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Ovatodiolides: Scalable Protection-free Syntheses, Configuration Determination and Biological Evaluation against Hepatic Cancer Stem Cells

Junhong Xiang, Yahui Ding*, Jiaxin Li, Xiuhe Zhao, Yuanjun Sun, Da Wang, Liang Wang*, and Yue Chen*

Abstract: A concise, scalable and 6-steps (longest linear sequence) synthetic route to ovatodiolide scaffolds was developed for the first time. This protecting group-free route features a tandem ROM/RCM reaction to install the macrocycle fused butenolide ring and a tandem allylboration/lactonization to build the α-methylene-γ-lactone. Our syntheses have enabled the determination of the hitherto unknown stereochemical configurations of this family of natural products. A preliminary SAR was concluded based on the cellular biological tests of 4 natural ovatodiolides and 3 analogues. Further assays indicated that the synthetic natural product isoovatodiolide can significantly decrease the population of hepatic cancer stem cells and reduce the tumorsphere forming capability of HepG2 cells.

Cancer stem cells (CSCs), defined by their ability to dedifferentiate and self-renew, play a key role in carcinogenesis [1]. For example, only 100 cancer stem cells (characterized by the specific cell surface biomarker $CD133^+$) after injection are able to generate tumors in tested mice, while no tumors were observed after injection with 100,000 regular cancer cells[2]. Additionally, conventional chemotherapeutic agents are effective in ablating regular cancer cells while leaving CSCs intact, resulting in increased proportions of CSCs in cancers[3]. Therefore, targeting CSCs is considered to be a promising strategy for cancer treatment. However, compounds targeting CSCs are still rare [3].

Herbal medicines are a source of lead compounds for the identification of anti-CSCs therapeutics. For example, parthenolide (PTL, Figure 1A), an active component of the traditional western herb feverfew (*Tanacetum parthenium*), was found to selectively ablate leukemia stem cells[4]. We converted PTL into DMAMCL (registered as ACT001)[5]. ACT001 provided remarkable efficacy in brain glioblastoma models[6] and is currently in phase I clinical trial for advanced glioblastoma in Australia[7]. ACT001 has received orphan drug designation for glioblastoma multiforme from the FDA[8]. The clinical progress[9] of ACT001 encouraged us to further pursue widely used herbs containing natural products with anti-CSCs properties.

Ovatodiolide is a macrocyclic diterpenoid. The leaves of *Anisomeles indica Kuntzer*, a traditional Chinese medicine[10] used for treating hepatic diseases, are rich in ovatodiolide. Recently, ovatodiolide has attracted the interest of the pharmacological community. The interest stems not only from its antiproliferative and proapoptotic effects[11], but also from its compelling inhibitory activity towards various CSCs[11a-d]. As hepatocellular carcinoma (HCC) is one of the deadliest cancers[12], the molecular mechanisms underlying the pathogenesis of HCC are of vital interest to researchers. Although it remains largely undefined, accumulating evidence from recent studies suggests HCC may be attributed to a stem cell pathology[13], and hepatic CSCs may play the critical roles in HCC[14]. Therefore, the inhibitory effects against hepatic CSCs makes ovatodiolide not only an target for anti-CSCs medicinal chemistry, but also a new entity serving as a probe for investigation into the molecular mechanism specific to HCC.

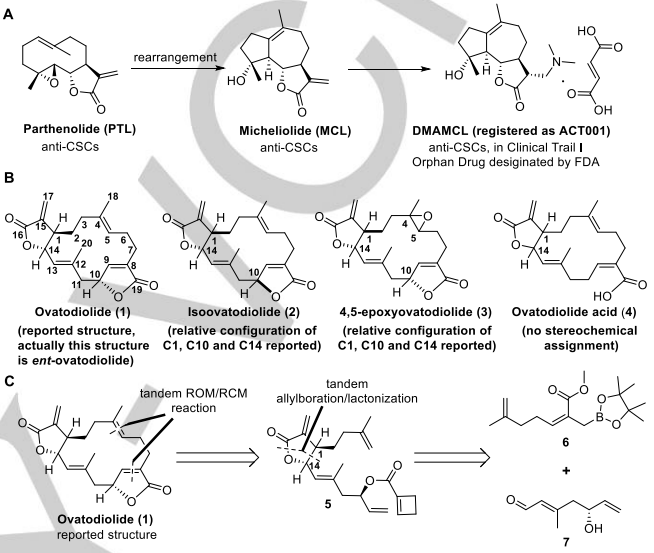

Figure 1. A) Anti-CSCs compounds PTL, MCL and clinical drug candidate ACT001. B) Natural products with ovatodiolide scaffold. C) Retrosynthetic analysis of ovatodiolide.

