kelly and Hartwell (1954). Podophyllum resin (300 g) was dissolved in 600 ml of hot absolute ethanol, filtered through a plug of cotton into 900 ml of toluene and allowed to cool to room temperature. This solution was applied to a flash chromatography column packed with 2 kg of alumina basic (Brockman activity 1) in toluene and eluted with 40% EtOH/toluene in 500 ml fractions. The fractions containing podophyllotoxin were identified by TLC, concentrated *in vacuo* and POD recrystallized from toluene. Purity was assessed by TLC, mp, ^{1}H NMR, and MS. From 300 g of resin approximately 80 g of analytically pure POD was obtained.

mp: 110-115 °C (lit.: 114-118 °C, Merck Index, 9th ed.)
TLC: ($CHCl_3$:MeOH / 95:5) Rf= 0.43
MS: (DCI) m/z 432 [$M^+ + NH_4^+$], 415 [$M^+ + 1$] 414 [M^+], 397 [M^+ - C4-OH], 247 [ABCD$^+$ ring system].
^{1}H NMR: (AC 200) δ 2.87 (d, 2H, CH-2 & 3), 3.81 (s, 6H, 3' & 5'-OCH_3), 3.86 (s, 3H, 4'-OCH_3), 4.1 (t, 1H, CH_2-11), 4.6 (m, 2H, CH-1 & CH_2-11), 4.75 (d, 1H, CH-4), 6.0 (d, 2H,-O-CH_2-O-), 6.42 (s, 2H, CH-2' & CH-6'), 6.56 (s, 1H, CH-8), 7.16 (s, 1H, CH-5).

2.2.4 *tert*-Butyldiphenylsilyl-5'-thymidine (1.1)

The synthesis was carried out according to the method described by Hanessian and Lavallee (1975). To a solution of thymidine (9.9 g, 40.9 mmol) in 50 ml of dry THF was added 2.2 equivalents of dry imidazole (6.2 g, 81.8 mmol). Next, 1.1 equivalents of *tert*-butylchlorodiphenylsilane (tBDPCS) (13.2 ml, 45.4 mmol) was added dropwise to the

stirring solution at room temperature. After stirring overnight under anhydrous conditions the reaction mixture was decanted into 400 ml of ice cold water and extracted with chloroform (3 x 150 ml). The organic phase was dried over anhydrous sodium sulphate (Na_2SO_4) and concentrated *in vacuo*. The resulting gummy yellow substance was purified by silica gel column chromatography, yielding 15.3 g of compound 1.1 (71% yield). The identity and purity of this compound was deduced from the following analytical methods.

mp: 157-163 °C
TLC: ($CHCl_3$:MeOH / 95:5) Rf= 0.50
MS: (DCI) m/z 481 [M^+ + 1], 405 [M^+ - C_6H_5], 127 [thymine$^+$].
^{1}H NMR: (AM 500) δ 1.07 (s, 9H, t-butyl: -C(CH_3)$_3$), 1.59 (s, 3H, CH_3-7'''), 2.14-2.44 (m, 2H, CH_2-2''), 3.8 (d, 1H, CH_2-5''), 3.9 (d, 1H, CH_2-5''), 4.03 (m, 1H, CH-4''), 4.5 (d, 1H, CH-3''), 6.4 (t, 1H, CH-1''), 7.3-7.4 (m, 6H, aromatic groups), 7.5 (d, 1H, CH-6'''), 7.6 (d, 4H, aromatic groups), 9.6 (broad s, 1H, NH-3''').
^{13}C NMR: (AM 500) δ 12.0 (C-7'''), 19.3 (t-butyl: -C(CH_3)$_3$), 27.0 (t-butyl: -C(CH_3)$_3$), 111.2 (C-2'''), 127.9-135.3 (aromatic groups), 150.7 (C=O, C-2'''), 164.0 (C=O, C-4''').

2.2.5 Podophyllotoxin-4-(3''-thymidyl-5''-*tert*-butyldiphenylsilane) (1.2)

To a solution of POD (4.5 g, 0.011 mol) in 35 ml of 1,2-dichloroethane (DCE) was added 1.5 eq. of *tert*-butyldiphenylsilyl-5'-thymidine (8.0 g, 0.017 mol). The solution was cooled to -15 °C and 3.0 eq. of boron trifluoride etherate (4.0 ml, 0.032 mol) was slowly added to the stirring solution. The reaction temperature was maintained between -10 and -20 °C for 2 h until the reaction was stopped by the dropwise addition of 4.5 eq. of pyridine (4.0 ml, 0.049 mol). After warming to room temperature the solution was diluted with $CHCl_3$ and washed with deionized water (3 x 50 ml). The organic phase was

dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product, after purification by silica gel chromatography ($CHCl_3$ to 3% MeOH/$CHCl_3$), afforded 7.56 g of analytically pure sample of compound 1.2 (80% yield). The identity and purity of this compound was inferred from the following analytical methods.

mp: 132-135 °C

TLC: ($CHCl_3$:MeOH / 95:5) Rf= 0.60

MS: (FAB^+) m/z 877 [M^+ + 1], 635 [M^+-tBDPS], 397 [POD^+ - C4-OH], 229 [M^+ - ring E - thymidyl moiety].

^1H NMR: (AM 500) δ 1.11 (s, 9H, t-butyl: -$C(CH_3)_3$), 1.69 (s, 3H, CH_3-7'''), 2.02-2.30 (m, 2H, CH_2-2''), 2.8 (m, 1H, CH-3), 3.4 (dd, 1H, CH-2), 3.71 (s, 6H, 3' & 5'-OCH_3), 3.78 (s, 3H, 4'-OCH_3), 3.8 (dd, 1H, CH_2-5''), 4.0 (dd, 1H, CH_2-5''), 4.1 (m, 2H, CH_2-11), 4.39 (m, 1H, CH-3''), 4.44 (d, 1H, CH-4), 6.22 (m, 1H, CH-1''), 6.54 (s, 1H, CH-8), 6.72 (s, 1H, CH-5), 7.3-7.5 (m, 6H, aromatic groups), 7.6 (m, 4H, aromatic groups), 8.6 (broad s, 1H, NH-3''').

^{13}C NMR: (AM 500) δ 12.2 (C-7'''), 27.0 (t-butyl: -$C(\underline{C}H_3)_3$),38.1 (C-3), 56.2 (3' & 5'-OCH_3), 60.7 (4'-OCH_3), 111.2 (C-2''), 128.1 & 128.2 (aromatic groups), 128.6 (C-4'), 130.3 & 130.4 (aromatic carbons), 150.1 (C=O, C-2'''), 163.5 (C=O, C-4'''), 174.4 (C=O, C-9).

2.2.6 Podophyllotoxin-4-(3''-thymidine) (1.3)

To a solution of compound 1.2 (7.6 g, 8.79 mmol) in 40 ml of THF was added 1.1 eq. of 1M tetra-n-butylammonium fluoride (9.5 ml, 9.5 mmol) in THF. After stirring overnight at room temperature the THF was removed by rotary evaporation, the crude product taken up in a small volume of $CHCl_3$, and washed with deionized water (2 x 50 ml). The organic phase was dried over anhydrous Na_2SO_4, concentrated *in vacuo* and purified by silica gel column chromatography ($CHCl_3$ to 3% MeOH/$CHCl_3$). The pure fractions yielded 5.4 g of compound 1.3 (96% yield). The identity and purity of this compound was inferred from the following analytical methods.

mp: 164-168 °C

TLC: ($CHCl_3$:MeOH / 95:5) Rf= 0.44

MS: (DCI) m/z 639 [M^+ + 1], 638 [M^+], 530 [M^+ - thymidine + NH_3], 432 [POD + NH_4^+], 243 [thymidine$^+$].

^{1}H NMR: (AM 500) δ 1.86 (s, 3H, CH_3-7'''), 2.17-2.34 (m, 2H, CH_2-2''), 2.8 (m, 1H, CH-3), 3.4 (dd, 1H, CH-2), 3.69 (s, 6H, 3' & 5'-OCH_3), 3.75 (s, 3H, 4'-OCH_3), 3.8 (dd, 1H, CH_2-5''), 4.0 (d, 1H, CH_2-5''), 4.0 (d, 1H, CH-4''), 4.22-4.31 (m, 2H, CH_2-11), 4.5 (s, 1H, CH-1), 4.6 (m, 2H, CH-3'' & CH-4), 5.9 (s, 2H, C-12), 6.0 (t, 1H, CH-1''), 6.51 (s, 1H, CH-8), 6.83 (s, 1H, CH-5), 7.34 (s, 1H, CH-1'''), 9.3 (broad s, 1H, NH-3''').

^{13}C NMR: (AM 500) δ 12.2 (CH_3-7'''), 38.1 (C-3), 56.2 (3' & 5'-OCH_3), 60.7 (4'-OCH_3), 111.2 (CH-2'''), 128.7 (C-4'), 150.4 (C=O, C-2'''), 163.9 (C=O, C-4'''), 174.4 (C=O, C-9).

2.2.7 1-Bromo-1-desoxy-4'-demethylepipodophyllotoxin (2.0a)

The synthesis of 2.0a and 2.0b was carried out according to the method described by Kuhn *et al.* (1969). To a suspension of POD (80 g, 0.19 mol) in 1000 ml of DCE and 80 ml of diethyl ether at 0 °C was passed anhydrous HBr gas until 200 g was adsorbed. After stirring for 21 h the solvent was removed via rotary evaporation and the brominated compound was recrystallized from acetone yielding 45.4 g of light brown crystals (49% yield). The identity and purity of this compound was determined by the following analytical methods.

mp: 181-189 °C (rep. 186-195 °C, Kuhn *et al.*, 1969)

TLC: ($CHCl_3$:MeOH / 90:10) Rf= 0.85

^{1}H NMR: (EM 390) δ 3.8 (s, 6H, 3' & 5'-OCH_3), 4.4 (d, 2H, CH_2-11), 4.7 (d, 1H, CH-4), 5.7 (d, 1H, CH-1), 6.0 (s, 2H,-O-CH_2-O-), 6.42 (s, 2H, CH-2' & CH-6'), 6.6 (s, 1H, CH-8), 7.0 (s, 1H, CH-5).

2.2.8 4'-Demethylepipodophyllotoxin (DMEP) (2.0b)

To a suspension of compound 2.0a (45.4 g, 0.086 mol) in 350 ml of acetone was added 1.2 eqivalents of barium carbonate (20.4 g, 0.10 mol) dissolved in 50 ml of distilled water. After stirring at 40 °C for 1.5 h the barium salts were filtered off and the crystals washed with acetone (3 x 50 ml). The filtrate was concentrated *in vacuo* yielding 26.9 g of pale yellow crystals (35% yield). The purity and identity of this compound was assessed by the following analytical methods, including x-ray crystallography (Sicheri *et al.*, 1992a).

mp: 234-237 °C (rep. 228-330 °C, Kuhn *et al.*, 1969)
TLC: ($CHCl_3$:MeOH / 90:10) Rf= 0.74
MS: (DCI) m/z 418 [M^+ + $NH4^+$], 401 [M^+ + 1], 400 [M^+], 383 [M^+ - OH], 247 [M^+ - ring E].
^{1}H NMR: (EM 390) δ 2.5 (m, 2H, CH-2 & 3), 3.7 (s, 6H, 3' & 5'-OCH_3), 4.4 (d, 2H, CH_2-11), 4.7 (m, 1H, CH-4), 5.4 (d, 1H CH-1), 6.0 (s, 2H,-O-CH_2-O-), 6.3 (s, 2H, CH-2' & CH-6'), 6.6 (s, 1H, CH-8), 7.0 (s, 1H, CH-5).

2.2.9 4'-Benzyloxycarbonyl-4'-demethylepipodophyllotoxin (4'-CBZ-DMEP) (2.1)

The synthesis of 4'-CBZ-DMEP was carried out as described by Kuhn and von Wartburg (1969). To a stirring suspension of DMEP (10 g, 0.025 mol) in 150 ml of DCE was added 1.5 eq. of pyridine (2.5 ml, 0.038 mol). This solution was cooled to -20 °C and 1.5 eq. of benzyloxycarbonyl chloride (5.4 ml, 0.038 mol) was added dropwise while maintaining the temperature at -20 °C. After stirring the reaction mixture for 2 h, 100 ml of water was added and stirred for a further 5 min. The organic layer was washed

with water (2 x 100 ml), dried over anhydrous Na_2SO_4 and concentrated *in vacuo* yielding a thick gummy residue which, after one crystallization from methanol gave 11.7 g of 4'-CBZ-DMEP (88% yield). The identity and purity of this compound was determined using the following analytical methods.

mp: 185-190 °C (rep. 205-207 °C, Kuhn and von Wartburg, 1969)
TLC: (DCE:MeOH / 90:10) Rf= 0.64
MS: (DCI) m/z 552 [M^+ + $NH4^+$], 535 [M^+ + 1], 534 [M^+], 418 [M^+ - CBZ + NH_4^+]
^{1}H NMR: (EM 390) δ 2.5 (s, 2H, CH-2), 3.3 (d, 1H, CH-3), 3.6 (s, 6H, 3' & 5'-OCH_3), 4.2 (s, 2H, CH_2-11), 4.5 (d, 1H, CH-1), 4.75 (d, 1H, CH-4), 5.2 (s, 2H, O=C-CH_2-CBZ), 5.9 (s, 2H,-O-CH_2-O-), 6.3 (s, 2H, CH-2' & CH-6'), 6.5 (s, 1H, CH-8), 6.8 (s, 1H, CH-5), 7.3 (s, 6H, aromatic protons of CBZ group).

2.2.10 4'-Benzyloxycarbonyl-4'-demethyl-4-[3''-(5''-*tert*-butyldiphenylsilyl)-thymidyl]-epipodophyllotoxin (2.2)

To a stirred suspension of 4'-CBZ-DMEP (3.2 g, 6.1 mmol) in 20 ml of 1,2-dichloroethane was added 1.5 eq. of *tert*-butyldiphenylsilyl-5'-thymidine (4.4 g, 9.2 mmol). The solution was cooled to -15 °C and 2.6 eq. BF_3-OEt_2 (2.0 ml, 16.0 mmol) in 2.0 ml of DCE was added drop by drop. After stirring for 1 h at -15 °C the reaction was stopped by dropwise addition of 4 eq. of pyridine (2.0 ml, 0.024 mol). Upon warming to room temperature 100 ml of water was added and the solution stirred for another 5 min. The organic layer was washed with water (2 x 100 ml), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude product, after purification by silica gel chromatography, afforded an analytically pure sample of compound 2.2 yielding 5.2 g

(87% yield). The purity and identity of this compound was deduced from the following analytical methods.

mp: 205-208 °C

TLC: ($CHCl_3$:MeOH / 90:10) Rf= 0.74

MS: (FAB^+) m/z 997 [M^+ + 1], 862 [M^+ - CBZ], 740 [M^+ - 5''-O-*t*BDPS], 607 [M^+ - 5''-O-*t*BDPS - CBZ], 517 [DMEP-CBZ^+ - C4-OH].

^1H NMR: (AM 500) δ 1.11 (s, 9H, t-butyl: -$C(CH_3)_3$), 1.69 (s, 3H, CH_3-7'''), 2.02-2.30 (m, 2H, CH_2-2''), 2.8 (m, 1H, CH-2), 3.3 (dd, 1H, CH-3), 3.65 (s, 6H, 3' & 5'-OCH_3), 3.8 (dd, 1H, CH_2-5''), 4.0 (m, 2H, CH_2-2'' & CH_2-5''), 4.1 (m, 2H, CH_2-11), 4.38 (m, 1H, CH-3''), 4.43 (d, 1H, CH-1) 4.62 (d, 1H, CH-4), 5.24 (O=C-CH_2-CBZ), 6.0 (d, 2H, CH_2-12), 6.22 (m, 1H, CH-1''), 6.24 (s, 2H, CH-2' & 6'), 6.54 (s, 1H, CH-8), 6.72 (s, 1H, CH-5), 7.3-7.5 (m, 10H, aromatic protons of tBDPCS), 7.6 (m, 5H, aromatic protons of CBZ), 8.6 (broad s, 1H, NH-3''').

^{13}C NMR: (AM 500) δ 12.2 (CH_3-7'''), 27.0 (t-butyl: -$C(\underline{C}H_3)_3$), 38.0 (C-3), 56.2 (3' & 5'-OCH_3), 70.5 (O=C-CH_2- CBZ), 128.0 & 128.1 (aromatic groups), 128.7 (C-4), 130.3 & 130.4 (aromatic carbons), 150.1 (C=O, C-2'''), 151.4 (C=O, CBZ), 163.4 (C=O, C-4'''), 174.3 (C=O, C-9).

2.2.11 4'-Benzyloxycarbonyl-4'-demethyl-4-(3''-thymidyl)-epipodophyllotoxin (2.3)

To a solution of compound 2.2 (5.1 g, 5.2 mmol) in 25 ml of THF was added 1.1 eqivalents of tetra-n-butylammonium fluoride (5.7 ml, 5.7 mmol) at room temperature. After stirring overnight the reaction mixture was concentrated *in vacuo*, the gummy components taken up in 100 ml of $CHCl_3$, washed with water (3 x 100 ml) and the organic layer dried over anhydrous Na_2SO_4. After purification by silica gel column chromatography, compound 2.3 was determined to be analytically pure yielding 1.5 g (38% yield). The identity and purity of this compound was inferred from the following analytical techniques.

mp: 212-217 °C

TLC: ($CHCl_3$:MeOH / 95:5) Rf= 0.61

MS: (DCI) m/z 760 [M^+ + 1], 650 [M^+ - C_7H_7O], 552 [M^+ - thymidine + NH_3], 516 [DMEP-CBZ$^+$], 418 [DMEP + NH_4^+], 400 [DMEP$^+$].

^{1}H NMR: (AM 500) δ 1.87 (s, 3H, CH_3-7'''), 2.2 (m, 1H, CH_2-2''), 2.3 (m, 1H, CH_2-2''), 2.85 (m, 1H, CH-2), 3.4 (m, 1H, CH-3), 3.63 (s, 6H, 3' & 5'-OCH_3), 3.8 (dd, 2H, CH_2-5''), 4.0 (d, 1H, CH_2-4''), 4.2 (m, 2H, CH_2-11), 4.48 (m, 1H, CH-3''), 4.59 (d, 1H, CH-1) 4.61 (d, 1H, CH-4), 5.22 (O=C-CH_2- CBZ), 5.95 (s, 2H, CH_2-12), 6.0 (t, 1H, CH-1''), 6.23 (s, 2H, CH-2' & 6'), 6.51 (s, 1H, CH-8), 6.84 (s, 1H, CH-5), 7.3-7.4 (m, 5H, aromatic protons of CBZ), 9.2 (broad s, 1H, NH-3''').

^{13}C NMR: (AM 500) δ 12.2 (CH_3-7'''), 38.1 (C-3), 56.2 (3' & 5'-OCH_3), 70.5 (O=C-CH_2- CBZ), 128.9 (C-4), 138.3 (aromatic carbons), 150.4 (C=O, C-2'''), 151.5 (C=O, CBZ), 163.8 (C=O, C-4'''), 174.6 (C=O, C-9).

