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Total synthesis of natural derivative and artificial analogs of 13-oxyingenol and their biological evaluation

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We have established an efficient synthetic methodology for 13-oxyingenol natural derivative (13-oxyingenol 13-dodecanoate 20-hexanoate), featuring a ring-closing olefin metathesis reaction for the "direct" construction of a highly strained *inside–outside* framework and a Mislow–Evans-type [2,3]-sigmatropic rearrangement for the stereoselective introduction of the hydroxy group at C5. We also synthesized artificial analogs of 13-oxyingenol and ingenol by using our synthetic strategy. *In vitro* activation assays of protein kinase C (PKC) α and δ revealed that the dodecanoyl group at O13 on 13-oxyingenol analogs had a significant role in PKCδ activation. The PKCα- or PKCδ-activating 13-oxyingenol and ingenol analogs induced both distinct morphological changes and increases CD11b expression in HL-60 cells, which would be typical signs of HL-60 cell differentiation to macrophage-like cells, as expected by previous reports. Intriguingly, however, similar differentiation phenotypes were observed with the use of 13-oxyingenol natural derivatives and 13-oxyingenol 13-dodecanoate showing a remarkably less potent PKCα or PKCδ activation ability, which PKC inhibitor Gö6983 diminished. This indicated the involvement of other PKC isozymes or related kinase activities. 13-Oxyingenol analogs, which induced HL-60 cell differentiation, also induced HL-60 cell death, similar to the action of a phorbol ester, a strong PKC activator.

Introduction

13-oxyingenol 13-dodecanoate 20-hexanoate (13-oxyingenol natural derivative; 13-oxyingenol derivative) (**1**) was isolated from *Euphorbia kansui* plants by Uemura et al. (Figure 1).[1] The structural features of **1** is a high degree of oxygenation and a highly strained unique bicyclo[4.4.1]undecane skeleton with *trans* intrabridgehead stereochemistry at the BC rings, which is called an *inside–outside* framework. Ingenol (**3**) has the same framework of **1**,[2] and ingenol 3-angelate (Picato®) (**4**) was approved by the FDA as a topical treatment for solar keratosis.[3] In 1996, Fujiwara et al. reported the structure–activity relationship study of **1**.[4] As a results, a 13-oxyingenol analog, RD4-2227 (**2**), showed a potent inhibitory effect at picomolar concentrations on HIV-1 (HTLV-III$_B$) replication in MT-4 cells (EC_{50} = 140 pM). This is stronger than the inhibitory effect of **3** (5,200,000 pM) or of anti-HIV drug zidovudine (Retrovir®) (4,100 pM) under the same assay conditions.

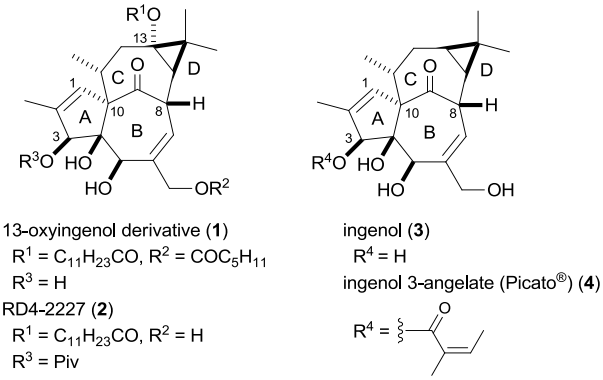

Fig. 1 Structures of 13-oxyingenols and ingenols.

Ingenol analogs are reported to be involved in the modulation of protein kinase C (PKC) enzyme activity.[5] PKCs are a family of serine/threonine kinases and are key mediators of many intracellular signal transduction pathways involved in cell growth, differentiation, carcinogenesis and cell death.[6] PKCs are categorized into three classes: conventional (cPKCs), novel (nPKCs), and atypical (aPKCs). The binding of ligands such as endogenous second messenger diacylglycerol (DAG) and tumor promoter phorbol esters (e.g., phorbol 12-myristate 13-acetate; PMA) to the C1 domain in cPKCs and nPKCs, but not in

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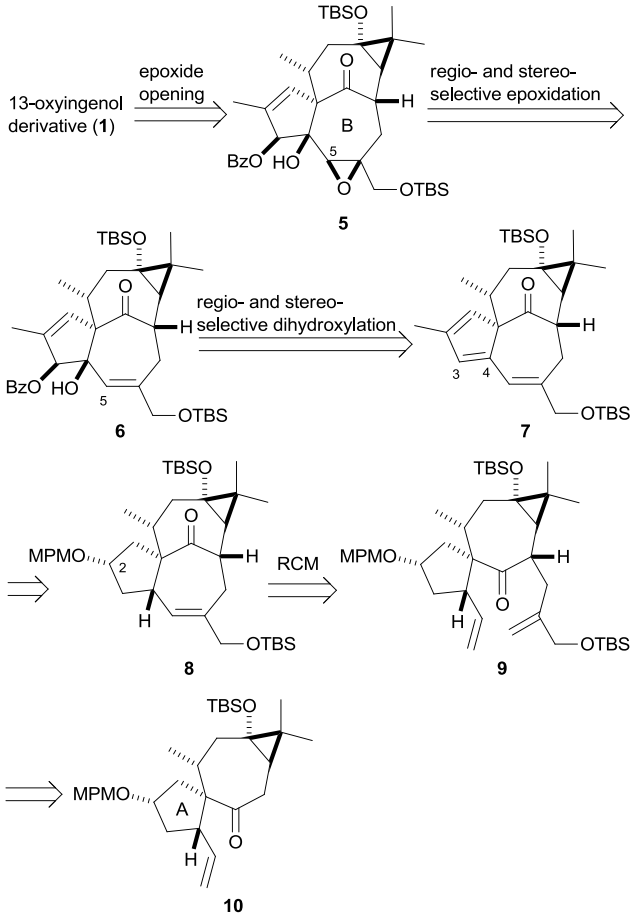
aPKCs, triggered the enhancement of enzymatic activity. The crystal structure of the PKCδ C1 domain with phorbol ester revealed crucial hydrogen bonding interactions between the hydrophilic cleft of the C1 domain and phorbol esters.[7] Some ingenol derivatives, especially those that have an acyl group on the hydroxyl group at C3, were reported to be C1 domain ligands and to activate PKC, which was explained by their structural similarities with phorbol esters. For 13-oxyingenol analogs, RD4-2227 (**2**) was identified as a potent nontoxic PKC activator (no toxicity was observed in mice up to 0.1 mg $kg^{-1}day^{-1}$ by intraperitoneal injection).[4a] Non-tumor-promoting PKC activators, such as bryostatin 1,[8] prostratin,[9] and aplysiatoxin analogs,[10] have been attracting attention as potential therapeutic agents for acquired immune deficiency syndrome (AIDS), tumors, and Alzheimer's disease. Thus, the activities of 13-oxyingenol analogs, which have an additional functionalization site at the C13 position in 13-oxyingenol as compared to ingenol analogs, have been of great interest. However, the structure–activity relationships of 13-oxyingenol derivatives are still largely unknown, which hampers further rational molecular design and obscures the merit of 13-oxyingenols for use as parent compounds. We anticipated that the development of an efficient synthetic methodology for **1** would enable us to provide a practical synthetic route for research supply of its analogs and to conduct further biological studies in order to uncover the characteristic features of **1**.

The unique stereostructures of the ingenol family have made them attractive targets for total synthesis.[11] Four groups have achieved the total synthesis of ingenol (**3**): Winkler,[12] Tanino–Kuwajima,[13] Wood,[14] and Baran[15]. Also, several groups have reported the synthesis of the ingenol skeleton.[16] Whereas many groups have constructed the highly strained *inside–outside* framework of **3** by using rearrangement or fragmentation reactions, we developed a "direct" construction method using the ring-closing olefin metathesis (RCM) reaction.[17] We then achieved the formal synthesis of optically active **3** using this methodology as a key step.[18] Wood et al. accomplished the total synthesis of ingenol by using similar approach in 2004.[14] We also have investigated the synthesis of 13-oxyingenol derivative (**1**).[19] In 2012, we preliminarily reported the total synthesis of **1**.[20] The highlights of this approach are the use of an RCM reaction for the direct construction of an *inside–outside* framework and a Mislow–Evans-type [2,3]-sigmatropic rearrangement[21] for the stereoselective introduction of a hydroxy group at C5. We describe herein the history of the total synthesis of 13-oxyingenol derivative (**1**). Furthermore, we discuss the synthesis and activities against PKC of the C3-acyloxy 13-oxyingenol and ingenol analogs.

