7-17-2007

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Published in final edited form as:
Biochemistry. 2007 July 17; 46(28): 8217–8225. doi:10.1021/bi700272u.

Topoisomerase II-Drug Interaction Domains: Identification of Substituents on Etoposide that Interact with the Enzyme†

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Abstract

Etoposide is one of the most successful chemotherapeutic agents used for the treatment of human cancers. The drug kills cells by inhibiting the ability of topoisomerase II to ligate nucleic acids that it cleaves during the double-stranded DNA passage reaction. Etoposide is composed of a polycyclic ring system (rings A–D), a glycosidic moiety at the C4 position, and a pendant ring (E–ring) at the C1 position. Although drug-enzyme contacts, as opposed to drug-DNA interactions, mediate the entry of etoposide into the topoisomerase II-drug-DNA complex, the substituents on etoposide that interact with the enzyme have not been identified. Therefore, saturation transfer difference [^{1}H]-nuclear magnetic resonance spectroscopy and protein-drug competition binding assays were employed to define the groups on etoposide that associate with yeast topoisomerase II and human topoisomerase IIα. Results indicate that the geminal protons of the A–ring, the H5 and H8 protons of the B–ring, as well as the H2' and H6' protons and the 3'– and 5'–methoxyl protons of the pendent E–ring interact with both enzymes in the binary protein-ligand complexes. In contrast, no significant nuclear Overhauser enhancement signals arising from the C–ring, the D–ring, or the C4 glycosidic moiety were observed with either enzyme, suggesting that there is limited or no contact between these portions of etoposide and topoisomerase II in the binary complex. The functional importance of E–ring substituents was confirmed by topoisomerase II-mediated DNA cleavage assays.

Etoposide is one of the most successful chemotherapeutic agents used for the treatment of human cancers (1–4). The drug currently is in its third decade of clinical use and is front line

†This work was supported by National Institutes of Health grant GM33944 (NO), National Science Foundation grant MCB-0334785 (DEG), and American Cancer Society grant RPG-96-001-05-CDD (DBB). DBB was an Alfred P. Sloan Research Fellow, and AMW and RPB were trainees under National Institutes of Health grant T32 CA09582.

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SUPPORTING INFORMATION AVAILABLE

A representative [^{1}H]-NMR spectrum of etoposide with complete assignments for all of the visible protons is available free of charge via the Internet at http://pubs.acs.org.

therapy for a variety of malignancies, including leukemias, lymphomas, and several solid tumors (1–4). Until the development of paclitaxel and related compounds, etoposide was the most widely prescribed anticancer drug in the world.

The cellular target for etoposide is topoisomerase II (1–5). This essential enzyme relaxes, unknots, and untangles DNA by passing a double helix through a transient double-stranded break that it generates in a separate segment of DNA (6–12). Etoposide acts specifically by inhibiting the ability of topoisomerase II to ligate DNA molecules that it has cleaved (13,14). This drug-induced inhibition leads to the accumulation of covalent, topoisomerase II-cleaved DNA complexes (*i.e.*, *cleavage complexes*) (5). When DNA tracking enzymes such as polymerases or helicases collide with these complexes, they convert them to permanent enzyme-linked double-stranded breaks in the genetic material (6–12). These breaks destabilize the genome and when present in sufficient concentrations, trigger programmed cell death pathways (9,15–21).

Several lines of evidence indicate that interactions between topoisomerase II and etoposide, as opposed to drug-DNA interactions, are critical for drug activity and mediate the entry of etoposide into the ternary enzyme-drug-DNA complex. First, mutation of specific residues in topoisomerase II dramatically affects the ability of etoposide to increase levels of enzyme-DNA cleavage complexes (4,9,22–28). Second, etoposide binds weakly (if at all) to DNA in the absence of topoisomerase II (29). Third, the drug binds to yeast topoisomerase II and human topoisomerase IIα in the absence of nucleic acids and a mutant yeast enzyme (yTop2H1011Y) that is resistant to etoposide displays a reduced binding affinity for the agent (30,31). Fourth, etoposide displays a similar kinetic affinity for topoisomerase II-DNA cleavage complexes formed at sites with markedly different levels of scission enhancement (32). Finally, DNA breaks accumulate more rapidly when etoposide is incubated with topoisomerase II prior to the addition of DNA (as compared to the opposite order of addition) (32).

Hundreds of etoposide derivatives have been analyzed in an effort to establish structure-activity relationships within this drug class (33–42). Despite the importance of protein-drug interactions, none of these studies have identified any of the substituents on etoposide that interact with topoisomerase II. Therefore, the present study utilized saturation transfer difference [^{1}H]-nuclear magnetic resonance (STD [^{1}H]-NMR) spectroscopy (43–47) and protein-drug competition binding assays to define the groups on the drug that associate with the type II enzyme. Results indicate that substituents on the A–, B–, and E–rings of etoposide interact with yeast topoisomerase II and human topoisomerase IIα. The functional importance of E–ring substituents was confirmed by topoisomerase II-mediated DNA cleavage assays.

EXPERIMENTAL PROCEDURES

Materials

Negatively supercoiled pBR322 plasmid DNA was prepared using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Etoposide was purchased from Sigma. Teniposide was provided by Bristol-Meyers Squibb. The synthesis of etoposide derivatives with modified E-ring substituents will be described elsewhere. TOP-53 was provided as a dichloride salt by Taiho Pharmaceuticals. All drugs were stored at 4 ° C as 20 mM stock solutions in 100% DMSO. Drugs used for NMR experiments were stored in 100% d-DMSO. [^{3}H]etoposide was obtained from Moravek Biochemicals as a 1.5 mM stock in 100% ethanol. D_2O (99.9%) was purchased from Aldrich. All other chemicals were analytical reagent grade.

Purification of Type II Topoisomerases

Highly concentrated yeast topoisomerase II and human topoisomerase IIα were expressed in *Saccharomyces cerevisiae* and purified as described (26,48,49). The yeast and human enzymes were purified from 50–60 g of frozen wet-packed yeast JEL 1 cells transformed with YEpGAL1TOP or YEpWOB6, respectively. In the final step of the purification, the type II topoisomerases were eluted from phosphocellulose columns (P81, Whatman) with buffer containing 10 mM sodium phosphate, pH 7.7, 750 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, and 5% d-glycerol in D_2O. Typical yields exceeded 1 mg of yeast topoisomerase II or 0.2 mg of human topoisomerase IIα per g of wet-packed yeast cells.

