STUDIES WITH PLANT CELL CULTURES OF THE CHINESE HERBAL PLANT, *TRIPTERYGIUM WILFORDII*. SYNTHESIS AND BIOTRANSFORMATION OF DITERPENE ANALOGUES.*

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Abstract - Synthetic routes to a series of diterpene analogues from dehydroabietic acid (**1**) are presented. These synthetic compounds are then utilized as substrates in biotransformation experiments with the cells of a stable cell line (coded as TRP4a) of the Chinese herbal plant, *Tripterygium wilfordii*. The enzyme-catalyzed conversions of the substrate, isotriptophenolide (**12**), afford a series of novel diterpene analogues to be evaluated in pharmacological screening programs as antineoplastic and immunosuppressive agents. Of particular interest are the quinone epoxides (**14**) and (**15**) for comparison with the highly active diterpene triepoxides, triptolide and tripdiolide. Some screening data obtained with the latter compounds and relating to their anti-cancer and immunosuppressive activity are also presented.

Tripterygium wilfordii Hook F, a perennial twining vine found mainly in the mountainous areas of south-eastern China, has served as an important herb in Chinese traditional medicine for several hundred years. The powdered root of this plant, known as Lei Gong Teng (rank grass), has been used

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* Dedicated to Dr. Shigeru Oae, Professor Emeritus, Tsukuba University, on the occasion of his 77th birthday.

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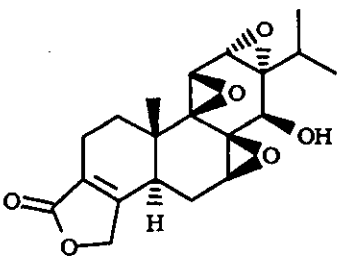
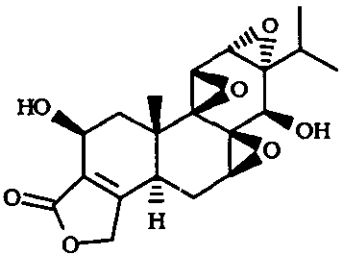
for crop protection against chewing insects,[1] while tablets, derived from a water/chloroform extract of the roots (a so-called total multi-glycoside or GTW) have been used in clinical treatment of rheumatoid arthritis, skin disorders, in male fertility control as well as in treatment of cancer and inflammatory diseases.[2-4] This remarkably broad spectrum of pharmacological activities of the plant constituents has stimulated research in our laboratory to develop a stable plant cell culture line (coded as TRP4a) of *T. wilfordii*, which can be grown in bioreactors from which larger quantities of secondary metabolites can be isolated[5,6] as pure components, and their pharmacological activities evaluated. In the recent study,[6] 28 diterpenes have been isolated and characterized. A number of these have been submitted for pharmacological screening (see later) and information concerning structure-activity relationships within the areas of immunosuppression and cytotoxicity is being accumulated. The purpose of the present study, discussed below, is to afford, via a combination of synthesis and enzyme-catalyzed biotransformations, additional novel diterpene analogues for further screening and to establish their relationship with the pharmacologically active natural products, triptolide and tripdiolide.

Of the various metabolites obtained from the TRP4a cell line, the diterpene triepoxides, triptolide and tripdiolide revealed significant activity as cytotoxic, immunosuppressive and anti-spermatogenic agents. It was therefore of interest whether novel analogues of this structural template could be obtained in order to assess the pharmacological importance of the triepoxide functionality. The approach selected was to synthesize appropriate "substrates" and subsequently to incubate these in whole cell fermentations and/or in the presence of TRP4a cell line derived enzymes, in order to achieve biotransformation to novel end products.

The synthetic routes to two substrates, the butenolide (**9**) and isotriptophenolide (**12**) from the readily available dehydroabietic acid (**1**) are presented in Schemes 1 and 2.

