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SYNTHESIS AND BIOLOGICAL ACTIVITY OF 3',4',5'-TRIHYDROXY ETOPOSIDE

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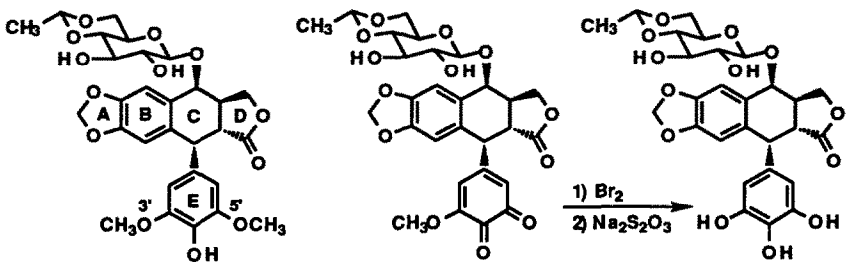
ABSTRACT: *The E-ring 3',4',5'-trihydroxy etoposide analog* ***3*** *of the clinical antitumor agent etoposide* ***1*** *was synthesized from the corresponding etoposide ortho-quinone* ***2****. This analog is twice as potent as etoposide in its ability to elicit topoisomerase II mediated DNA double strand breaks. However, our in vitro cytotoxicity assay, using human colon tumor cells grown in culture, and our in vivo P388 murine leukemia screen reveal considerable loss of potency and/or activity vis-a-vis etoposide.*

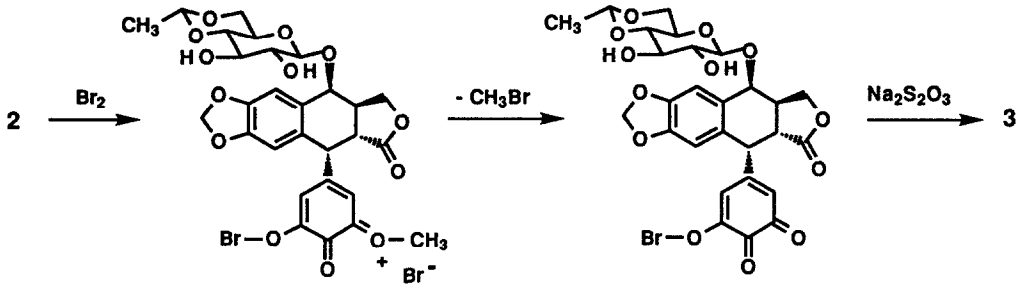
Etoposide (Vepesid,VP-16, **1**), the most widely used of anticancer agents, recently surpassed cisplatin (Platinol) as the leading drug in this field in terms of total annual sales. Etoposide is approved in the United States for the treatment of small cell lung cancer and testicular cancer. The oral activity of etoposide offers a particular advantage for chronic daily dosing on an outpatient basis, and its synergy with other antineoplastic drugs such as cisplatin has expanded its use for other oncological indications as well. We have previously reported our work on the further validation of the mechanism of action of **1** and its related congeners[1,2], the principle operative modalities being topoisomerase II (topo II) inhibition and bioactivation to its corresponding ortho-quinone **2** via oxidative demethylation. From a structure activity relationship (SAR) perspective, the E-ring 3',4',5'-trihydroxy etoposide analog **3** is an appealing target due to its greater hydrogen bonding capabilities and the expected ease of bioactivation to its corresponding ortho quinone. We now report the synthesis of **3**, as well as its topo II mediated DNA strand cleavage, and *in vitro* and *in vivo* biological activities.