Cleavage of Plasmid DNA by Yeast Topoisomerase II

DNA cleavage reactions were carried out as described (26). Reaction mixtures contained 150 nM yeast topoisomerase II and 5 μM negatively supercoiled pBR322 in a total of 20 μL of 10 mM sodium phosphate, pH 7.7, 100 mM KCl, 0.1 mM NaEDTA, 5 mM $MgCl_2$ containing etoposide, teniposide, TOP-53, hydroxyphenyl-etoposide, or phenyl-etoposide (up to 300 μM) solubilized in 100% DMSO (all reactions contained 10% DMSO final concentration). DNA cleavage was initiated by the addition of enzyme and reaction mixtures were incubated for 6 min at 28 °C to establish DNA cleavage/religation equilibria. Cleavage complexes were trapped by the addition of 2 μL of 5% SDS and 1.5 μL of 250 mM NaEDTA, pH 8.0. Proteinase K was added (2 μL of 0.8 mg/mL) and reaction mixtures were incubated for 30 min at 45 °C to digest the topoisomerase II. Samples were mixed with 2 μL of 30% sucrose, 0.5% bromophebol blue, and 0.5% xylene cyanol FF in 10 mM Tris-HCl, pH 7.9, heated for 2 min at 45 °C, and subjected to electrophoresis in a 1% agarose gel in 40 mM Tris-acetate, 2 mM NaEDTA containing 0.5 μg/mL ethidium bromide. Cleavage was monitored by the conversion of negatively supercoiled DNA to linear molecules. DNA was visualized by medium wavelength UV light and quantified using an Alpha Innotech digital imaging system.

STD [^{1}H]-NMR Spectroscopy

All NMR experiments are performed at 283 K using a Bruker Avance DRX 500-MHz spectrometer equipped with a 5-mm BBI probe. NMR buffers were prepared in 99.9% D_2O and contained 10 mM sodium phosphate, pH 7.7, 250 mM KCl, 0.1 mM Na_2EDTA, and 5 mM $MgCl_2$. NMR samples (500 μL) contained 5 μM yeast topoisomerase II or human topoisomerase IIα and 100–400 μM etoposide or selected analogs, and were maintained at 5 °C until data were obtained. STD-NMR experiments employed a pulse scheme similar to that reported by Mayer and Meyer (46). The gradient pulse that was applied was 1 ms at 30% with a 500 μs recovery delay. The water signal was suppressed by tailoring a watergate pulse sequence to the beginning of the f2 presaturation STD-pulse program. For each experiment (on and off resonance irradiation), a total of 2000 scans were collected with a 3 s relaxation delay between each scan. On and off-resonance irradiations were performed at 0.5 and 17 ppm, respectively. Difference spectra were prepared by subtracting the on-resonance spectrum from the off-resonance spectrum. Signals resulting in the difference spectrum represent the NOE difference signals generated by the transfer of irradiation energy from the enzyme to the bound ligand. Ligand protons in close spatial proximity with the enzyme displayed larger NOE signals. Mapping of the NOE signals with their proton assignments on the ligand revealed the ligand binding epitope to the target topoisomerase II. Spectra were processed using Bruker XWINNMR (v3.2) software.

Topoisomerase II-Drug Binding

Competition binding studies were performed using a nitrocellulose filter binding technique. Nitrocellulose membranes (0.45 μm HA; Millipore) were soaked in binding buffer (10 mM sodium phosphate, pH 7.7, 250 mM KCl, 0.1 mM NaEDTA, and 5 mM $MgCl_2$) for 10 min.

Reaction mixtures contained 1.6 μM yeast topoisomerase II and 20 μM [^{3}H]etoposide, as well as 0–100 μM etoposide, TOP-53, hydroxyphenyl-etoposide or phenyl-etoposide in a total of 60 μL of binding buffer. Samples were incubated for 6 min at 30 °C and applied to the nitrocellulose membranes in vacuo. Filters were immediately washed three times with 1 mL of ice-cold binding buffer, dried, and submerged in 8 mL of scintillation fluid (Econo-Safe; Research Products International). Radioactivity remaining on membranes was quantified using a Beckman LS 5000 TD scintillation counter. The amount of radioactive etoposide remaining on the filter in the absence of enzyme was subtracted prior to binding calculations.

RESULTS

Interaction of Etoposide with Topoisomerase II

Etoposide, a semisynthetic derivative of podophyllotoxin, is an important anticancer agent (1–4). It is composed of a polycyclic ring system (rings A–D), a glycosidic moiety at the C4 position, and a pendant ring (E–ring) at the C1 position (Figure 1) (1–4). The drug kills cells by inhibiting the ability of topoisomerase II to ligate nucleic acids that it cleaves during the double-stranded DNA passage reaction (13,14). As a result of etoposide action, high levels of topoisomerase II-associated DNA breaks accumulate in treated cells (1–5). The effects of etoposide on DNA cleavage mediated by yeast topoisomerase II are shown in Figure 2.

In an effort to identify the substituents that influence drug activity, numerous derivatives of etoposide have been synthesized (33–42). Most of the modifications have focused on the glycosidic moiety and the E–ring (35,37–40). Many of these changes have profound effects on the cellular activity of etoposide. However, for most substitutions, it is not known whether alterations in drug potency or efficacy are caused by changes in drug uptake/efflux or metabolism, as opposed to a direct effect on topoisomerase II-mediated DNA ligation. Even in the small number of cases in which the effects of drug derivatives on the purified enzyme have been assessed, it has not been possible to ascribe a specific function to any given substitutent on etoposide (35,39,40).

Given the wide clinical use of etoposide, it is important to understand how the drug interacts with its protein target. Therefore, STD [^{1}H]-NMR spectroscopy (43–47) was employed to identify the substituents on etoposide that interact with topoisomerase II. Since drug-enzyme contacts, as opposed to drug-DNA interactions, mediate the entry of etoposide into the topoisomerase II-drug-DNA complex (4,9,22–32), the present study focused on interactions between etoposide and topoisomerase II in the binary protein-ligand complex.

In the STD [^{1}H]-NMR technique (43–47), topoisomerase II is selectively saturated with magnetization by irradiating the sample at a frequency at which no drug protons resonate (on-resonance frequency). Magnetization is spread very rapidly throughout the protein by intramolecular spin diffusion. Substituents on the drug (*i.e.*, ligand) that interact with topoisomerase II are progressively saturated with magnetization via intermolecular, through-space, dipole-dipole interactions. The NMR spectrum generated following on-resonance irradiation is subtracted from an off-resonance (or reference) spectrum. This off-resonance spectrum is taken following saturation of the sample with a magnetization frequency that is different from the resonance frequencies of either topoisomerase II or the drug. The resulting difference spectrum contains only the signals of the drug substituents that interact with the enzyme and are saturated through the intermolecular transfer of magnetization from the protein (43–47).

NMR samples contained 5 μM yeast topoisomerase II and 200 μM drug, and spectra were recorded at 15 °C. The buffer used for spectroscopy contained 10 mM sodium phosphate and 250 mM KCl, as opposed to 10 mM Tris-Cl and 100 mM KCl, which generally is used to assess

topoisomerase II-mediated DNA cleavage (26). The use of phosphate eliminated the proton spectrum of the Tris buffer, which overwhelmed many of the drug peaks. In addition, the increased salt was required in order to maintain enzyme solubility at the high protein concentration and low temperature used for NMR. As seen in Figure 2, topoisomerase II maintained high DNA cleavage activity in the NMR buffer. Under the experimental conditions employed, enzyme-drug samples were stable for at least 24 h as assessed by NMR spectroscopy and DNA cleavage assays (data not shown).