However, to the best of our knowledge, the total synthesis of ovatodiolide or other ovatodiolides (Figure 1B) has not yet been reported, which, along with those undetermined absolute stereochemistry, poses a barrier to extensive structure-activity relationship (SAR) studies and the development of superior ovatodiolide-based drugs. Considering this, we became interested in synthesis of ovatodiolides.

Structurally, ovatodiolide owns a unique 5/14/5 ring system featuring a butenolide moiety and a trans α-methylene-γ-lactone. The stereochemical assignment of ovatodiolide reported in recent literature[11b, 11d, 15] is C1-*R*, C10-*R*, C14-*S*. Therefore, we chose this enantiomer as our target molecule. Retrosynthetically (Figure 1C), an tandem ROM/RCM reaction developed by Grubbs[16] was anticipated to be the key step in our route. Next, the α-methylene-γ-lactone could be assembled from allylboronate **6** and aldehyde **7** through a tandem

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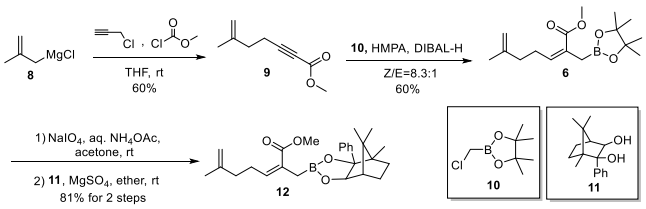
allylboration/lactonization reaction, which would enable the introduction of the C1 and C14 stereocenters and the exocyclic α-methylene unit simultaneously.



Scheme 1. Syntheses of **6** and **12**.

Our study commenced with the preparation of Z-allylboronate **6** (Scheme 1). We expected to produce **9** employing a multicomponent reaction. To our delight, the reaction proceeded smoothly to afford **9**. Compound **9** was then treated with DIBAL-H/HMPA followed by borylation with **10**[17], delivering allylboronate as a separable mixture of Z and E isomers in a ratio of 8.3:1.

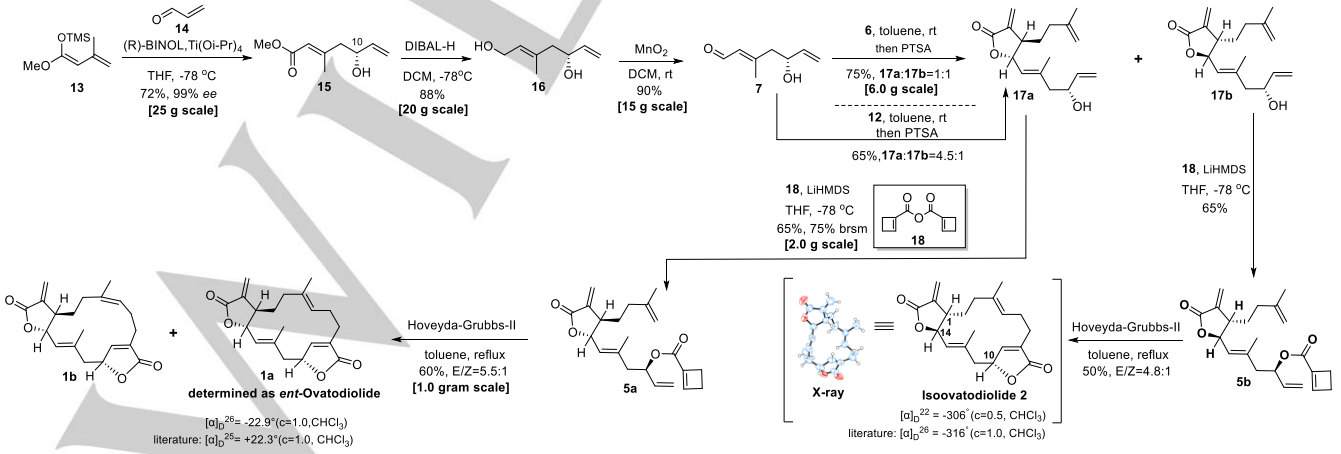
We then moved on to the synthesis of aldehyde **7** (Scheme 2). An asymmetric Mukaiyama-type aldol reaction between **13** and **14**[18] led to **15** with high stereoselectivity. The absolute configuration of C10 in **15** was determined as R employing Mosher ester method (see SI). Reduction of **15** with DIBAL-H provided diol **16**, which was subsequently subjected to selective allylic oxidation to produce aldehyde **7**.

