AcOEt and evaporated under reduced pressure. The residue was dissolved in 80% formic acid (1.2 mL). After being kept at room temperature for 5 h, the mixture was evaporated under reduced pressure. The residue was coevaporated three times with distilled water to remove the last traces of formic acid. The residue was chromatographed on a column of C-18 with solvent system III by using a medium pressure reverse-phase chromatography. The fractions containing **3** were collected and evaporated under reduced pressure. Rechromatography on a C-18 column with water–acetonitrile (90:10) followed by lyophilization from its aqueous solution to **3** (21.7 mg, 32%): ^{1}H NMR (270 MHz, D_2O): δ 1.10–1.53 (9H, m, CH_2 of biotin, CH_3 of POEt, $J_{POCH_2CH_3} = 6.9$ Hz), 1.76–192 (3H, m, 3″Ha, 4″H), 2.01–2.05 (2H, m, $COCH_2$), 2.20 (1H, m, 3″H), 2.53–2.79 (2H, m, SCH_2), 2.99–3.06 (1H, m, SCH), 3.20–3.35 (6H, m, 5″H, NCH_2), 3.45–3.56 (8H, m, OCH_2-CH_2O, OCH_2), 3.73–3.83 (2H, m, NCH of biotin), 3.97–4.20 (5H, m, 5′H, 2″H, CH_2 of POEt), 4.37–4.42 (1H, m, 4′H), 4.47–4.50 (1H, m, 3′H), 4.96–5.02 (1H, m, 2′H, $J_{2',3'} = 5.6$ Hz), 5.77 (1H, 2d, 1′H, $J_{1',2'} = 2.0$ Hz), 8.17 (1H, s, 2H); ^{13}C NMR (D_2O): δ 17.96, 18.06, 26.43, 26.44, 27.78, 30.26, 30.52, 32.38, 38.02, 41.51, 42.07, 42.08, 42.29, 48.86, 57.86, 62.75, 64.55, 64.65, 64.74, 65.01, 65.56, 65.58, 65.64, 65.67, 67.81, 67.85, 67.88, 67.90, 67.92, 67.94, 71.40, 71.79, 72.08, 72.16, 72.29, 72.39, 73.26, 73.29, 84.42, 84.44, 84.47, 84.50, 84.55, 84.56, 84.60, 88.83, 88.86, 110.09, 110.11, 110.13, 142.81, 151.44, 152.33, 154.93, 154.99, 157.77, 167.54, 178.42, 178.48, 178.98. ^{31}P NMR (D_2O): 10.26, 10.28; ESI-mass *m/z* calcd for $C_{34}H_{55}N_{11}O_{13}PS$ 888.3439; observed [M+H] 888.3446.

4.1.11. Assay of in vitro antitumor activity. The tetrazolium-based semi-automated colorimetric assay (MTT assay) developed by Carmichael et al.[16] was modified and used to determine the in vitro antitumor activity of phosmidosine analogs. The activity was determined by using mouse leukemia L1210 cells and human epidermoid carcinoma KB cells. Roswell Park Memorial Institute Medium 1610 supplemented with 10% heat-inactivated fetal bovine serum and 50 μ/mL of kanamycine was used as the cell culture medium. Tumor cells (2×10^3 cells/well) plated into flat-bottomed 96-well plates (NUNC, Roskilde, Denmark) were incubated in a CO_2 gas incubator at 37 °C for 72 h in 200 μL of medium containing various concentrations of the test compounds. Cell growth was measured by using MTT reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St. Louise, Missouri, USA). After the addition of 25 μL of MTT solution (2 mg/mL), each well was incubated at 37 °C for an additional 4 h. Then the medium was removed and 200 μL of dimethyl sulfoxide (DMSO) were added. After mixing with a mechanical plate mixer for 5 min, absorbance at 540 nm was measured with Immuno Reader NJ-2000 (Nippon InterMed, Tokyo, Japan). The percentage of cell growth inhibition was calculated by the following formula: % inhibition = [1 − OD of sample wells/OD of control wells] × 100. The IC_{50} (μM) was given as the concentration at 50% inhibition of cell growth. Its value was determined graphically from the dose-response curve with at least three drug concentration points.