Results and disscussion

Construction of the A-ring portion of 13-oxyingenol derivative (1)

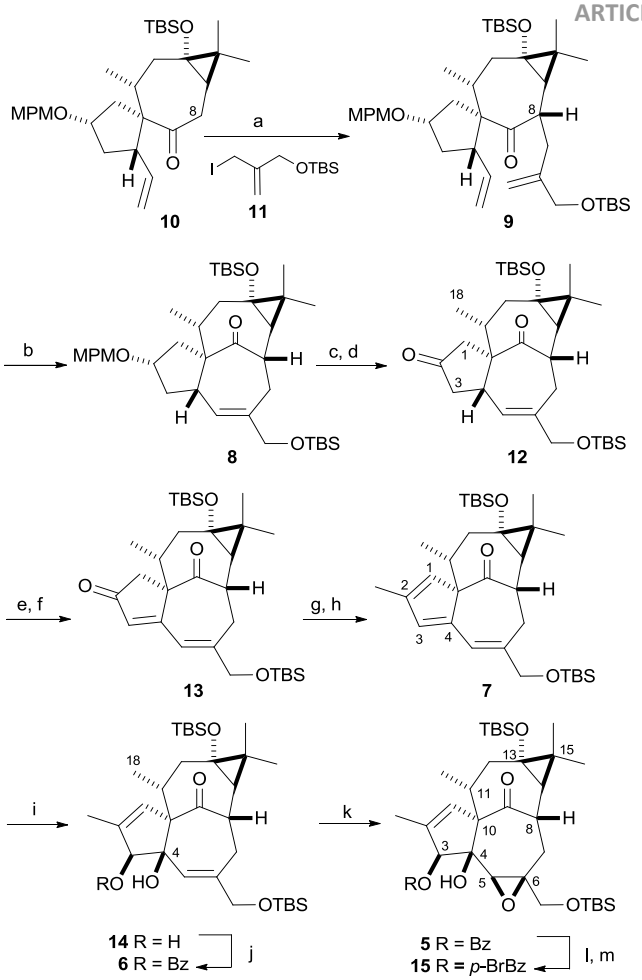
The retrosynthetic pathway of 13-oxyingenol derivative (**1**) is shown in Scheme 1. We planned to construct the B-ring portion by using ring-opening reaction of the epoxy group in **5**. To introduce the requisite functional groups at the A-ring portion, we decided to use the introduced hydroxy group at C2. The secondary hydroxy group at the C5 in the B-ring portion would be introduced by using regio- and stereoselective epoxidation of allylic alcohol **6** and the subsequent ring-opening reaction of the epoxy group in **5**. Allylic alcohol **6** was prepared by regio- and stereoselective dihydroxylation at the most strained $\Delta^{3,4}$ olefin in triene **7**, which might be obtained from tetracyclic ketone **8**. The *inside–outside* framework of **8** was prepared by using an RCM reaction of diene **9**. Diene **9** can be synthesized from spiro-ketone **10**.



Scheme 1 Retrosynthetic pathway of 13-oxyingenol derivative (**1**).

The synthesis of epoxide **5** started from the spiro-ketone **10**, which is our previous intermediate (Scheme 2).[19c] Thus, the introduction of an allyl group to the C8 position of spiro-ketone **10** with iodide **11** gave diene **9**. Diene **9** was treated with the second-generation Hoveyda–Grubbs (HG-II) catalyst (**37**)[22] to afford tetracyclic ketone **8**, which possessed the *inside–outside* framework. We attempted the stereoselective introduction of the functional groups at the A- and B-rings from **8**. The removal of the MPM group of **8** followed by oxidation of the resulting secondary hydroxy group with Dess–Martin periodinane[23] gave diketone **12**. We next tried to

synthesize enone **13** from **12**. Regioselective deprotonation at the H3 in **12** and silylation of the resultant enolate with TMSCl gave an enol silyl ether as the sole product, which was oxidized by using Ito–Saegusa oxidation[24] to afford **13** (95% yield in two steps). In this deprotonation of **12**, deprotonation at the H1 did not proceed, presumably due to the steric hindrance of the methyl group at the C18. Enone **13** was converted into an enol triflate, which was subjected to the Negishi coupling[25] reaction with Me_2Zn to afford triene **7**. Regio- and stereoselective dihydroxylation of **7** with a stoichiometric amount of OsO_4 gave diol **14**. The high regio- and stereoselectivity could be explained by the shielding of the $\Delta^{1,2}$ olefin by the steric hindrance of the C18 methyl group and by the enhancement of the reactivity by the pyramidal distortion of $\Delta^{3,4}$ olefin. We succeeded in synthesizing the A-ring portion of 13-oxyingenol derivative (**1**) by using the C2 hydroxy group. Next, we attempted to functionalize the B-ring portion of **1**. Protection of the secondary hydroxy group of **14** with a benzoyl group gave allylic alcohol **6**, which was converted into epoxide **5** as a single isomer. In this epoxidation, stereoselectivity was controlled by the tertiary hydroxy group at the C4 and the cage structure of **6**. The stereochemistry of **5** was established by X-ray crystallographic analysis of crystalline *p*-bromobenzoate **15** (Figure 2).



Scheme 2 Synthesis of epoxide **5**. *Reagents and conditions*: (a) **11**, LDA, THF, HMPA, –10 °C, 90%; (b) HG-II (**37**), toluene, reflux, 98%; (c) DDQ, ^{t}BuOH, phosphate buffer (pH = 6.6), CH_2Cl_2, rt, 61%; (d) Dess–Martin periodinane, CH_2Cl_2, rt, 87%; (e) TMSCl, LHMDS, Et_3N, THF, –78 °C; (f) $Pd(OAc)_2$, MeCN, rt, 95% in two steps; (g) Tf_2NPh, LHMDS, THF, 0 °C; (h) $Pd(PPh_3)_4$, Me_2Zn, THF, rt, quant in two steps; (i) OsO_4, py, THF, 0 °C, then $NaHSO_3$ aq, rt, 99%; (j) BzCl, Et_3N, DMAP, CH_2Cl_2, 0 °C, 94%; (k) ^{t}BuOOH, $VO(acac)_2$, benzene, rt, quant; (l) K_2CO_3, MeOH, rt; (m) *p*-BrBzCl, Et_3N, DMAP, CH_2Cl_2, 83% in two steps. TBS = *tert*-butyldimethylsilyl, LDA = lithium diisopropylamide, THF = tetrahydrofuran, HMPA = hexamethylphosphoramide, DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, TMS = trimethylsilyl, Ac = acetyl, Ph = phenyl, LHMDS = lithium bis(trimethylsilyl)amide, Tf = trifluoromethanesulfonyl, DMAP = *N*,*N*-dimethylaminopyridine, Bz = benzoyl, acac = acetylacetonyl.

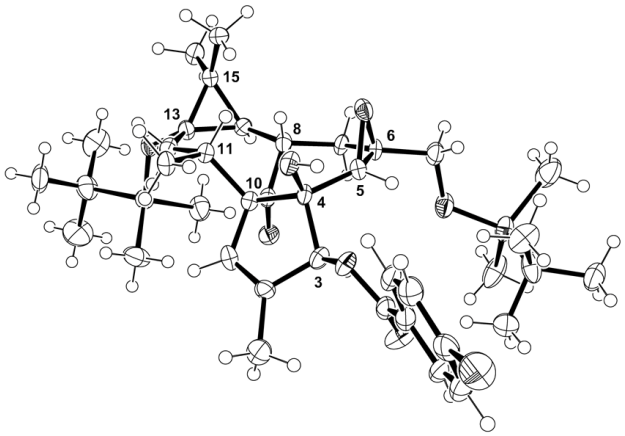
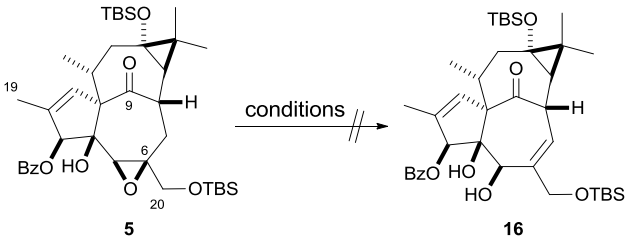


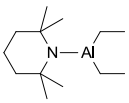
Fig. 2 X-ray crystallographic structure of **15** (CCDC 1508569)