2.2.12 4'-Demethyl-4-(3''-thymidyl)-epipodophyllotoxin (2.4)

Compound 2.3 (1.48 g, 1.9 mmol) was dissolved in 15 ml of acetone and 100 ml of absolute ethanol and the flask was flushed with hydrogen gas four times. To the stirring solution was added 10% Pd/C catalyst (200 mg), and the flask was flushed with hydrogen gas three times. A balloon filled with hydrogen gas was attached to the flask in order to maintain a constant atmosphere of hydrogen, and the reaction mixture was allowed to stir overnight at room temperature. The solution was filtered through a bed of Celite, concentrated *in vacuo* and recrystallized from MeOH yielding 1.1 g of compound 2.4 (88% yield). The purity and identity of this compound was determined from the following analytical methods, including x-ray crystallography (Sicheri *et al.*, 1991).

mp: 226-229 °C

TLC: ($CHCl_3$:MeOH / 95:5) Rf= 0.41

MS: (DCI) m/z 642 [M^+ + NH_3], 624 [M^+], 516 [M^+ - thymine], 418 [M^+ - thymidine + NH_3], 400 [M^+ - thymidine]; (FAB$^+$) m/z 625 [M^+ + 1], 624 [M^+].

^{1}H NMR: (AM 500) δ 1.78 (s, 3H, CH_3-7'''), 2.2 (m, 2H, CH_2-2''), 2.8 (m, 1H, CH-2), 3.3 (dd, 1H, CH-3), 3.61 (s, 6H, 3' & 5'-OCH_3), 3.98 (s, 1H, CH_2-4''), 4.2 (t, 1H, CH_2-11), 4.34 (s, 1H, CH_2-3''), 4.43 (s, 1H, CH_2-11), 4.5 (d, 1H, CH-1), 4.75 (d, 1H, CH-4), 5.20 (t, 1H, CH-6'''), 6.0 (d, 2H, CH_2-12), 6.12 (t, 1H, CH-1''), 6.17 (s, 2H, CH-2' & 6'), 6.52 (s, 1H, CH-8), 7.04 (s, 1H, CH-5), 8.23 (s, 1H, NH-3''').

^{13}C NMR: (AM 500) δ 12.2 (CH_3-7'''), 37.1 (C-3), 56.0 (3' & 5'-OCH_3), 129.6 (C-4), 150.5 (C=O, C-2'''), 163.7 (C=O, C-4'''), 174.6 (C=O, C-9).

2.2.13 2'-Deoxy-5'-*tert*-butyldiphenylsilyladenosine (3.1)

The synthesis was carried out according to the method described by Hanessian and Lavallee (1975). To a solution of 2'-deoxyadenosine (10 g, 0.040 mol) dissolved in 50 ml of DMF was added 2.2 equivalents of dry imidazole (5.96 g, 0.088 mol), which caused the solution to turn slightly yellow. Next, 1.5 equivalents of *t*BDPCS (15.5 ml, 0.060 mol) was added, causing the solution to turn bright yellow, and allowed to stir overnight. The reaction mixture was then decanted into 500 ml of ice cold, deionized water and extracted with chloroform (3 x 200 ml). The organic phase was dried over anhydrous sodium sulphate and concentrated *in vacuo*. The crude product, after purification by silica gel column chromatography, afforded an analytically pure sample of compound 3.1 yielding 9.32 g (48%). The identity and purity of this compound was determined using the following analytical methods.

mp: 192-194 °C
TLC: ($CHCl_3$:MeOH / 95:5) Rf= 0.91
MS: (FAB^+) m/z 490 [M^+ + 1], 136 [adenine$^+$].
^{1}H NMR: (AC 200) δ 2.2 (m, 1H, CH-2'), 3.45 (dd, 2H, CH_2-5'), 3.66 (m, 1H, CH-3'), 4.19 (broad s, 1H, C3'-OH), 5.0 (m, 1H, CH-4'), 6.05 (t, 1H, CH-1'), 6.33 (broad s, 2H, NH_2), 6.9 (m, 6H, aromatic protons), 7.2 (m, 4H, aromatic protons), 7.64 (s, 1H, purine proton), 7.76 (s, 1H, purine proton).

2.2.14 4'-Benzyloxycarbonyl-4'-demethyl-4-[3''-(5''-*tert*-butyldiphenylsilyl)-2''-deoxyadenosyl]-epipodophyllotoxin (3.2)

To a stirred suspension of dry 4'-DMEP-CBZ (2.95 g, 5.51 mmol) in 25 ml of 1,2-dichloroethane was added 1.5 equivalents of 2'-deoxy-5'-*tert*-butyldiphenylsilyl-adenosine (4.04 g, 8.27 mmol). The solution was cooled to -15 °C and 2.5 equivalents of BF_3-OEt_2 (1.7 ml, 13.8 mmol) was added dropwise. After stirring for 1.5 h at -15 °C, was slowly added 2.5 equivalents more of BF_3-OEt_2 (1.36 ml, 1.09 mmol) to the stirring solution. After 1 h at -10 °C the reaction was stopped by the dropwise addition of 5 equivalents of pyridine (2.2 ml, 27.6 mmol). After warming to room temperature, the organic phase was washed with deionized water (3 x 100 ml), dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography yielding 3.23 g of analytically pure sample of compound 3.2 (78% yield). The identity and purity of compound 3.2 was assessed using the following analytical methods.

mp: 141-144 °C
TLC: ($CHCl_3$:MeOH / 90:10) Rf= 0.80
MS: (FAB^+) m/z 1006 [M^+ + 1].
^{1}H NMR: (AC 200) δ 1.09 (s, 9H, ^{t}Bu), 2.4 (m, 1H, CH-2''), 2.85-2.92 (m, 2H, CH-2), 3.4 (dd, 2H, CH-3), 3.68 (s, 6H 3' & 5'-OCH_3), 3.7 (m, 1H, CH-5''), 4.2 (d, 1H, CH-4), 4.2 (m, 3H, CH-3'' & CH_2-11), 4.4-4.8 (m, 3H, CH-4'', CH-1, & CH-4), 5.2 (s, 2H, -O-$C\underline{H}_2$-bz of CBZ), 5.7 (broad s, 2H, NH_2), 5.94 (d, 2H, CH-12), 6.3 (m, 3H, CH-1'', CH-2'& CH-6'), 6.56 (s, 1H, CH-8), 6.89 (s, 1H, CH-5), 7.3-7.43 (m, 10H, aromatic protons of silyl protective group), 7.64-7.68 (m, 5H, aromatic protons of CBZ protective group), 7.96 (s, 1H, purine proton), 8.29 (s, 1H, purine proton).

2.2.15 4'-Benzyloxycarbonyl-4'-demethyl-4-[3''-(2''-deoxyadenosyl)]-epipodophyllotoxin (3.3)

To a solution of compound 3.3 (2.96 g, 2.94 mmol) dissolved in 15 ml of THF was added 1.5 equivalents of tetra-n-butylammonium fluoride (1.28 ml, 4.41 mmol) at room temperature. After stirring overnight the reaction mixture was concentrated *in vacuo*, the gummy components taken up in 30 ml of $CHCl_3$, washed with deionized water (3 x 100 ml) and the organic layer dried over anhydrous Na_2SO_4. After purification by silica gel column chromatography, compound 3.3 was determined to be analytically pure yielding 1.64 g (72% yield). The identity and purity of this compound was inferred from the following analytical techniques.

mp: 150-153 °C
TLC: ($CHCl_3$:MeOH / 90:10) Rf= 0.65
MS: (FAB^+) m/z 768 [M^+ + 1].
^1H NMR: (AC 200) δ 2.2 (m, 1H, CH-2''), 2.8-3.1 (m, 2H, CH-2), 3.5 (dd, 2H, CH-3), 3.68 (s, 6H 3' & 5'-OCH_3), 3.8 (m, 1H, CH-5''), 4.1 (m, 1H, CH-4), 4.2-4.4 (m, 3H, CH-3'' & CH_2-11), 4.6-4.8 (m, 3H, CH-4'', CH-1, & CH-4), 5.26 (s, 2H, -O-$C\underline{H}_2$-bz of CBZ), 5.91 (broad s, 2H, NH_2), 5.98 (d, 2H, CH-12), 6.2 (t, 1H, CH-1''), 6.26 (s, 2H, CH-2' & CH-6'), 6.58 (s, 1H, CH-8), 6.83 (s, 1H, CH-5), 7.25-7.43 (m, 5H, aromatic protons of CBZ protective group), 7.84 (s, 1H, purine proton), 8.32 (s, 1H, purine proton).

2.2.16 X-ray Crystallography

X-ray crystallography was carried out in collaboration with Frank V. Sicheri and Dr. D.S.C. Yang, Department of Biochemistry, McMaster University. 4'-Demethylepipodophyllotoxin was crystallized from a 4:1 ethanol/water solution by slow evaporation at 4°C, forming rodlike crystals with approximate dimensions of 0.3 mm x

0.3 mm x 0.2 mm. 4'-Demethyl-4-(3''-thymidyl)-epipodophyllotoxin (compound 2.4) was crystallized from absolute ethanol by slow evaporation at 4°C and formed transparent plate like crystal having approximate dimensions of 0.3 mm x 0.3 mm x 0.15 mm. The crystals were mounted on glass fibres and measurements collected on a Rigaku AFC6R as described by Sicheri *et al.* (1991, 1992a).

2.2.17 Cell Culture and Cell Lines

The various CHO cell lines used in this investigation (Table 2.1) have been described earlier (Gupta, 1981a; Gupta *et al.*, 1982; Gupta, 1983a,b, 1989) and serve as a sensitive tool by which one may determine the biological activity of various classes of drugs. CHO cell lines were grown in monolayer cultures at 37 °C in humidifiers with a fixed atmosphere of 95% air and 5% CO_2. The growth medium was α-modified minimum essential medium (α-MEM; Stanners *et al*, 1971) suplemented with 5% fetal calf serum. The parental cell line from which mutants were selected is auxotrophic for proline and referred to as Wild Type (WT). The selection and various characteristics of these mutants have been described earlier (Gupta, 1981a, 1983b; Gupta and Ross, 1989). The specific drug resistant phenotype of these mutant cell lines is completely stable and these cell lines are routinely grown and maintained in growth medium in the absence of any selective drugs.

Table 2.1: CHO cell lines employed in analysis of POD-nucleoside analogs

Cell Line	Phenotype and origin
WT (Pro^-)	Proline-requiring CHO cell line established by Kao and Puck (1967), used for various mutant selections (Gupta, 1981; Gupta & Siminovitch, 1977, 1978, 1980).
EOT	Pro^- Emt^{rI} EMT^{rII} Oua^R Thg^R, a multiply marked CHO line derived from WT (Pro^-) line selected for resistance to emetine (two steps), ouabain, and 6-thioguanine.
$Pod^{RI}16$	POD-resistant clonal lines selected from EOT cells (Gupta, 1981).
$Pod^{RII}6$	Two step POD-resistant mutants selected from $Pod^{RI}16$ cells (Gupta, 1981).
Vpm^R5	VP16/VM26-resistant clonal lines selected from WT cells (Gupta, 1983b).

Adapted from Gupta *et al.* (1982).

2.2.18 Measurement of Drug of Resistance

The degree of resistance of mutant cell lines towards various drugs was determined by seeding approximately 250 and 500 cells (in 0.5 ml of growth medium) into the wells of 24 well tissue culture dishes (Costar, Falcon), containing 0.5 ml of increasing drug dilutions (including the control which contained no drug) made twice the final concentretion desired in the growth medium (Gupta, 1981; Gupta & Siminovitch, 1980). After incubating for 5-7 days at 37 °C the plates were washed with water, stained with 0.5% methylene blue in 50% methanol, and the number of colonies estimated. The

relative plating efficiencies (i.e. percent survival) were calculated as the ratios of the percentage of colonies at a given drug concentration to that obtained in the absence of the drug. The D_{10} value of a drug towards a cell line refers to the dose of the drug that reduces the plating efficiency of a cell line to 10% of that observed in the absence of the drug. These values were verified by semiquantitative measurements using 60 mm diameter plastic tissue culture dishes containing 5 ml of medium. After 7-10 days of incubation at 37 °C the colonies were stained as above and aggregates of 25 or more cells were counted as colonies. The D_{10} values, as determined from these latter experiments, did not vary significantly from those obtained using the 24 well dishes.

2.2.19 Mitotic Index

To examine the effects of the various derivatives on mitotic index, approximately 1 x 10^5 cells/ml were grown on glass coverslips in 35 mm plastic culture dishes 24 h prior to the experiment. When the cells were in exponential growth the medium was removed and replaced with 1 ml of medium containing increasing concentrations of the analogues. After incubating at 37 °C for two hours the cells were treated with an equal volume of 0.075 M solution of KCl to facilitate swelling of the cells. After 15 min. the cells were slowly fixed with a solution of methanol : acetic acid (3:1). The coverslips were allowed to air dry and the cells were then fixed with 4% Giemsa in phosphate buffer (pH 6.8) for approximately 5 minutes. The fixed-stained cells were examined at random under the light microscope and the number of metaphases per 100 cells was calculated.

2.2.20 Tubulin Purification

Tubulin was purified from fetal calf brains (Highland Packers Ltd., Stoney Creek) in PEMG buffer (0.1 M PIPES-NaOH, pH 6.6, 1 mM EGTA, 1 mM $MgSO_4$, and 0.1mM GTP) according to the procedure of Vallee (1986). Fetal calf brains were obtained immediately after slaughter were kept in an ice-water slurry for the 45 min. trip from the slaughter house to the lab. The entire procedure was carried out at 4 °C. Blood clots and fat was removed from 5 calf brains and approximately 600 g of cerebral cortex was added to 900 ml of PEMG buffer with 63 µl of MeSH (1 mM). The brain tissue was cut into small pieces with surgical scissors and the resulting solution homogenized for 4 sec. in a Waring commercial blender set at low speed. The homogenate, transferred in about 10 aliquots, was subjected to approximately four passes in a 250 ml Teflon-in-glass homogenizer (Glenco) until the homogenate appeared smooth. The homogenate was centrifuged in six 290 ml centrifuge bottles at 0 °C in GSA rotor (Dupont-Sorval) at 12,000 rpm for 90 min. The supernatant (cytosolic extract) was decanted carefully to avoid resuspending the pellet; final volume 550 ml.

To the cytosolic extract was added 86 mg of GTP (0.3 mM) and 700 mg of ATP (2.5 mM). The cytosolic extract was incubated at 37 °C in two 500 ml flasks with gentle swirling for 45 min to induce tubulin polymerization, which was evident from the increased viscosity of the solution. The polymerized solution was poured into four pre-weighed 250 ml centrifuge bottles and centrifuged at 37 °C for 45 min at 12,000 rpm in a pre-warmed GSA rotor. The pellets were pink and transparent in appearance (yield:

18.7 g; 34 mg/ml).

The microtubule pellets were resuspended in 70 ml of ice-cold PEMG buffer and homogenized in a Teflon-in-glass homogenizer (2 passes) at 4 °C. The solution was incubated on ice for 30 min to depolymerize microtubules, then centrifuged at 18,000 rpm in an SS-34 rotor (Dupont-Sorval) for 30 min at 0 °C. The supernatant from the previous step, which contains soluble tubulin, was collected and incubated in 8 preweighed 12 ml centrifuge tubes for 15 min at 37 °C then centrifuged at 18,000 rpm in an SS-34 rotor for 30 min at 37 °C. The supernatant was carefully aspirated from the transparent, gelatinous pellets (yield: 2.2 g; 0.33 mg/ml). Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard. Tubulin purity was empirically assessed after each round of polymerization by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, Fig. 2.4).

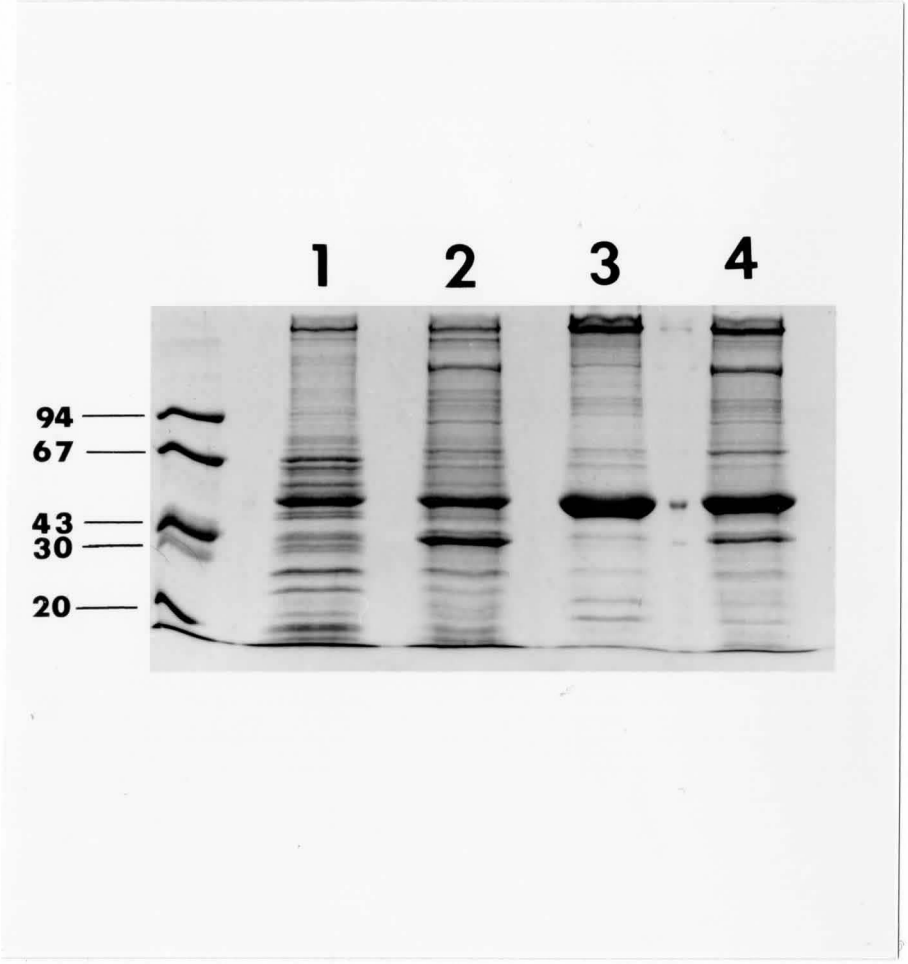


Figure 2.4: SDS-PAGE of fetal calf brain tubulin purification steps. Approximately 10μg of protein was boiled in running buffer then loaded onto the gel. Lane 1: supernatant from first polymerization step. Lane 2: protein pellet from first round of polymerization. Lane 3: microtubule pellet from third round of polymerization (this fraction was used in the tubulin binding assay). Lane 4: protein pellet from second round of tubulin purification.