With substrates **6** and **7** in hand, we focused on the tandem allylboration/lactonization reaction (Scheme 2, see SI for optimization details, Table S1). Gratifyingly, the allylboration proceeded smoothly in 7 days followed by rapid lactonization with a catalytic amount of PTSA[17, 19] to deliver a mixture of diastereoisomers (**17a** and **17b**, 1:1, 6.0-gram scale, 75% combined yield). The lack of diastereoselectivity in this transformation indicated that the chiral center C10 is too remote to impose any effect on the two transition states leading to **17a** and **17b**. Developed by Tang and coworkers[19], chiral phosphoric acid (CPA)-catalyzed asymmetric allylboration/lactonization may serve as a solution to bias the conformational preference. However, after evaluating a broad range of CPAs (Table S1, entries 5–13), it was found that even though the yield remained constant, only a slight increase in the d.r. was observed (Table S1, entries 6 and 9).

Thus, we turned our attention to an alternative pathway (Scheme 1). Replacing the pinacol group in substrate **6** with chiral auxiliary **11**[17, 20] might be a solution. In a two-step protocol, we were able to cleave the pinacol followed by condensation with **11**[17] to produce compound **12**. The reaction between **7** and **12** was initially conducted with PTSA in toluene and conveniently afforded **17a** and **17b** in a good combined yield (65%) with acceptable diastereoselectivity (d.r.=4.5:1, Scheme 2). To our delight, the desired isomer **17a** was obtained as the major product.

For structural diversification to facilitate future biological studies, **17a** and **17b** were advanced together. Compound **17a** was converted into **5a** under the reported esterification conditions[16c, 21] with cyclobut-1-enecarboxylic anhydride. Having rapidly obtained **5a**, there is only one chemical manipulation left. Various conditions were evaluated (see SI for optimization details, Table S2) for the key ROM/RCM reaction and finally intermediate **5a** at a concentration of 0.0015 M in toluene together with the loading of 0.1 equiv of Hoveyda-Grubbs-II were identified as the optimal condition. Employing this condition, compounds **1a** and **1b** were obtained in 60% combined yield in a ratio of 5.5:1, and notably, on a combined 1.0-gram scale.

After careful comparison, the NMR data of **1a** was consistent with the data reported for natural ovatodiolide[22]. However, the optical rotation of **1a** is opposite that of natural ovatodiolide, which led us to suspect that either the optical rotation data recorded or the reported stereochemical assignment of ovatodiolide was incorrect. To verify our hypothesis, we obtained natural ovatodiolide (**1**) by extracting the leaves of *Anisomeles indica* followed by chromatography (we also separated out ovatodiolide acid (**4**), but we did not identify isoovatodiolide (**2**) even in 100 grams of crude extract). The optical results suggest that synthetic compound **1a** is



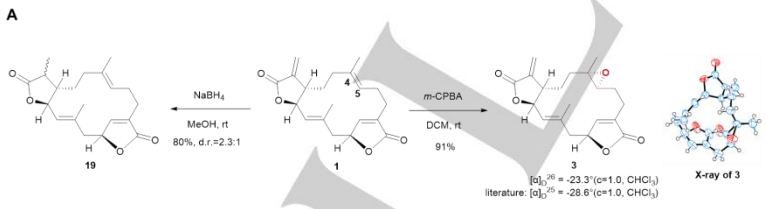
Scheme 2. Syntheses of **1a** and **2**.