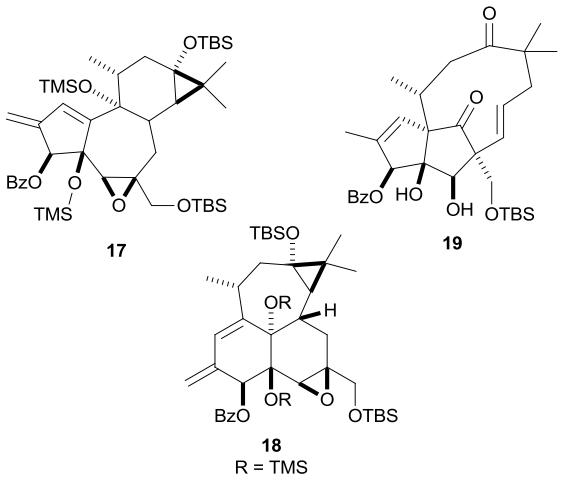
With a fully substituted tetracyclic compound **5** in hand, we next tried the ring-opening reaction of the epoxy group in **5** (Table 1). In entries 1 and 2, the ring-opening reaction of the epoxy group was investigated by using TfOH as a Brønsted acid or $Al(O^iPr)_3$ as a Lewis acid.[26] However, the desired compound **16** was not obtained. Next, the ring-opening reaction was examined by using TMSOTf and DBU (entry 3).[27] Again, however, the reaction did not give **16**, but rather undesired compounds **17** and **18**. The production of these undesired compounds can be explained as follows (Scheme 3). First, the carbonyl group at the C9 in **5** was activated by TMSOTf as **20**, and allylic cations **21** and **22** were generated by the migration of blue or green bonds, respectively. The methyl proton at the C19 in the resulting cations **21** and **22** were removed by DBU to give compounds **17** and **18**, respectively. Because the desired epoxide-opening reaction under acidic conditions did not afford **16**, we next investigated the reaction under basic conditions (entries 4 and 5). The ring-opening reaction of **5** with LDA was attempted, but **16** was not obtained (entry 4). This reaction afforded a complex mixture containing byproducts, such as those that seemed to form via the allylic anion at the C19 in **5**. In entry 5, the treatment of **5** by an aluminum amide[28] did not give the desired ring-opening compound **16**. We next attempted the ring-opening reaction under radical conditions (entry 6). Treatment of **5** with Cp_2TiCl[29] gave only the undesired compound **19** and not the desired **16**. A plausible reaction pathway for the formation of **19** is shown in Scheme 4. The generated tertiary radical at the C6 in **23** attacked to the carbonyl group to give oxy-radical **24**. The fragmentation of **24** and the subsequent ring-opening reaction of the cyclopropane ring formed compound **19**. The structure of **19** was determined by X-ray crystallographic analysis.[30] Despite these attempts, we could not establish reaction conditions for the ring-opening reaction of the epoxy group in **5**. Therefore, we reexamined the strategy of constructing the A- and B-ring portions of 13-oxyingenol derivative (**1**).

Table 1 Ring-opening reaction of epoxide **5**



5 **16**

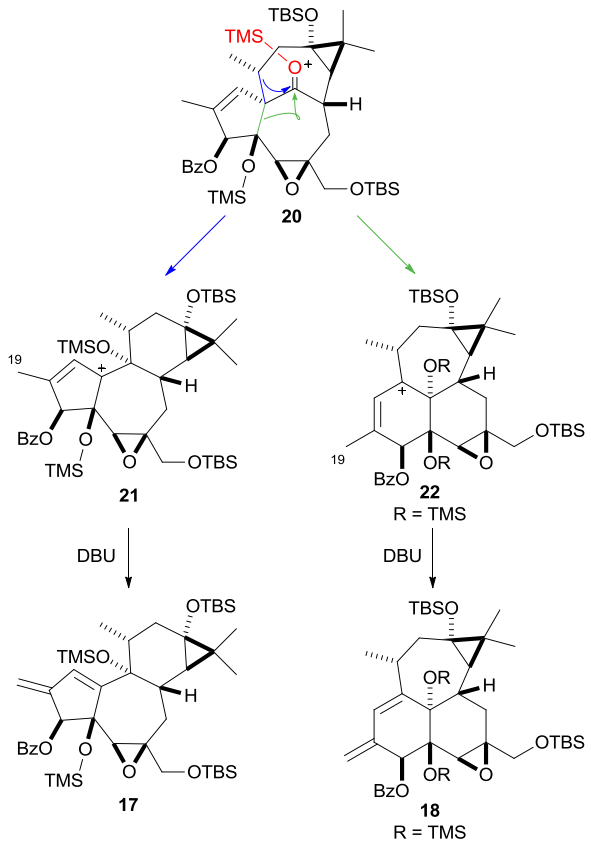
entry	conditions	result
1	TfOH, CH_2Cl_2, 0 °C	decomposition
2	$Al(O^iPr)_3$, toluene, reflux	decomposition
3	TMSOTf, 2,6-lutidine, DBU, rt	mixture of compounds **17** and **18**
4	LDA, THF, 0 °C	complex mixture
5	 , toluene, 0 °C	decomposition
6	Cp_2TiCl, THF, rt	compound **19**



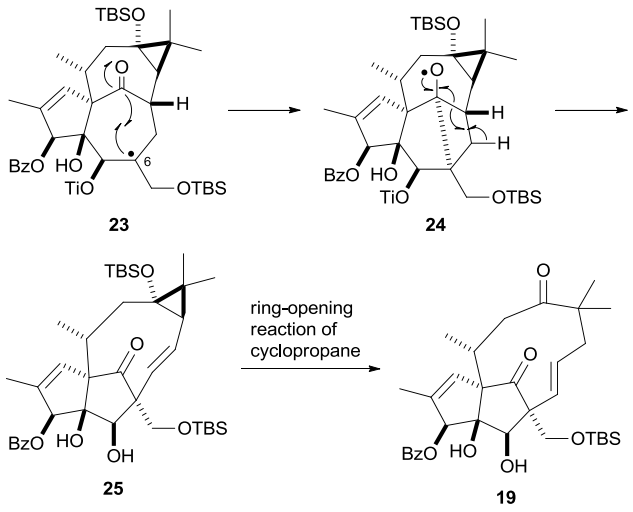
17 **19**

18
R = TMS

DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, Cp = cyclopentadienyl.



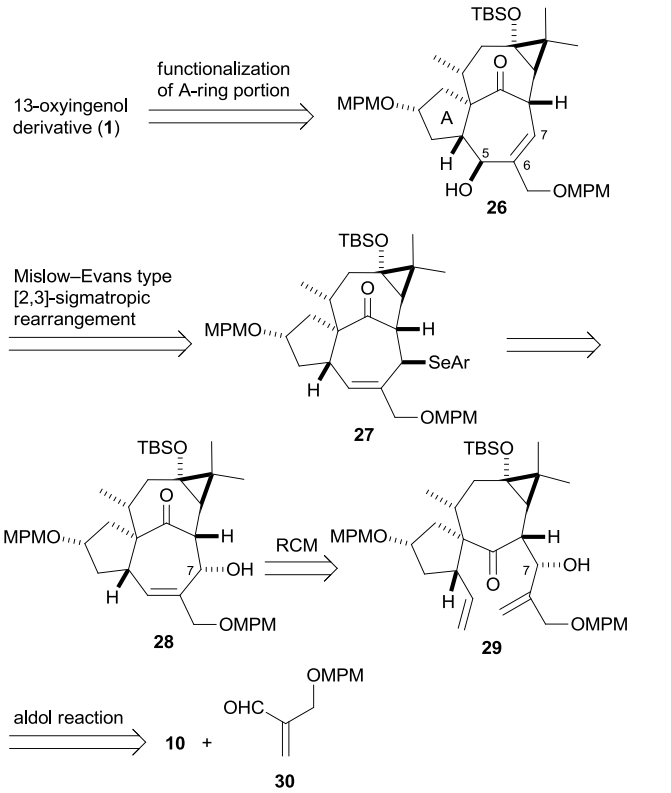
Scheme 3 Proposed reaction mechanism for the formation of compounds **17** and **18**.



Scheme 4 Proposed reaction mechanism of formation of compound **19**.