2.2.21 Competition Assay

Competition of tritium labelled podophyllotoxin ([^{3}H]POD) with the thymidine derivatives binding to tubulin was performed according to the method of Cortese *et al.* (1977). The incubation medium contained PEMG buffer plus 0.75 μM tubulin, 10 μM [^{3}H]POD, and increasing concentrations of unlabelled POD derivative, giving a total reaction mixture volume of 150 μl. All competition assays were carried out in plastic 1.5 ml Eppendorf tubes. Tubulin was maintained at 0 °C until all components of the reaction mixture, except the unlabelled ligand, were added. Compounds with low water solubility were added to the incubation mixtures in DMSO at a final concentration of 1% or less. The competing ligand was added to the incubation medium after the reaction mixture had warmed to room temperature (2 min.) to prevent its precipitation. The final reaction mixture was allowed to incubate for 30 min. in a gently rocking water bath at 37 °C. The reactions were stopped by rapidly cooling the tubes to 0 °C in an ice-water slurry.

[^{3}H]Podophyllotoxin (1 mCi/ml) binding was evaluated by the DEAE-filter disc method of Cortese *et al.* (1977), however, one filter paper was used for each assay instead of two. The DE81 paper discs (Whatman) were washed with cold PEMG buffer (4 °C) by suction, such that the papers were not allowed to dry out. Over a period of 1 min. the total volume (150 μl) from each reaction mixture was adsorbed to a filter, then allowed to incubate for 30 min. at room temperature. The filters were washed four times with 4 ml of cold PEMG buffer by suction. The discs were counted in 5 ml of CytoScint to a counting error of less than 1%. Identical blanks lacking only tubulin or containing BSA in place of tubulin amounted to less than 1% of the total radioactivity applied.

2.2.22 Molecular modelling studies on IRIS computer

Molecular modelling was carried out using the *Biograf* version 2.2 (Molecular Simulations Inc., Pasadena, CA) at an IRIS personal workstation (Silicon Graphics Inc., Mountain View, CA). The generic forcefield used in the energy calculations was DREIDING (Mayo *et al.*, 1990). The models of compound 2.4 and DMEP were generated from x-ray crystal coordinates (Sicheri *et al.*, 1991, 1992a). The structure of VP16-213 was built from the crystal structure of DMEP and its conformation minimized with no constraints using the Q equilibrium routine of the *Biograf* software.

3.0 RESULTS

3.1 Cross Resistance Patterns of Thymidine Analogues Towards Various CHO Mutant Cell Lines.

In order to determine whether the thymidine derivatives of POD and DMEP displayed POD-like or VM26-like activity, cross-resistance studies were performed using first step and second step CHO mutants resistant to POD ($Pod^{RI}16$, and $Pod^{RII}6$, respectively; Gupta *et al.*, 1982, Gupta, 1983a) and single-step mutants resistant to the DNA topoisomerase II inhibitors VP16-213 and VM26, denoted as $Vpm^{R}5$ (Gupta, 1983b). These mutants exhibit highly specific cross-resistance to POD derivatives showing POD- and VM26-like activities, and their cross resistance pattern provides a sensitive and reliable assay to determine whether a given POD derivative displays POD-like or VM26-like activity (Gupta & Singh, 1984; Gupta *et al.*, 1987a). As a control, the toxicity of these compounds were compared with the POD- and VP16-213/VM26-sensitive WT cell line. Furthermore, resistance of the POD derivatives was examined with the multiply marked EOT-3 cell line to confirm that different genetic lesions played no significant role in resistance patterns observed using the Pod^{R} and Vpm^{R} mutants. As mentioned, the Pod^{R} mutants possess genetic lesions which affect the electrophoretic

mobility of two major cellular proteins associated with microtubules, designated P1 and P2 (Gupta *et al.*, 1982, 1985, 1989, & 1990).

The dose-response curves of the parental sensitive cells (WT), multiply marked EOT-3, $Pod^{RI}16$, $Pod^{RII}6$, and $Vpm^{R}5$ cells towards POD and VM26 are shown in figures 3.1 and 3.2, respectively. Wild-type, EOT-3, and $Vpm^{R}5$ cell growth, determined by relative plating efficiency was affected by concentrations of POD as low as 5 ng/ml (i.e. 12 nM). However, the first-step and second-step mutants selected for resistance to POD were affected at much higher drug concentrations (Fig. 3.1). The D_{10} values calculated for the WT, $Pod^{RI}16$, and $Pod^{RII}6$ cells were found to be 0.02, 0.10, and > 0.24 μM, respectively (see Table 3.1). Conversely, only the $Vpm^{R}5$ mutants showed increased resistance to the TOPO II poison VM26 (Fig. 3.2), with D_{10} values of 0.15 μM in the WT, $Pod^{RI}16$, and $Pod^{RII}6$ cells (Table 3.1). These findings are consistent with previous studies where these mutants were characterized (Gupta *et al.*, 1982; Gupta, 1983a, 1983b) and used as a screening assay for structure/activity relationships (Gupta *et al.*, 1985, 1987; Gupta & Chenchaiah, 1987; Pamidi *et al.*, 1991).

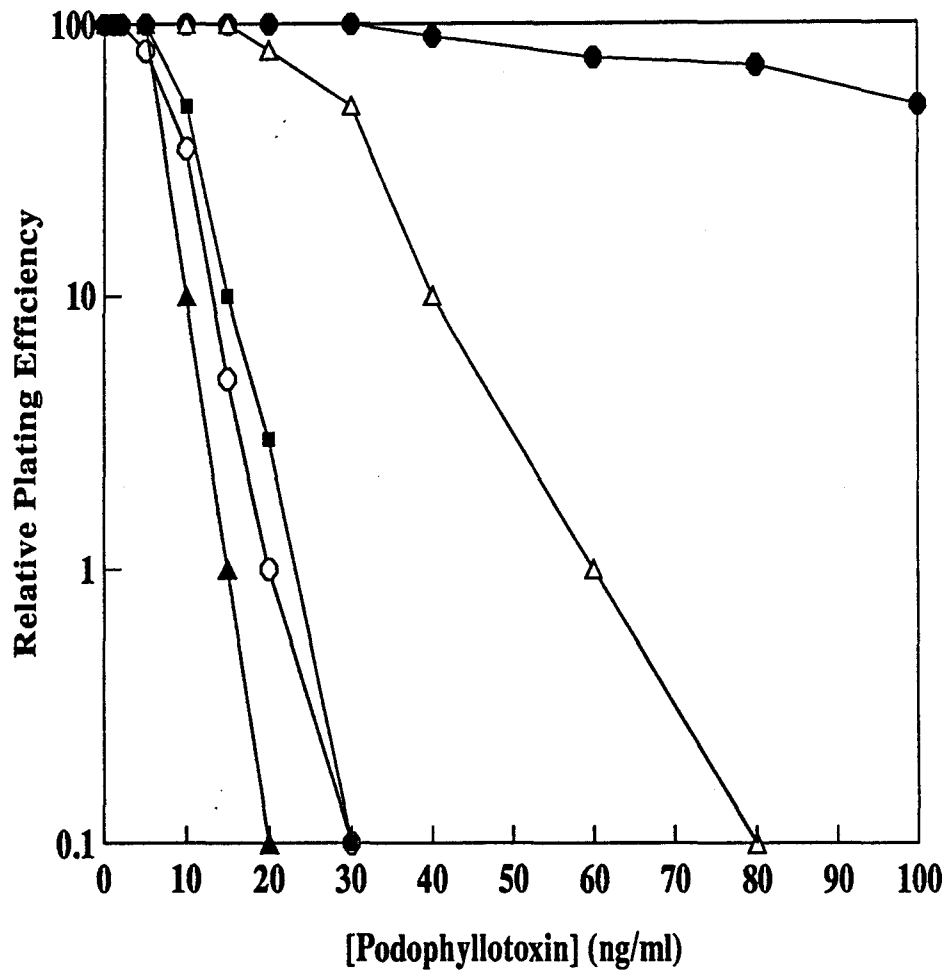


Figure 3.1: Dose-response curves illustrating the cross-resistance of Pod^R and Vpm^R CHO mutants toward the tubulin polymerization inhibitor, podophyllotoxin. WT cells (filled triangles), EOT-3 line (filled squares), $Pod^{RI}16$ line (open triangles), $Pod^{RII}6$ line (filled circles) and, Vpm^R5 line (open circles).

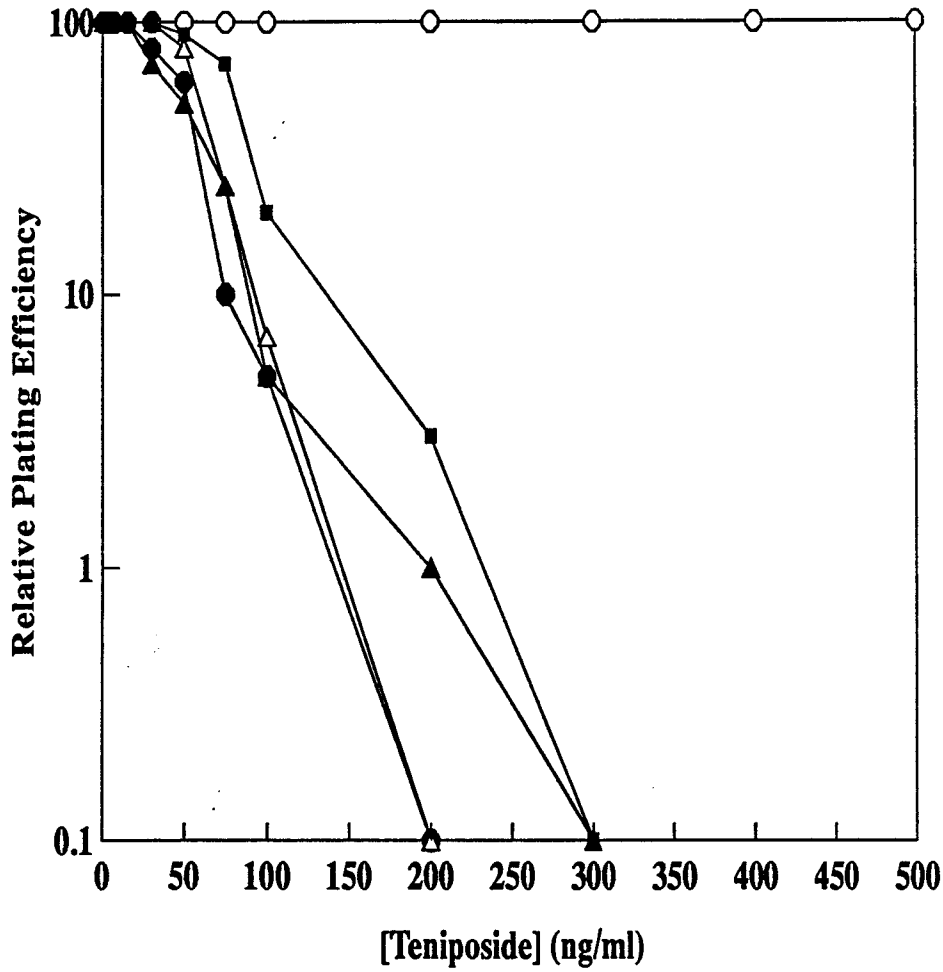


Figure 3.2: Dose-response curves illustrating the cross-resistance of Vpm^R and Pod^R CHO mutants toward the DNA topoisomerase II inhibitor, 4'-demethylepipodophyllotoxin thenylidene ß-D-glucoside (VM26). WT cells (filled triangles), EOT-3 line (filled squares), $Pod^{RI}16$ line (open triangles), $Pod^{RII}6$ line (filled circles) and, Vpm^R5 line (open circles).

Dose-response curves for the thymidine series of POD derivatives are shown in figures 3.2 to 3.9. As can be seen, the $Pod^{RI}16$ and $Pod^{RII}6$ cells exhibit proportionately increased resistance towards all derivatives except compound 1.3. Conversely, the $Vpm^{R}5$ cell line, which is highly resistant to VM26, displayed no resistance towards these compounds. These cross-resistance patterns indicate that the thymidine derivatives possess POD-like antimitotic activity and display no VM26-like activity. The lack of cellular toxicity of compound 1.3 in the cross-resistance assay may be partially due to its poor solubility in buffer (see discussion). From the dose-response curves in figures 3.2 to 3.9 the D_{10} values of the cell lines towards these compounds have been determined and are presented in Table 3.1. These results provide strong evidence that the thymidine derivatives of POD and DMEP behave like POD, although they are substantially less toxic than POD and many of its analogues. Like DMEP, the thymidine derivatives of DMEP are approximately 10-fold less toxic than compound 1.2, which is a derivative of POD. This reinforces previous findings showing that the presence of a 4' methoxy group on POD increases its affinity for tubulin (Kelleher, 1977, 1978; Jardine, 1980; Gupta, 1983a). Without this methoxy group there appears to be no affect on the antimitotic activity of derivatives with bulky substituents attached to 4' position (e.g. the CBZ group of compounds 2.2 and 2.3). This observation is consistent with the results of Gupta and Chenchaiah (1987) who report a similar trend with semi-synthetic C4 esters of DMEP.

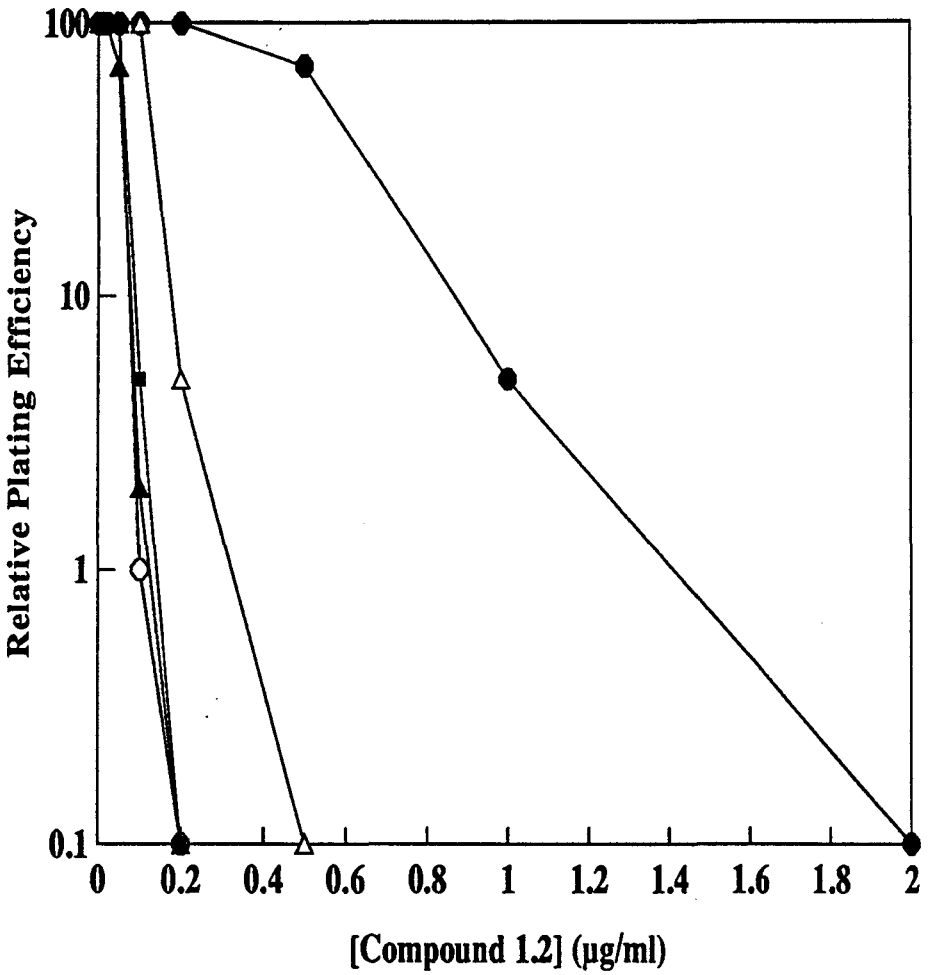


Figure 3.3: Dose-response curves illustrating the cross-resistance of Pod^R and Vpm^R CHO mutants toward podophyllotoxin-4-(3''-thymidyl-5''-*tert*-butyldiphenylsilane) (compound 1.2). WT cells (filled triangles), EOT-3 line (filled squares), $Pod^{RI}16$ line (open triangles), $Pod^{RII}6$ line (filled circles) and, Vpm^R5 line (open circles).

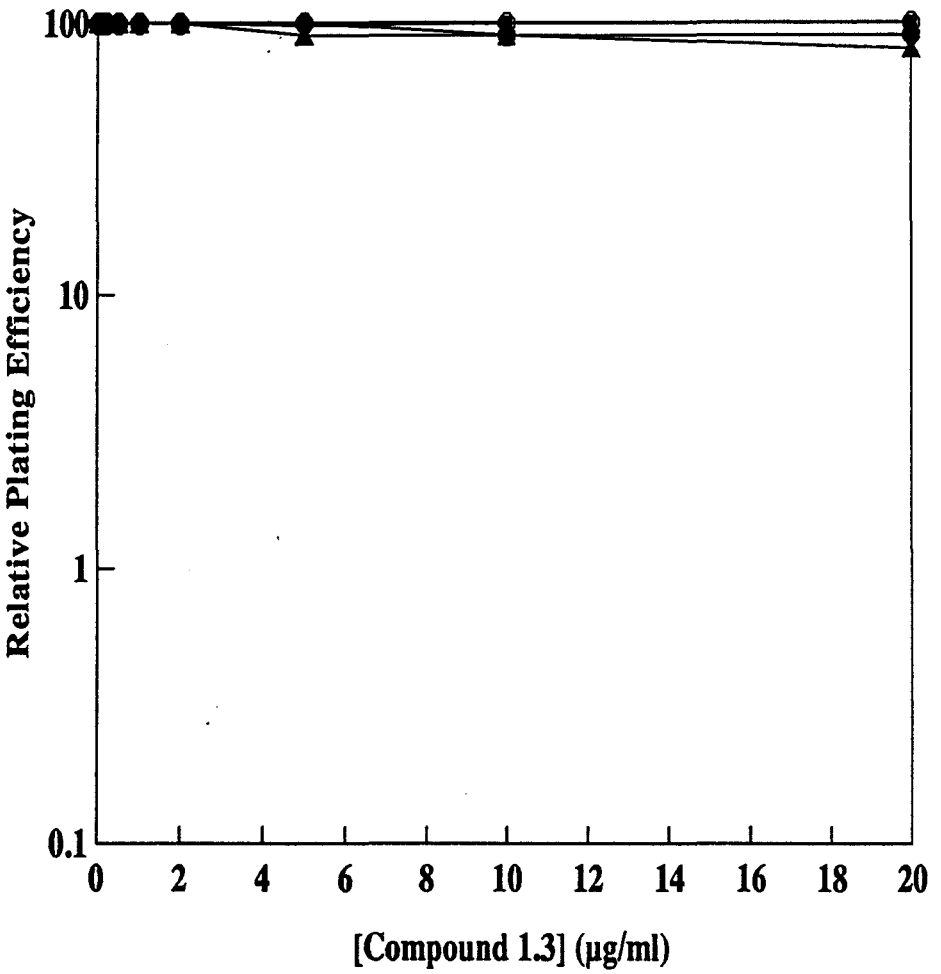


Figure 3.4: Dose-response curves illustrating the cross-resistance of Pod^R and Vpm^R CHO mutants toward podophyllotoxin-4-(3''-thymidine) (compound 1.3). WT cells (filled triangles), EOT-3 line (filled squares), $Pod^{RI}16$ line (open triangles), $Pod^{RII}6$ line (filled circles) and, Vpm^R5 line (open circles).