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Structure–activity relationships of epolactaene derivatives: structural requirements for inhibition of Hsp60 chaperone activity

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Abstract—Epolactaene is a microbial metabolite isolated from the fungal strain *Penicillium* sp. It arrests the cell cycle at the G_0/G_1 phase and induces the outgrowth of neurites in human neuroblastoma SH-SY5Y cells. In this communication, we report the structure–activity relationships (SARs) of new epolactaene derivatives, including those lacking the epoxylactam moiety and having various side chains. These derivatives were evaluated for their ability to inhibit the growth of human cancer cell lines. They were also analyzed for their ability to affect human heat shock protein 60 (Hsp60), which we have already identified as a protein that binds to epolactaene. We also identified the important structural framework of epolactaene/ETB (epolactaene tertiary butyl ester) for not only binding to Hsp60 but also inhibiting Hsp60 chaperone activity.

1. Introduction

Epolactaene is a microbial metabolite isolated by Kakeya et al. from the fungal strain *Penicillium* sp. BM 1689-P.[1] It was originally isolated for its effectiveness in promoting neurite outgrowth and arresting the cell cycle at the G_0/G_1 phase in a human neuroblastoma cell line.[2] Epolactaene contains characteristic structures that invoke certain biological activities, such as a highly oxidized γ-lactam and a conjugated (*E*,*E*,*E*)-triene in the side chain. It also has electrophilic characteristics in its α,β-unsaturated ketone, epoxide, and hemiaminal carbon, which are potentially reactive with biological nucleophiles, such as the sulfhydryls of cysteines. Because of epolactaene's interesting biological properties and its highly unusual structure, it has been an attractive target for organic chemists, and several groups, including our own, have undertaken its total synthesis.[3–8] In addition to the synthesis research, several reports have screened epolactaene analogues to investigate its biological activity and mode of action.[9,10] We also investigated the interaction between epolactaene and a proteasome complex, because lactacystin, a potent proteasome inhibitor, promotes neurite outgrowth and inhibits cell cycle progression in both the G_0/G_1 and G_2/M phases in mouse neuroblastoma Neuro 2A cells.[11,12] However, epolactaene did not inhibit the proteasome peptidase activities in vitro at a dose sufficient to inhibit growth in SH-SY5Y cells.[13]

In our recent report, we revealed that epolactaene binds to human Hsp60 and inhibits Hsp60 chaperone activity.[13] The Hsp60 family is known as a molecular chaperone assisting in protein folding.[14] In addition, mammalian Hsp60 has been reported to be involved in several biological processes, such as apoptosis,[15–18] immunoregulatory function,[19,20] and cell spreading.[21] Despite its importance, mammalian Hsp60 has been little studied compared with members of the prokaryotic Hsp60 family, such as GroEL.[14] In this report, we study the structure–activity relationships (SARs) of epolactaene derivatives to clarify the structural requirements for exhibiting biological activities. We prepared new epolactaene derivatives with various epoxylactam rings and side-chain moieties. We investigated these

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analogues for their ability to inhibit cell growth. Furthermore, we disclosed the key structural that enables epolactaene to inhibit human Hsp60 chaperone activity.

2. Chemistry

Epolactaene (Fig. 1, Epo) is a fungal metabolite that contains an epoxide inside a γ-lactam ring and an alkyl side chain bearing a triene moiety. Because each moiety could possibly induce biological activities, we are interested in clarifying the roles of these moieties in epolactaene's biological activity. For this purpose, we synthesized nine epolactaene derivatives with different epoxy lactam moieties and side-chain structures (Fig. 1).