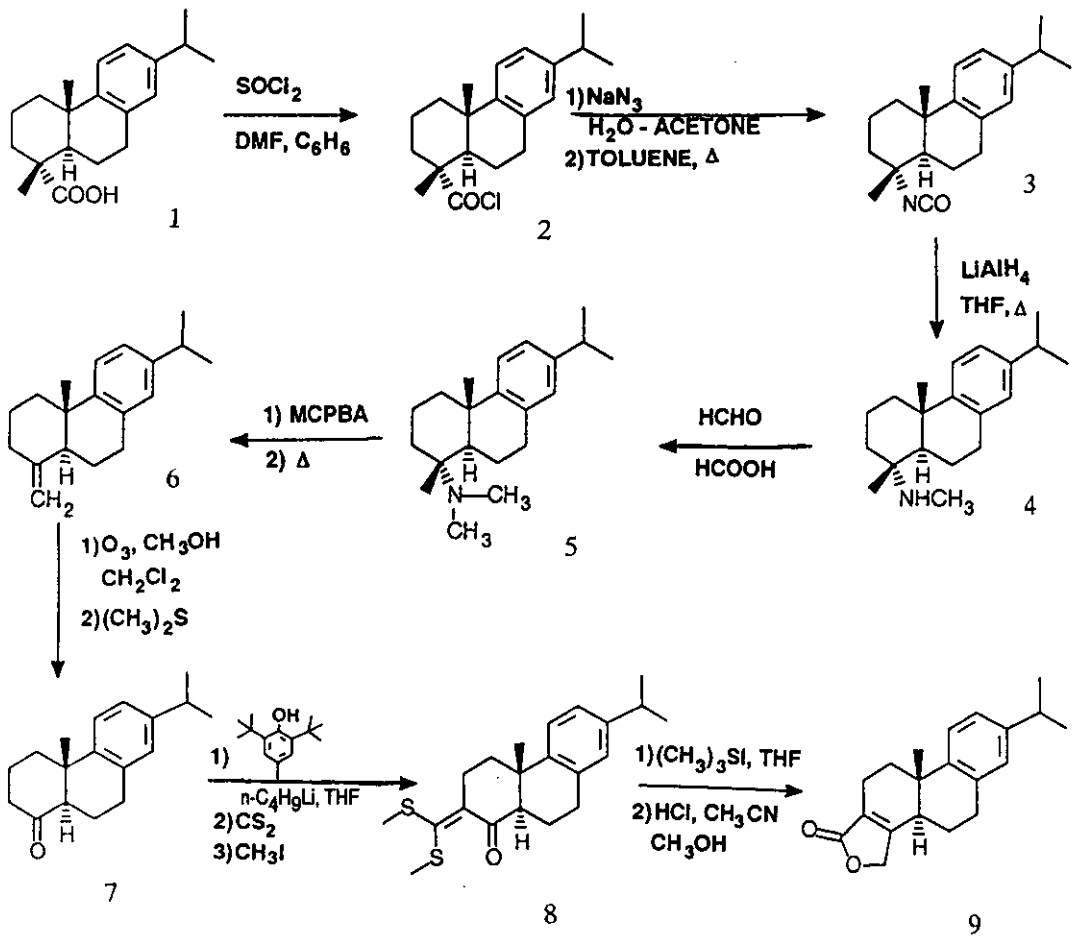


Triptolide

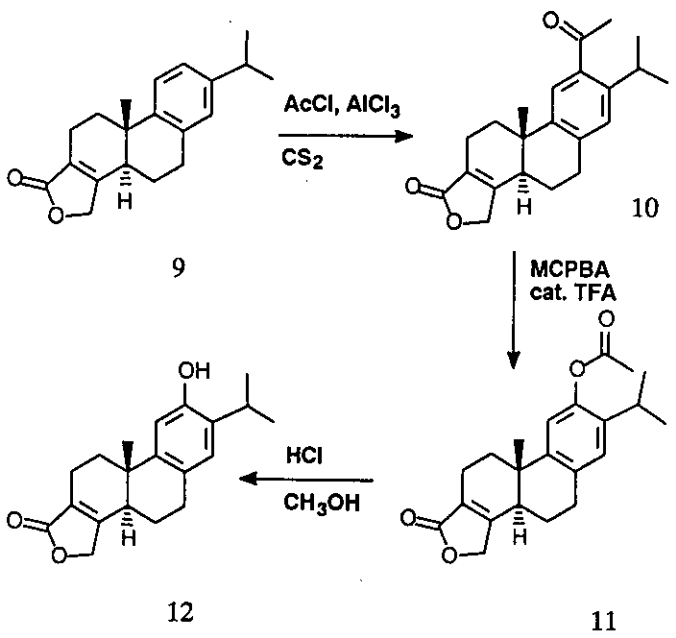


Tripdiolide

The conversion of **1** to the exocyclic olefin (**6**) was achieved *via* standard reactions, as noted. The intermediate acid chloride (**2**), obtained in quantitative yield, was converted directly to the acid azide and the latter upon heating rearranges to the isocyanate (**3**). The latter, after isolation but without purification, was reduced to the amine (**4**) which was again methylated to **5** and finally converted to **6**. The overall yield of **6** from **1** was 71%. Although for preparative purposes, the conversion, **1** -> -> **6**, was performed without purification of intermediates, the subsequent conversions from **6** to butenolide (**9**) require rigorous purification of ketone (**7**) and (**8**) in order to achieve the high yields indicated. Removal of the methylene unit in **6** by ozonolysis afforded the crucial intermediate ketone derivative (**7**), a crystalline white powder (mp 40-42°C, 82% yield).



Scheme 1. Synthesis of butenolide (**9**) from dehydroabietic acid (**1**).



Scheme 2. Synthesis of isotriptophenolide (**12**) from butenolide (**9**).

In order to direct anion formation to the secondary carbon (C-3), a sterically hindered phenoxide base is utilized. The resultant anion, upon reaction with carbon disulfide and methyl iodide, affords crystalline ketene thioketal (**8**) (mp 68-70°C, 95% yield).

Treatment of **8** with the sulfonium ylid (trimethylsulfonium iodide, n-BuLi, THF, -60°C) converted the ketone function to an epoxide, which without isolation, was treated with methanolic HCl (40 h, room temperature) to provide the butenolide (**9**) as colorless needles (mp 98-100°C, 59% overall yield from **8**). This efficient route provided **9** in an overall yield of 35% from dehydroabietic acid (**1**).

The other synthetic substrate, isotriptophenolide (**12**) became available via the synthetic route summarized in Scheme 2.