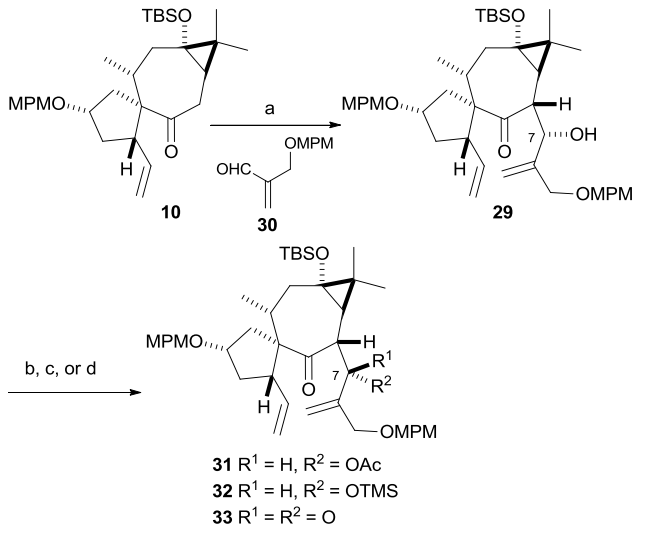
Construction of the B-ring portion of 13-oxyingenol derivative (1)

The modified retrosynthetic pathway of 13-oxyingenol derivative (**1**) is shown in Scheme 5. As mentioned above, we established the functionalization of the A-ring portion. In this section, we mainly describe the construction of the B-ring portion of **1**. We planned to introduce the $\Delta^{6,7}$ olefin and chiral secondary hydroxy group at the C5 in the B-ring portion by using Mislow–Evans-type [2,3]-sigmatropic rearrangement.[21,31] This strategy has the benefit of constructing the $\Delta^{6,7}$ olefin and the C5 stereocenter at once. This precursor **27** might be obtained from alcohol **28** by S_N2 reaction with a selenide anion at the C7. Alcohol **28** was synthesized by an aldol reaction between **10** and the unsaturated aldehyde **30** for the introduction of the hydroxy group at the C7 and the ring-closing olefin metathesis reaction of the resultant **29**.



Scheme 5 Modified retrosynthetic pathway of 13-oxyingenol derivative (**1**).

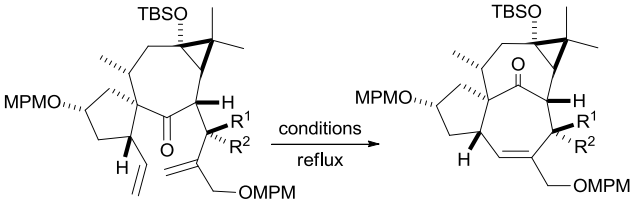
We prepared precursors for the RCM reaction, such as **29**, from spiro-ketone **10** (Scheme 6). The aldol reaction between **10** and unsaturated aldehyde **30**[32] gave allylic alcohol **29** as a single diastereomer. The stereochemistry of C7 in **29** was determined after the RCM reaction. Aldol **29** was converted into acetate **31**, TMS ether **32**, and unsaturated ketone **33** as the precursors for the RCM reaction.



Scheme 6 Syntheses of precursors for the RCM reaction. *Reagents and conditions*: (a) **30**, NaHMDS, THF, –78 °C; (b) Ac_2O, DMAP, py, 50 °C, 83% in two steps for **31**; (c) TMSCl, imidazole, DMF, rt, 47% in two steps for **32**; (d) Dess–Martin periodinane, CH_2Cl_2, rt, 81% in two steps for **33**. MPM = *p*-methoxybenzyl, NaHMDS = sodium bis(trimethylsilyl)amide, DMF = *N*,*N*-dimethylformamide.

Next, the RCM reactions of the precursors were optimized (Table 2). We examined the RCM reactions of precursors **29** and **31**–**33** with HG-II catalyst (**37**).[22] First, the RCM reaction of allylic alcohol **29** was attempted, but the yield was moderate (54%) (entry 1). Because this reaction gave spiro-ketone **10**, we supposed that a retro-aldol reaction occurred under this RCM condition. We next investigated the RCM reactions with acetate **31** and the TMS ether **32**. However, the yields were still moderate (entry 2: 64%, entry 3: 49%). These results indicated that the RCM reaction was most efficiently effected by using **31**. Therefore, we next screened solvents by using **31** as a precursor. In entry 4, we attempted the RCM reaction in boiling benzene, but the yield was low. Halogenated aromatic solvents, such as 1,2-dichlorobenzene (reaction at 120 °C) and trifluorobenzene (reaction at reflux) (entries 5 and 6) did not improve the yields. We also screened an assortment of Ru catalysts. The RCM reaction with highly active Ru catalyst **38**[33] afforded tetracyclic ketone **34** in 51% yield (entry 7), whereas the RCM reaction with the less-hindered Ru catalyst (Stewart–Grubbs catalyst) **39**[34] did not yield the desired compound **34** (entry 8). These results indicated that the use of acetate **31** cannot improve the yield of the RCM reaction. Next, we tried the RCM reaction with α,β-unsaturated ketone **33** as a precursor, because the steric hindrance around the C7 of the α,β-unsaturated ketone group at C7 is smaller in **33** than in acetate **31** and because the reactivity of the enone is suitable for the RCM reaction. The reaction of α,β-unsaturated ketone **33** with HG-II catalyst (**37**) in boiling toluene proceeded smoothly to afford the desired tetracyclic ketone **36** in 86% yield (entry 9). On the basis of these results, we established an optimized precursor and reaction conditions for the RCM reaction.

Table 2 Optimization of RCM reaction

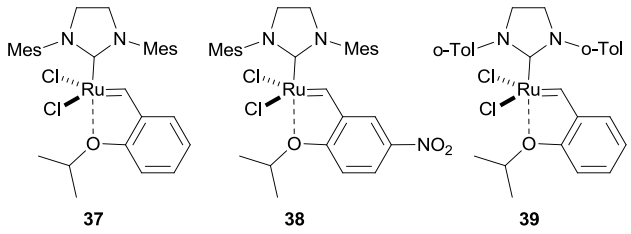


29 R^1 = H, R^2 = OH
31 R^1 = H, R^2 = OAc
32 R^1 = H, R^2 = OTMS
33 R^1 = R^2 = O

28 R^1 = H, R^2 = OH
34 R^1 = H, R^2 = OAc
35 R^1 = H, R^2 = OTMS
36 R^1 = R^2 = O

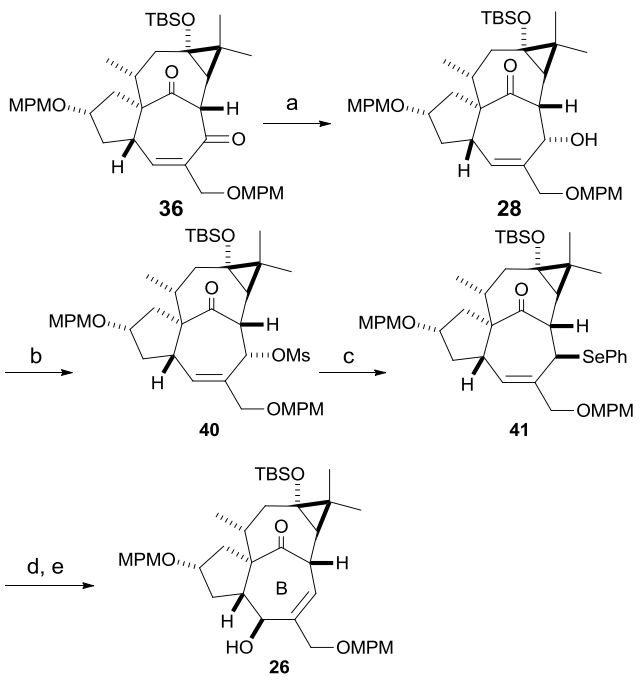
entry	precursor of RCM	catalyst	solvent	yield
1	**29**	**37**	toluene	54%
2	**31**	**37**	toluene	64%
3	**32**	**37**	toluene	49%
4	**31**	**37**	benzene	0%
5[a)]	**31**	**37**	1,2-dichlorobenzene	25%
6	**31**	**37**	trifluorobenzene	0%
7	**31**	**38**	toluene	51%
8	**31**	**39**	toluene	0%
9	**33**	**37**	toluene	86%

a) This reaction was carried out at 120 °C.