Epolactaene tertiary butyl ester (ETB) was prepared so that we could observe the importance of the ester moiety. ETB was synthesized by the same procedures as those of Epo.[7] Bio-ETB as a probe was synthesized from ETB by the following reactions: (1) treatment of ETB with CF_3CO_2H afforded carboxylic acid, (2) coupling of the carboxylic acid with (11-hydroxyundecyl)carbamic acid *tert*-butyl ester by the use of EDC·HCl and DMAP gave amide, (3) treatment of the amide with CF_3CO_2H afforded amine, (4) coupling of the amine with (+)-biotin by the use of EDC·HCl and DMAP gave bio-ETB (74% yield in four steps). As to the importance of the lactam ring moiety, the complete loss or alteration of the lactam moiety gave rise to Epo-E, Epo-F, and Epo-G. They were prepared from tetrahydropyran-2-ol by highly stereoselective reactions, the details of which have already been reported.[7] For the side-chain derivation, compounds Epo-J, Epo-K, Epo-L, and Epo-M were synthesized. Epo-J, Epo-K, and Epo-L were prepared from the corresponding aldehydes by the diastereoselective reactions shown in Scheme 1. The aldehydes were treated with a newly developed Horner–Emmons reagent in the presence of *t*-BuOK in THF–HMPA to afford β-ketonitriles **2**. The Knoevenagel condensation between β-ketonitriles **2** and (*S*)-2-triethylsiloxypropanal prepared from (*S*)-methyl lactate proceeded in the presence of a catalytic amount of ethylenediammonium diacetate, affording the adducts **3** as single *E*-isomers, which were treated with TrOOLi at −78 °C to afford epoxides **4** with high diastereoselectivity. Both the bulky nucleophile (TrOOLi) and the TES protecting group are essential for the high selectivity. The deprotection of the TES group with AcOH and NH_4F in aq THF, hydrolysis of the nitrile by silica gel on TLC, and ammonolysis of the lactones formed **6** by NH_4OH in MeOH afforded the hydroxyamide **7**. The mild hydrolysis of the nitrile by silica gel should be ascribed to the intramolecular assistance of the hydroxy group. The final oxidation was achieved using SO_3·pyridine,[22] DMSO, and NEt_3 in CH_2Cl_2, affording Epo-J, Epo-K, and Epo-L. Finally, Epo-M was synthesized from Epo-L by hydrogenolysis catalyzed by Pd/C under a hydrogen atmosphere in an 84% yield.

3. Biological results

3.1. Effects of epolactaene derivatives on cell viability

We analyzed the SAR of the epolactaene derivatives for their inhibition of the growth of human neuroblastoma SH-SY5Y and human T-lymphoma Jurkat cells. The cell viability was assessed by MTT assay.[13] The 50% growth-inhibitory concentrations (IC_{50}s) are listed in Table 1. ETB was found to be as effective as epolactaene, whereas compound Epo-E, lacking the γ-lactam, resulted in the great loss of activity. The change to a lactone or reductive opening of the lactam ring also reduced activity greatly (compounds Epo-F and Epo-G). Furthermore, compounds Epo-J and Epo-L, each with a long hydrophobic alkyl chain lacking triene and ester moieties, were as active as epolactaene, aside from the less active Epo-K. Epo-M, with an α,β-saturated ketone in the side chain, retained a potent growth-inhibitory effect. From these results, we designed a biotin-conjugated epolactaene that retained its biological activity. Because the methyl ester moiety could be substituted by a bulky group such as tertiary butyl ester, we conjugated a biotin linker at the ester