Friedel-Crafts acylation of butenolide (**9**) with acetyl chloride in refluxing carbon disulfide provided the crystalline ketone (**10**) (mp 153-155°C, essentially quantitative yield). The Baeyer-Villiger reaction of **10** with MCPBA in methylene chloride (room temperature) afforded a high yield (>90%) of acetate (**11**) and the latter upon acid hydrolysis afforded **12** (overall yield of 84% from **9**) as a crystalline solid (mp 198-200°C). For the sake of brevity, characterization data of the synthetic compounds shown in Schemes 1 and 2 and those in Scheme 3 are not presented here. These data will

be presented in a future detailed publication.

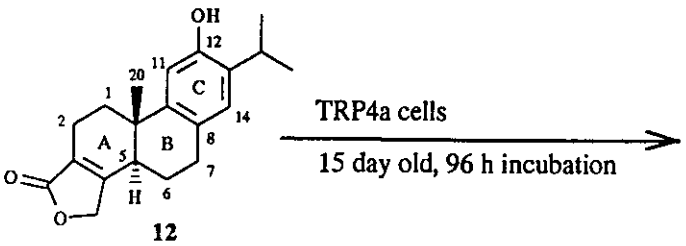
With isotriptophenolide (**12**) on hand, the evaluation of the enzyme-catalyzed bioconversion of this substrate with TRP4a cells was undertaken. There are many factors which can result in a lack of incorporation or biotransformation of exogenous substrates: the specificity of the enzyme system; the impermeability of the cellular membrane towards the exogenous material, poor solubility in the largely aqueous culture medium, age of culture at which maximum enzyme activity occurs, etc. Of particular relevance to our objectives, was the evaluation of "epoxidase" activity since it is of considerable interest to derive novel diterpene epoxides structurally related to the triptolide-tripdiolide series. Since previous studies[5,6] had revealed that the natural diterpene triepoxides were maximizing in "older" (generally 15-21 days old) growing cells of the TRP4a cell line, it appeared that the "epoxidase" enzymes were likely produced during the later stages of cell growth. On this basis, studies involving the biotransformation of **12** with 15 and 21 day old cells were undertaken. A number of experiments were completed but only one typical study is presented to indicate the various modes of biotransformation which can be achieved with TRP4a cells. Isotriptophenolide (**12**, 100 mg) was dissolved in ethanol (50 ml) and added to two 1 *l* conical shake flasks containing TRP4a cell cultures (15 days old, 2 x 550 ml). The culture was harvested after incubation for 96 h, cells and broth extracted with ethyl acetate and the combined extracts were subjected to column chromatography. Eight products along with unreacted starting material were isolated and characterized (total recovery 89%). Table 1 and Scheme 3 summarize the various biotransformation products obtained.