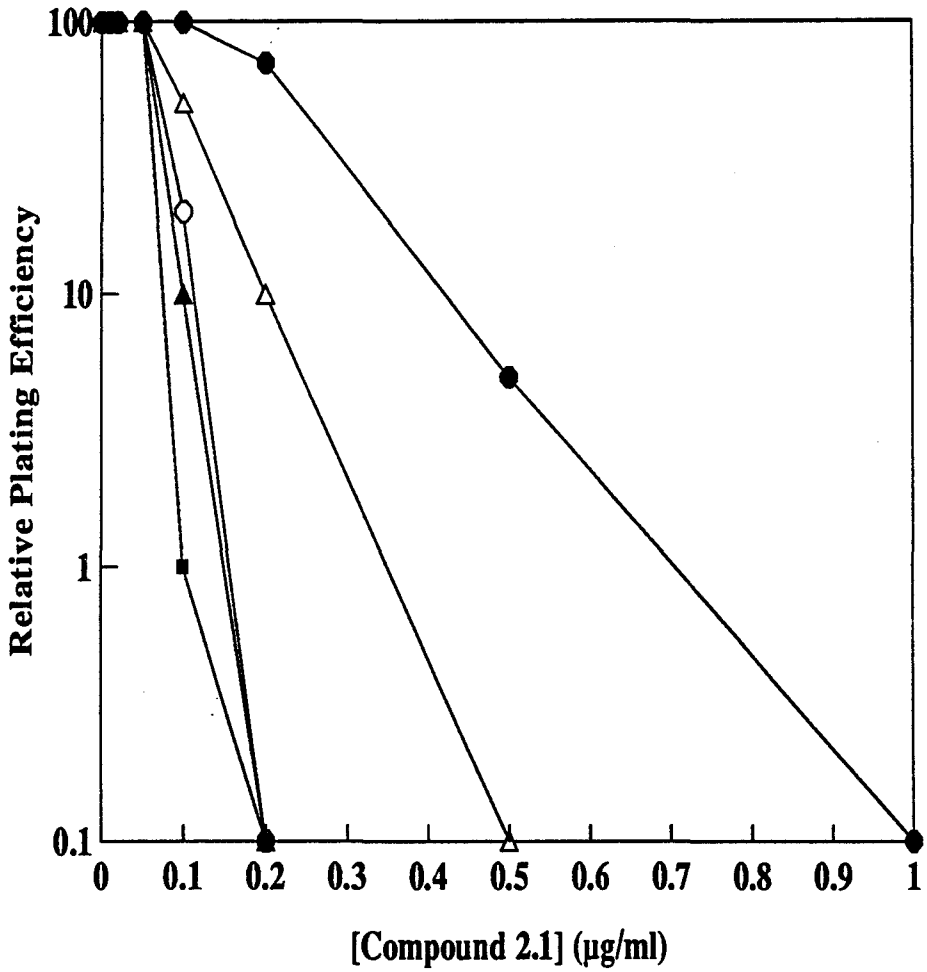


Figure 3.5: Dose-response curves illustrating the cross-resistance of Pod^R and Vpm^R CHO mutants toward 4'-benzyloxycarbonyl-4'-demethyl-epipodophyllotoxin (compound 2.1). WT cells (filled triangles), EOT-3 line (filled squares), $Pod^{RI}16$ line (open triangles), $Pod^{RII}6$ line (filled circles) and, Vpm^R5 line (open circles).

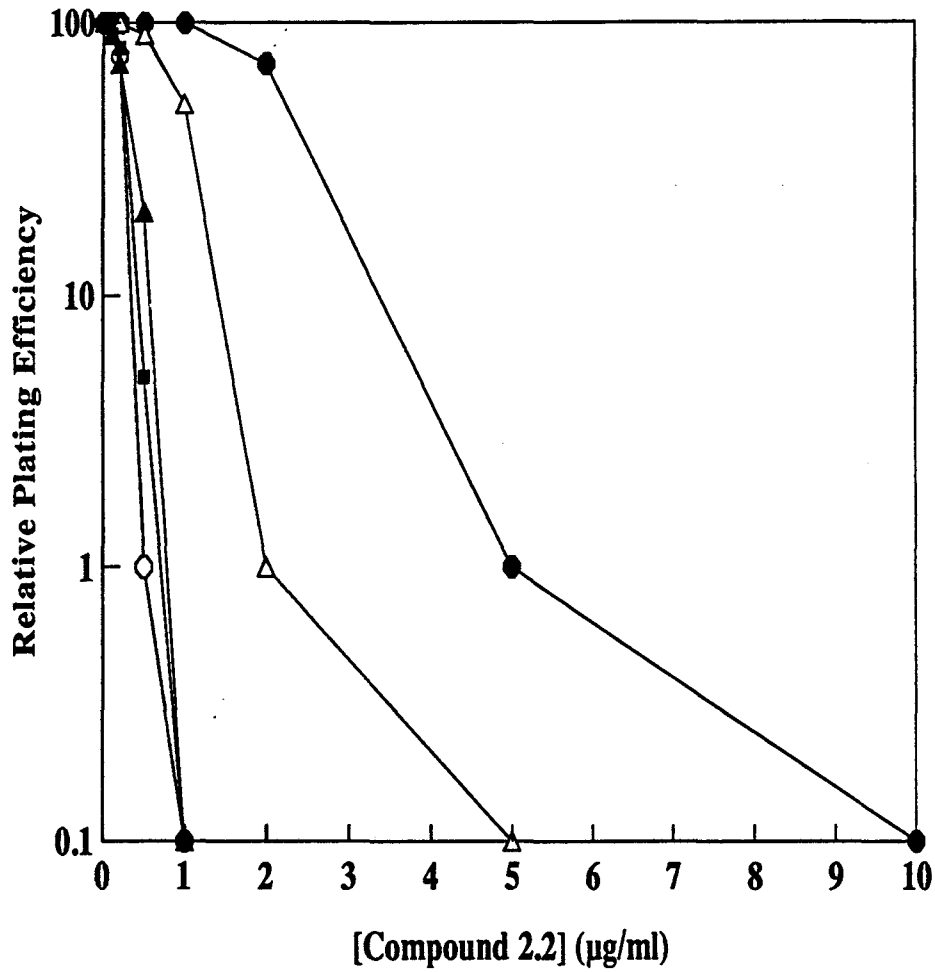


Figure 3.6: Dose-response curves illustrating the cross-resistance of Pod^R and Vpm^R CHO mutants toward 4'-benzyloxycarbonyl-4'-demethyl-4-[3''-(5''-*tert*-butyldiphenylsilyl)-thymidyl]-epipodophyllotoxin (compound 2.2). WT cells (filled triangles), EOT-3 line (filled squares), $Pod^{RI}16$ line (open triangles), $Pod^{RII}6$ line (filled circles) and, Vpm^R5 line (open circles).

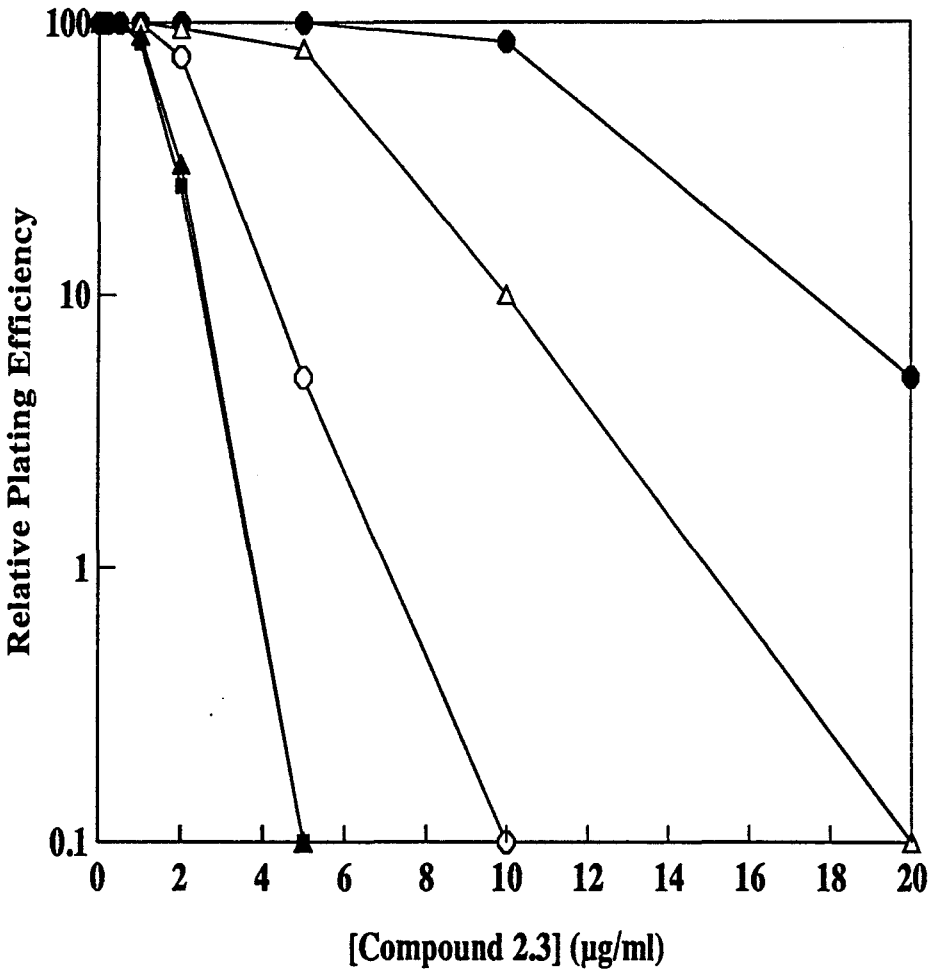


Figure 3.7: Dose-response curves illustrating the cross-resistance of Pod^R and Vpm^R CHO mutants toward 4'-benzyloxycarbonyl-4'-demethyl-4-(3''-thymidyl)-epipodophyllotoxin (compound 2.3). WT cells (filled triangles), EOT-3 line (filled squares), $Pod^{RI}16$ line (open triangles), $Pod^{RII}6$ line (filled circles) and, Vpm^R5 line (open circles).

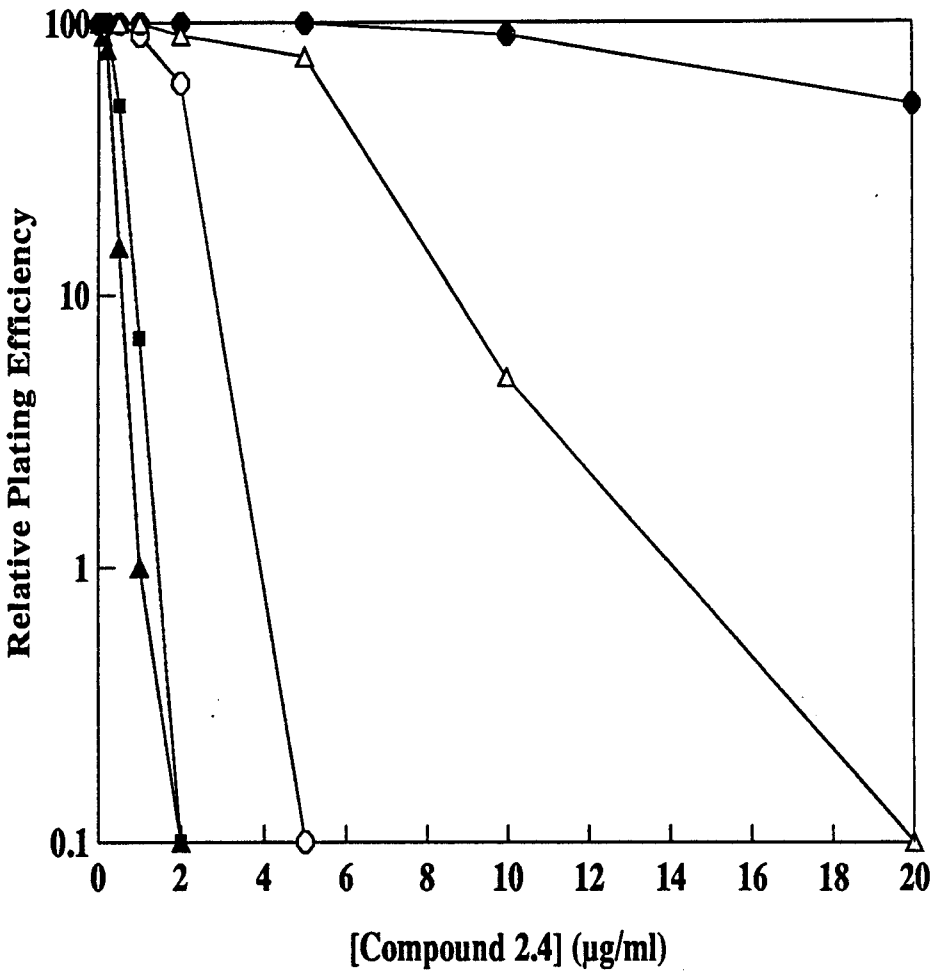


Figure 3.8: Dose-response curves illustrating the cross-resistance of Pod^R and Vpm^R CHO mutants toward 4'-demethyl-4-(3''-thymidyl)-epipodophyllotoxin (compound 2.4). WT cells (filled triangles), EOT-3 line (filled squares), $Pod^{RI}16$ line (open triangles), $Pod^{RII}6$ line (filled circles) and, Vpm^R5 line (open circles).

Table 3.1: D_{10} values for the thymidine series of epipodophyllotoxin derivatives.

Podophyllotoxin Analogue	D_{10} Values for cell lines			Podophyllo-toxin-like activity
	WT (μmol/L)	Pod^{RI}-16 (μmol/L)	Pod ^{RII}-6 (μmol/L)	
Podophyllotoxin	0.02	0.10	>0.24	Yes
Teniposide	0.15	0.15	0.15	No
1.2	0.12	0.24	1.06	Yes
1.3	----	----	----	No
2.1	0.19	0.28	0.75	Yes
2.2	1.2	1.5	3.0	Yes
2.3	6.9	12.4	24.8	Yes
2.4	6.8	16.1	>30	Yes

The 2'-deoxyadenosine derivatives of DMEP (compounds 3.2 and 3.3) were found to be inactive in the cross-resistance assay (data not shown). Because no cellular toxicity was observed for these compounds in the WT and mutant cell lines their biological activity was not investigated any further.

3.2 Mitotic Index of the thymidine series of epipodophyllotoxin derivatives.

It has been previously shown that antimitotic compounds such as podophyllotoxin increase the number of cells blocked in mitosis, whereas the epipodophyllotoxin derivatives that interact with TOPO II show no increase in the mitotic index (Stahelin, 1970; Grieder *et al.*, 1974; Krishan *et al.*, 1975; Gupta &

Singh, 1984; Gupta & Chenchaiah, 1987). Generally, such compounds display a marked increase in the mitotic index at concentrations 3 to 5 times higher than the D_{10} values observed with the same cell lines (Gupta & Singh, 1984). This variance may arise from the high concentration of drug in the cell. Treatment of WT CHO cells with POD, DMEP-CBZ, compounds 1.2, 2.2, and 2.4 increased the mitotic index approximately ten times in parallel with the dose-response curves. Furthermore, there was a marked increase in the levels of drug required to elevate the mitotic index in the $Pod^{RII}6$ cell line. It was also observed that a proportion of the CHO cells became multinuclear, which suggests that the blocked cells either revert or progress to an interphase state without completing mitosis or cytokinesis (Jordan *et al.*, 1991). In all of these experiments the mitotic index never surpassed a value of 10 percent. This was most likely due to the rounding up of cells during mitosis and their subsequent loss of adhesion to the glass coverslips. These results strongly support the finding that the thymidine derivatives of POD inhibit microtubule assembly and display POD-like activity as observed in the cross resistance studies. Compound 1.3, which displayed no biological activity in the cross resistance assay, showed no increase in mitotic index of WT and $Pod^{RII}6$ CHO cells.

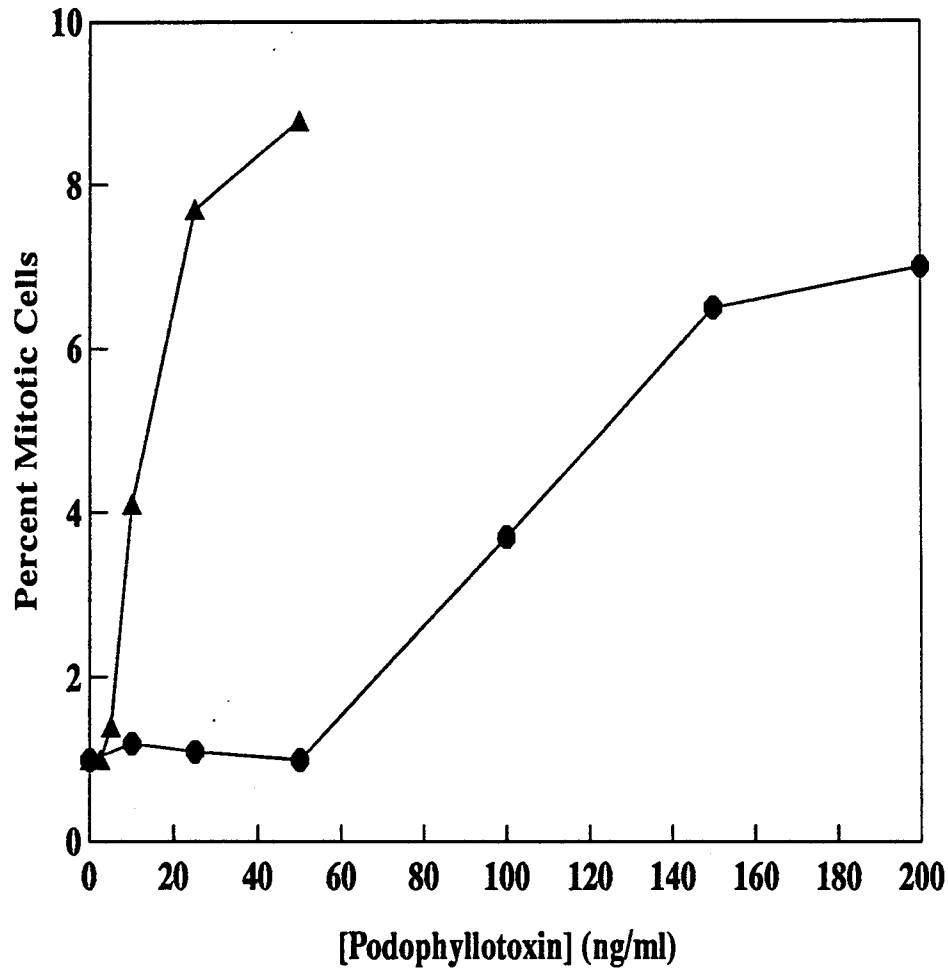


Figure 3.9: Mitotic indicies of WT (triangles) and Pod^{RII}6 (circles) CHO cells in the presence of increasing concentrations of podophyllotoxin.