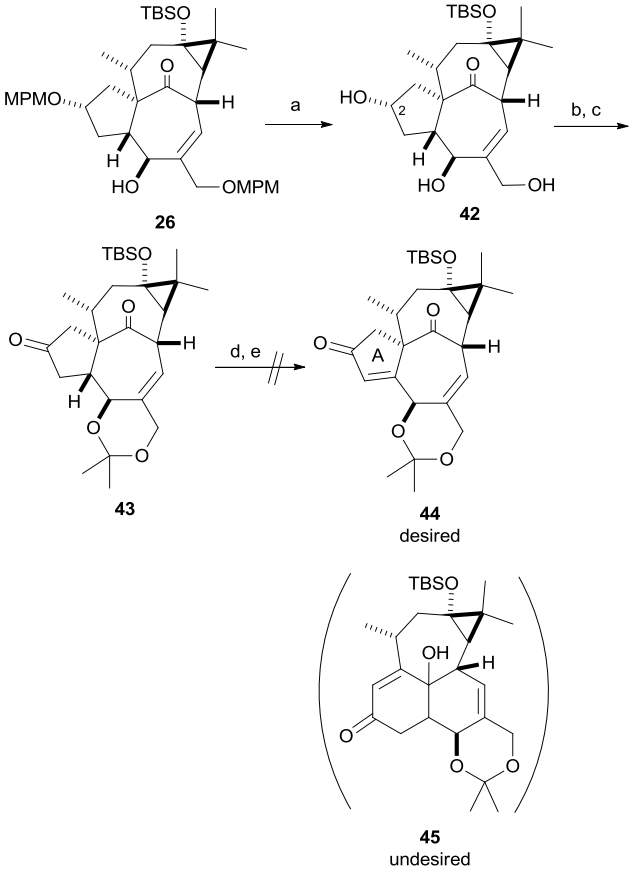
Mes = mesityl, Tol = *p*-tolyl.

With tetracyclic ketone **36**, which has an oxygen functional group at the C7, in hand, we examined the construction of the B-ring portion. Reduction of the carbonyl group at the C7 in **36** proceeded from the convex face in a highly stereoselective manner to give allylic alcohol **28** (Scheme 7). Mesylation of the resultant hydroxy group of **28** by the Tanabe method[35] afforded mesylate **40**, and the introduction of a phenylselenyl group by the selenide anion prepared from $(PhSe)_2$ and $NaBH_4$ gave selenide **41**. Oxidation of **41** by *m*-CPBA gave a selenoxide, which was treated with $P(OMe)_3$ to afford [2,3]-sigmatropic rearrangement product **26**. Thus, we established the construction of the B-ring portion by using [2,3]-sigmatropic rearrangement as a key step.



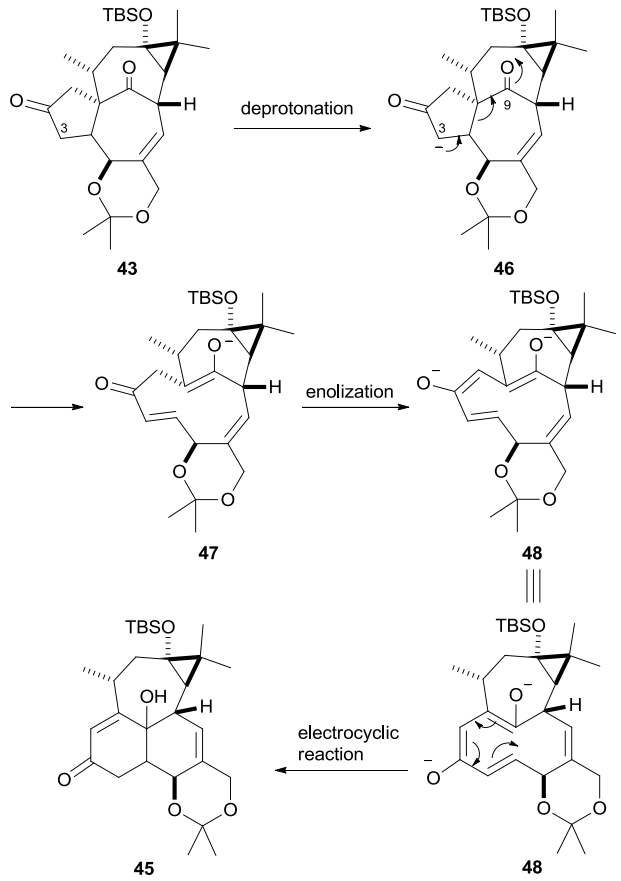
Scheme 7 Synthesis of allylic alcohol **26**. *Reagents and conditions*: (a) DIBAL, toluene, –78 °C, 96%; (b) MsCl, $Me_2N(CH_2)_3NMe_2$, toluene, 0 °C; (c) $(PhSe)_2$, $NaBH_4$, THF, EtOH, rt, 96% in two steps; (d) *m*-CPBA, THF, –78 °C; (e) $P(OMe)_3$, MeOH, 0 °C, 76% in two steps. Ms = methanesulfonyl, *m*-CPBA = *m*-chloroperbenzoic acid.

Next, we tried to construct the A-ring portion of 13-oxyingenol derivative (**1**) using a similar strategy as that shown in Scheme 2. Removal of two MPM groups in **26** afforded triol **42** (Scheme 8). The 1,3-diol group in **42** was protected as an acetonide, and the remaining secondary hydroxy group at the C2 was oxidized by Dess–Martin periodinane to give diketone **43**.[36] We next attempted the Ito–Saegusa oxidation of **43**. However, we did not obtain the desired enone **44**, but rather an undesired compound **45**.



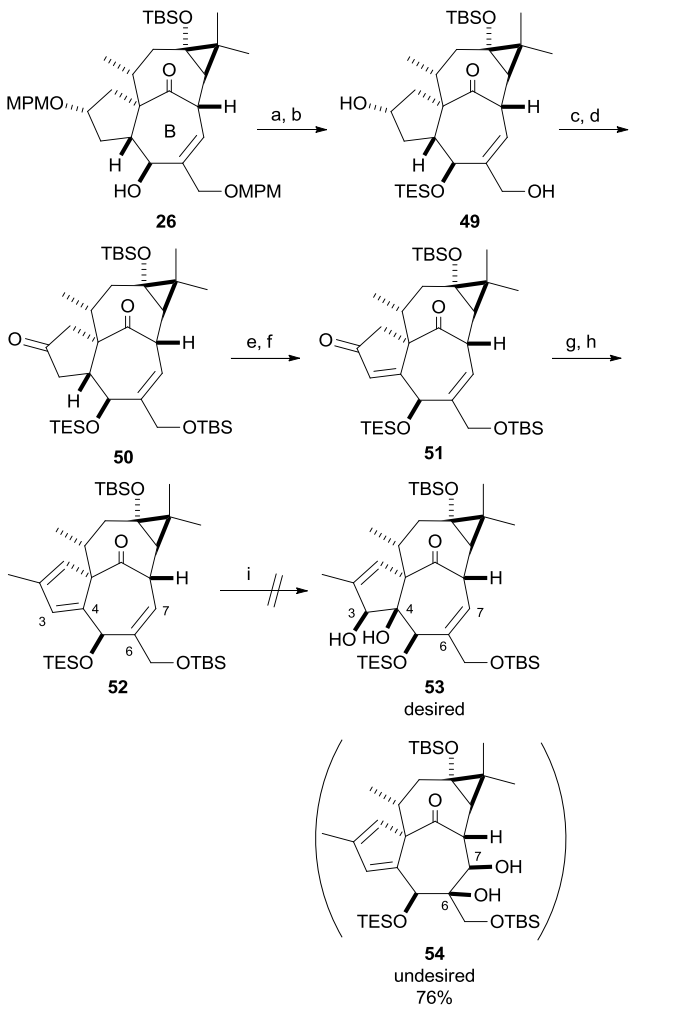
Scheme 8 Attempt to functionalize the A-ring portion. *Reagents and conditions*: (a) DDQ, ^tBuOH, phosphate buffer (pH = 6.6), CH_2Cl_2, rt, 52%; (b) 2-methoxypropene, PPTS, benzene, rt; (c) Dess–Martin periodinane, PPTS, CH_2Cl_2, rt, 90% in two steps; (d) TMSCl, LHMDS, Et_3N, THF, –78 °C; (e) $Pd(OAc)_2$, MeCN, rt. PPTS = pyridinium *p*-toluenesulfonate.

We can interpret the mechanism underlying the undesired reaction in the following way (Scheme 9): deprotonation of H3 in diketone **43** gave anion **46**, the fragmentation of which afforded enolate **47**. Enolate **47** was then transformed into dienolate **48**, which was converted into **45** by an electrocyclic reaction. We thought that this undesired reaction was due to the highly strained pentacyclic skeleton of **43**. Therefore, we tried to convert the acetonide group into another protecting group to decrease the strain.