As the structural variety of biotransformation products indicates, the enzymes produced by the TRP4a cell line possess a diverse capability. However, it was most interesting that the "epoxidase" activity

Table 1. Compounds isolated from biotransformation of **12** (100 mg) in TRP4a cell culture.

Compound	12[a]	13	14 + 15	16	17	18	19	20
(mg)	59	9	15	1	3	2	1	2

[a] Recovered starting material



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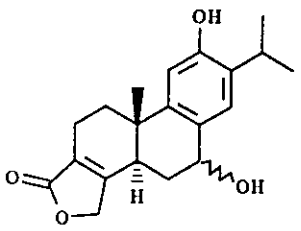
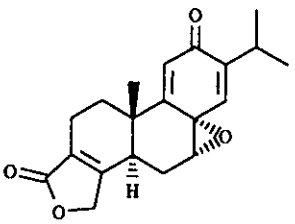


13 7α-OH
7β-OH



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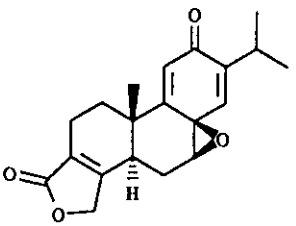

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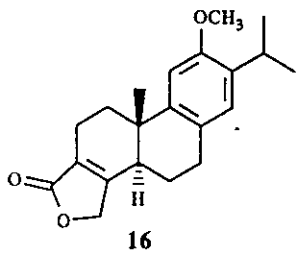

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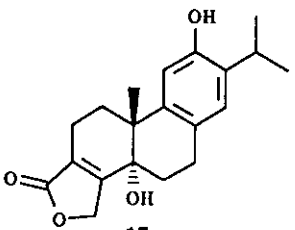

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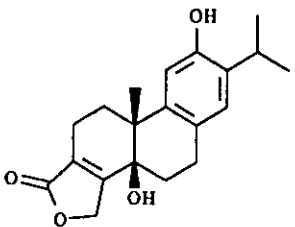

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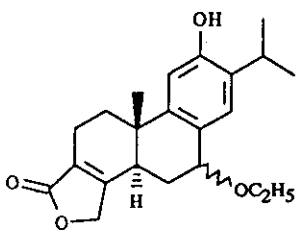
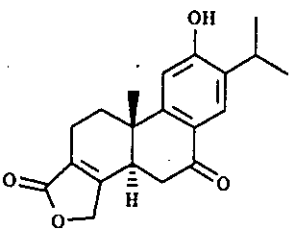


19 7α-OC_2H_5
7β-OC_2H_5



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Scheme 3. Biotransformation of isotriptophenolide (**12**) with TRP4a cells.

desired is indeed predominant and the isomeric quinone epoxides (**14**) and (**15**) are the major components in the biotransformation mixture. Subsequent experiments under similar conditions with substrate (**12**) but with 21 day old cells and longer (7 day) incubation times afforded a combined yield of 54% of the two isomeric epoxides (**14**) and (**15**). Clearly further experiments to optimize the fermentation parameters are essential but it is obvious that the TRP4a cell line possesses a significant level of "epoxidase" activity. Since the TRP4a cell line has been successfully grown in large bioreactors,[5,6] it is clear that **14** and **15** could be obtained in gram quantities, if desired.

It is significant to note that extensive studies directed toward a chemical conversion of **12** to **14** and/or **15** have not been successful. Clearly, the combination of synthetic chemistry with plant cell culture methodology is of advantage in this instance.

With the novel diterpene epoxides (**14**) and (**15**) available from the biotransformation experiments, it was of interest to utilize them as starting materials in further epoxidations (chemical and/or biological) of the "quinoid" double bonds to afford diepoxide and/or triepoxide analogues of the triptolide-tripdiolide series. Such analogues would afford the opportunity to achieve a better understanding in terms of structure-activity relationships. Results relating to these studies will form the subject of future publications.

Pharmacological screening of triptolide, tripdiolide, butenolide (**9**) and isotriptophenolide (**12**) in the murine P388 lymphocytic leukemia system (Table 2) and in six human cancer cell lines grown *in vitro* (Table 3) have been completed by Professor George R. Pettit and his colleagues, Cancer Research Institute, Arizona State University, Tempe, Arizona.