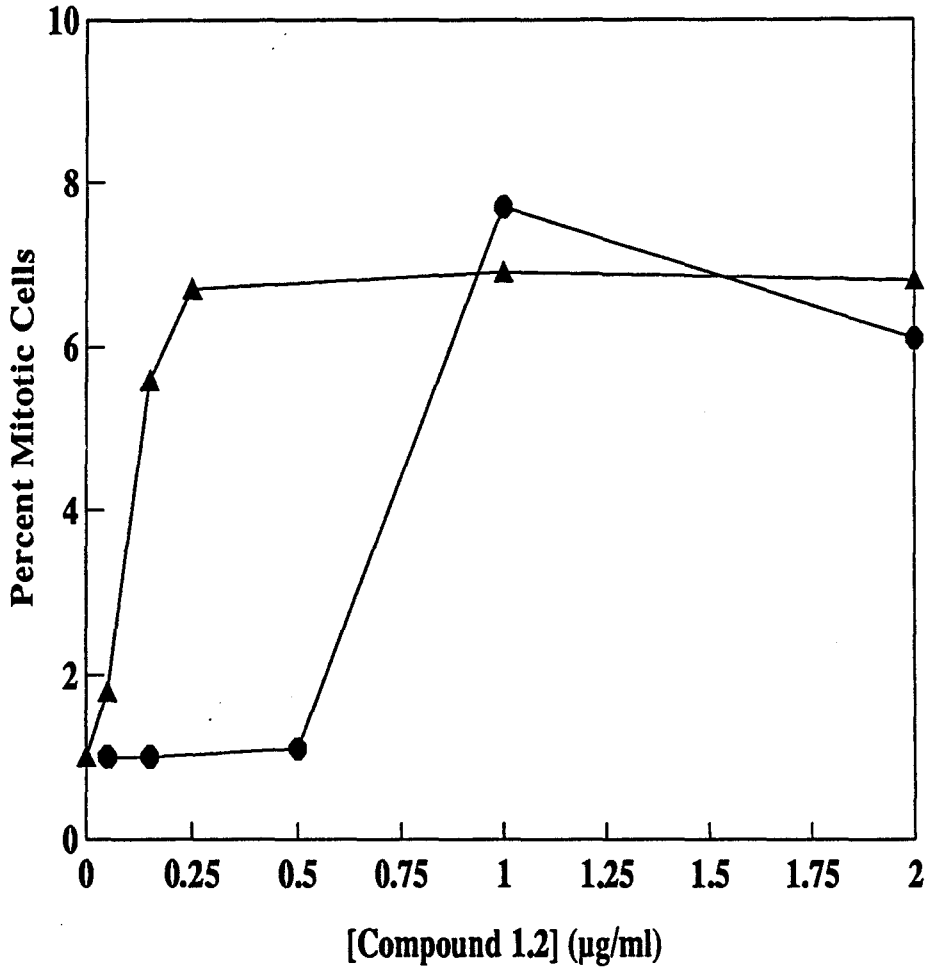


Figure 3.10: Mitotic indicies of WT (triangles) and Pod^{RII}6 (circles) CHO cells in the presence of increasing concentrations of podophyllotoxin-4-(3''-thymidyl-5''-*tert*-butyldiphenylsilane) (compound 1.2).

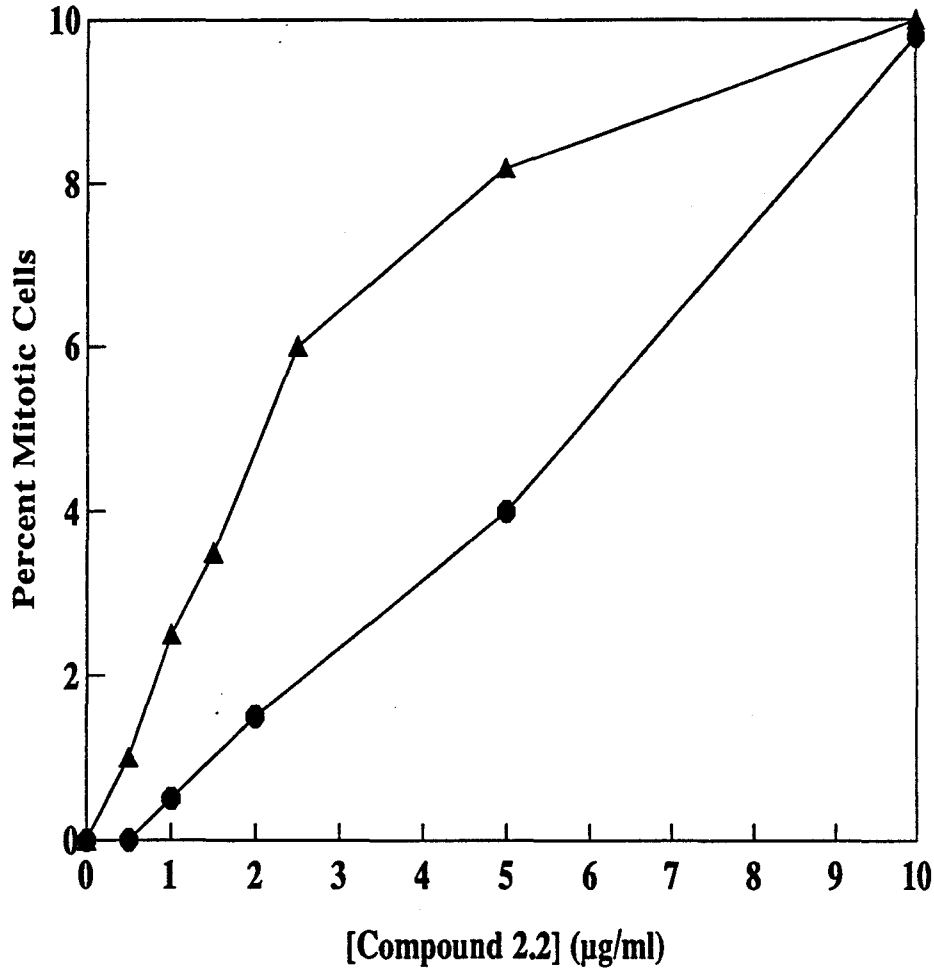


Figure 3.11: Mitotic indicies of WT (triangles) and Pod^{RII}6 (circles) CHO cells in the presence of increasing concentrations of 4'-benzyloxycarbonyl-4'-demethyl-4-[3''-(5''-tert-butyldiphenylsilyl)-thymidyl]-epipodophyllotoxin (compound 2.2).

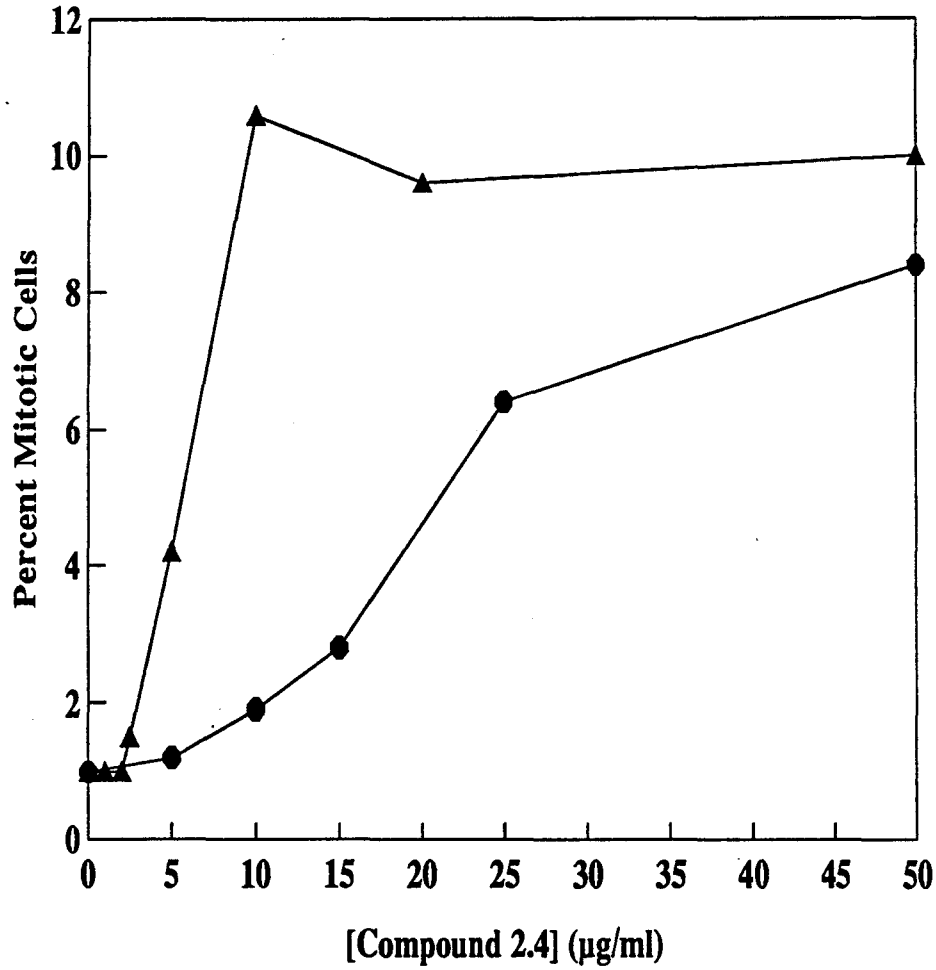


Figure 3.12: Mitotic indicies of WT (triangles) and Pod^{RII}6 (circles) CHO cells in the presence of increasing concentrations of 4'-demethyl-4-(3''-thymidyl)-epipodophyllotoxin (compound 2.4).

3.3 Competition of [^{3}H]-Podophyllotoxin Binding to Purified Calf Brain Tubulin

Podophyllotoxin competitively inhibits the binding of colchicine to tubulin (Wilson & Friedkin, 1967; Wilson, 1970; Bhattacharyya & Wolf, 1974; Cortese *et al.*, 1977; Kelleher, 1977). This has been attributed to the presence of the trimethoxyphenyl ring in both compounds (Bhattacharyya & Wolf, 1974). Bryan (1972) postulated that the positive enthalpy and entropy changes associated with colchicine binding to tubulin indicate a hydrophobic or nonpolar binding site. Although colchicine studies on tubulin function dominate the literature, POD has been shown to have a greater affinity for vertebrate tubulins (Wilson & Bryan, 1974). In this study, the only compound found to competitively inhibit the binding of [^{3}H]POD to purified tubulin was compound 1.2. Unfortunately, the binding of compound 1.2 was too weak to displace all of the [^{3}H]POD (see Fig. 3.13) and no dissociation constant could be calculated. Infact, only about 50% of the [^{3}H]POD could be displaced by compound 1.2, and the concentration at which this occurred was 100 μM. This is about 800 times the D_{10} value calculated for the WT CHO cell line. Typically, the cross-resistance assay is much more sensitive than determining the affinity of various compounds by the tubulin binding assay (e.g. see Gupta, 1983a). None of the other thymidine derivatives, which had considerably lower toxicity than compound 1.2 were able to out compete the binding of [^{3}H]POD with purified tubulin (data not shown). Because the 2'-deoxyadenosine derivatives were found to be inactive

in the CR assay, they were not tested for their ability to out compete binding of [^{3}H]POD to purified calf brain tubulin.

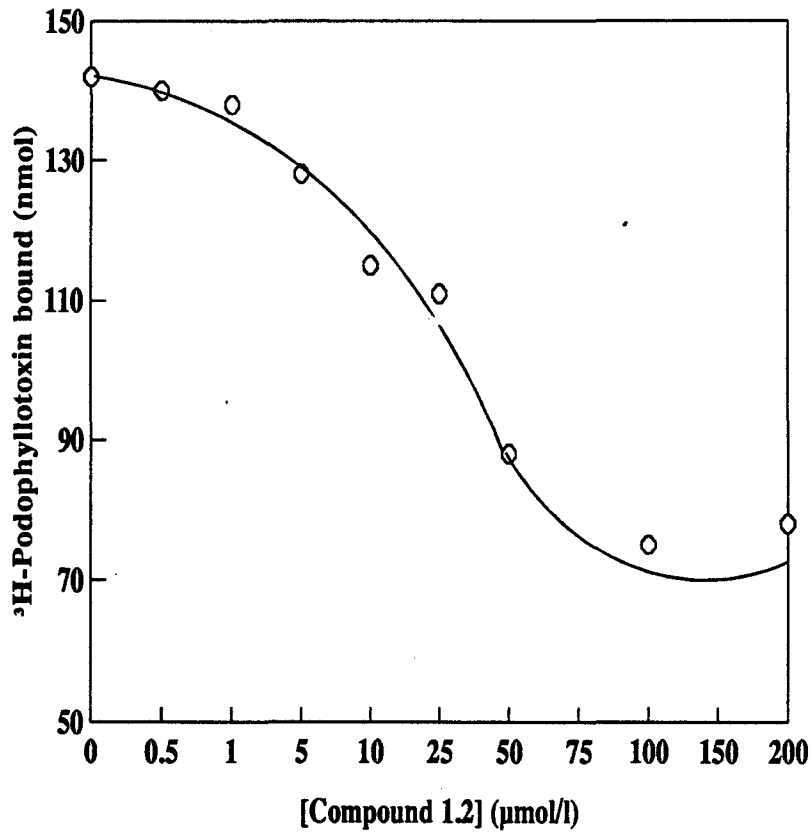


Figure 3.13: Competition curve of [^{3}H]Podophyllotoxin binding to purified calf brain tubulin tubulin with podophyllotoxin-4-(3''-thymidyl-5''-*tert*-butyldiphenylsilane) (compound 1.2).

4.0 DISCUSSION

The central theme of this thesis is to advance contemporary knowledge of the structure/activity relationships between podophyllotoxin and its analogues showing POD-like antimitotic activity, and POD derivatives which exhibit DNA topoisomerase II inhibitory activity. Two DMEP derivatives, VP16-213 and VM26, exhibit the latter type of activity and have been established as excellent anticancer drugs (Rozencweiz *et al.*, 1977; Dorr & Fritz, 1980; Issell *et al.*, 1984). Only semi-synthetic glycoside and galactoside derivatives of DMEP have been established as TOPO II inhibitors (Stähelin & von Wartburg, 1991; Pamidi *et al.*, 1991). However, Thurston *et al.* (1986) have synthesized a number of DMEP derivatives and found that DMEP-4-ethylether inhibits TOPO II with potency comparable to that of VP16-213. Thus, with the synthesis of a series of thymidine derivatives of POD and DMEP, more insight into the design of effective anticancer agents has hopefully been achieved.

Previously, synthesis of compounds modified at carbon 4 of DMEP has focused on targeting these derivative to the nuclear enzyme DNA topoisomerase II, with very little attention paid to the structural features that either enhance or diminish POD-like antimitotic activity (Stähelin & von Wartburg, 1989). Because the POD derivatives which inhibit TOPO II have proven more clinically useful as anticancer agents than their

antimitotic counterparts, there is very limited information on the structure/activity relationships that discriminate between these two types of biological activity. As a rational approach to drug design it may be useful to understand not only the structure/activity relationships among compounds showing either type of activity, but also the structural features which discriminate between these types of activity. Granted that such an ambitious undertaking would involve the synthesis and systematic evaluation of many more compounds with varying physical and chemical properties, a simple investigation such as this may provide a starting point for future research into the intriguing problem of differentiating the structural basis for such a unique divergence in biochemical specificity.

4.1 Synthetic Chemistry

Initially, we were interested in delineating the role of the glycoside moiety of VP16-213 and VM26 and whether it was absolutely necessary for TOPO II inhibitory activity. To address this question we synthesized a series of thymidine and 2'-deoxyadenosine derivatives of POD and DMEP using the Lewis acid, boron trifluoride etherate, catalyzed condensation reaction developed by von Wartburg and coworkers (Kuhn & von Wartburg, 1968; Kuhn *et al.*, 1968). The strategy of chemical syntheses with polyfunctional molecules is primarily dependent upon the availability of the appropriate protecting groups (Greene, 1981). In nucleoside chemistry the proper protection of the sugar hydroxyl groups is a challenging problem which requires selective

deblocking that does not interfere with other protective groups. Protection of the POD molecule was not necessary since the only functional group (the C4 hydroxy) was utilized in the conjugation reaction. However, the C4' hydroxyl group of DMEP was protected with CBZ according to the method of Kuhn and von Wartburg (1969), and eventually deprotection via catalytic hydrogenation over palladium catalyst. The use of sterically hindered silanes for the protection of hydroxyl groups has been reported (Corey & Venkateswarlu, 1972; Hanessian & Lavallee, 1975; Torisawa *et al.*, 1983), including with nucleosides (Ogilvie, 1973; Ogilvie *et al.*, 1974). We chose *tert*-butyldiphenylchlorosilane (*t*BDPCS) because of its preferential silylation of primary over secondary alcohols and its stability in the presence of acid (Hanessian & Lavallee, 1975). Protection of thymidine with *t*BDPCS gave 71% yield, whereas with 2'-deoxyadenosine a yield of only 48% was obtained. Selective removal of the silyl group with the fluoride ion of tetra-n-butylammonium fluoride generally afforded high yields for both thymidine and 2'-deoxyadenosine derivatives. Compound 2.3, however was recovered in suprisingly low yield after purification by silica gel column chromatography, a fact which was likely due to moisture contamination in the reaction mixture. Problems were encountered in the attempted conjugation of POD with 2'-deoxyadenosine, in that the reaction product seemed to overlap with POD on the TLC plate. While several attempts were made to separate the reaction product from POD (*viz* straight phase and reverse phase TLC, recrystallization, and HPLC), it was impossible to purify the compound. It is quite likely that this reaction failed to conjugate the nucleoside and POD molecules in the presence

of BF_3 etherate catalyst. The DMEP series of 2'-deoxyadenosine derivatives was more successful as two intermediates were purified. Because of their low yields there was not enough of compound 3.3 to carry out the final step in this series (i.e. removal of the CBZ protective group to give compound 3.4). The identity and purity of all nine nucleoside derivatives was inferred using NMR and mass spectrometry.

4.2 Cross-Resistance And Mitotic Index Studies

The biological activity of the thymidine analogues were first assessed in the cross-resistance assay employing a set of Pod^R and Vpm^R mutants of CHO cells. Because these mutants exhibit highly specific and mutually exclusive cross-resistance to compounds possessing POD-like or VP16/VM26-like activities, this assay is an excellent preliminary test for determining mechanism of action and obtaining toxicity data (Gupta, 1983 & 1990). Furthermore, D_{10} values calculated from dose-response curves (Table 3.1) with WT and mutant cell lines provides valuable information regarding cellular toxicity and structure/activity relationships among these compounds. Representative dose-response curves of the thymidine series of POD derivatives are shown in Figures 3.3 and 3.4. The dose-response curves for POD and VM26 are shown in Figures 3.1 and 3.2, respectively. In agreement with Gupta *et al.* (1982) $Pod^{RI}16$ and $Pod^{RII}6$ mutants exhibit proportionately increased resistance to POD while remaining sensitive to the TOPO II inhibitor (and POD derivative) VM26. Conversely, the Vpm^R5 mutant cell line exhibits resistance to VM26 while remaining sensitive to POD and all but one of the thymidine analogues. In Figure

3.3 it can be seen that compound 1.2 displays a similar pattern of cross-resistance as POD, however, it shows only about one-fifth of the cellular toxicity as POD. Compound 1.3 is completely inactive by this assay, notwithstanding its poor solubility in the culture medium; a property which may account for its lack of biological activity. This poor solubility may hinder its uptake into the cells. Alternatively, the free primary hydroxyl group on the thymidyl moiety may interact unfavourably with the POD binding site when conjugated with POD. Increasing the concentration of organic solvent could be an effective method of circumventing this problem, but may lead to misleading results due to solvent toxicity effects. Thus, from these experiments it appears that condensation of a 5'-protected thymidine group with POD produces a molecule with POD-like antimitotic activity, however, showing significantly less biological activity than POD. Upon removal of bulky silyl protective group this activity is lost and the molecule appears to be inactivated.