As a prelude to STD [^{1}H]-NMR experiments, proton resonances of etoposide were assigned by 1-D NMR analysis (50) (a spectrum that includes these assignments is shown in supplemental Figure S1). A representative STD [^{1}H]-NMR experiment that analyzed the binding of etoposide to yeast topoisomerase II is shown in Figure 3. The difference spectrum, which reveals the nuclear Overhauser enhancment (NOE) signals from the bound drug, is seen at the top. Results indicate that substituents on the A–, B–, and E–rings interact with topoisomerase II in the binary protein-ligand complex. More specifically, NOE signals were observed for the geminal protons of the A–ring (6.0 ppm), the H5 and H8 protons of the B–ring (7.0 and 6.6 ppm, respectively), as well as the H2’ and H6’ protons (6.4 ppm) and the 3’– and 5’–methoxyl protons of the pendent E–ring (3.8 ppm). In contrast, no significant NOE signals arising from the C–ring, the D–ring, or the C4 glycosidic moiety were observed, suggesting that there is limited or no contact between these portions of etoposide and the enzyme in the binary complex.

One caveat should be noted, however. Resonances for the three hydroxyl groups present in etoposide, the 2”–OH and 3”–OH of the glycosidic moiety and the 4’–OH of the E–ring, were obscured by the water peak and could not be visualized in any of the NMR spectra.

To determine whether conclusions based on yeast topoisomerase II could be extended to mammalian systems, interactions between etoposide and human topoisomerase IIα were analyzed by STD [^{1}H]-NMR spectroscopy. The NOE signals in the difference spectrum generated for the binary topoisomerase IIα–etoposide complex were similar to those described above for yeast topoisomerase II (Figure 3, third spectrum from top). Thus, it appears that the same etoposide substituents that interact with yeast topoisomerase II also interact with the human enzyme.

C4 Substituents

On the basis of the STD [^{1}H]-NMR data, it appears that the glycosidic moiety of etoposide, which is attached to the C–ring at the C4 position, does not interact with yeast topoisomerase II or human topoisomerase IIα in the binary enzyme-drug complex. To characterize the effects of alternative substituents at the C4 position, interactions between the yeast enzyme and two etoposide derivatives were characterized by STD [^{1}H]-NMR. The first was teniposide, which, like etoposide, also is in clinical use (1,2). Teniposide contains the C4 substituent of etoposide, except that the 8”-methyl group is replaced by a thiophene moiety (see Figure 1). In all other respects, the two compounds are identical. NMR signals derived from the 8”-thiophene protons are shifted well downfield to a region of the spectrum that is devoid of protein peaks (6.96–7.37 ppm). Although teniposide is ~10–fold more potent than etoposide in human cells, the two drugs are equipotent in purified systems (see Figure 2). Thus, it is believed that the effects of the thiophene moiety are primarily physiological (uptake, metabolism, etc.) in nature (1,2).

As seen in Figure 4 (top spectrum), difference spectrum NOE signals from the A–, B–, and E–ring substituents of teniposide were similar to those of etoposide. In addition, no NOE signals from the glycosidic protons of the three thiophene protons were observed. These findings suggest that the C4 glycosidic moieties of etoposide and teniposide do not interact with topoisomerase II in the drug-enzyme complex. In support of this conclusion, the ability of

etoposide and teniposide to enhance topoisomerase II-mediated DNA cleavage is identical (see Figure 2), despite the difference in the 8”-substituents of these two drugs.

The second derivative that was assessed was TOP-53. In contrast to etoposide and teniposide, TOP-53 contains a flexible dicationic amino-alkyl chain at the C4 position (see Figure 1) (51). Although TOP-53 is structurally identical to these compounds at all other positions, it exhibits a significantly greater ability (~4–fold higher potency) to stimulate DNA cleavage mediated by yeast (see Figure 2) or human type II topoisomerases (52). This finding implies that substituents at the C4 position have the capacity to alter drug actions in the ternary complex. Therefore, to determine whether the presence of the amino-alkyl chain of TOP-53 affects the interaction of the drug with the enzyme, drug-topoisomerase II interactions were assessed by STD [^{1}H]-NMR.

As found for etoposide and teniposide, NOE signals were observed for the geminal protons of the A–ring, the H5 and H8 protons of the B–ring, as well as the H2’ and H6’ protons and the 3’– and 5’–methoxyl protons of the pendent E–ring (Figure 4, third spectrum from top). However, the NOE signals for all of these positions on TOP-53 were ~2-fold stronger than those observed for the other podophyllotoxins. In addition, NOE signals for every CH_2 group of the amino-alkyl side chain of TOP-53 (2.27–3.05 ppm) were observed (Figure 4).

These findings suggest that TOP-53 binds to yeast topoisomerase II more tightly or with a closer geometry than does etoposide. Unfortunately, the STD [^{1}H]-NMR technique employed cannot distinguish between these two possibilities. Therefore, nitrocellulose filter binding competition assays were utilized to address this issue. In these experiments, the ability of non-radioactive etoposide and TOP-53 to compete with [^{3}H]etoposide for binding to yeast topoisomerase II was determined. As seen in Figure 5, the concentration of TOP-53 that was required to displace ~50% of the bound [^{3}H]etoposide was ~4–fold lower than needed by etoposide. These data demonstrate that the affinity of TOP-53 for topoisomerase II is greater than that of etoposide, and likely accounts for the enhanced potency of the etoposide derivative. Furthermore, they imply that this enhanced binding is due to the presence of the additional contacts between the C4 amino-alkyl moiety of TOP-53 and the enzyme that were identified by STD [^{1}H]-NMR.

E–Ring Substituents

Early structure-activity studies suggested that E–ring substituents on etoposide were important for drug function against mammalian type II topoisomerases (35,39,40). As seen in Figure 2, this conclusion was confirmed for drug-induced DNA cleavage by yeast topoisomerase II. Removal of the 3’- and 5’-methoxyl groups from the E–ring of etoposide (hydroxyphenyl-etoposide; see Figures 1 and 2) decreased drug activity ~60%. Further removal of the 4’-hydroxyl group (phenyl-etoposide; see Figures 1 and 2), decreased drug activity to nearly baseline.

The NMR experiments discussed above indicate that the E–ring of etoposide is intimately associated with topoisomerase II in the binary complex. Therefore, the effects of the 3’- and 5’-methoxyl, and the 4’-hydroxyl substituents on the interaction between etoposide and yeast topoisomerase II were monitored by STD [^{1}H]-NMR. NOE signals for the E–ring H2’ and H6’ protons were observed for hydroxyphenyl-etoposide and phenyl-etoposide (Figure 6, top and third spectra, respectively). In addition, signals were observed for the H3’ and H5’ protons that replaced the corresponding methoxyl substituents in hydroxyphenyl-etoposide and phenyl-etoposide and the H4’ proton that replaced the hydroxyl group in phenyl-etoposide. As observed for etoposide, the only other NOE signals seen corresponded to the geminal protons of the A–ring and the H5 and H8 protons of the B–ring. It should be noted that NOE signals for both drug derivatives were somewhat diminished as compared to the parent compound.

