# Isolation, Semisynthesis, and Molecular Modeling of Deoxypodophyllotoxin Analogs for an Anti-oral Cancer Agent

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Oral cancer is one of the 10 most general cancers in the world, often detected late and associated with a poor prognosis, a lack of specific biomarkers, and expensive treatments. Oral cancers are mostly oral squamous cell carcinomas (OSCC) with sophisticated biological and clinical behaviors.[1] In general, the important risk factors of oral cancer are tobacco, alcohol, ultraviolet (UV) light exposure, and human papillomavirus infection.[2,3] Despite advanced cancer identification and therapy, there has been little improvement in the 5-year survival rate of oral cancer patients over the last few decades.[4]

Therefore, we have been searching for lead compounds from natural products as potential preclinical candidates for oral cancer therapy for the last few years. Recently, we reported that deoxypodophyllotoxin (DPT, **1**), isolated from *Anthriscus sylvestris*, showed a potent cytotoxic effect on oral cancer cells.[5] DPT is known to have many bioactivities such as antiproliferative,[5] antitumor,[6] antiplatelet aggregation,[7] antiviral,[8] insecticidal,[9] anti-inflammatory, anti-allergic,[10] and liver protective activities.[11] The main mechanism of action of DPT is the inhibition of tubulin polymerization, which leads to cell cycle arrest at the G2/M phase and apoptosis by caspase 3 and 7 activation.[12] Unfortunately, although DPT has a strong *in vitro* antitumor effect, it may be limited by *in vivo* insolubility in water. The absence of a hydroxyl group at position 4 in DPT blocks the synthesis of water-soluble derivatives, which limits its clinical treatment.[13] Therefore, DPT analogs need to be developed to increase the water solubility and maintain its efficacy. Herein, we report the isolation, semisynthesis, and molecular modeling of DPT analogs for new anti-oral cancer agents (Figure 1).

We isolated DPT (**1**),[5] yatein (**2**),[14] and nemerosin (**3**)[15] by cytotoxicity-guided fractionation from the roots of *A. sylvestris* identified by comparison of their spectroscopic data with previously reported values in the literature. The semisynthesis of DPT and podophyllotoxin (PT) derivatives is shown in Schemes 1 and 2. Treatment of DPT with boron trichloride yielded catechol compound 6 and its 4′-demethyl compound 7.[16] Reduction of the lactone ring with KOH led to compound **8**. Demethylation of the 4′-methoxyl group with 30%-HBr-AcOH afforded 4′-demethylated DPT, compound **9**.[16]

Compounds **10** and **11** were prepared from treatment of PT (**4**) by a methanesulphonic acid/sodium iodide system in $CH_2Cl_2$, followed by weak basic hydrolysis.[17] Compounds **10** and **11** were then oxidized by pyridinium dichromate (PDC) in $CH_2Cl_2$, leading to compounds **12** and **13**. PDC was more convenient than Dess–Martin periodinane.[18] Compounds **14** and **15** were synthesized with PT (**4**) and trimethylsilyl cyanide in the presence of boron trifluoride diethyl etherate.[19] Using trimethylsilyl cyanide in the presence of boron trifluoride diethyl etherate as a nucleophilic agent was more effective than any other materials, such as KCN, HCN, or $Hg(CN)_2$.[19,20] The C4-configuration of semisynthetic compounds was deduced from the reaction mechanism.[21]

We evaluated the antitumor activities of prepared compounds (**1**–**15**) against oral cancer HN22 and HSC4 cell lines by MTT assay. The results are presented in Table 1. Almost all compounds except yatein (**2**) and nemerosin (**3**) were more effective than positive control, 5-fluorouracil. In general, the efficacy in the HN22 cell line tended to be better than in the HSC4 cell line. DPT showed the strongest inhibitory effect, with an $IC_{50}$ value of 6.52 nM for HN22 cells and 7.26 nM for HSC4 cells. PT (**4**) and picropodophyllotoxin (PPT, **5**) had moderate activity, whereas yatein (**2**) and nemerosin (**3**), which had open C rings, exhibited micromolar levels of efficacy. In particular, nemerosin (**3**), with a double bond, had double the efficacy of yatein (2). Therefore, cleavage of the C-ring seemed to have the most important effect on efficacy. The difference in efficacy between PT (**4**) and PPT (**5**) seemed to be due to the difference in the absolute structure of the D-ring, but the difference is only about twofold, so this structural difference did not have a significant effect. Catechol compounds **6** and **7**, which had opened A rings, had decreased activity in the HN22 cell line like that of PT. Compounds **6** and **7** showed significant differences in efficacy between the two cell lines. 6,7-Demethylene compounds, compounds **6** and **7** are known to significantly decreased the inhibition of tubulin polymerization.[16] The efficacy of compound **8**, opened D ring, was similar to that of PT, and the