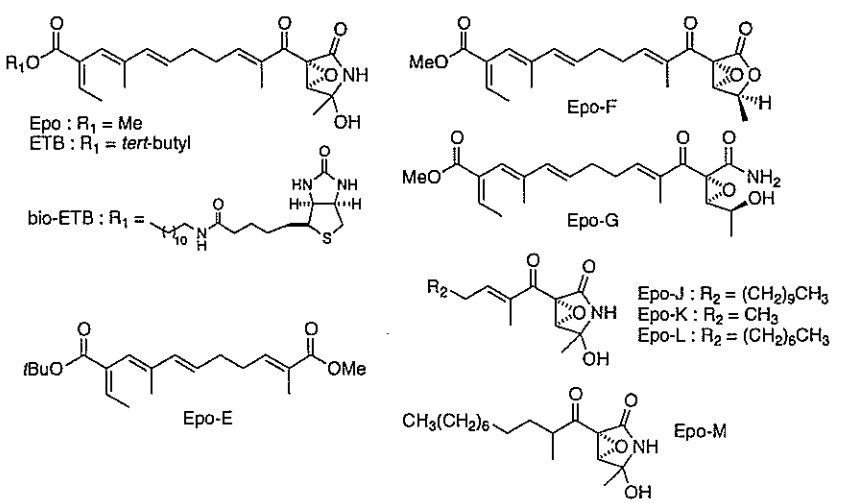
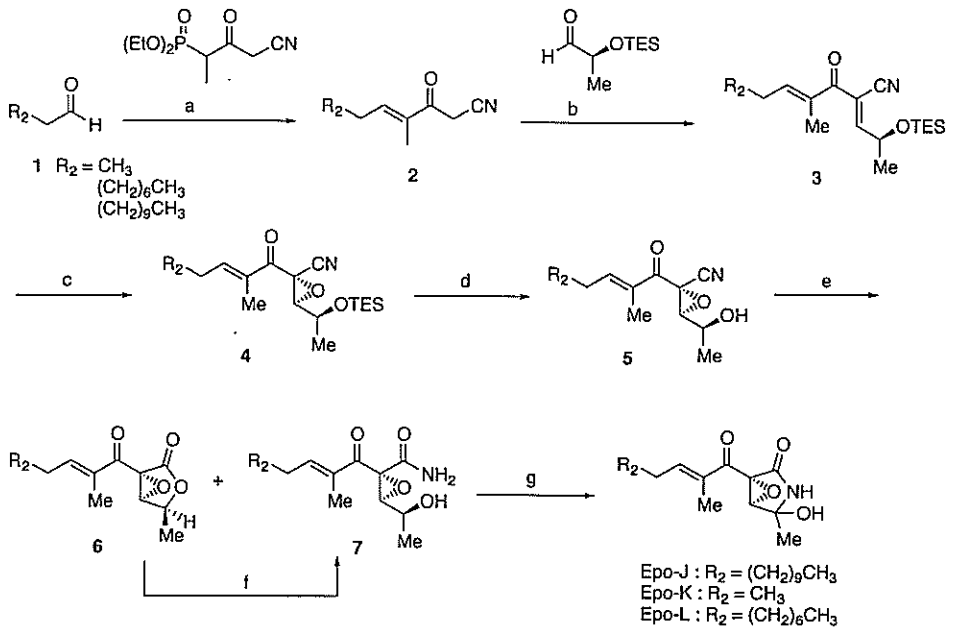


Figure 1. Structures of epolactaene and its derivatives used in this study.



Scheme 1. Synthesis of Epo-J, Epo-K, and Epo-L. Reagents and conditions: (a) *t*-BuOK, THF–HMPA (92–98%); (b) cat. ethylenediammonium diacetate, MeOH (83–89%); (c) TrOOH, *n*-BuLi, THF, −78 °C (78–84%); (d) AcOH, NH_4F, THF, H_2O; (e) TLC on silica gel; (f) NH_4OH, MeOH (71–77% from 4); (g) SO_3·Py, DMSO, NEt_3, CH_2Cl_2 (70–76%).