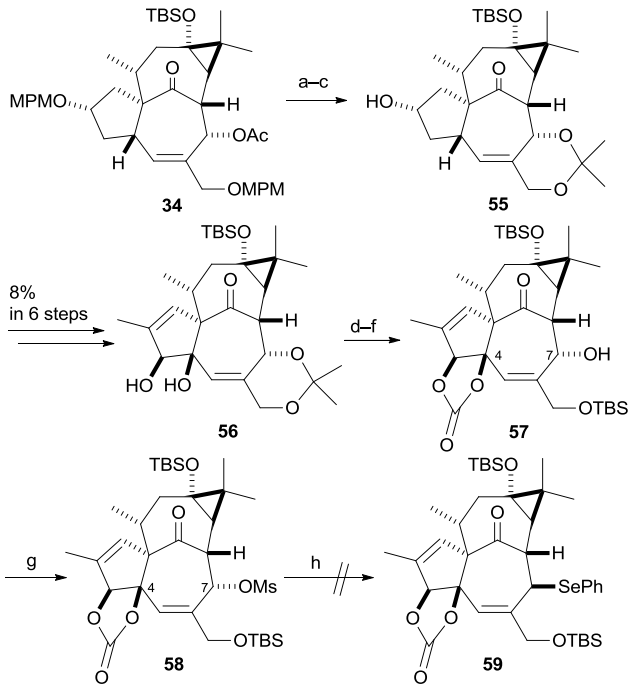
Scheme 9 Proposed reaction mechanism of formation of compound **45**.

Protection of the secondary hydroxy group in **26** with a TES group and removal of two MPM groups afforded diol **49** (Scheme 10). The primary alcohol in **49** was protected as a TBS group, and the remaining secondary hydroxy group at the C2 was oxidized by Dess–Martin periodinane to give diketone **50**. Diketone **50** was oxidized to enone **51** by using Ito–Saegusa oxidation. In this case, a rearrangement compound, such as compound **45**, was not afforded. Enone **51** was converted into an enol triflate, which was transformed into triene **52** by using Negishi coupling. Next, the regio- and stereoselective dihydroxylation of **52** with a stoichiometric amount of OsO_4 was carried out. However, the desired diol **53** was not obtained, but rather the undesired compound **54**, which was oxidized at the $\Delta^{6,7}$ olefin, was obtained. We thought that steric hindrance of the TES group in **52** interfered with the electrophilic attack of OsO_4 at the $\Delta^{3,4}$ olefin.



Scheme 10 Study of functionalization of the A-ring portion. *Reagents and conditions*: (a) TESOTf, 2,6-lutidine, CH_2Cl_2, 0 °C, 89%; (b) DDQ, ^tBuOH, phosphate buffer (pH = 6.6), CH_2Cl_2, rt, 84%; (c) TBSCl, Et_3N, DMAP, CH_2Cl_2, rt, quant; (d) Dess–Martin periodinane, CH_2Cl_2, rt, 91%; (e) TMSCl, LHMDS, Et_3N, THF, –78 °C; (f) $Pd(OAc)_2$, MeCN, rt, 50% in two steps; (g) Tf_2NPh, LHMDS, THF, 0 °C; (h) $Pd(PPh_3)_4$, Me_2Zn, THF, rt, 77% in two steps; (i) OsO_4, THF, py, 0 °C then $NaHSO_3$, rt. TES = triethylsilyl, py = pyridine.

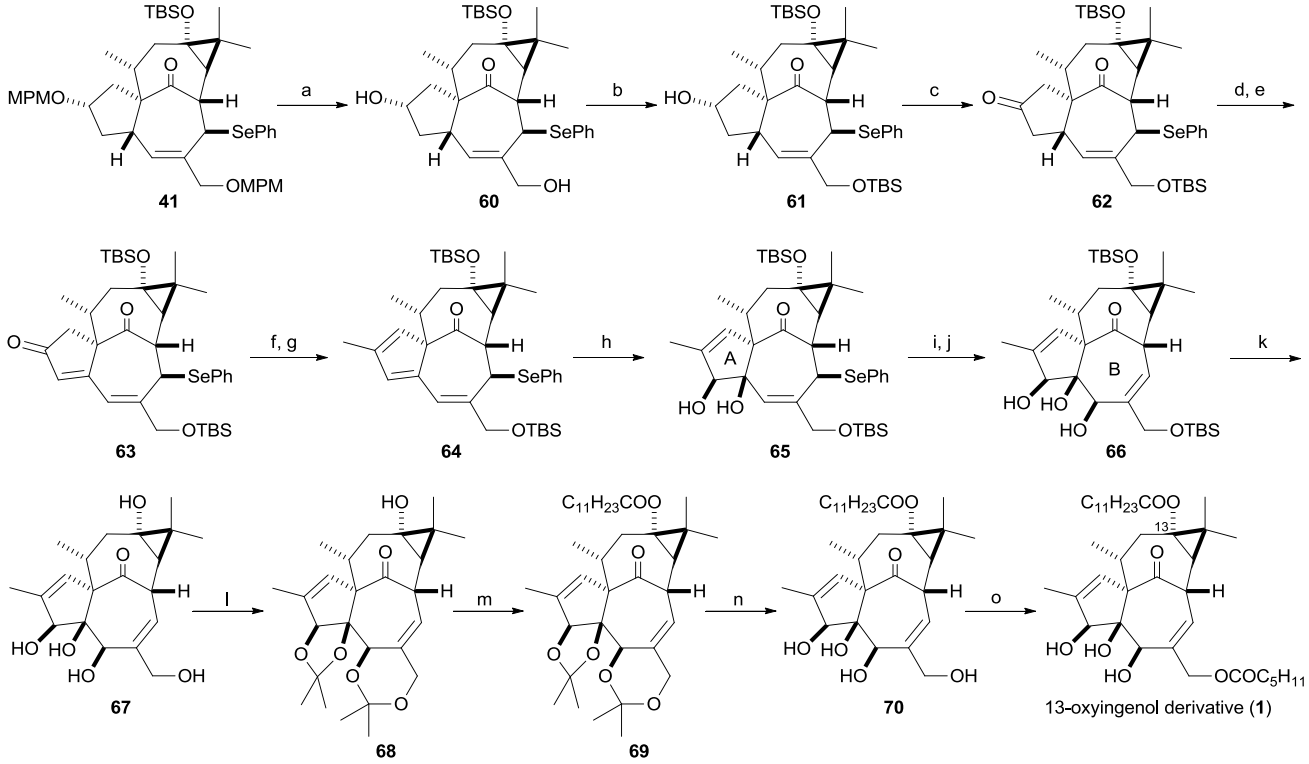
We found it difficult to introduce functional groups at the A-ring portion after the construction of the B-ring portion. Thus, we next tried to construct the A-ring portion before constructing the B-ring portion. Removal of two MPM groups in **34** and subsequent hydrolysis of the acetyl group gave a triol, which was converted into acetonide **55** (Scheme 11). Acetonide **55** was transformed into diol **56** by using our established synthetic strategy (Scheme 2). Protection of the diol group in **56** with a cyclic carbonate group and removal of the TBS group gave a diol, which was transformed into alcohol **57** by selective silylation of the primary hydroxy group. We next examined the introduction of a phenylselenyl group at the C7 in **57**. The mesylation of **57** afforded mesylate **58**, which was treated with $(PhSe)_2$ and $NaBH_4$. However, selenide **59** was not obtained. This suggested that steric hindrance of the cyclic carbonate group in **58** interfered with the nucleophilic attack by a selenide anion.



Scheme 11 Attempt to functionalize the B-ring portion. *Reagents and conditions*: (a) DDQ, ^{t}BuOH, phosphate buffer (pH = 6.6), CH_2Cl_2, rt; (b) 1 M LiOH aq, THF, MeOH, rt; (c) 2-methoxypropene, PPTS, benzene, rt, 87% in three steps; (d) carbonyl diimidazole, benzene, rt; (e) PPTS, EtOH, 50 °C; (f) TBSCl, Et_3N, DMAP, CH_2Cl_2, rt, 60% in three steps; (g) MsCl, $Me_2N(CH_2)_3NMe_2$, toluene, 0 °C; (h) $(PhSe)_2$, $NaBH_4$, THF, EtOH, rt to reflux.