Table 2. Evaluation of compounds in the Murine P388 lymphocytic leukemia system.

Compound	ED_{50} (μg/ml)
Triptolide	< 0.01
Tripdiolide	0.0040
Butenolide (**9**)	7.5
Isotriptophenolide (**12**)	3.6

Legend: ED_{50} - concentration of compound required to kill 50% of cells, μg/ml.

As Table 2 indicates, the most potent compounds inhibiting the growth of murine lymphocytic leukemia cells *in vitro* are triptolide and tripdiolide. The two other compounds, lacking the epoxide functionality, are less active although ED_{50} values less than 10 micrograms per ml are still observed. The pharmacological data obtained from the evaluation of the above-noted compounds in human cancer cell lines grown *in vitro* are shown in Table 3.

GI_{50} values represent the concentration of a compound required for 50% growth inhibition. TGI (total growth inhibition) values give the concentration of compound required for 100% growth inhibition and

Table 3. Evaluation of compounds for activity in human cancer cell lines *in vitro*.

GI_{50} (50% growth inhibition; μg/ml)						
Compound #	Ovarian[a] OVCAR-3[b]	CNS[a] SF-295[b]	Renal[a] A498[b]	Lung-NSC[a] NCI-H460[b]	Colon[a] KM20L2[b]	Melanoma[a] SK-MEL-5[b]
Triptolide	0.0013	0.0064	0.0040	<0.0010	0.00086	0.0014
Tripdiolide	0.0023	0.013	0.017	<0.0010	0.0013	0.0023
9	4.5	3.7	3.5	2.5	1.7	3.1
12	5.6	7.3	5.1	3.5	4.3	3.3

TGI (total growth inhibition, μg/ml)						
Compound #	Ovarian[a] OVCAR-3[b]	CNS[a] SF-295[b]	Renal[a] A498[b]	Lung-NSC[a] NCI-H460[b]	Colon[a] KM20L2[b]	Melanoma[a] SK-MEL-5[b]
Triptolide	0.65	>10	0.045	0.0019	2.8	0.011
Tripdiolide	0.0055	>10	0.069	0.0031	>10	0.025
9	>10	>10	>10	6.4	4.7	>10
12	>10	>10	>10	>10	>10	>10

LC_{50} (50% cells killed, μg/ml)						
Compound #	Ovarian[a] OVCAR-3[b]	CNS[a] SF-295[b]	Renal[a] A498[b]	Lung-NSC[a] NCI-H460[b]	Colon[a] KM20L2[b]	Melanoma[a] SK-MEL-5[b]
Triptolide	>10	>10	>10	>10	>10	>10
Tripdiolide	>10	>10	>10	>10	>10	>10
9	>10	>10	>10	>10	>10	>10
12	>10	>10	>10	>10	>10	>10

[a]Cell type [b]Cell line

LC_{50} (50% cell kill) values give the concentration of compound required to kill 50% of all cells. All concentrations are in μg/ml culture. It is clear that significant activity is found in several of the compounds, particularly the triepoxides, triptolide and tripdiolide, which are highly potent. It should be noted that earlier studies by Kupchan and coworkers[7] had shown that the diterpene triepoxides possessed cytotoxic activity *in vivo* against L-1210 and P-388 leukemias in mice. Butenolide (**9**) and isotriptophenolide (**12**) have exhibited lower potency. It should be noted that butenolide (**9**) which lacks hydroxyl or epoxide functionality in rings B and/or C is more potent than the ring C diterpene analogue, isotriptophenolide (**12**).

Although the structure-activity relationship for this family of diterpenes is still incomplete, there is a strong suggestion that the presence of epoxide functionality in rings B and/or C is important for promoting antineoplastic activity. Future studies with the quinone epoxides (**14**) and (**15**) as well as other related analogues, will shed more light within this area.

In evaluation of the above-noted compounds as immunosuppressive agents, triptolide and tripdiolide revealed strong immunosuppressive activity while butenolide (**9**) and isotriptophenolide (**12**) were inactive. Again, it should be noted that the screening data are incomplete, but the presence of epoxide functionality appears highly important for promoting this type of activity.

ACKNOWLEDGEMENT

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