Table 1. IC_{50} acting against SH-SY5Y and Jurkat cell viability, competitive binding to Hsp60, and inhibition of Hsp60 chaperone activity on MDH reactivation by epolactaene derivatives

Compound	IC_{50} (μM)[a]		Binding competition[b]	In vitro MDH reactivation %[c]
	SH-SY5Y	Jurkat		
Epo	3.9	1.5	++	7.2±0.9
ETB	1.1	2.0	+++	1.9±6.8
Epo-E	>300.0	90.0	–	85.9±5.3
Epo-F	>130.0	20.0	+	53.1±11.8
Epo-G	38.0	20.0	–	62.7±8.1
Epo-J	5.7	1.2	+	31.2±6.2
Epo-K	18.0	8.0	–	35.1±2.4
Epo-L	13.0	1.5	+++	13.2±3.1
Epo-M	n.d.[d]	3.0	+++	70.0±8.9
Bio-ETB	6.5	3.0		n.d.

[a] IC_{50}: 50% inhibitory concentration.
[b] Binding competition percent of each derivative was generated relative to untreated sample as the control and shown as follows: +++, >90%; ++, 60–90%; +, 20–60%; –, <20%. Experimental conditions: see text.
[c] MDH reactivation % by BSA was subtracted as the baseline, and then the reactivation derived using untreated Hsp60 was taken as 100%. Experimental condition: see text.
[d] Not determined.

position (bio-ETB in Fig. 1). This biotin-labeled epolactaene retained its growth-inhibitory effect (Table 1).

3.2. Competitive effects by epolactaene derivatives on bio-ETB binding to human Hsp60

Epolactaene has electrophilic structures in its α,β-unsaturated ketone, epoxide, and hemiaminal carbon, which are potentially reactive with biological nucleophiles, such as the sulfhydryls of cysteines. Indeed, as we reported recently, human Hsp60 Cys-442 was the crucial amino acid residue for binding to epolactaene.[13]

To observe the structural requirement for interactions with Hsp60, we assessed the performance of epolactaene derivatives in a binding experiment. We utilized bio-ETB as an active molecular probe and analyzed the ability of each derivative to compete bio-ETB binding to recombinant Hsp60-His_6 protein. Human Hsp60-His_6 (14 μM in phosphate buffered saline (PBS)) pretreated with 1.3 equiv of each derivative at 4 °C. After 2 h, 2 equiv of bio-ETB was added to the mixture (the final concentration of Hsp60 was 2.8 μM) and incubated at 4 °C for another 0.5 h. SDS-PAGE loading buffer was added, and the resulting mixture was analyzed by

SDS-PAGE followed by blotting using HRP-conjugated streptavidin. The loss of labeling, that is, competition, showed the degree to which each derivative blocked the binding of bio-ETB with Hsp60. As summarized in Table 1, ETB almost completely blocked the binding of bio-ETB. Epo-L and Epo-M also competed remarkably, and there were no big differences in their inhibitory activities. Epo, Epo-F, and Epo-J also competed the binding, although they were less effective than ETB. Epo-E, Epo-G, and Epo-K, however, did not block the binding. We recently revealed that epolactaene/ETB selectively bind to Cys-442 of Hsp60, although human Hsp60 has two other cysteines, Cys-237 and Cys-447.[13] To confirm that the observed competitive binding of Epo-L and Epo-M was through Cys-442 of Hsp60, we also analyzed the competitive effect using the Ala mutant of both Cys-237 and Cys-447 of Hsp60. The competitive binding experiment by Epo-L and Epo-M using the double Ala mutant of Cys-237 and Cys-447 in Hsp60-His_6 gave the same results as those with the wild-type Hsp60-His_6 protein (data not shown).