  

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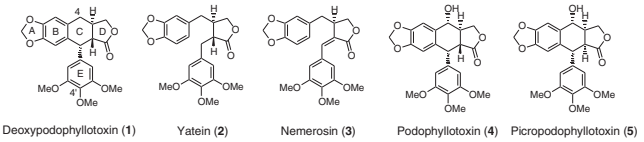
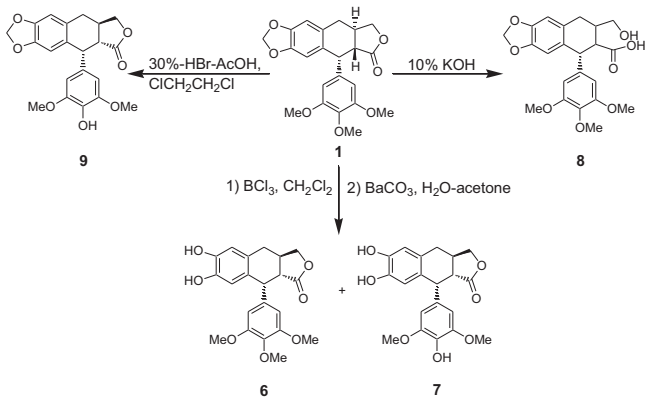
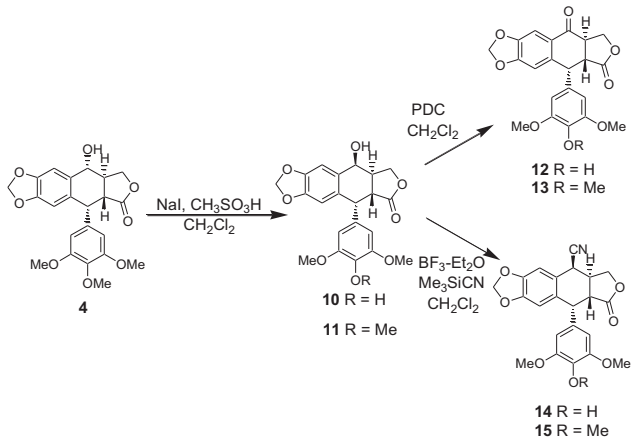



**Figure 1.** Chemical structures of compounds (**1**–**3**) isolated from *A. sylvestris*, podophyllotoxin (**4**), and picropodophyllotoxin (**5**).




**Scheme 1.** Synthetic pathway for DPT derivatives.




**Scheme 2.** Synthetic pathway for PT derivatives.

carboxylic acid group of the opened D ring showed the possibility of another structural transformation. The efficacy of 4′-demethylated DPT, compound **9**, was about half that of DPT, but 10 times more potent than that of PT. It showed the increased water solubility and the best potency besides DPT. However, an *in vivo* experiment in BDF1 mice bearing murine Lewis lung carcinoma cells demonstrated a loss of antitumor activity. Compound **9** needed esterification of the phenolic hydroxyl group with carbamic, carbonic, and amino acids to increase antitumor activity.[22] Compound **11**, epipodophyllotoxin, showed better efficacy than that of PT, and 4′-demethylated epipodophyllotoxin, compound **10**, exhibited a worse effect than that of PT. Like the relationship between DPT (**1**) and 4′-demethylated DPT (**9**), the 4′-demethylated epipodophyllotoxin (**10**) and 4-cyano-4-deoxy-4′-demethylated epipodophyllotoxin (**14**) were less effective than epipodophyllotoxin (**11**) and 4-cyano-4-deoxy-epipodophyllotoxin (**15**). However, in the case of 4′-demethylated podophyllotoxone (**12**) and podophyllotoxone (**13**), 4′-demethylated podophyllotoxone (**12**) was more effective than podophyllotoxone (**13**). Podophyllotoxone (**13**) is known as an anti-prostate agent and inhibitor of the tubulin polymerization.[24] 4-Cyano-4-deoxy-4′-demethylated epipodophyllotoxin (**14**) was reported to exhibit an anti-cancer effect against $L_{1210}$ (mouse lymphocytic leukemia) and KB (human nasopharyngeal cancer) cell lines.[19] Although 4-cyano-4-deoxy-epipodophyllotoxin (**15**) showed efficacy similar to PT and compound 8, its water solubility was similar to that of DPT, which reduced the possibility of another structural transformation. From these results, the structural modification of the hydroxyl group at position 4′ of similar compound **9** could be expected to yield a new anti-oral cancer agent with increased water solubility and an improved pharmacokinetic profile. In addition, the carboxylic acid due to D-ring opening like compound **8** and the hydroxyl group at position 4′ of the like 4′-demethylated podophyllotoxone (**12**) also offer potential as prodrugs. Cleavage of the C ring was confirmed to have one of the biggest roles in the deterioration of efficacy. DPT derivatives are known for their various anticancer activities, but their role in oral cancer treatment is uncertain. We confirmed that DPT analogs were effective in treating oral cancer and the findings provide a starting point for further optimization of DPT derivatives as anti-oral agents.