actually ent-ovatodiolide, and the stereochemical assignment of ovatodiolide reported was misleading. Similarly, compound **17b** was carried through the same reaction sequence to afford compound **2**. X-ray analysis of **2**, combined with a comparison of optical data[22a], confirmed **2** as isoovatodiolide with the opposite configuration at C10 compared to ovatodiolide. Further transformation of the isolated ovatodiolide **1** with *m*-CPBA produced 4,5-epoxyovatodiolide (**3**), another natural product[22b], whose absolute configuration was confirmed via X-ray analysis (Figure 2A). Direct reduction of the α-methylene of **1** afforded a mixture of isomers (**19**) in a ratio of 2.3:1.

To preliminarily evaluate the anti-HCC effect and gain insight into the SAR, compounds **1**, **2**, **3**, **4**, **1a**, **1b** and **19** were subjected to cellular tests against 2 hepatic cancer cell lines (Figure 2B). Interestingly, *ent*-ovatodiolide (**1a**) and natural ovatodiolide (**1**) showed comparable activities. This discovery for the two enantiomers will need extra caution during further investigations into the target identification. Isoovatodiolide (**2**) was the most potent compound tested in this study. Epoxidation of the C4-C5 double bond led to an approximately 3-fold reduction in the inhibitory activity compared to that of ovatodiolide. Ovatodiolide acid (**4**) showed remarkably lower inhibitory activity, indicating that the butenolide ring is essential. Reduction of the α-methylene unit diminished the potency, which implied that the α-methylene is important. The modest inhibitory activity towards solid cancer cells *in vitro* is prevalent[23] in compounds with α-methylene-γ-lactone units, as exemplified by the active component of ACT001, MCL (Figure 1A) showed IC_{50} values of approximately 20 μM against various glioblastoma cells[6]. The reason for this may due to its molecular mechanism to form a covalent bond with multitarget proteins in diverse oncogenic signaling pathways, such as Wnt/β-catenin[11d], ROS[11f], NF-κB[11e]and JAK/STAT[11g] validated by recent research, rather than a single-target cytotoxic agent. The most attractive feature of ovatodiolide is the selective inhibitory activity towards CSCs. To further test the anti-hepatic CSCs effects, we first analyzed the inhibitory activity of **1**, **2**, **3**, **1a** and **1b** against the well recognized hepatic CSCs in HepG2 cells characterized by the $CD133^+$ biomarker. Based on the results at respective concentrations of 5 μM (Figure 2C), ovatodiolide (**1**) resulted in minimal changes to the expression of CSC marker CD133. To our delight, *ent*-ovatodiolide (**1a**) and 4,5-epoxyovatodiolide (**3**) were both superior to ovatodiolide in decreasing CD133 protein expression. Notably, isoovatodiolide exhibited significantly selective cytotoxicity, with the significantly downregulation of CD133. In contrast, the anti-CSC clinical drug candidate ACT001 was ineffective in this test.

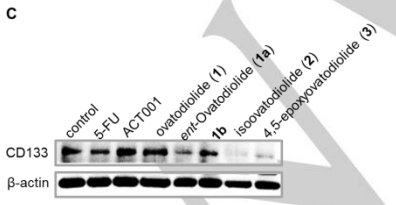
Because tumorspheres are 3D representations of CSCs *in vitro*, evidenced by their properties of high proliferative and tumorigenic potential. We generated hepatic tumorspheres and subjected them to the treatment with compounds **1**, **2**, **3**, **1a** and **1b** at their respective concentrations of 5 μM. The results indicated that treatment with ovatodiolide induced a dramatic 11.7-fold reduction in tumorsphere formation ability compared to the untreated control, which is consistent with the findings by Tsai[11d]. The performance of isoovatodiolide was even better than ovatodiolide in this test by decreasing 15-fold compared to the untreated control. ACT001 slightly reduced the tumorsphere formation ability. These results confirmed that both ovatodiolide and its analogues can ablate hepatic CSCs, providing us with a new chemical pool for identifying anti-CSCs compounds for further medicinal purpose.