3.3. Inhibitory effects of epolactaene derivatives on human Hsp60 chaperone activity

Next, we investigated the inhibitory effects of epolactaene derivatives against human Hsp60 chaperone activity. Hsp60 chaperone activity was measured by analyzing the chaperonin-assisted refolding of pig mitochondrial malate dehydrogenase (MDH).[13] Briefly, MDH was denatured in 10 mM HCl for 2 h at room temperature and diluted to a concentration of 100 nM in a buffer (0.1 M Tris(hydroxymethyl)aminomethane–HCl, pH 7.6, 7 mM KCl, 7 mM $MgCl_2$, 10 mM dithiothreitol) containing reconstituted chaperones (2.1 μM Hsp60 treated with 1.3 equiv epolactaene derivatives for 15 h at 4 °C, and 4.2 μM Hsp10). The refolding reaction was started by the addition of 2 mM ATP at room temperature. We tested the reactivation of MDH, which is dependent on the chaperone activity of Hsp60, treated with each derivative. The reactivation (%) of MDH by BSA was subtracted as the baseline, and then reactivation derived using Hsp60 treated without derivative was taken as 100%. The data are summarized in Table 1. Epolactaene, ETB, and Epo-L significantly inhibited reactivation. Epo-F, Epo-J, and Epo-K blocked reactivation slightly or moderately, though Epo-E did not effectively interfere with the chaperone activity. Epo-M showed weak inhibition relative to the extent of its binding competition compared with Epo-L. This loss in the inhibition of Epo-M invoked us the importance of α,β-unsaturated ketone that is the only difference between Epo-L and Epo-M. In the recent paper, we have already shown that epolactaene/ETB covalently binds with Hsp60 through Cys-442 and this binding was suggested to be responsible for the effective inhibition on the chaperone activity. There are several moieties in epolactaene that is potentially reactive with cysteine, that is, α,β-unsaturated ketone, epoxide, and hemiaminal carbon. To examine the possible reaction site on epolactaene, we analyzed the binding between bio-ETB and Hsp60. When we incubated Hsp60 already bound with bio-ETB in a buffer A (8 M guanidium–HCl, 0.5 M Tris(hydroxymethyl)aminomethane–HCl, pH 8.5, 1% dithiothreitol), the biotin-labeled amount of Hsp60 reduced (Fig. 2). It demonstrated that the binding between bio-ETB and Hsp60 could be reversible in buffer A, thus Michael addition to α,β-unsaturated ketone is reasonable.

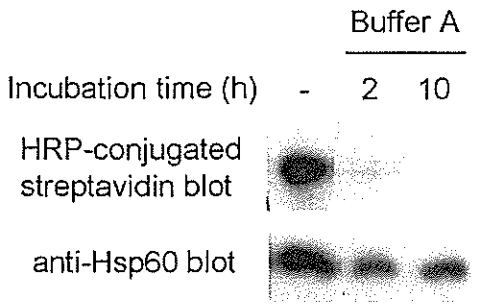


Figure 2. Reversible binding between bio-ETB and Hsp60 protein. Hsp60-His_6 protein (14 μM) in PBS was pretreated with 1.5 equiv bio-ETB. The mixture was then diluted 10-fold in buffer A (8 M guanidium–HCl, 0.5 M Tris(hydroxymethyl)aminomethane–HCl, pH 8.5, 1% dithiothreitol) and incubated for indicated time at 37 °C. The mixture was separated by SDS-PAGE and analyzed by Western blotting using HRP-conjugated streptavidin (*upper panel*) and anti-Hsp60 antibody (*lower panel*).

4. Discussion

In the present study, we described the SARs of new epolactaene derivatives. We evaluated the ability of each to inhibit the growth of human cancer cell lines, to compete the binding of biotin-conjugated epolactaene with human Hsp60, and to inhibit Hsp60's chaperone activity. The results for growth inhibition suggest that the γ-lactam moiety is important for the biological activity. Triene and ester moieties may not be always necessary, although the lack of a long side chain leads to a great loss of the growth-inhibitory effect.