To understand the mechanism of action, we attempted the docking simulations and the molecular dynamics simulation to predict the tubulin–ligand complex. Known as the mechanism of action of DPT, the occupied colchicine

**Table 1.** $IC_{50}$ values of compounds in HN22 and HSC4 cell lines.

| Compound | HN22 (nM) | HSC4 (nM) | logS[a] |
|---|---|---|---|
| DPT (**1**) | 6.52 | 7.26 | −5.57 |
| Yatein (**2**) | $3.92 \times 10^4$ | $5.44 \times 10^4$ | |
| Nemerosin (**3**) | $1.73 \times 10^4$ | $2.89 \times 10^4$ | |
| PT (**4**) | 122 | 161 | −4.75 |
| PPT (**5**) | 245 | 243 | −4.75 |
| **6** | 115 | 945 | −4.68 |
| **7** | 114 | 987 | −3.99 |
| **8** | 97 | 153.6 | −4.58 |
| **9** | 12 | 17.3 | −4.88 |
| **10** | 143 | 229.2 | −4.06 |
| **11** | 84.3 | 99.2 | −4.75 |
| **12** | 76.2 | 89.3 | −4.76 |
| **13** | $2.5 \times 10^3$ | $3.5 \times 10^3$ | −5.45 |
| **14** | 398.2 | 820 | −4.71 |
| **15** | 92.7 | 151.7 | −5.41 |
| 5-Fluorouracil[b] | $6.1 \times 10^3$ | $1.7 \times 10^3$ | |

[a] logS was developed by SILICOS-IT. The predicted values are the decimal logarithm of the molar solubility in water.[23]
[b] Positive control.

domain prevented microtubule assembly by maintaining a curved αβ heterodimer structure.[24] However, the complex DPT structure was not demonstrated in the X-ray experiment. According to the computational result, DPT was located at the αβ interface and was bound to the β-subunit (Figure 2(a)). The T5 loop of the α-subunit (αT5) came closer to the T7 loop of the β-subunit (βT7) and looked like a lid on the binding pocket. An interaction between αT5 and βT7 could induce the curved tubulin. DPT was totally surrounded by H7 and H8 helices and the strand S8 and S9 of the β-subunit driven by the hydrophobic interactions. In more detail, the A, B, C, and D rings of DPT were deeply supported by the Leu255 of helix H8. The hydrophobic *E*-ring of DPT was contacted by the hydrophobic side chains: Cys241 of helix H7, Ala316 and Val318 of strand S8, and Ala354 of strand S9. Compared to the tubulin-PT complex seen on X-rays,[25] it was similar to the binding interaction of the DPT-tubulin complex. It would not be established by the structure–activity relationship. However, based on an analysis of the side chain complex contacts, additional interactions of the binding site can be considered. When a substituent of DPT was expended with a functional group, it tried to find accessible amino acids to make a hydrogen bond, an ionic bond, or van der Waals interaction. Notably, Asn258 of helix H8 could be an important key in forming the extra-hydrogen bonding such as proton acceptor or donor. It could be related to the structure–activity relationship of PT with 4α-hydroxyl group and compound **11** with 4*β*-hydroxyl group. The configuration of C-4*β* was shown to positively affect anticancer efficacy. To form extra-hydrogen bonding with Asn258, it should be extended at the C-4*β* position of DPT and two specific derivatives could be proposed, 4*β*-carboxyl-4-deoxypodophyllotoxin (model 1), and 4*β*-carboxyl-4′-hydroxyl (demethylated)-deoxypodophyllotoxin (model 2) (Figure 2 (b)). Although the synthesis of the model compounds[21] and the anticancer effect of model 2 against some cancer cell lines[19] were already reported, anti-oral cancer effect and the mechanism of action such as destabilizer of tubulin did not yet have investigated. When the designed compound model 1 was modeled by the docking simulation, it formed a hydrogen bond with the side chain of Asn258 (Supporting Information Figure S1). Specifically, the *β* direction of the C-ring was in significant contact with helix H8. Furthermore, when the activity and predicted water solubility of DPT (**1**) was compared with compound **9**, the substituted 4′-hydroxyl group of the E-ring improved the water solubility and slightly was affected for the antitumor activity (Table 1). Then, the two-position substituted compound (model 2) adding a C-4*β* carboxyl group and a 4′-hydroxyl group on the E-ring increased pharmacokinetic optimization. The predicted log S value was about −3.98, indicating that it could be more soluble than the synthesized compounds.