The NMR results indicate that despite the loss of critical substituents, the pendant E–rings of hydroxyphenyl-etoposide and phenyl-etoposide still maintain a close association with topoisomerase II in the binary complex. This finding suggests that the 3'– and 5'–methoxyl groups and the 4'-hydroxyl of the E–ring are not required for drug binding. Therefore, nitrocellulose filter binding competition assays were employed to further define the contribution of these groups to drug binding. As seen in Figure 5, the ability of hydroxyphenyl-etoposide and phenyl-etoposide to compete with [^{3}H]etoposide for binding to yeast topoisomerase II was similar to that of etoposide. On the basis of these results, we conclude that the 3'– and 5'– methoxyl groups and the 4'–hydroxyl of the etoposide E–ring play critical functional roles for drug action, but do not mediate binding of the drug to the type II enzyme.

DISCUSSION

Although etoposide is one of the most widely prescribed drugs used for the treatment of human cancers (1–4), the specific ring substituents that mediate its interactions with topoisomerase II have been impossible to define. However, the use of STD [^{1}H]-NMR has enabled drug-enzyme interactions to be characterized at the proton level. Results indicate that protons on the A–ring, B–ring, and pendent E–ring are in close contact with yeast topoisomerase II and human topoisomerase IIα in the binary enzyme-ligand complex. These findings are summarized in Figure 7, which highlights hydrogens that interact with topoisomerase II in red and those for which no interactions were observed in green.

It has long been known that the glycosidic moiety of etoposide plays important physiological roles (1–4). The presence of this group keeps etoposide from interacting with tubulin and substitution of a thiophene for the 8"–methyl alters cellular uptake of the drug. Beyond these physiological functions, it is not clear whether the glycosidic moiety of etoposide plays any direct role in enhancing topoisomerase II-mediated DNA cleavage. To this point, no contacts were observed between topoisomerase II and any glycosidic protons and substitution of the thiophene at the 8" position had no effect on DNA scission. It is notable, however, that substitution of the glycosidic moiety with a flexible and charged amino-alkyl side chain, as in TOP-53, significantly enhanced drug-enzyme binding and drug activity against topoisomerase II. Furthermore, contacts were observed between the enzyme and every observable proton of this side chain in the binary complex. Therefore, while the C–4 glycosidic moiety does not appear to mediate interactions between etoposide and topoisomerase II, other substituents at this position have the capacity to influence drug actions against its enzyme target.

Previous studies indicate that E–ring substituents are important for etoposide function (35, 37,39,40). Indeed, as seen in Figure 2, removal of the 3'– and 5'–methoxyl groups or the 4'–hydroxyl moiety significantly impairs the ability of etoposide to enhance DNA cleavage mediated by yeast topoisomerase II. As determined by STD [^{1}H]-NMR, every substituent on the E–ring is intimately associated with the protein in the binary complex. Since the E–ring has free rotation about the 1'–linkage to the C–ring, the H2' and H6' protons, as well as the 3'– and 5'–methoxyl groups are chemically equivalent and cannot be distinguished by NMR. A previous study by Long and co-workers found that removal of one of the two methoxyl groups had no obvious effect on levels of DNA scission (37). Thus, as shown in the shaded region in Figure 7, we propose that contacts between etoposide and topoisomerase II extend to only a portion of the E–ring.

Although the 3'–methoxyl, 5'–methoxyl, and 4'–hydroxyl moieties of the E–ring all are important for etoposide function (35,39,40), none of them appear to contribute significantly to drug-enzyme binding. In fact, when these groups were replaced with hydrogen atoms, NOE signals were observed by STD [^{1}H]-NMR for all of the resulting protons. This finding suggests that protein associations with the E–ring may be mediated by stacking interactions rather than

any specific group on the ring. In the absence of additional structural information, it is not known whether the 3'–methoxyl, 5'–methoxyl, or 4'–hydroxyl groups contribute to drug function through direct interactions with individual amino acids in topoisomerase II, or by subtly changing the overall geometry of the etoposide-enzyme-DNA complex.

In the absence of topoisomerase II, etoposide exhibits little if any ability to bind to DNA (29). However, the fact that the drug displays a DNA cleavage site specificity (preferring a cytosine at the base immediately 5' to the scissile bond (5,53) implies that etoposide interacts with DNA in the ternary topoisomerase II-drug-nucleic acid complex. If this is the case, which portions of etoposide contact DNA in the active site of topoisomerase II? Given the strong contacts between the enzyme and the A–, B–, and E–rings we believe that these portions of the drug are unlikely to interact with DNA. Furthermore, substitution of a thiophene for the 8"–methyl in teniposide or an amino alkyl chain in TOP-53 does not alter the DNA cleavage specificity of etoposide, and the amino alkyl substitution at the C4 position interacts strongly with topoisomerase II (5,52,53). Consequently, we also believe that the glycoside moiety of etoposide probably does not contact DNA. Therefore, we propose that if any portions of etoposide interact with DNA in the active site of topoisomerase II, the C– and D–rings are the most likely candidates. Future studies with the ternary enzyme-drug-DNA complex will be required to address this hypothesis directly.

Finally, it should be noted that there is a strong correlation between results obtained from STD [^{1}H]-NMR experiments and DNA cleavage assays. Alterations in etoposide substituents that contact the enzyme in the binary complex diminished the ability of the drug to enhance topoisomerase II-mediated DNA cleavage. Conversely, alterations in drug substituents that increased the number of contacts between the enzyme and etoposide raised levels of DNA scission. Therefore, STD [^{1}H]-NMR results with the binary enzyme-drug complex appear to have predictive value that may contribute to the future development of etoposide derivatives with greater activity against topoisomerase II.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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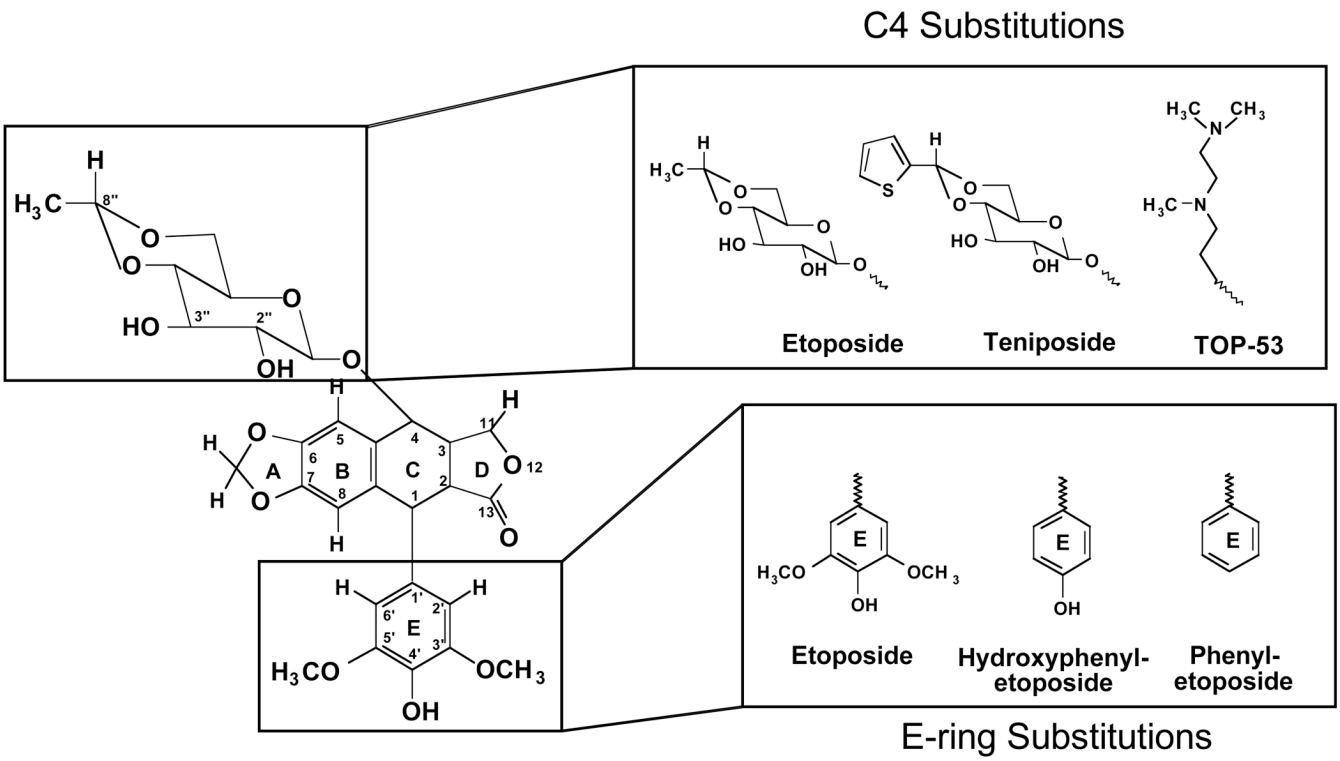