The DMEP series of thymidine derivatives also exhibited POD-like activity *in vivo*, but were significantly less active than compound 1.2. Typically, DMEP derivatives modified at the carbon 4 position, which act as inhibitors of tubulin polymerization, show no reduction in activity relative to the parent DMEP molecule (Gupta & Chenchaiah, 1987). These results are in agreement with earlier studies using deoxypodophyllotoxin which proclaim the nonessential nature of the C4 hydroxyl group (Kelleher, 1977; Loike *et al.*, 1978; Gupta, 1983a). In one study the presence of bulky substituents containing electron-donating groups was observed to enhance the POD-like activity determined in

both the cross-resistance assay and in mitotic index experiments (Gupta & Chenchaiah, 1987). None of the thymidine derivatives synthesized in this study were observed to enhance the POD-like antimitotic activity of the parent POD molecule, however, some empirical trends were observed. The presence of the bulky, hydrophobic *tert*-butyldiphenylsilyl protective group at the 5'' position amplified the potency of both compounds 1.2 and 2.2 in the cross-resistance assay. In fact, compound 1.2 had about the same toxicity as VM26 when comparing the molar D_{10} values for WT cells (0.12 μM and 0.15 μM, respectively), while compound 2.2 was roughly 10 times less toxic than compound 1.2 and 60 times less toxic than POD. However, compound 2.2 was about 7 times more toxic than its analogues (2.3 and 2.4) whose *t*BDPS protective group was removed. Empirically, this observation shows the potency of these thymidine derivatives increases with increasing hydrophobicity, lending support to the hydrophobic nature of the POD binding site on tubulin (Bryan, 1972). Interestingly, the 2'-deoxyadenosine derivatives were observed to be biologically inactive against WT and mutant ($Pod^{RII}6$ and $Vpm^{R}5$) CHO cells up to concentrations where their solubility becomes poor (data not shown). Based on this preliminary screen, the 2'-deoxyadenosine derivatives were excluded from further biological studies. Perhaps the purine ring system is too bulky and/or contains the wrong charge distribution to facilitate favourable interactions with tubulin or TOPO II. It is also quite possible that these compounds are not even getting into the cells. All of the thymidine derivatives synthesized were far less toxic than the parent POD and DMEP molecules, while the 2'-deoxyadenosine derivatives were non-

toxic to CHO cells. There appears to be a steric limit for which C4 derivatives of POD and DMEP can be active. For example, ester derivatives having relatively small substituents attached to the C4 position show very little decrease in biological activity (Gupta & Chenchaiah, 1987), while thymidine (pyrimidine) derivatives exhibit very weak POD-like activity, and the bulkier 2'-deoxyadenosine derivatives are completely inactive.

Table 4.1: Relative toxicity and cross-resistance of the Pod^R mutants towards the thymidine and various other analogues.

Podophyllotoxin Analogue	D_{10} value[a] for Pod^S cells	Relative degree of resistance[c]		Activity of analogues relative to
	(μM)	$Pod^{RI}16$	$Pod^{RII}6$	POD[b]
Podophyllotoxin	0.02	5.0	>12	1.0
Epipodophyllotoxin[d]	0.11	1.8	5.6	0.18
Deoxypodophyllotoxin[d]	0.0075	2.7	6.7	2.7
4'-Demethylpodophyllotoxin[d]	0.038	2.7	8.3	0.53
4'-Demethylepipodophyllotoxin[d]	0.3	1.7	3.3	0.067
DMEP-CBZ (compound 2.1)	0.19	1.5	4.0	0.11
Compound 1.2	0.12	2.0	9.0	0.17
Compound 2.2	1.2	1.3	2.5	0.017
Compound 2.3	6.9	1.8	3.6	0.0029
Compound 2.4	6.8	2.4	>4.4	0.0029
Podophyllotoxin-ß-D-glucoside[d]	7.8	2.8	6.7	0.0025
VM26 (Teniposide)	0.15	1.0	1.0	---

[a] Determined as described in Materials and Methods, section 2.3.1.

[b] Assuming the molar D_{10} value of POD to be 1, the relative toxicities of the other analogues showing POD-like activity have been calculated.

[c] A compound is considered to possess POD-like activity if the Pod^{RI} and Pod^{RII} mutants exhibit increasing degrees of resistance towards it.

[d] Data taken from Gupta (1983a).

The fact that the DMEP derivatives were less toxic than the POD derivatives supports previous studies where DMEP has been show to interact with tubulin at the POD binding site with a lower affinity than POD (Kelleher, 1977; Loike *et al.*, 1978). The presence of the bulky CBZ protective group at the 4' position does not alter the activity of compound 2.4 relative to 2.3, which can be seen by comparison of their D_{10} values (see Table 3.1). This observation is consistent with previous investigations that found no difference in the interaction of 4'-demethylpodophyllotoxin with tubulin relative to POD, hinting that the presence of a 4' methoxy substituent not essential for its interaction with the receptor site (Kelleher, 1977; Loike *et al.*, 1978; Gupta, 1983a). Furthermore, Gupta and Chenchaiah (1987) observed a slight increase in the toxicity of DMEP blocked at the 4' position with CBZ relative to DMEP in the cross-resistance assay. There appears to be a slight increase in the resistance of the mutant cell lines ($Pod^{RI}16$ and $Pod^{RII}6$) relative to the WT line for the thymidine-DMEP derivative with the free 4' hydroxyl group (compound 2.4) compared with compound 2.3 which has the bulky CBZ protective group at position 4' (Table 4.1). This trend has been observed before with compounds showing weak activity relative to the parent POD or DMEP molecules (Gupta, 1983a; Gupta & Chenchaiah, 1987). One might reason that in the mutant lines the genetic lesion responsible for the increased resistance to POD might be physically hindering its interaction with the binding site on the tubulin dimer. In the case of the Pod^{R} mutants utilized in this investigation, it is likely an alteration in the microtubule associated protein, P1, impedes the interaction of POD with its receptor site on tubulin (Gupta *et al.*, 1982;

Gupta, 1990). Thus, if this mutation leads to a conformation change in P1, bulkier molecules may be more hindered and find it more difficult to interact with tubulin.

In order to confirm that the thymidine derivatives showing POD-like antimitotic activity in the CR assay were in fact behaving like spindle poisons, the effect of these compounds on the mitotic index of WT and $Pod^{RII}6$ cells was examined. The mechanism by which POD and its derivatives arrest cells in metaphase is the same as that of colchicine, where there is an inhibition of the assembly of tubulin into microtubules (Wilson *et al.*, 1974; Stähelin & von Wartburg, 1989). During mitosis microtubules, which make up the mitotic spindle, are responsible for rearrangement of the chromosomes in the metaphase plate and their subsequent separation during anaphase (Dustin, 1984; Darnell *et al.*, 1986). The inability of cells to form MTs prevents them from dividing, and they remain arrested in metaphase with clumped chromosomes until they eventually die (Dustin, 1984; Stähelin & von Wartburg, 1989). When all of the MTs are disassembled, the chromosomes become scattered throughout the cytoplasm and those which are no longer attached to the spindle often appear thicker and shorter (Dustin, 1984).

The mitotic indices of WT and $Pod^{RII}6$ cells with increasing concentrations of POD and compounds 1.2, 2.2, and 2.4 are shown in Figures 3.9, 3.10, 3.11, and 3.12, respectively. As seen, both the WT and $Pod^{RII}6$ cells show a dose-dependent increase in mitotic index similar to the dose-response curves in the CR assay. These results support previous investigations where the mitotic index has been used to determine whether a

compound has spindle poison activity (Hartwell & Schrecker, 1958; Dustin, 1984; Gupta, 1985; Gupta & Chenchaiah, 1987). Similar to the dose-response data (summarized in Table 3.1) the $Pod^{RII}6$ cells display an increased resistance to these compounds in blocking mitosis. Furthermore, the compounds which are most potent *in vivo* require a substantially larger increase in concentration to produce the same relative mitotic index in the mutant cell lines. For example, the relative degree of resistance of the $Pod^{RII}6$ mutants compared with the WT cells for compounds 1.2 and 2.2 was 9.0 versus 2.5, respectively (see Table 4.1). The mitotic index data for compound 1.2 shows that the WT cells reach a mitotic index of 6.5 at 0.2 μg/ml whereas the $Pod^{RII}6$ cells attain this value at approximately 0.9μg/ml (Figure 3.10), or a relative increase of 4.5. Compound 2.2 increases the mitotic index of WT cells to a value of 8 at 5μg/ml while $Pod^{RII}6$ cells reach this value at approximately 8.5μg/ml (Figure 3.11), or a relative increase of 1.7. This large discrepancy between compounds 1.2 and 2.2 agrees with the dose-response studies condensed in table 3.1 and accounts for the decreased affinity the DMEP derivatives have for tubulin relative to POD (see competition assay section). This relationship between the relative degrees of resistance has been noted for many other POD derivatives which exhibit lower cellular toxicity and weaker interactions with tubulin (Gupta, 1983a; Gupta & Chenchaiah, 1987).

4.3 Competitive Inhibition of [^{3}H]POD Binding to Tubulin

Of all the thymidine derivatives of epipodophyllotoxin synthesized, only compound 1.2 displayed competitive inhibition of [^{3}H]POD binding to semi-purified tubulin. This competition did not completely annihilate POD binding to tubulin, since only about 50% of the radiolabelled POD was displaced (Fig. 3.13). All of the other thymidine derivatives were unable to out compete the binding of [^{3}H]POD to tubulin. Because these compounds were found to be of substantially lower potency, as dictated by the CR assay and mitotic index experiments, their affinity for tubulin may be too low to be detected as antimitotic compounds by the competition assay. That many of these nucleoside derivatives do not out compete the binding of [^{3}H]POD and display marginal degrees of resistance between first-step and second-step mutants resistant to POD (Table 4.1), their toxicity may be partially attributable to inhibition of other cellular targets. For example, Loike and Horwitz (1976) have demonstrated that POD and VP16-213, two compounds which share the same parent ring system but have different cellular targets, inhibit nucleoside transport in HeLa cells at very high concentrations. Clearly, these thymidine derivatives display POD-like antimitotic activity, as determined by the CR assay and mitotic index experiments. However, one cannot exclude the possibility that other cellular processes are being inhibited at the same time, especially at high drug concentrations. This observation reinforces the sensitivity of the CR assay as a screening mechanism for the detection of compounds believed to be inactive by conventional methods.

4.4 Molecular Modelling Studies

In approaching the structure/activity relationships among the thymidine derivatives we solved the crystal structures of DMEP (Sicheri *et al.*, 1992a) and compound 2.4 (Sicheri *et al.*, 1991). These structures provide a three-dimensional representation of the compounds, which is the basis of our approach to the structure activity studies in this thesis. In order to visualize the structural variations between the thymidine derivatives and the TOPO II targeting drugs, VP16-213 was modelled (utilizing the crystal coordinates of DMEP) and its conformation refined by energy minimization (Mayo *et al.*, 1990) using the Molecular Design and Analysis Program (Molecular Simulations, Inc., Pasadena, CA). Overlaying the crystal structures of DMEP and compound 2.4 shows very minimal deviation in the parent DMEP molecule (not shown). Because of its rigid structure, there is negligible conformational variation between the parent DMEP molecules of VP16-213 and the thymidine derivatives (e.g. compound 2.4). When the crystal structure of compound 2.4 is overlapped with the modelled VP16-213 there is significant conformational variation in the substituents attached at C4. In figure 4.1 it can be seen that the glucosidic moiety of VP16-213 lies closer to the planar ABCD fused ring system of the parent DMEP molecule than the thymidyl moiety of compound 2.4. Electrostatic potential calculations on these molecules provide a more detailed understanding of the these empirical relationships. For example, there is an asymmetric charge distribution over the dioxole ring (ring A) of VP16-213 which is absent in the antimitotic compounds 2.4 and DMEP. Because the glucosidic moiety lies closer to the parent DMEP ring

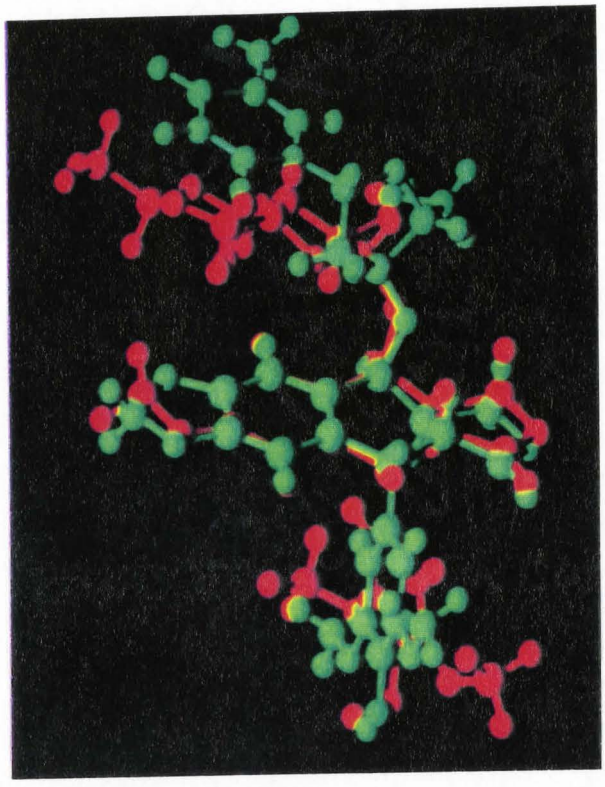


Figure 4.1: Overlay of ball-and-stick models of VP16-213 (red) and compound 2.4 (green) illustrating the steric differences between the C4 substituents. Note that there is very little variation in the rigid DMEP ring system. Images generated using the Molecular Design and Analysis Program (Molecular Simulations, Inc., Pasadena, CA) at an IRIS Workstation (Silicon Graphics, Inc., Mountain View, CA).

system and its van der Waal radii overlap with those on the dioxole ring, the asymmetric charge distribution may be a consequence of this interaction. Its is possible that this interaction helps to stabilize the VP16-213 molecule, perhaps "locking" it in a conformation that prevents its interaction with tubulin while facilitating its interaction

with the TOPO II cleavable complex. The van der Waal radii of the thymidyl moiety do not overlap with the parent ABCD ring system of DMEP which would allow free rotation about the C4-O-C2'' bonds. If this were the case, then the thymidyl derivatives would have to assume the proper conformation facilitating their interaction with tubulin at the POD binding site. The bulkiness of the thymidyl moiety may hinder to some extent the interaction of these compounds with tubulin. Such steric hinderance would help explain their low toxicity and weak interactions with purified tubulin relative to POD. Because the POD binding site is believed to lie in a hydrophobic pocket of tubulin (Bryan, 1972), the higher activities observed with the more hydrophobic intermediates of the thymidine series may be attributable to hydrophobic interactions at this site. Calculation of the water accessible surface for VP16-213 (Fig. 4.2a) illustrates the close association of the glycosidic moiety with the parent DMEP ring system. In this representation the green colouration indicates a neutral charge distribution, while the blue and red areas represent slightly positive and slightly negative charges, respectively. Conversely, the water accessible surface of compound 2.4 (Fig. 4b) shows less association between the thymidyl moiety and the planar DMEP ring system as well as a water accessible cleft between these regions of the molecule. The area between the DMEP ring system of VP16-213 and its glucoside moiety is very hydrophobic (Fig. 4.2a) and may also contribute to stabilization of the TOPO II targeting drugs, VP16-213 and VM26. It should also be noted that there is a more defined charge distribution over the thymidyl system than over the glycoside group of VP16-213. This is interesting because derivatives of VP16-213

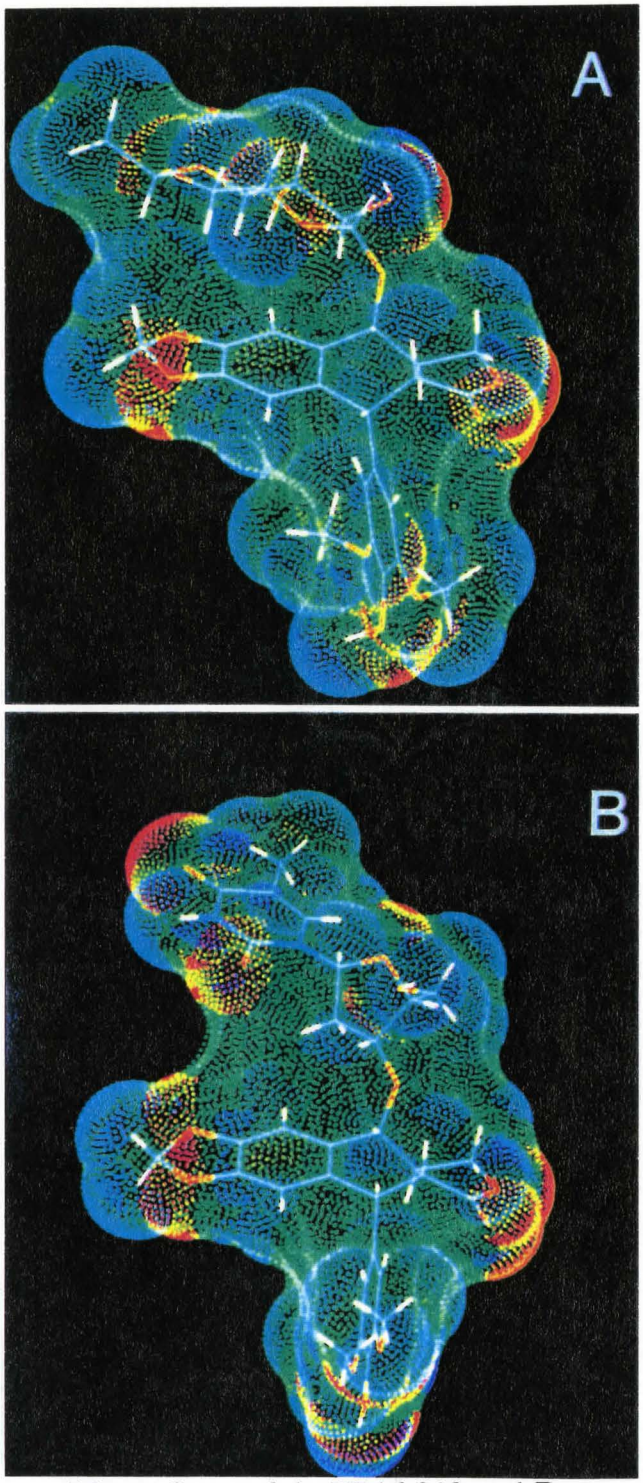


Figure 4.2: Water accessible surfaces of **A**, VP16-213 and **B**, compound 2.4. Green colouration represents neutral areas, red colouration represents a slightly negative charge distribution, and blue represents a slightly positive charge on the molecule.

which show strong TOPO II inhibitory activity contain bulky, hydrophobic groups substituted at the methyl position (Gupta *et al.*, 1987). Furthermore, it appears from this and many previous studies that the TOPO II binding site is much more selective and difficult to target than the POD binding site of tubulin, although both proteins must share some degree of structural homology at their receptor sites. Perhaps the binding site for these anticancer drugs on TOPO II requires a very specific and complimentary charge distribution over the targeting drug to facilitate its molecular recognition and subsequent inhibition of this enzyme. Thus, from this investigation there appears to be important electrostatic and hydrophobic interactions to consider when targeting POD derivatives to either tubulin or TOPO II. This study should shed more light on understanding, at the molecular level, the structural requirements which discriminate between the two types of biological activities associated with POD and its derivatives.