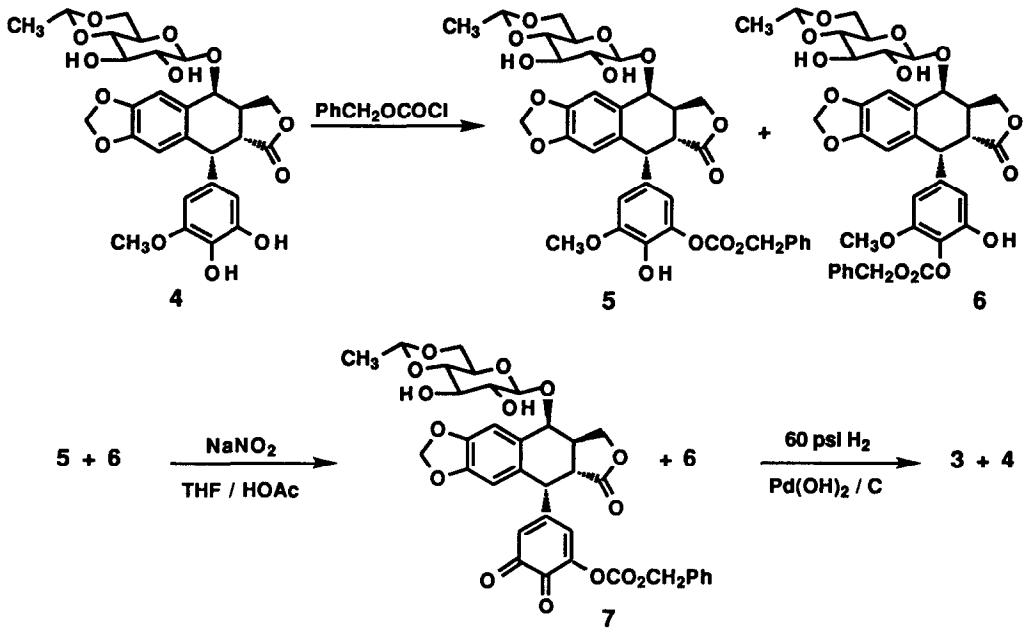
1 (VP-16) **2** **3**

Bromination of the etoposide ortho-quinone[3] **2** (1.0 equiv Br_2, 1:1 dioxane:H_2O, 25 °C / 30 min), followed by reductive workup (aqueous $Na_2S_2O_3$, CH_2Cl_2) gives a 51% yield of the trihydroxy analog **3**, following purification by flash chromatography[4] on silica gel (10% MeOH / CH_2Cl_2). The surprising reactivity of this electrophilic ortho-quinone **2** with molecular bromine proceeds by an unknown mechanism.[5] We propose the sequence shown in **Scheme I** below as one reasonable possibility, although we have no experimental data towards its verification. The structure of 3',4',5'-trihydroxyetoposide **3** is fully secured by ^1H NMR, ^{13}C NMR, mass spectroscopy, and independent synthesis as described below.[6]

SCHEME 1



Reduction of the etoposide ortho-quinone **2** to its known hydroquinone **4**[7], followed by non-regioselective acylation with benzyl chloroformate ($EtN(i\text{-}Pr)_2$, CH_3CN / THF, 0 °C) yields an inseparable and undetermined mixture of presumed mono-carbonates **5** and **6**. Direct treatment of this mixture with $NaNO_2$ (THF / HOAc, 0 °C to 25 °C over 5 h) followed by extractive workup (aq $NaHCO_3$ / CH_2Cl_2) and hydrogenolysis (20% $Pd(OH)_2/C$, 60 psi H_2, EtOAc, 44 h) provides the desired trihydroxy analog **3** in addition to hydroquinone **4**. A single chromatography for the overall process produces an 11% yield of pure **3** (25% based on recovered **4**), which is identical in all respects to the material produced via the more efficient one step bromination route (*vide supra*). Presumably, this latter synthetic scheme proceeds via oxidation ($NaNO_2$) of mono carbonate **5** to its corresponding ortho-quinone **7**. Hydrogenolysis then converts **7** to the desired trihydroxy analog **3**, and also transforms mono carbonate **6** to hydroquinone **4**.[8]



We have previously described the details of the *in vivo* P388 murine leukemia assay[1,2]. The data for compounds **1-4** are summarized in **Table I**. Significant activity in the P388 model is defined as a T/C of >125%. Since etoposide (**1**) itself is often exceptionally active in this assay, it was sometimes administered initially on day 5 instead of on day 1. Inspection of the data in **Table I** clearly shows that the 3',4',5'-trihydroxy analog **3** is less potent than VP-16, nontoxic at the highest dose tested (150 mg/kg/injection, which is greater than the maximum tolerated dose of VP-16), and less active at the doses evaluated. The P388 data for the etoposide ortho-quinone **2** and hydroquinone **4** are also included in Table I. Both **2** and **4** display comparable potency and activity to VP-16.

Table I. Anti-P388 Leukemia Activity of Compounds 1-4.

Expt No.	Compound	Dose, mg/kg per inj	T/C (LTS)[a]	Max T/C (LTS)[a] for 1: dose, mg/kg per inj
1	2	120[b]	447 (1/4)	
		80[b]	>621 (3/4)	>595 (2/4), 60[b]
		40[b]	274	
2	3	150[c]	150	
		75[c]	130	230, 100[c]
		38[c]	100	
3	4	100[b]	344 (1/4)	
		50[b]	>413 (2/4)	>563 (4/4), 60[b]
		25[b]	250	

[a]T/C refers to the percent of the median survival time of drug-treated mice compared to saline-treated controls. Long-term survivors (LTS) were mice alive/total on day 30 when the experiment was terminated. [b]Administered ip on days 1 and 5. [c]Administered ip on day 5 and 8.

Compounds **1-4** were evaluated for their abilities to stabilize the transient intermediate formed between DNA and topo II during DNA strand passage. Knotted P4 phage DNA was incubated with 20 or 5 units of purified topoisomerase II in the presence of 1, 3, 10, 30, and 100 uM of drug for 30 minutes, and then incubated with proteinase K and SDS for 10 minutes. The samples were then subjected to agarose gel electrophoresis for 15 hours, and the gels stained with ethidium bromide. The resulting fluorescent DNA bands were photographed. Negatives of the gels were scanned to quantify the amounts of linear DNA, reflecting stablizied intermediate, in each sample. The data is summarized in **Table II**. The 3',4',5'-trihydroxy etoposide analog **3** is about twice as potent as etoposide for eliciting topo II induced double stranded DNA breaks. The quinone **2** and hydroquinone **4** also are more potent than etoposide in this regard.