FIGURE 1.
Structures of etoposide and etoposide derivatives with C4 or C1 (E–ring) substitutions that were employed in the present study.

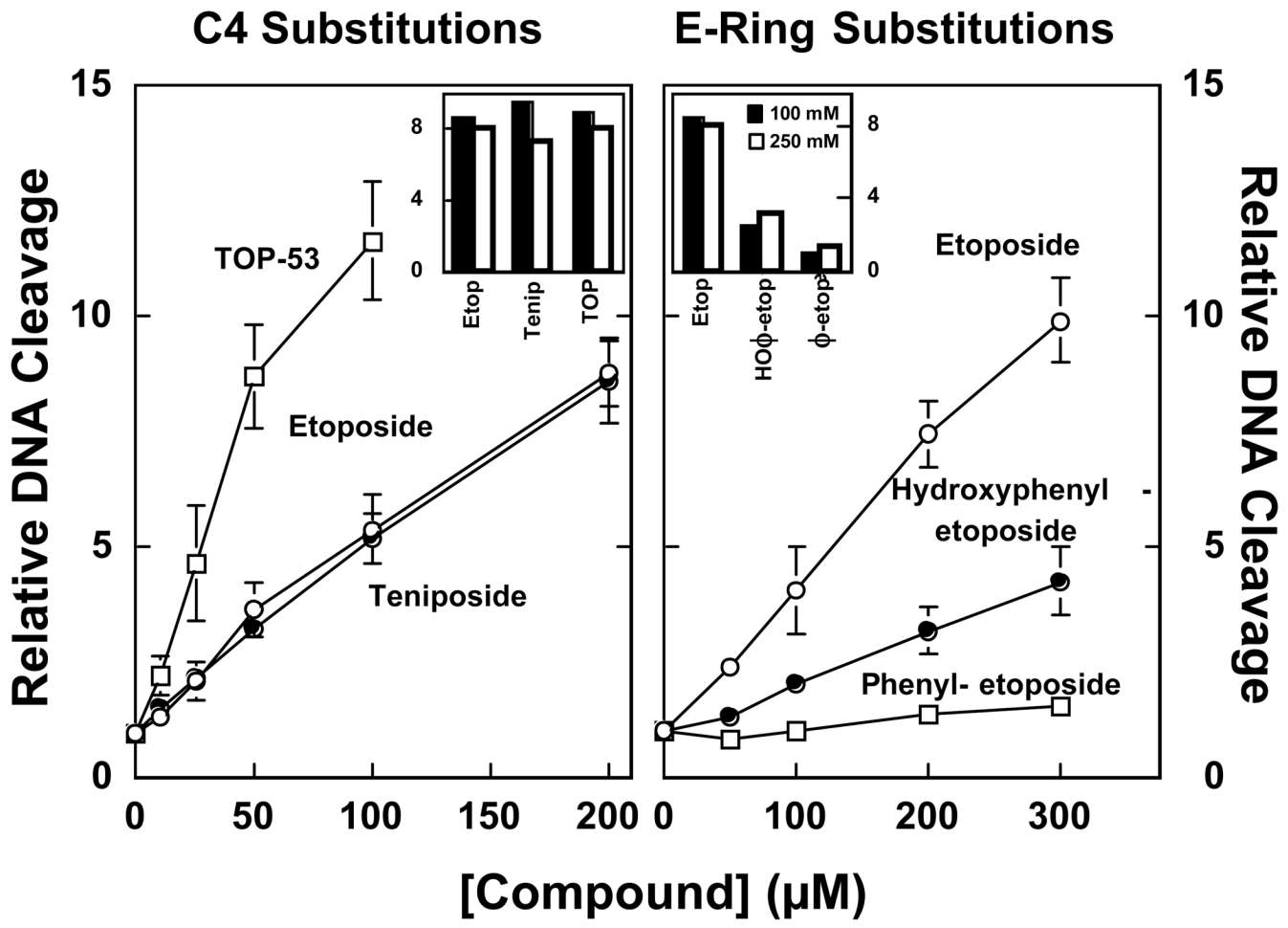

FIGURE 2.

Etoposide derivatives stimulate DNA cleavage mediated by yeast topoisomerase II. Levels of DNA cleavage were expressed as a fold–enhancement over reactions that were carried out in the absence of drug. Cleavage titrations using etoposide derivatives with C4 substitutions (left panel) or E–ring substitutions (right panel) are shown. Left: Assay mixtures contained 0–200 μM etoposide (open circles) or teniposide (closed circles), or 0–100 μM TOP-53 (open squares). Higher concentrations of TOP-53 generated multiple cleavage events per plasmid and therefore are not shown. Right: Assay mixtures contained 0–300 μM etoposide (open circles), hydroxyphenyl-etoposide (closed circles), or phenyl-etoposide (open squares). Error bars represent the standard deviation of three independent experiments. Insets show cleavage reactions carried out in cleavage buffer containing 100 mM KCl (filled bars) or 250 mM KCl (open bars) the presence of 200 μM etoposide (Etop), teniposide (Tenip), hydroxyphenyl-etoposide (HO-φ-etop), or phenyl-etoposide (φ-etop), or 100 μM TOP-53 (TOP).