4.5 Conclusions And Future Work

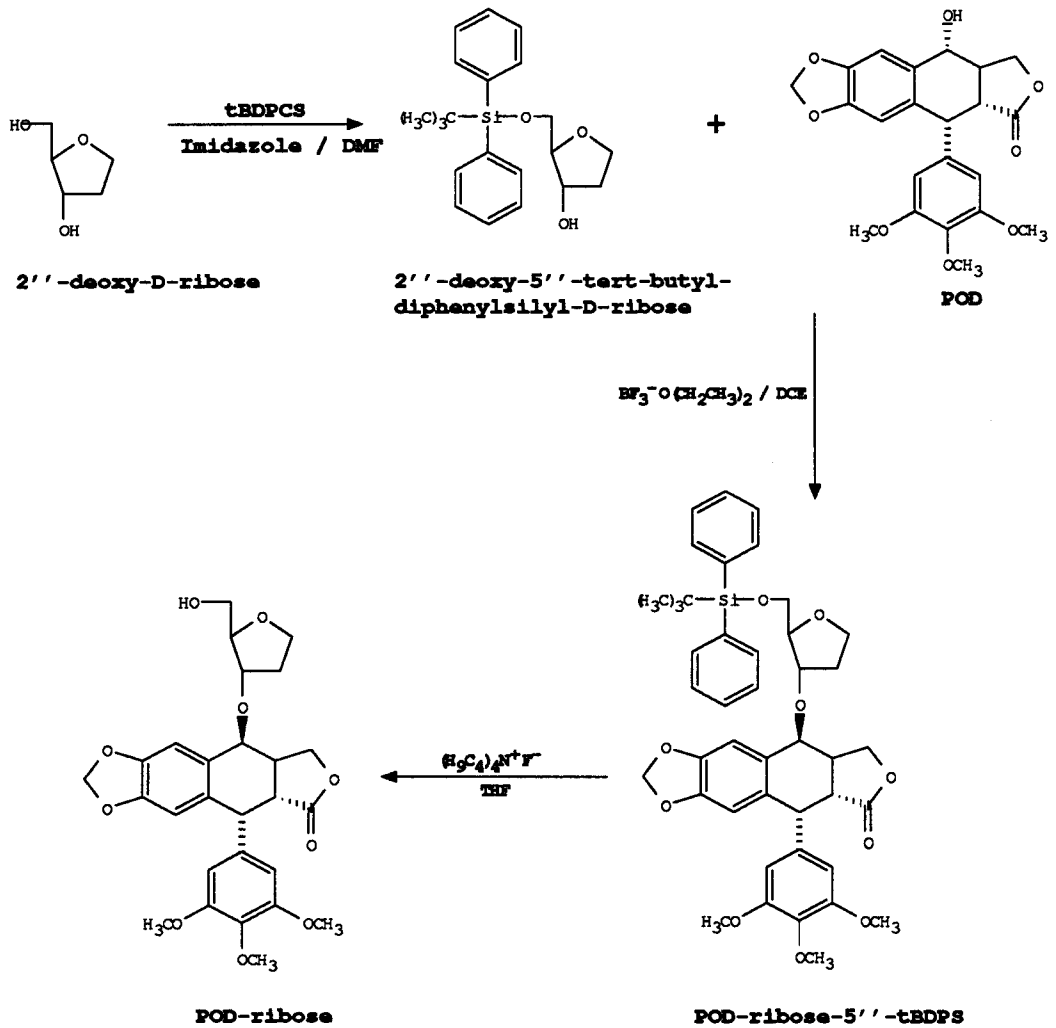
From this investigation the thymidine derivatives were determined to possess antimitotic activity with a mechanism of action similar to POD. This was based on two criteria; (i) the cross-resistance assay, and (ii) by mitotic index experiments. To more precisely ascertain the effects of these compounds on cell division, the ratio of cells in anaphase and metaphase should be determined at increasing drug concentrations. An increase in the number of cells in metaphase and a proportional increase in the number of anaphase cells would indicate that mitosis was slowed, whereas an increase in mitotic

cells and a decrease in anaphase cells would indicate a metaphase block (Jordan *et al.*, 1992). More detailed studies on the rate of uptake of these drugs into the cells should be addressed. This could be approached by radiolabelling the molecule with tritium at a non-exchangeable position on the POD molecule (e.g. C4'), then determining the rate of drug uptake in CHO cells. This would be especially interesting for the compounds which display weak activity, such as the 2'-deoxyadenosine derivatives. With the radiolabelled nucleoside derivative one could more easily study the interaction of these compounds with purified tubulin and perhaps generate some binding data. Only one nucleoside derivative (compound 1.2) was found to out compete the binding of [^{3}H]POD to purified tubulin. If these experiments were carried out using labelled derivatives that show weaker affinities for tubulin, their displacement by unlabelled POD or colchicine could be more easily studied. If these compounds show varying affinities for tubulin, then their binding data should compliment the dose-response and mitotic index experiments. That is, the less active compounds should display a lower affinity for tubulin than their more potent counterparts.

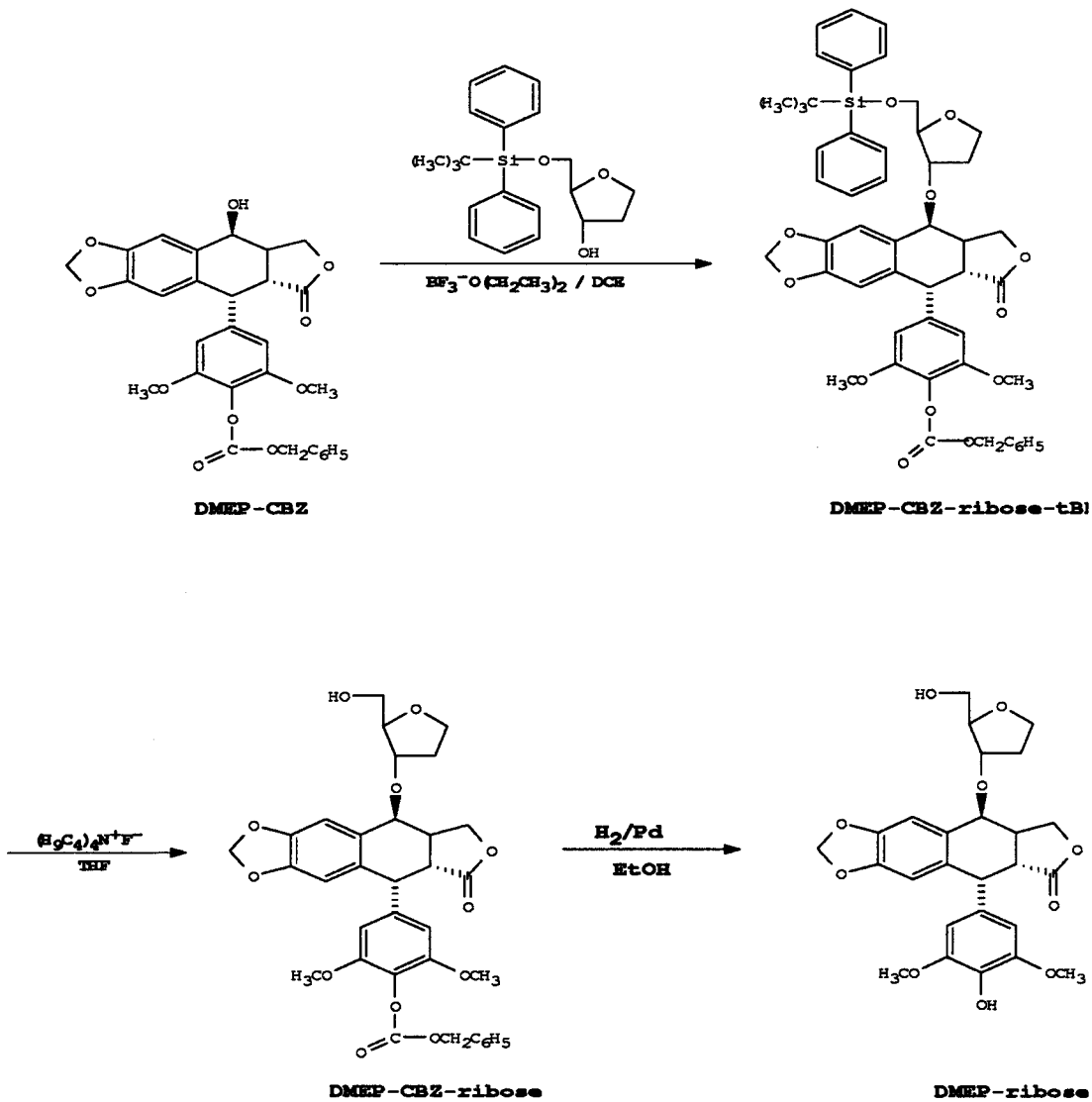
The nucleoside analogue-like properties of these compounds should also be investigated with mutant cells deficient in nucleoside metabolizing enzymes (e.g. thymidine kinase and adenosine kinase deficient mutants). If these mutants display sensitivity toward the nucleoside derivatives, then these compounds would likely possess a combination of two types of activity. Such an effect may be of interest to the clinician

where a combination of targets in one molecule may help decrease the development of resistance to chemotherapeutic agents.

One weakness of the current study is that replacement of the six carbon sugar (as in VP16-213 and VM26) with a five carbon sugar cannot be investigated without considering the attached base. To generate clearer structure/activity relationships between these compounds and to build on the data already obtained for the nucleoside derivatives, more compounds should be synthesized; namely ribose derivatives of POD and DMEP. Schemes 4.1 and 4.2 depict the proposed routes to the five-carbon ribose derivatives of POD and DMEP, respectively. These compounds would provide a better understanding of the activities observed with the nucleoside derivatives by logically building up from the smaller ribose to the larger nucleoside moiety. These compounds would also be useful in investigating the role of the five carbon sugar alone, without the added interference from the base, as in the nucleoside derivatives. Such a simple and rational approach to structure/activity studies should provide a better understanding of the biological activities observed with the nucleoside and glycoside derivatives of POD and DMEP. This would also be useful in the design of more effective and less toxic chemotherapeutic agents.

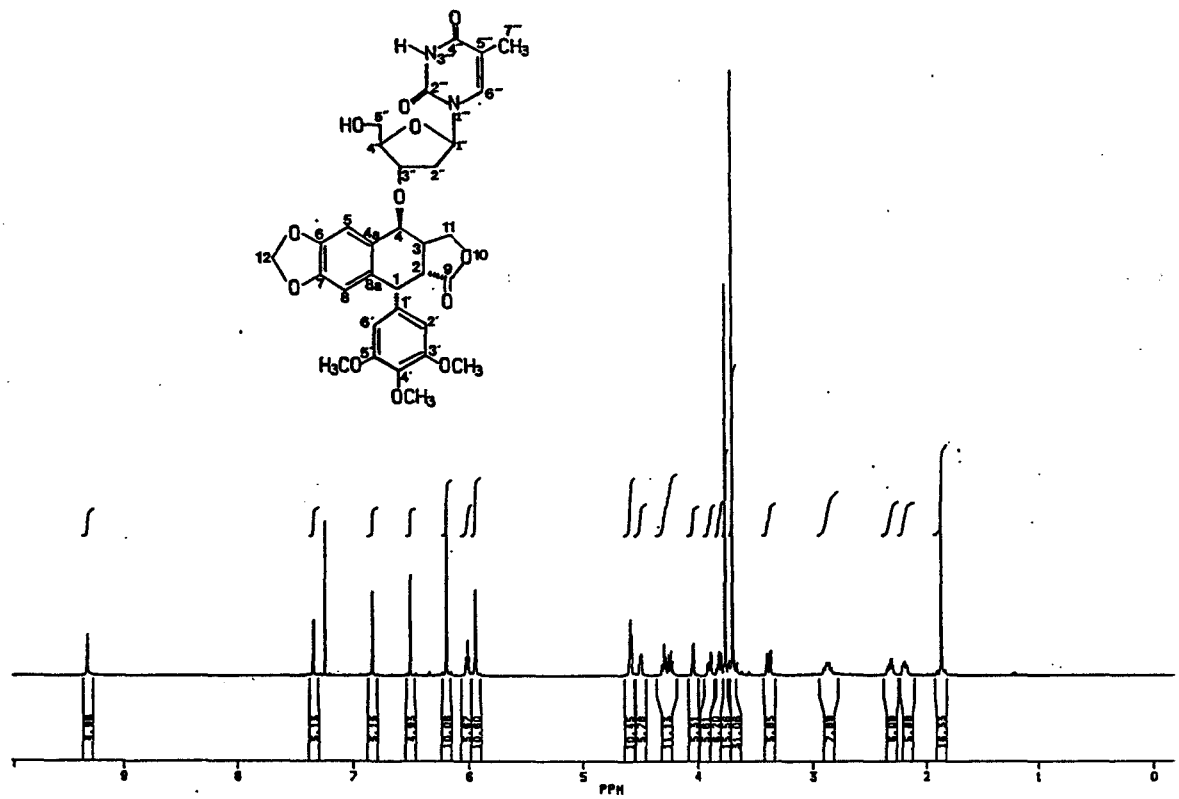


Scheme 4.1: Proposed chemical synthesis of ribose derivative of POD.

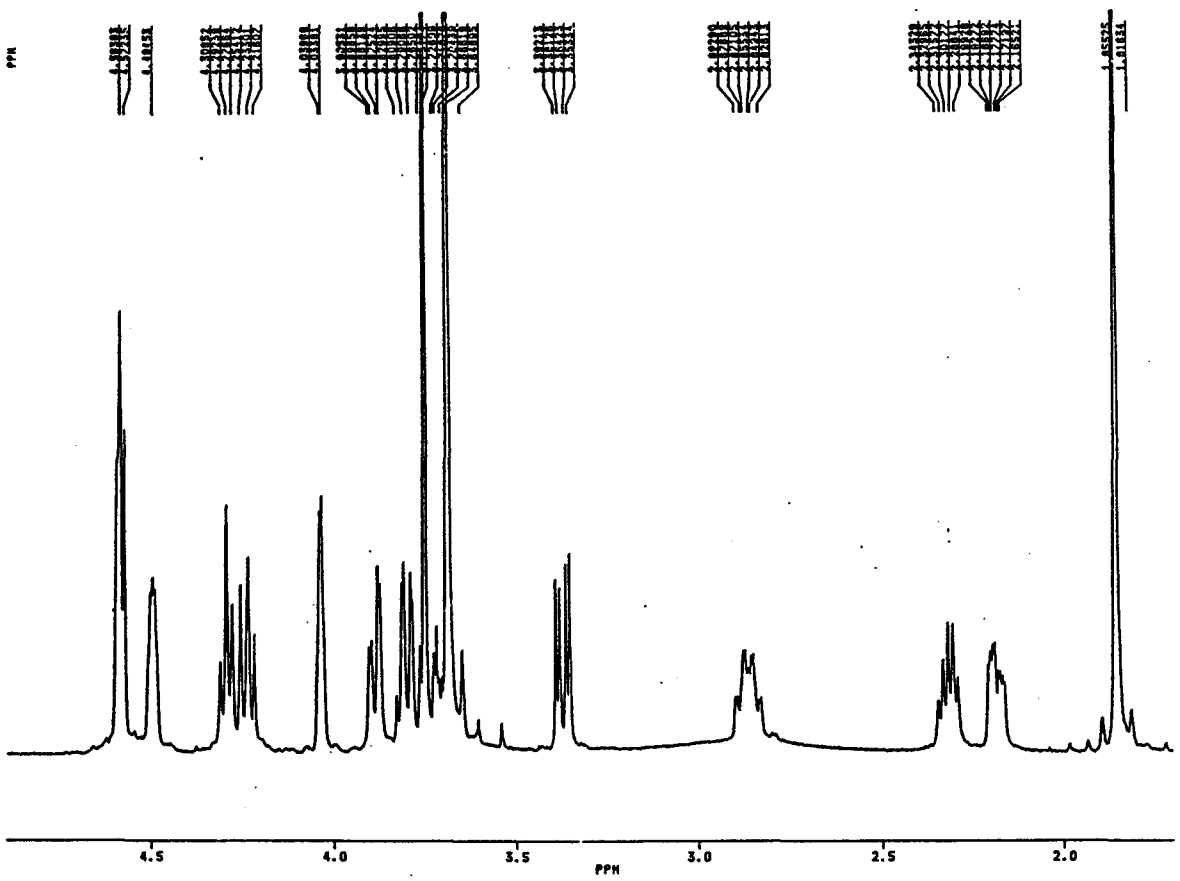


Scheme 4.2: Proposed chemical synthesis of ribose derivative of DMEP.