Table II. DNA-Topo II Intermediate Stabilization and In Vitro Cytotoxicity of Compounds 1-4.

Compound	DSB[a]	HCT-116 IC_{50} μg/mL[b]	HCT/VP35 IC_{50} μg/mL[b]	Resistance Ratio[e]
1	96	0.35*[c,d]	5.2*[c,d]	15
2	164	N.D.	N.D.	N.D.
3	182	79 (7.9)	68.9 (34)	0.87 (4.3)
4	152	35 (15)	N.D.	N.D.

[a]Double strand DNA breakage reflecting stabilization of Topo II-DNA intermediates. Values are expressed in ng of linear DNA per unit of enzyme per mM of drug. [b]The IC_{50} is the dose that reduces by 50%, after 72 h, cell growth in vitro as compared to controls. IC_{50} values of etoposide in the same assay are shown in parentheses. All values indicated are the average of at least two runs. The crystal violet assay was used except where indicated by an asterisk.[11] [c](*)The XTT assay[12] was employed. [d]The IC_{50} values for etoposide are the average of multiple runs. [e]The resistance ratio is a measure of the relative cytotoxic potency of the compound and is determined by dividingthe IC_{50} value for the etoposide resistant HCT/VP35 cell line by the IC_{50} value for the etoposide sensitive HCT116 cell line. The resistance ratio for etoposide in the same run is shown in parentheses.

The details of the experimental protocol for the *in vitro* cytotoxicity assay using etoposide sensitive (HCT-116) and resistant (HCT/VP35) cells grown in culture have been previously described.[1,2] The IC_{50} values and resistance ratios for compounds **1** and **3** are included in **Table II**. While the trihydroxy analog **3** does exhibit the ability to overcome resistance in the HCT/VP35 cell line, it is also 10X and 2X less potent than VP-16 in the HCT-116 and HCT/VP35 cell lines, respectively.

In conclusion, we have described the first synthesis and biological evaluation of the etoposide 3',4',5'-trihydroxy E-ring analog **3**. While this novel analog is inherently twice as potent as etoposide in its ability to elicit topo II mediated DNA double strand breaks, it is significantly less potent than VP-16 towards human colon tumor cells grown in culture. Moreover, the *in vivo* potency and activity of **3** are also significantly diminished vis-a-vis etoposide. **Thus, from a SAR perspective for topo II interaction, 3' and 5' E-ring hydroxyl groups are preferred over the corresponding E-ring methoxy substituents of etoposide.** Since our *in vitro* and *in vivo* assays reveal that the trihydroxy analog **3** displays considerable loss of potency and/or activity as compared to VP-16, we favor a rapid bioinactivation process to account for these phenomena. However, other pharmacokinetic parameters, such as clearance, metabolism, and biodistribution, may also play a sigificant role in this regard. The bioinactivation postulate gains support from our inability to oxidize **3** to its corresponding E-ring ortho-quinone[9], and from the greater chemical instability[10] of **3** as compared to etoposide. Further studies to improve upon the pharmacokinetic and stability problems associated with 3',4',5'-trihydroxy etoposide **3** using a prodrug approach are in progress.

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3. For the preparation of **2**, see Nemec, J. *U. S. Patent 4 609 644,* **Sept. 2, 1986**.

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5. An unusual example of reductive methylation of the related podophyllotoxin ortho-quinone has been described and its mechanism proposed. See, Ayres, D. C.; Ritchie, T. J. *J. Chem. Soc., Perkin Trans I* **1988**, 2573.

6. Data for **3**: 300 MHz ^{1}H NMR (CD_3OD); 6.94 (s, 1H), 6.46 (s, 1H), 6.04 (s, 2H), 5.91 (d, 2H, J=2.0 Hz), 4.96 (d, 1H, J=3.0 Hz,), 4.74 (q, 1H, J=5.0 Hz), 4.63 (d, 1H, J=7.5 Hz), 4.42-4.33 (m, 2H), 4.25-4.07 (m, 2H), 3.58-3.50 (m, 2H), 3.40-3.21 (m, 4H), 3.04-2.92 (m, 1H,), 1.32 (d, 3H, J=5.0 Hz). High resolution mass spectrum, calcd for $C_{27}H_{28}O_{13}$; 560.1530. Found; 560.1537.

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8. All new compounds gave satisfactory analytical and spectroscopic data in full accord with the assigned structures.

9. Although very rapid disappearance of **3** ensues under standard conditions used for converting **1** to **2** ($NaIO_4$ in aqueous CH_3CN or $NaNO_2$ in HOAc/ THF), no tractable products could be unambiguously identified in this regard.

10. Compound **3** is obtained as a white solid after initial purification but slowly darkens in air after several days.

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