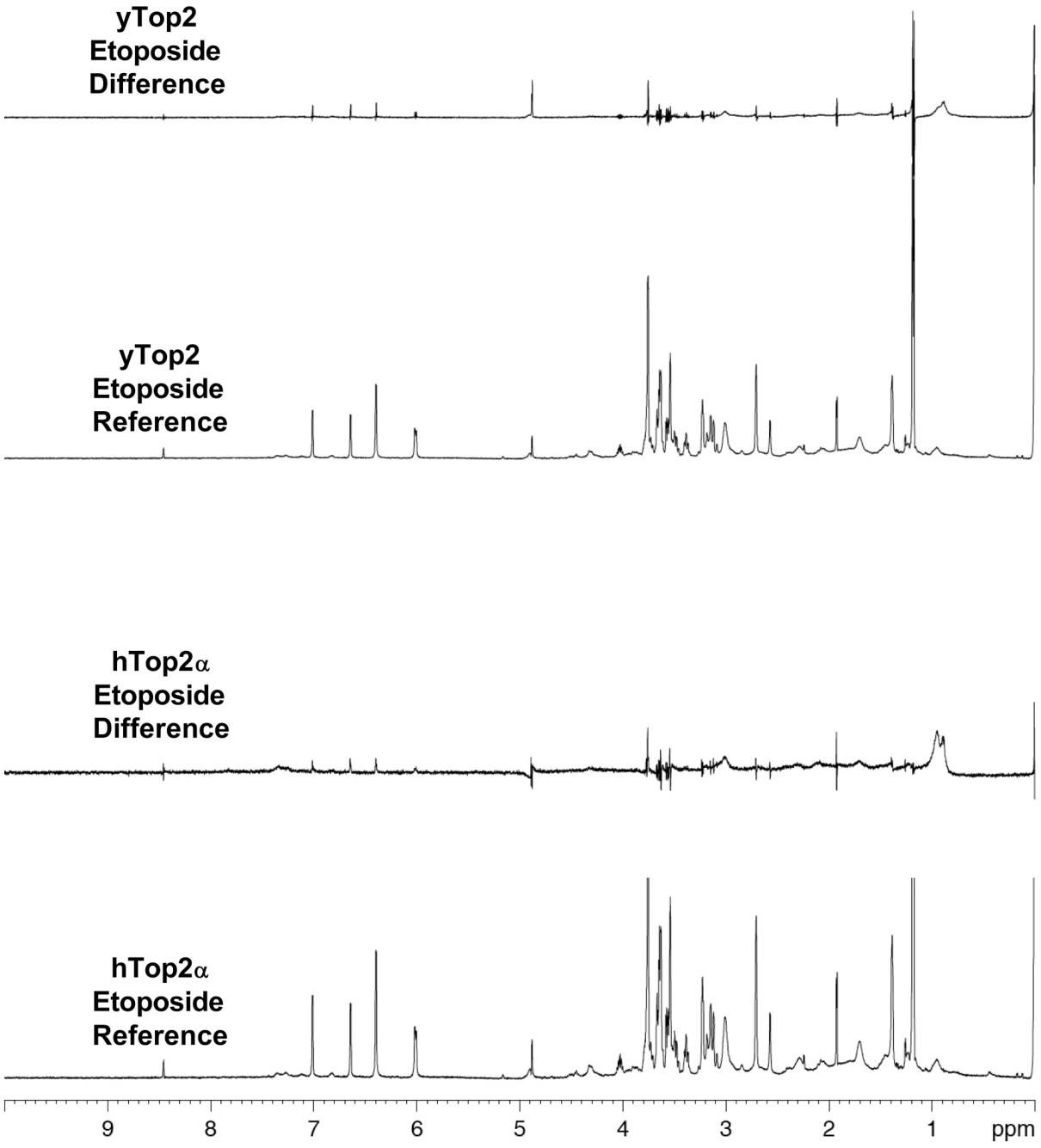

FIGURE 3.

Interaction of etoposide with yeast topoisomerase II and human topoisomerase IIα as determined by STD [^{1}H]-NMR spectroscopy. 1D [^{1}H]-NMR reference spectra of etoposide are shown in the second and fourth spectrum from the top. 1D STD [^{1}H]-NMR spectra of etoposide in the presence of yeast topoisomerase II (top spectrum) or human topoisomerase IIα (third spectrum from top) also are shown. Spectra are representative of at least three independent experiments.

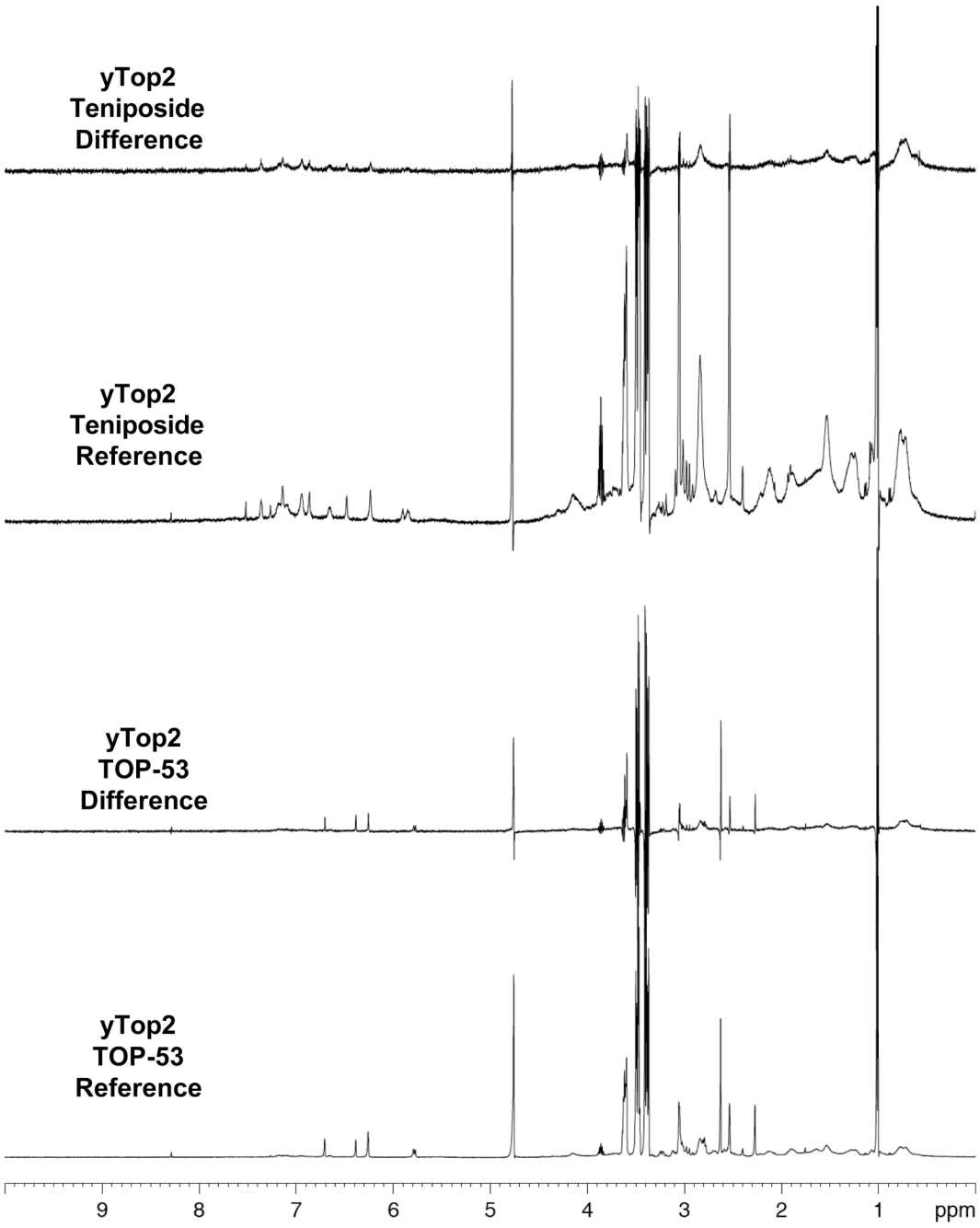

FIGURE 4.