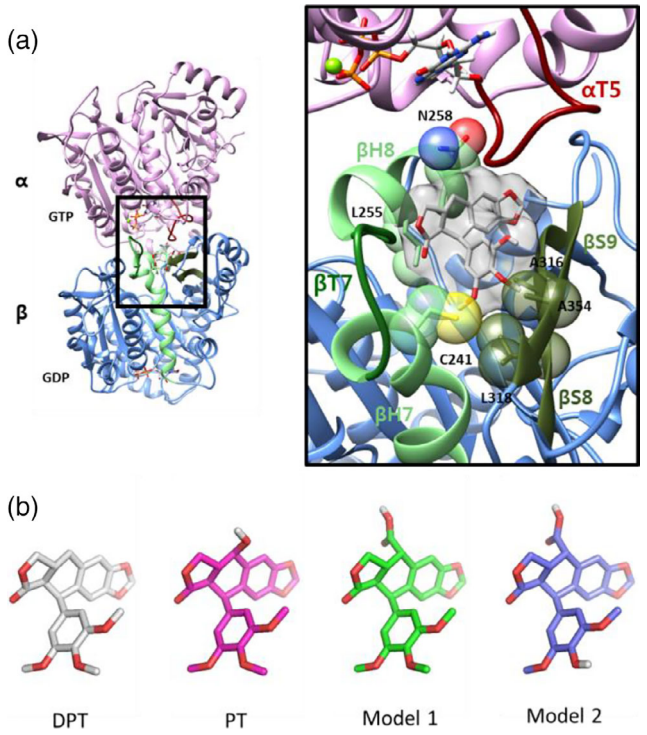



**Figure 2.** (a) The predicted binding pose of DPT in soluble tubulin and (b) 3-dimensional structures of the ligands, such as DPT, PT, model 1 (4*β*-carboxyl-4-deoxypodophyllotoxin), and model 2 (4*β*-carboxyl-4′-hydroxyl(demethylated)-deoxypodophyllotoxin). The ligand was located at the αβ interface of tubulin. The binding domain of DPT was identical to the colchicine domain. The T7 loop of the β-subunit surrounded the tetra-ring of DPT together with helix H8 (Leu255), and strands S9. The helix H7, and the strand S8 and S9 held the tri-methoxy benzene of DTP, such as the hydrophobic interactions; Cys241 of H7, Ala316 and Leu318 of S8, and Ala354 of S9 (sphere). In addition, the T5 loop of the α-subunit was flexible but seemed like a lid. Asn258 of H8 could be additional hydrogen bond sites as the proton donor and the proton acceptor, simultaneously. To form the hydrogen bond, we proposed the models 1 and 2 compounds with the carboxyl substituent at C-4. Adding the hydroxyl group, model 2 has better solubility than does model 1.

In conclusion, bioassay-guided fractionation of *A. sylvestris* led to compounds **1**–**3** with antitumor activity against oral cancer. DPT and PT derivatives were semisynthesized and molecular modeling was performed for the pharmacological and pharmacokinetic advanced DPT analog. The ring opening playing the most important role in the efficacy was identified as the cleavage of the C ring. The structure of compounds **8**, **9,** and **12** showed potential for the pharmacological and pharmacokinetic optimization. From these studies, we generated two advanced DPT analog structures with better pharmacological and/or pharmacokinetic potential. The synthesis and evaluation of these compounds, as well as mechanistic studies as anti-oral cancer agents, are currently underway.

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**Supporting Information.** Additional supporting information may be found online in the Supporting Information section at the end of the article.

  

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