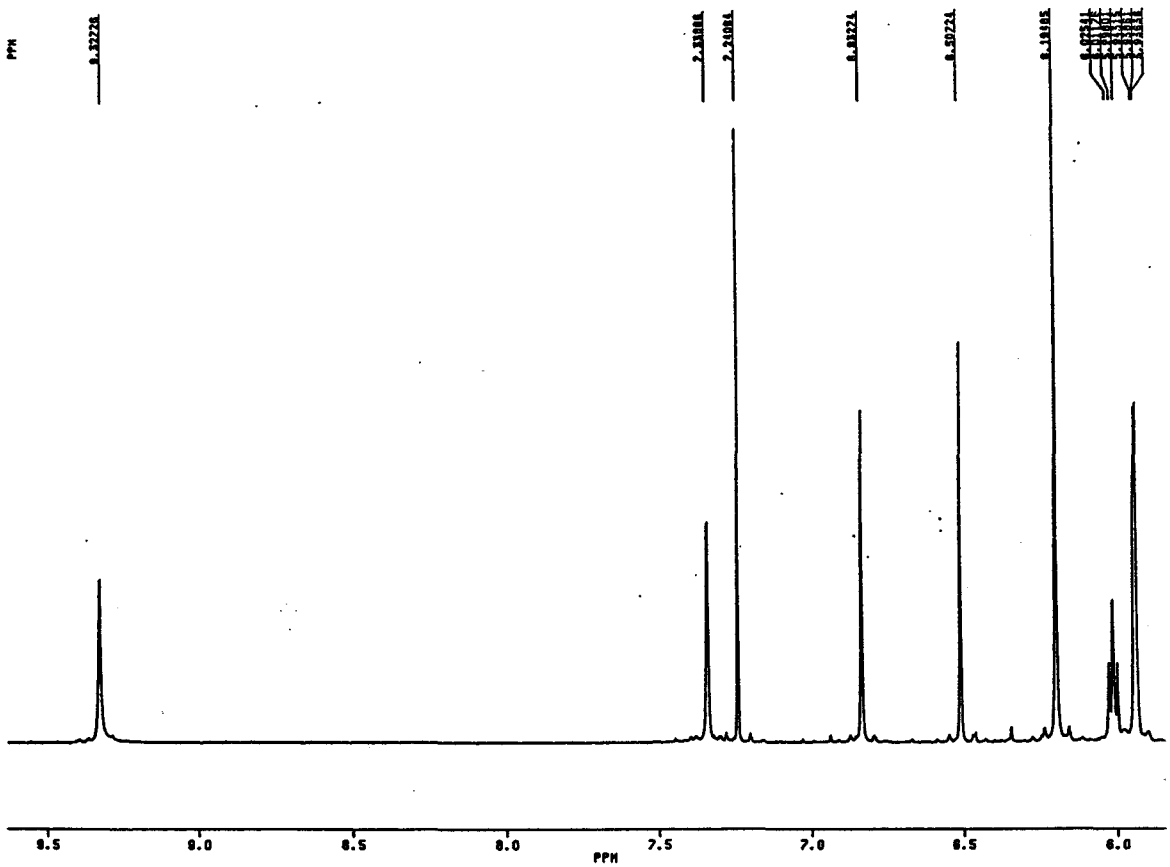
APPENDIX



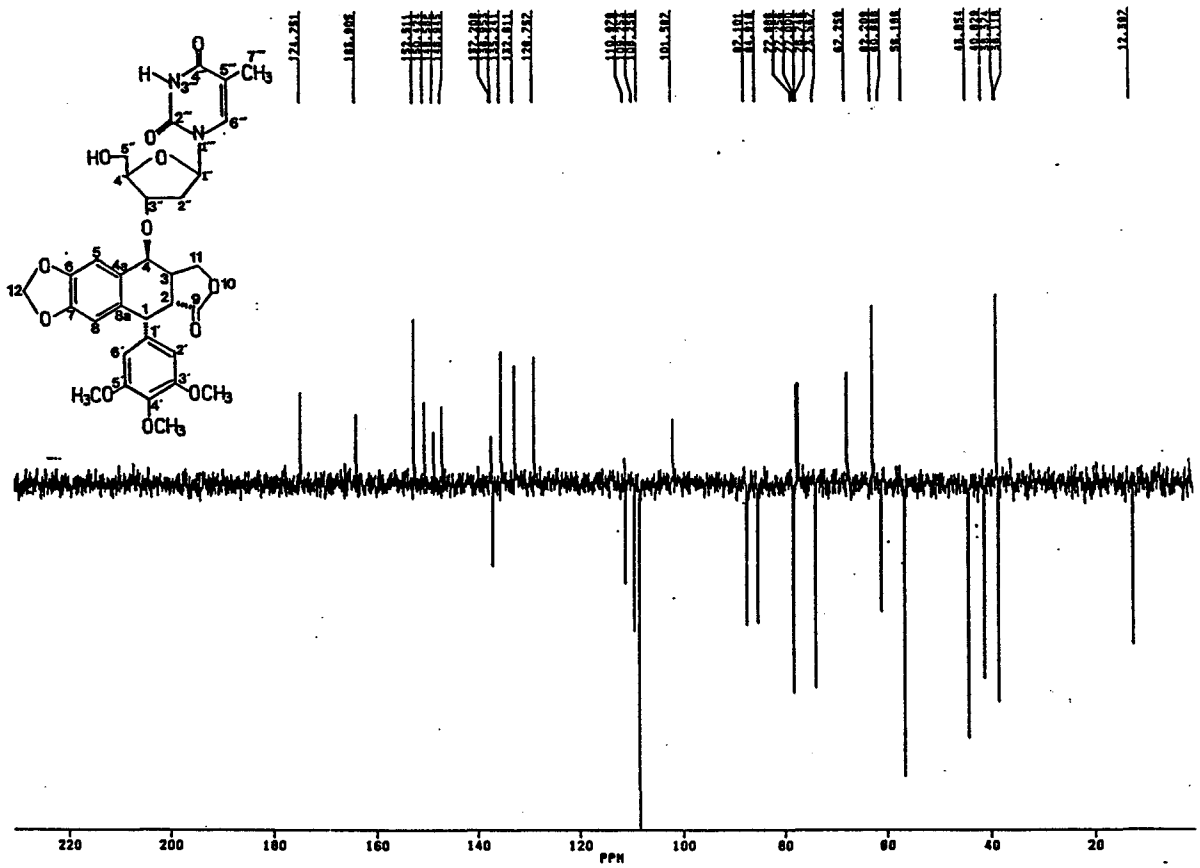
Spectrum 1.0: 500 MHz ^{1}H NMR spectrum of compound 1.3 in $CDCl_3$.



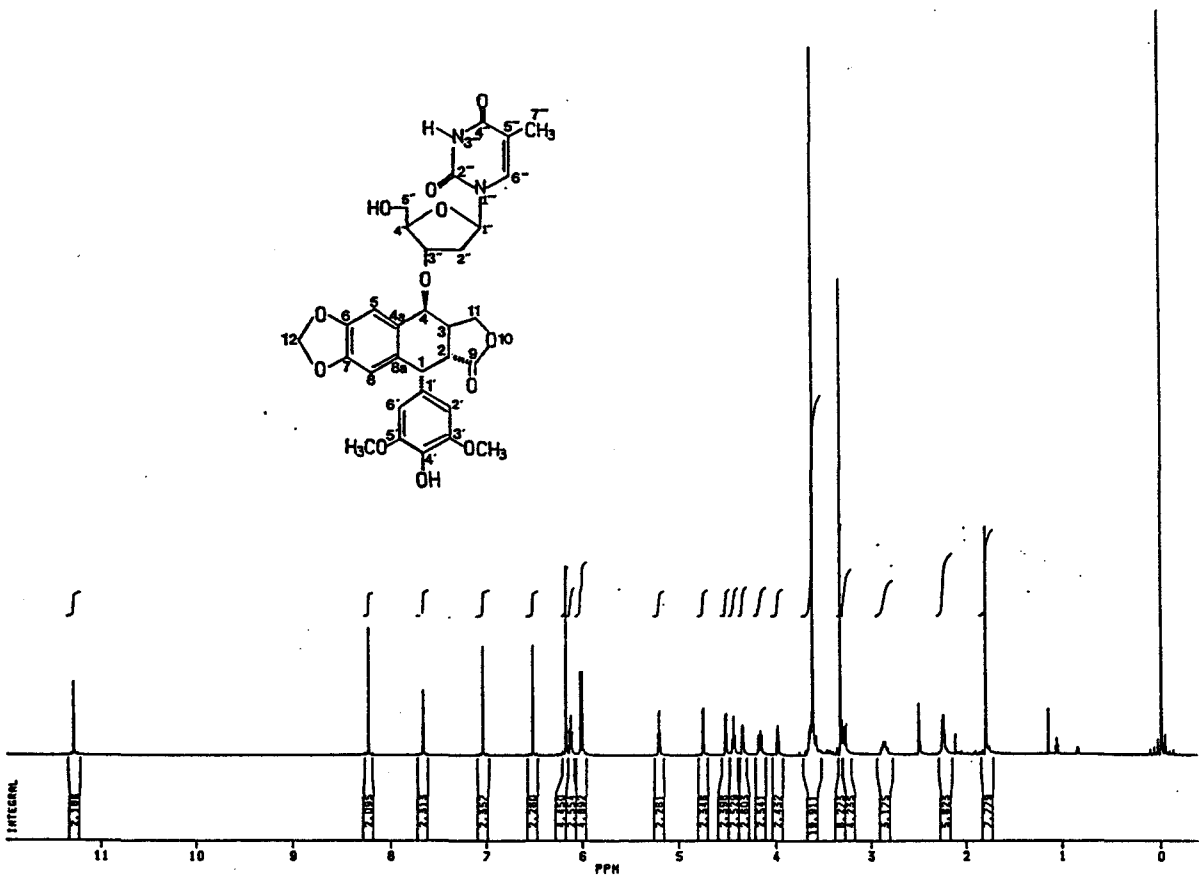
Spectrum 1.1: Expansion plot of ^{1}H NMR spectrum for compound 1.3 (1.0 to 5.0 ppm).



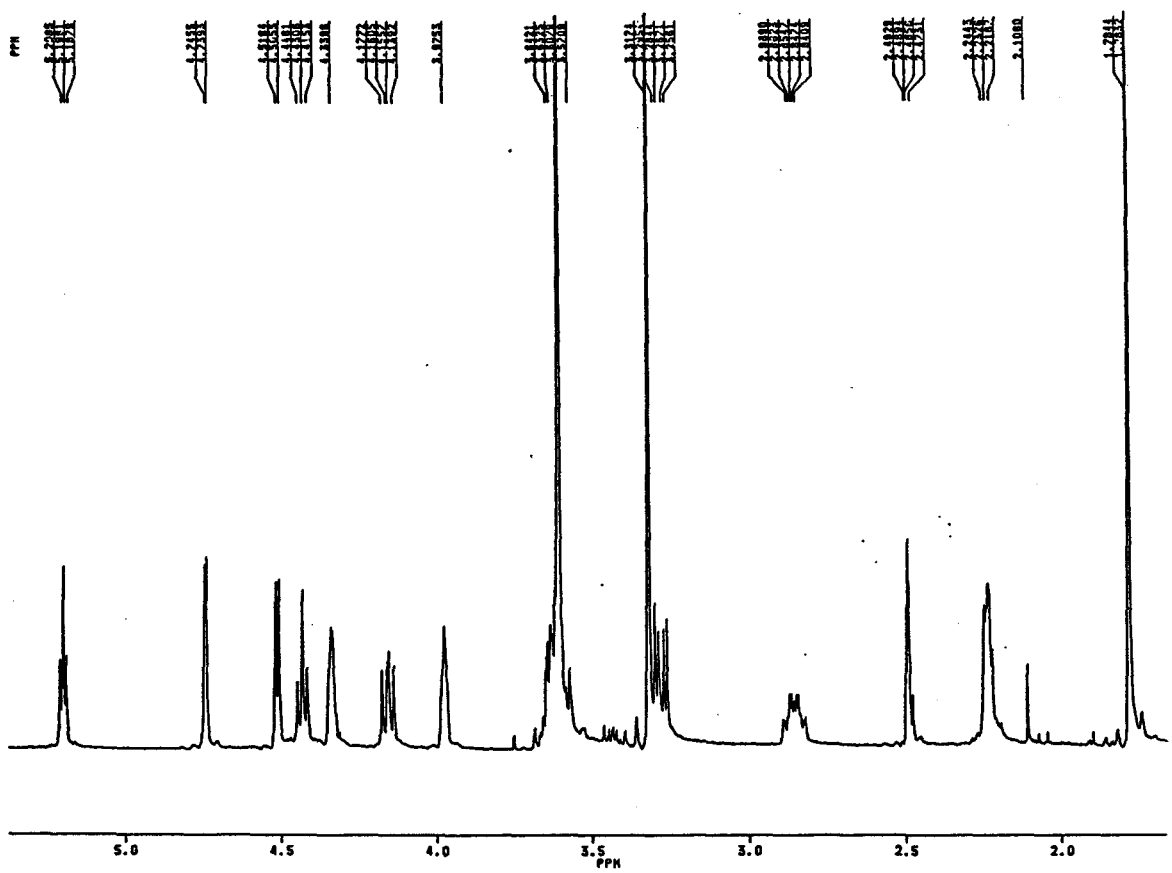
Spectrum 1.2: Expansion plot of ^{1}H NMR spectrum for compound 1.3 (6.0 to 9.5 ppm).



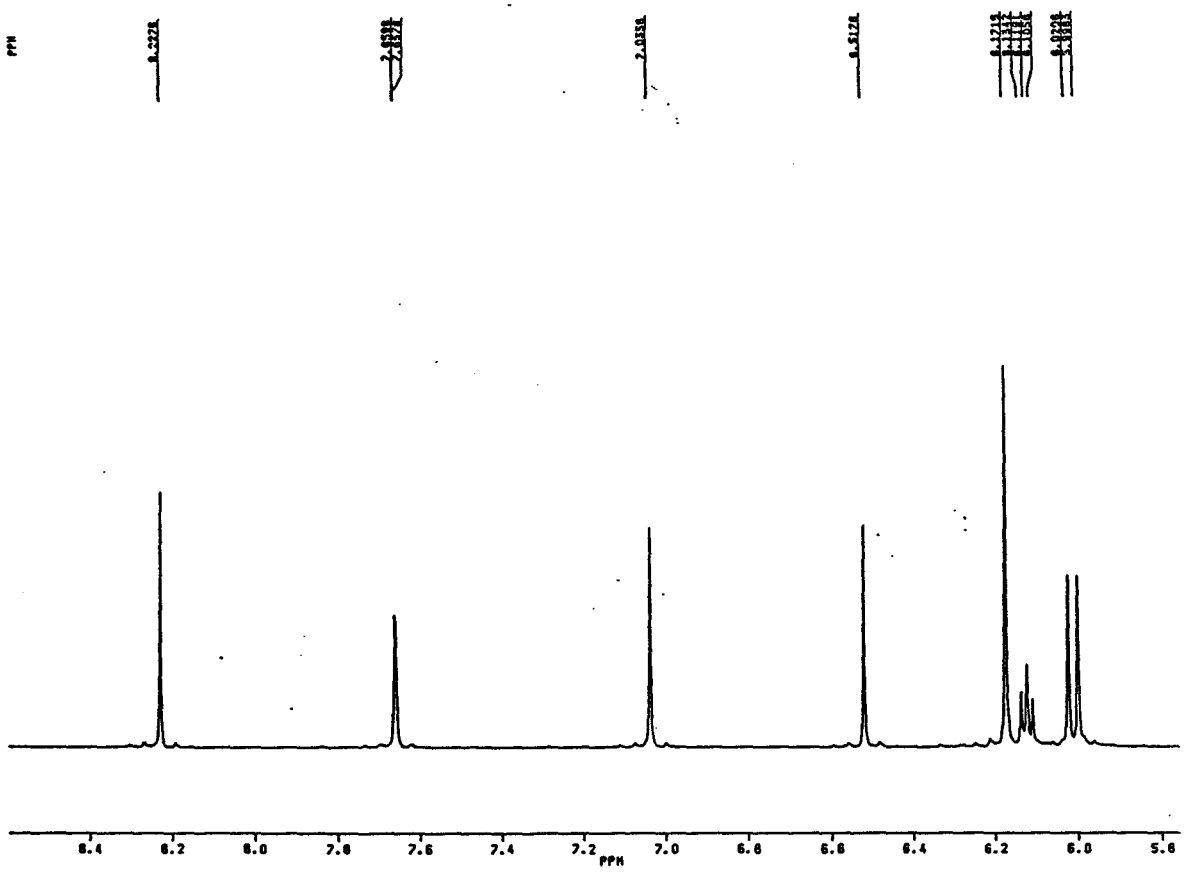
Spectrum 1.3: 500 MHz ^{13}C NMR spectrum of compound 1.3 in $CDCl_3$.



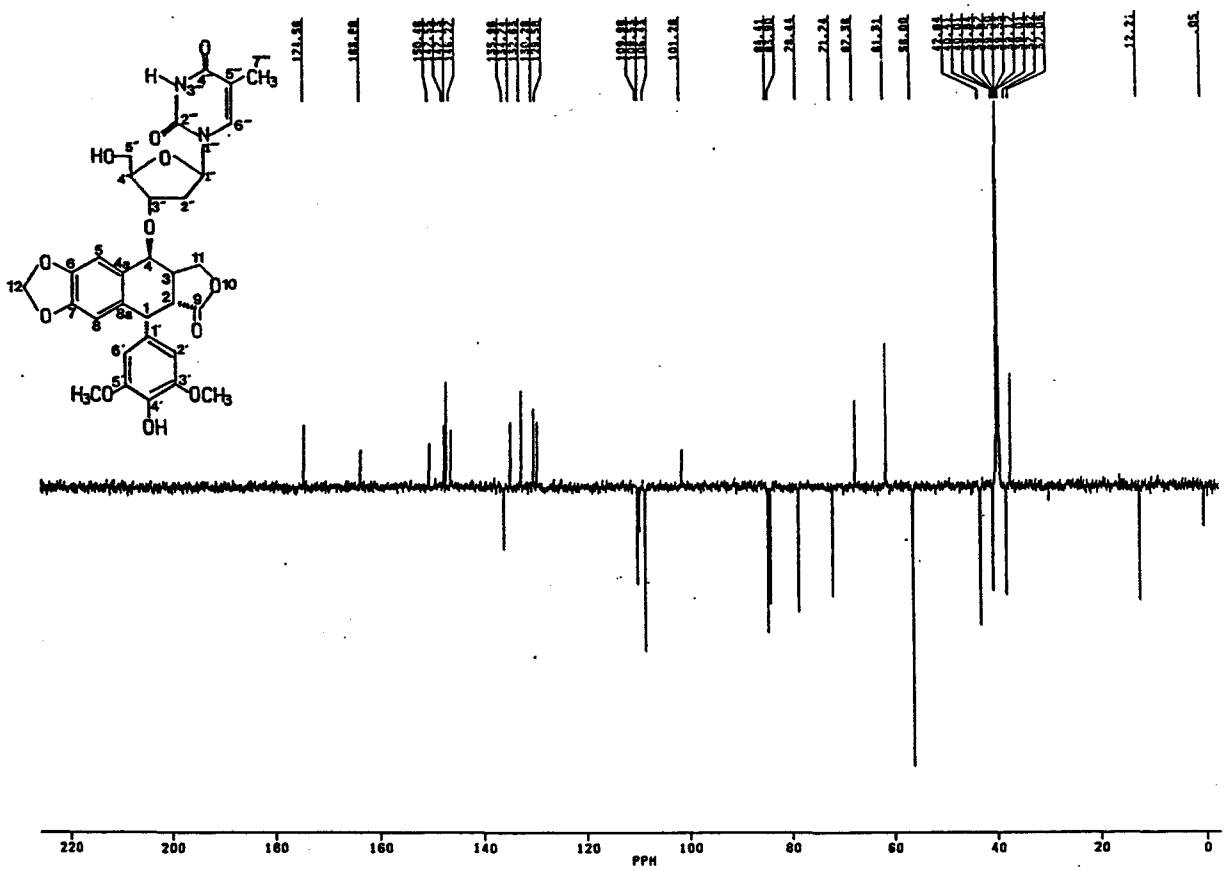
Spectrum 2.0: 500 MHz ^{1}H NMR spectrum of compound 2.4 in DMSO-d_6.



Spectrum 2.1: Expansion plot of ^{1}H NMR spectrum for compound 2.4 (1.0 to 5.5 ppm).



Spectrum 2.2: Expansion plot of ^{1}H NMR spectrum for compound 2.4 (5.8 to 8.4 ppm).



Spectrum 2.3: 500 MHz ^{13}C NMR spectrum of compound 2.4 in DMSO-d_6.

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