The potency of epolactaene derivatives for competing bio-ETB binding to Hsp60 showed almost the same tendency observed for growth inhibition. Although the extent of competition depends on the experimental conditions, such as preincubation and labeling times, the comparison of relative ability is worthwhile. The results from the competitive binding experiment again revealed the importance of the lactam ring. The failure of Epo-E and Epo-G to compete the binding would be attributable to the absence of the lactam ring. Although Epo-F inhibits the labeling of bio-ETB to an extent, it is likely that the lactone moiety gave rise to another reactive site that does not exist in the original epolactaene. Epo-K, which has a shorter side chain, did not inhibit the binding. It is also noteworthy that Epo-M blocked the binding as effectively as Epo-L did.

The SAR study of the inhibition of Hsp60 chaperone activity is very significant. The results almost correspond to those of the growth inhibition and binding exper-

iments, except for the results with Epo-M. Epo-M inhibited growth and competed the binding as effectively as ETB and Epo-L. However, the inhibitory effect of Epo-M on Hsp60 chaperone activity was much weaker than that of Epo-L. The only difference between Epo-L and Epo-M is that the former contains α,β-unsaturated ketone. This moiety should have a fundamental role in inhibiting chaperone activity. We have already shown that epolactaene/ETB covalently binds Hsp60 through Cys-442 and suggested that this binding is responsible for the chaperone activity inhibition.[13] There are several moieties in epolactaene that is potentially reactive with cysteine, that is, α,β-unsaturated ketone, epoxide, and hemiaminal carbon. The reversibility of the binding in a buffer A (Fig. 2) demonstrates the Michael addition to the α,β-unsaturated ketone is reasonable. Because the chaperone cycle of Hsp60, which is a homologue of GroEL, probably depends on its conformational change,[23] covalent modification of Hsp60 may be important for the effective inhibition of chaperone activity. However, we cannot completely exclude the possibility that Epo-M lost inhibitory effect on chaperone activity because of flexibility in the side chain. This result, that Epo-M effectively inhibits growth but not Hsp60 chaperone activity, also suggests that epolactaene's growth inhibition effect is not always dependent solely on the inhibition of Hsp60 chaperone activity. Because the SAR results from the Hsp60 binding experiment almost correspond to the growth-inhibitory effect, binding to Hsp60 may result in the alteration of Hsp60-associated proteins to affect cell viability. It is also possible that epolactaene modifies other proteins too that more effectively inhibit growth.

In conclusion, we identified epolactaene's structural requirement for biological activities, as follows. We have proven that epolactaene's unique lactam moiety is critical to the growth inhibition of human cancer cells and to the modification of human Hsp60. The ester and triene moieties are likely not essential, although the length of the alkyl side chain is important to the effectiveness of the biological activities. The SAR studies here also revealed that α,β-unsaturated ketone is important for the effective inhibition of human Hsp60 chaperone activity. As a result, we identified potent derivatives, such as ETB, Epo-L, and the unique analogue Epo-M, which may serve as probes in further studies of epolactaene's biological activities and the biological analysis of human Hsp60 functions.

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Fumagillin suppresses HIV-1 infection of macrophages through the inhibition of Vpr activity

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Abstract HIV-1 viral protein R (Vpr) is one of the human immunodeficiency virus type 1 encoded proteins that have important roles in viral pathogenesis. However, no clinical drug for AIDS therapy that targets Vpr has been developed. Here, we have established a screening system to isolate Vpr inhibitors using budding yeast cells. We purified a Vpr inhibitory compound from fungal metabolites and identified it as fumagillin, a chemical already known to be a potent inhibitor of angiogenesis. Fumagillin not only reversed the growth inhibitory activity of Vpr in yeast and human cells, but also inhibited Vpr-dependent viral gene expression upon the infection of human macrophages.