Interaction of teniposide and TOP-53 with yeast type II topoisomerase as determined by STD [^{1}H]-NMR spectroscopy. 1D [^{1}H]-NMR reference spectra of teniposide (second spectrum from top) and TOP-53 (fourth spectrum from top) are shown. 1D STD [^{1}H]-NMR spectra of teniposide (top spectrum) and TOP-53 (third spectrum from top) in the presence of yeast topoisomerase II also are shown. Spectra are representative of at least three independent experiments.

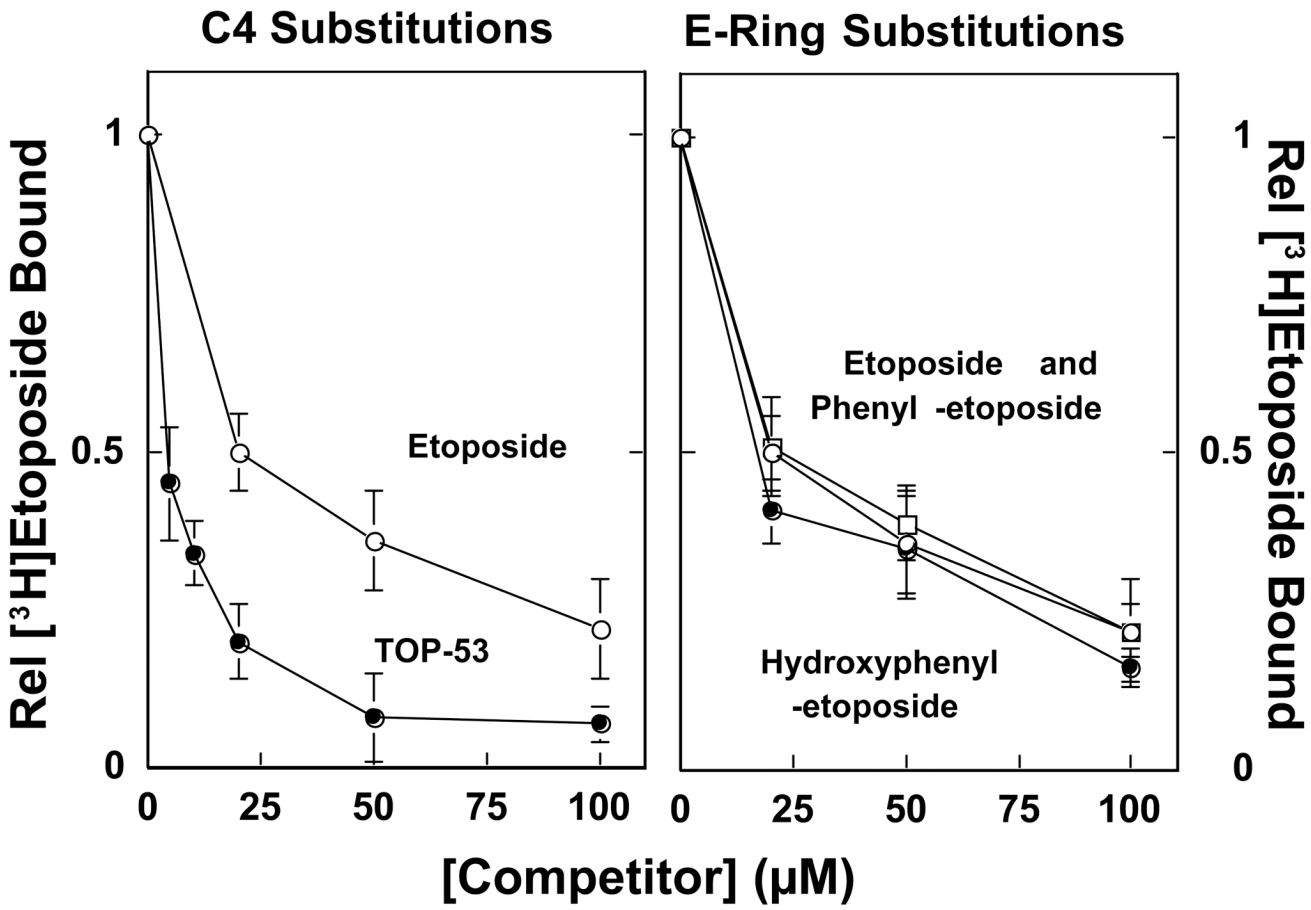

FIGURE 5.

Binding of etoposide and derivatives to yeast topoisomerase II. Left: Reaction mixtures contained 20 μM [^{3}H]etoposide and 0–100 μM etoposide (open circles) or TOP-53 (closed circles). Right: Reaction mixtures contained 20 μM [^{3}H]etoposide and 0–100 μM etoposide (open circles), hydroxyphenyl-etoposide (closed circles), or phenyl-etoposide (open squares). Levels of [^{3}H]etoposide binding to yeast topoisomerase II observed in the absence of competitor drug were set to 1. Error bars represent the standard deviation of three independent experiments.