Total synthesis of 13-oxyingenol derivative (1)

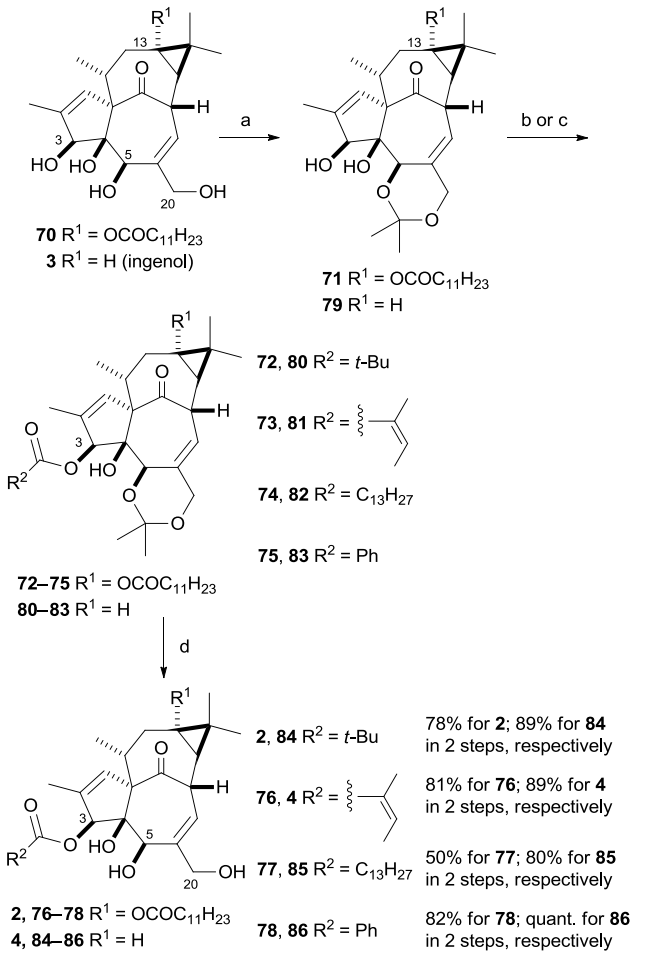
As described above, we constructed each of the A- and B-ring portions of 13-oxyingenol. However, we were unable to construct both of A- and B-rings in a molecule (Schemes 8 and 10). Therefore, we attempted to construct the A-ring portion at the next stage of the introduction of an aromatic selenyl group at the C7 (Scheme 12). Two MPM groups in selenide **41** were removed by using DDQ to afford diol **60**. Selective protection of the primary hydroxy group of **60** with a TBS group and the remaining secondary hydroxy group at the C2 was oxidized by Parikh–Doering oxidation[37] to give diketone **62**. Other oxidation conditions, such as Dess–Martin oxidation and Ley–Griffith oxidation,[38] gave Mislow–Evans-type rearrangement byproducts along with desired **62**. Diketone **62** was oxidized into enone **63** by using Ito–Saegusa oxidation. Enone **63** was treated with LHMDS and Tf_2NPh to form an enol triflate, which was subjected to Negishi coupling to afford triene **64**. Regio- and stereoselective dihydroxylation of the $\Delta^{3,4}$ olefin in triene **64** with a stoichiometric a mount of OsO_4 gave the desired diol **65**. During conversion of selenide **41** into **65**, the phenylselenyl group was not affected by oxidizing agents, such as DDQ, SO_3·pγ–DMSO, and OsO_4. We next examined the introduction of the C5 hydroxy group by using a Mislow–Evans-type rearrangement. The oxidation of the aromatic selenide group in **65** using *m*-CPBA and subsequent [2,3]-sigmatropic rearrangement with $P(OMe)_3$ afforded triol **66**. Thus, both the A- and B-ring portions of 13-oxyingenol derivative (**1**) were successfully constructed. The removal of two TBS groups in **66** afforded 13-oxyingenol (**67**), and four hydroxy groups at the A- and B-ring portions of **67** were protected as two acetonide groups to afford compound **68**. The introduction of the dodecanoyl ester group at the remaining tertiary hydroxy group at C13 in **68** gave **69**, which was hydrolyzed into 13-oxyingenol 13-dodecanoate (**70**). Finally, selective acylation of the primary hydroxy group in tetraol **70** formed 13-oxyingenol derivative (**1**). The spectral data (^{1}H NMR and ^{13}C NMR spectroscopy, HRMS, and optical rotation) of synthetic **1** were in full agreement with those of the natural product, thus completing the total synthesis (1.4% overall yield in 41 steps).



Scheme 12 Total synthesis of 13-oxyingenol derivative (**1**). *Reagents and conditions*: (a) DDQ, ^{t}BuOH, phosphate buffer (pH = 6.6), CH_2Cl_2, rt, quant; (b) TBSCl, Et_3N, DMAP, CH_2Cl_2, rt, 97%; (c) SO_3·py, DMSO, Et_3N, CH_2Cl_2, rt, quant; (d) TMSCl, LHMDS, Et_3N, THF, –78 °C; (e) $Pd(OAc)_2$, DMSO, rt, 64% in two steps; (f) Tf_2NPh, LHMDS, THF, –40 °C; (g) $Pd(PPh_3)_4$, Me_2Zn, THF, rt; (h) OsO_4, py, THF, then $NaHSO_3$, rt, 64% in three steps; (i) *m*-CPBA, THF, –78 °C; (j) $P(OMe)_3$, MeOH, 0 °C, 61% in two steps; (k) HF·py, THF, py, rt, quant; (l) 2,2-dimethoxypropane, PPTS, CH_2Cl_2, rt; (m) $C_{11}H_{23}CO_2H$, EDCI, DMAP, CH_2Cl_2, rt, 63% in two steps; (n) 1 M HCl, THF, rt, 99%; (o) $(C_5H_{11}CO)_2O$, Et_3N, CH_2Cl_2, –20 °C, 85%. DMSO = dimethylsulfoxide, EDCI = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

Synthesis of artificial analogs of the 13-oxyingenols and ingenols

To investigate the structure–activity relationship of 13-oxyingenol derivative (**1**), we prepared artificial analogs of 13-oxyingenol. Because RD4-2227 (**2**) and ingenol 3-angelate (**4**) show potent biological activity, we focused on the C3-acyloxy 13-oxyingenol analogs. We synthesized the C3-acyloxy analogs from our synthetic intermediate **70** (Scheme 13). Selective protection of the 1,3-diol group at C5 and C20 with an acetonide group gave mono acetonide **71.**[39] Selective acylation of the O3-hydroxy group and removal of the acetonide group afforded various C3-acyloxy 13-oxyingenol analogs, pivalate **2** (RD4-2227), angelate **76**, tetradecanoate **77**, and benzoate **78**. On the other hand, to examine the effect of the dodecanoyl ester group at the C13 in 13-oxyingenol analogs, C3-acyloxy ingenol analogs **4** and **84–86** were also prepared from ingenol (**3**)[40] by using the same strategy for the C3-acyloxy 13-oxyingenol analogs.



Scheme 13 Syntheses of the artificial analogs of 13-oxyingenol and ingenol. *Reagents and conditions*: (a) *p*-TsOH·H_2O, acetone, rt, 50% for **71**, 57% for **79**; (b) $(RCO)_2O$, Cs_2CO_3, MeCN, rt, for **72**–**74**, **80**–**82**; (c) BzCl, Et_3N, DMAP, CH_2Cl_2, rt, for **75** and **83**; (d) 0.5% HCl aq, MeOH, rt. Ts = *p*-toluenesulfonyl.

PKCα and PKCδ activation abilities of 13-oxyingenol natural derivative (1) and its artificial analogs

The enhancement of PKC activity by 13-oxyingenol and ingenol analogs was evaluated *in vitro* for PKCα (as a representative of cPKCs) and PKCδ (as a representative of nPKCs). First, the effects of the compounds (10 nM for PKCα and 100 nM for PKCδ) on the enzyme activities were examined. As shown in Table 3, analogs without the acyl group on the O3 position (R^2 = H) did not show significant activation of either PKCα or PKCδ, indicating that the 3-acyloxy group is essential for PKC activation as suggested previously.[4a,15c] Other derivatives showed the dose-dependent activation of PKCα and PKCδ (Figures S2 and S4). Regardless of the presence or absence of the 13-acyloxy group (R^1), a similar activation level of PKCα was observed for ingenol analogs (**4**, **84**–**86**) and 13-oxyingenol analogs (**2**, **76**–**78**). In contrast, it should be noted that PKCδ activity was clearly susceptible to the 13-acyloxy group. Though compounds **2**, **76**, and **78** exhibited remarkable activation of PKCδ, the corresponding ingenol analogs (**4**, **84**, and **86**) showed much lower activation. The bulkiness of the 3-acyl group (R^2) also seemed to affect PKCα activation ability, as we observed in our isobenzofuranone ligands before.[41] The branched pivaloyl derivatives (**2** and **84**) showed stronger activation than the straight chain myristoyl analogs (**77** and **85**). These results indicated that 13-oxyingenol analogs with a long acyl chain at the C13 position and a short bulky acyl group at the C3 position have the potential for broad activation ability toward both cPKCs and nPKCs. On the other hand, the activation abilities of compound **77** having two long straight acyl chains similar to DAGs were low, and indeed were lower than those of compound **85**, which lacks a long acyl chain at C13.