In conclusion, we have developed a scalable and enantioselective synthetic route to ovatodiolide scaffold featuring two tandem reactions. The route is 6 steps (longest linear), and it can be carried out without protection manipulations. X-ray analysis combined with comparison of optical rotation data allowed us to determine the unknown absolute stereochemistry of these natural products. It is envisioned that the synthetic route we developed towards *ent*-ovatodiolide and scarce isoovatodiolide is applicable not only to the synthesis of ovatodiolide, but also to the analogues unavailable from natural resource (e.g. **1b**). The preliminary SAR suggests that butenolide ring and α-methylene unit are essential for



B

compound	SMMC-7721(IC_{50}, μM)	HepG2(IC_{50}, μM)
1a	6.54±0.504	12.2±2.62
1b	8.12±1.37	10.8±1.07
1	5.59±1.40	10.7±2.64
2	4.33±1.46	7.13±3.28
3	13.3±1.16	27.7±15.7
4	>50	35.8±0.42
19	>50	>50



D

compound	Number of tumorspheres	Decrease fold
control	125.3±2.31	-
ACT001	107.7±12.58	1.2
1	10.7±2.51	11.7
1a	18.0±5.39	6.9
1b	40.3±8.38	3.1
2	8.3±0.57	15.0
3	49.7±6.80	2.5

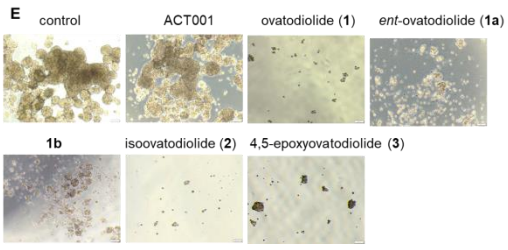


Figure 2. A) Syntheses of **3** and **19**. B) The IC_{50} values of tested compounds against SMMC-7721 and HepG2 cells. C) Western blot analysis of CD133 protein expression after treatment with 5-FU, ACT001, **1**, **1a**, **1b**, **2** and **3**. D) Quantitative analysis of tumorsphere formation of HepG2 cells. ACT001, **1**, **1a**, **1b**, **2** and **3** were used at the concentration of 5.0 μM respectively. E) Representative images of tumorspheres. The results shown represent the mean±SD values for three independent experiments.

maintaining inhibitory activity. More analogues to elucidate the SAR and to investigate the molecular mode of action are being synthesized and will be reported in due course. Importantly, we confirmed the anti-hepatic CSCs effects of ovatodiolide as well as other analogues. Isoovatodiolide proved to be more potent than ovatodiolide by significantly decreasing the population of hepatic CSCs and tumorspheres forming ability. Overall, the efficient synthetic sequence, along with its exciting anti-hepatic CSCs activity warrant the ovatodiolide scaffold as a promising start for the discovery of novel anti-CSCs drugs.

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Keywords: ovatodiolide • configuration determination • total synthesis • cancer stem cells • diterpenoid

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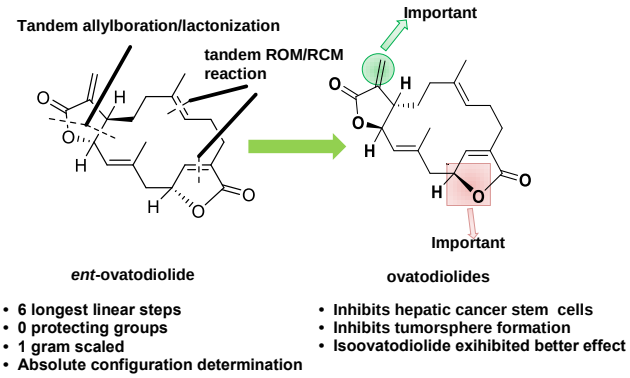
A concise, scalable and enantioselective synthetic route to ovatodiolides is reported for the first time. Ovatodiolides inhibit hepatic cancer stem cells and tumorsphere formation.



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