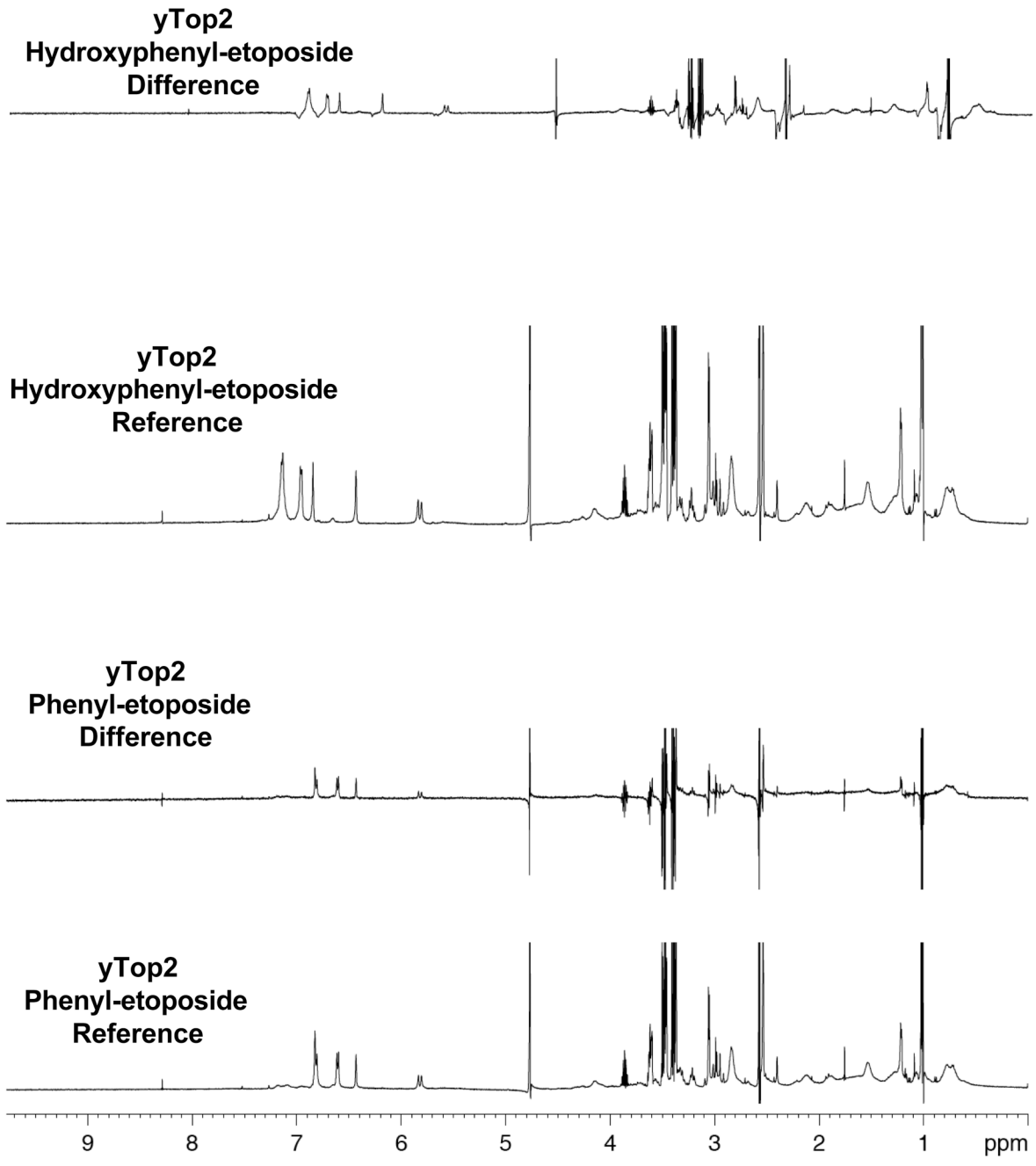

FIGURE 6.

Interaction of hydroxyphenyl-etoposide and phenyl-etoposide with yeast type II topoisomerase as determined by STD [^{1}H]-NMR spectroscopy. 1D [^{1}H]-NMR reference spectra of hydroxyphenyl-etoposide (second spectrum from top) and phenyl-etoposide (fourth spectrum from top) are shown. 1D STD [^{1}H]-NMR spectra of hydroxyphenyl-etoposide (top spectrum) and phenyl-etoposide (third spectrum from top) in the presence of yeast topoisomerase II also are shown. Spectra are representative of at least three independent experiments.

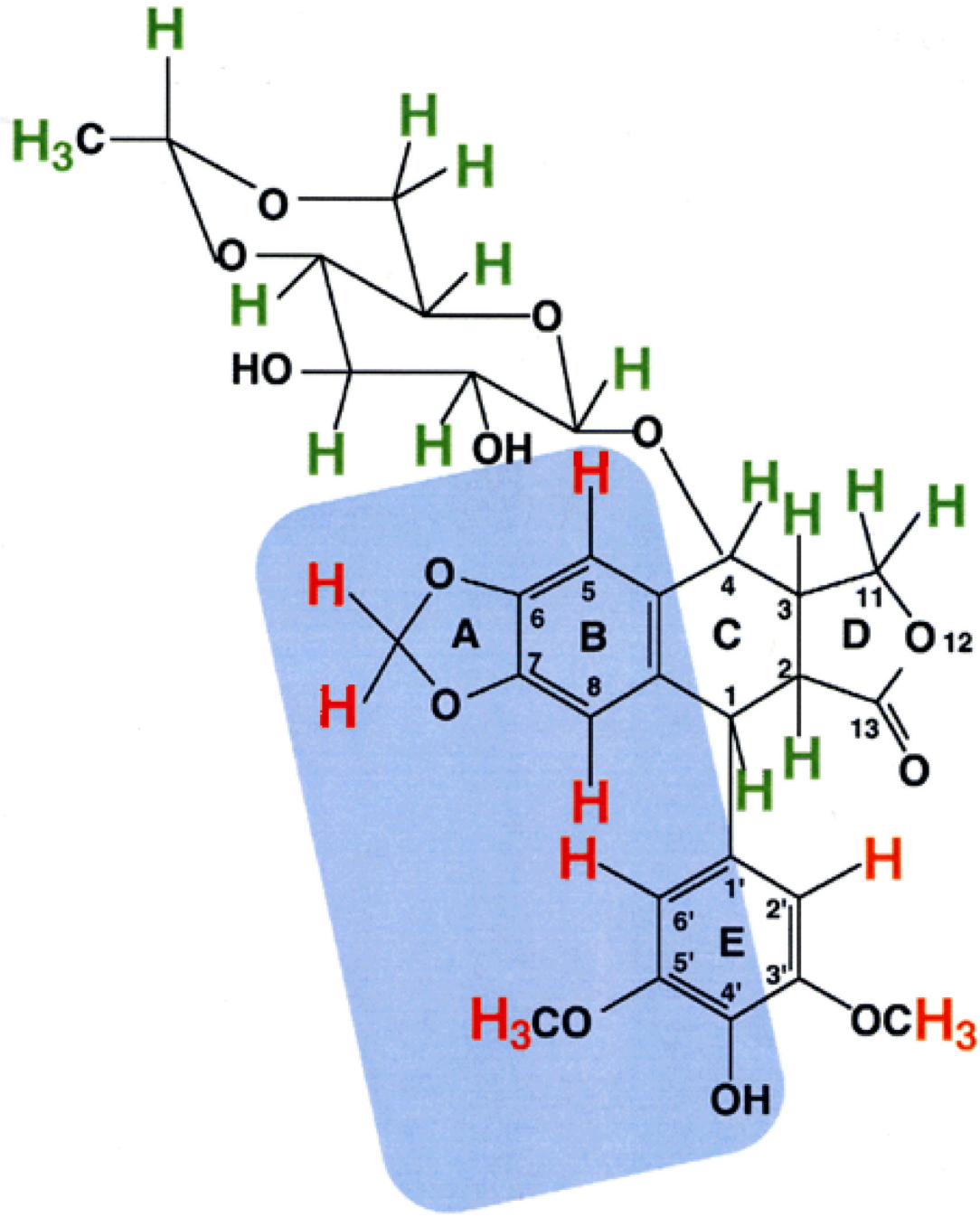

FIGURE 7.

Summary of etoposide substituents that interact with type II topoisomerases. Protons that interact with the enzyme are shown in red, those that do not are shown in green. Hydroxyl protons that were obscured by the water peak and could not be visualized are indicated in black. The shaded region on etoposide, including portions of the A–, B– and E–rings, is proposed to interact with topoisomerase II in the binary drug-enzyme complex.