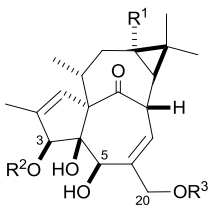
HL-60 cell differentiation and cell death

We further examined the biological effects of 13-oxyingenol analogs at the cellular level. It has been reported that ingenol 3-angelate (**4**) induced the differentiation and apoptosis of HL-60 cells, and that PKCδ would play a critical role in these effects.[42] Since CD11b was reported as an early marker of HL-60 cell differentiation, the expression levels of CD11b of HL60 cells treated with PMA and the ingenol and 13-oxyingenol analogs (1 μM, 24 hr) were measured by flow cytometry. The relative expression levels (%) compared to PMA are shown in Table 3. CD11b expression was greatly induced by all of the compounds except compounds **67** and **3** (see also Figure S6). Furthermore, morphological changes in HL-60 cells, cell aggregation, cell attachment and pseudopodia formation, induced by these ingenol and 13-oxyingenol analogs were confirmed by microscopic observations (Figure 3 and Figure S5).

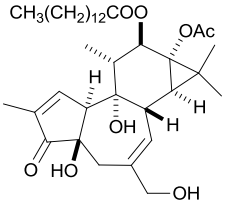
In addition, the induction of apoptosis by the treatment of these compounds (1 μM, 48 hr) was also quantified by measuring the sub-G1 population by flow cytometry. Similar to ingenol 3-angelate (**4**), these compounds induced apoptosis (Table 3 and S7).

Compounds **67** and **3**, each lacking the ability to active PKCα and δ, did not induce morphological changes, expression of CD11b, or the death of HL-60 cells. These results suggested that PKCδ activation might be involved in the mode of action of HL-60 differentiations and apoptosis induced by ingenol and 13-oxyingenol analogs, as reported previously.[42] However, 13-oxyingenol derivatives (**1**) and 13-oxyingenol 13-dodecanoate (**70**), each showing less potent PKCδ activation *in vitro* than PMA, significantly induced HL-60 cell differentiation and apoptosis (Table 3, Figures S3, S5, and S6), suggesting that these compounds induce activations of other PKC isozymes, although the possibility that migration of acyl groups in **1** and **70** altered these compounds to be stronger PKCδ activators could not be excluded.[39b] Therefore, the effects of broad-spectrum PKC inhibitor Gö6983 on CD11b expression were examined. Interestingly, Gö6983 suppressed the up-regulations of CD11b induced by **1** and **70** as well as other compounds (Figure S8). These results indicated that the activation of other PKC isozyme (or other kinases that Gö6983 inhibits) might contribute to HL-60 cells differentiation induced by these 13-oxyingenols.[15c]

Table 3 Biochemical and biological activities of 13-oxyingenol or ingenol analogs[a]



ingenol analogs



PMA

Compounds	R^1	R^2	R^3	PKCα [%]	PKCδ [%]	CD11b [%]	apoptosis [%]
13-oxyingenols							
1	$OCOC_{11}H_{23}$	H	COC_5H_{11}	19.9 (5.2)	14.2 (0.8)	152 (4.4)	40.8 (1.9)
67	OH	H	H	16.9 (4.9)	11.5 (1.1)	13.6 (3.6)	6.7 (0.88)
70	$OCOC_{11}H_{23}$	H	H	17.0 (1.6)	11.8 (2.0)	170 (1.1)	35.6 (1.3)
2	$OCOC_{11}H_{23}$	$COCMe_3$	H	96.6 (4.1)	124 (1.4)	131 (2.3)	40.6 (1.5)
76	$OCOC_{11}H_{23}$	COCMe=CHMe	H	98.3 (8.8)	118 (7.9)	138 (4.4)	40.2 (2.2)
77	$OCOC_{11}H_{23}$	$COC_{13}H_{27}$	H	69.7 (10.9)	82.2 (8.2)	162 (2.1)	37.4 (0.87)
78	$OCOC_{11}H_{23}$	COPh	H	88.3 (4.2)	109 (10.1)	130 (4.1)	41.3 (1.2)
ingenols							
3	H	H	H	12.1 (3.1)	8.0 (2.3)	14.6 (3.0)	9.2 (0.80)
84	H	COCMe3	H	119 (3.7)	59.6 (5.8)	128 (2.7)	42.5 (0.85)
4	H	COCMe=CHMe	H	98.0 (4.5)	37.6 (3.3)	141 (4.3)	41.2 (1.2)
85	H	$COC_{13}H_{27}$	H	88.3 (6.7)	103 (3.8)	130 (2.1)	43.4 (0.58)
86	H	COPh	H	85.7 (12.0)	42.0 (7.0)	126 (2.0)	36.5 (1.2)
PMA				100	100	100	43.7 (2.0)

[a] PKCα and PKCδ; The increased activity by the treatment of the compounds (10 nM for PKCα; 100 nM for PKCδ) are presented as relative acitivity (% of control PMA); CD11b; Expression levels of CD11b induced by the compounds (1 μM, 24 h) in HL-60 cells are presented as the relative activity (% of control PMA); apoptosis; Apoptosis-inducing activity of the compounds (1 μM) for HL-60 cells was shown as population of sub G1 cells.

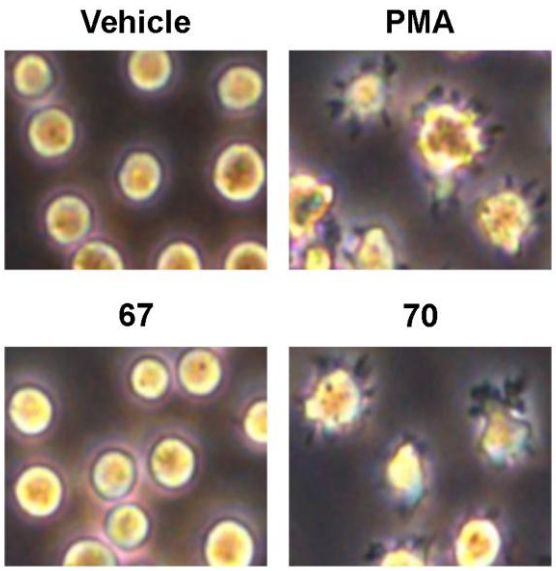


Fig. 3 Microscopic observations of HL-60 cells treated with 13-oxyingenol analogs. HL-60 cells were treated with PMA (1 μM) or 13-oxyingenol analogs (**67** or **70**, 1 μM) for 24 h.

Conclusions

We have achieved the total synthesis of 13-oxyingenol derivative (**1**), featuring the RCM reaction for the construction of an *inside–outside* framework and Mislow–Evans-type [2,3]-sigmatropic rearrangement for the stereoselective introduction of a hydroxy group at C5. The efficient functionalization of the A- and B-ring portions was established by using C2 and C7 hydroxy groups. Furthermore, we have investigated the structure–activity relationships (SAR) of 13-oxyingenol derivative (**1**) and ingenol (**3**). SAR studies of synthetic 13-oxyingenol analogs for PKCα and PKCδ activation reinforced the importance of esterification at the C3 hydroxyl group for PKC activation, and **2** and **76** proved to be the most powerful PKCδ activators. We also found that there are 13-oxyingenol analogs having a long hydrophobic alkyl chain that induced HL-60 cell differentiation and cell death, though they were poorer PKCα and PKCδ activators than others or PMA. This showed the potential of 13-oxyingenol as a new scaffold for the development of therapeutic agents against acute myeloid leukemia. Various artificial analogs of 13-oxyingenol prepared by our synthetic strategy of **1** enabled us to obtain these new findings.

Acknowledgements

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