Keywords: HIV-I; Vpr; AIDS; Small molecule; fumagillin; Cell cycle

1. Introduction

Viral protein R (Vpr) is a 96 amino acid, 14 kD nucleophilic protein that is incorporated into mature virions of human immunodeficiency virus type 1 (HIV-1). Vpr aids efficient translocation of the proviral DNA into the nucleus and is required for the HIV-1 infection of non-dividing cells such as macrophages [1–4]. Vpr is also involved in activation of viral transcription, induction of cell cycle G2 arrest and apoptosis of the host cells upon the HIV-1 infection (for recent reviews, see [5–7]). Although specific roles of these Vpr activities in viral pathogenesis and disease progression have not been elucidated, the effects of Vpr mutations found in AIDS patients suggest that Vpr is an important cytotoxic component of HIV-1 infection [8–10]. However, no Vpr targeted small molecule that can be used for AIDS therapy has yet been developed.

Fumagillin, a natural product of fungal origin, was discovered to act as a potent inhibitor of angiogenesis [11]. A semi-synthetic analog of fumagillin, TNP-470 (AGM-1470) is 50 times more active inhibitor of angiogenesis than its parent compound [11]. Since these compounds are covalent inhibitors selective for a protease, methionine aminopeptidases 2 (MetAp2), MetAp2 had been considered for the responsible molecular target for the inhibition of angiogenesis [12,13]. However, since there are two reports contradicting each other about the matter whether inhibition of angiogenesis by fumagillin is dependent on the MetAP2 activity or not, the molecular mechanism through which fumagillin inhibits angiogenesis remains to be elucidated [14,15].

In this study, we have established a screening system to isolate Vpr inhibitors. Using the system, we purified a Vpr inhibitory compound from fungal metabolites and identified it fumagillin. Fumagillin actually inhibited the HIV-1 infection of human macrophages. Our results demonstrate that fumagillin can be a lead compound for the development of the novel type of AIDS therapeutic drug that targets Vpr activity.

2. Materials and methods

2.1. Plasmids

For the expression of Vpr in yeast, *Xho*I–*Not*I fragments [16] of N-terminal FLAG-tagged HIV-1_{NL4-3}Vpr were blunted and inserted into *Bam*HI sites of copper inducible yeast expression vector, pYEX-BX (AMRAD BIOTECH, Vic., Australia). Plasmids, in which FLAG-Vpr transcription is driven in the reverse orientation under CUP1 promoter, were used as control plasmids. For the introduction of these plasmids into *URA3* cells, the *URA3* marker in pYEX-BX was changed to *HIS3*.

2.2. Yeast strains, culture and Vpr inhibitor screening

A multidrug sensitive yeast strain (MLC30) and MetAP2 deletion mutant strain (Δmap2, *map2*::*URA3*) were obtained from Dr. Tokichi Miyakawa (Hiroshima Univ., Higashi-Hiroshima, Japan) and Dr. Yie-Hwa Chang (St. Louis Univ. Sch. Med., MO), respectively [17,18]. For expression of Vpr, yeast cells were cultured in the SD medium (0.7% yeast nitrogen base (DIFCO), 2% glucose) containing amino acids minus selective amino acids and 0.5 mM $CuSO_4$. To silence the expression, $CuSO_4$ was removed and leucine was added to the culture. For the screening of Vpr inhibitor on agar plates, MLC30 cells with Vpr expression plasmids were cultured to log phase in the expression silencing media at 30 °C, washed, suspended in expression inducing medium at $OD_{600} = 0.5$ and cultured for an additional 30 min. Then, the culture was mixed with 9 volumes of expression inducing medium containing 2% agar (Phytagar, GIBCO) and 0.001% SDS, poured into plastic

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Abbreviations: HIV-1, human immunodeficiency virus type 1; Vpr, viral protein R; MetAP2, methionine aminopeptidases 2

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plates and solidified at room temperature. Paper filters (ϕ = 6 mm) containing extracts of the culture broth to be tested were put on the plates and the plates were incubated at 30 °C for several days.

2.3. Purification of fumagillin from a producing fungal strain

Fumagillin was isolated from the culture broth of a producing fungal strain using bioassay-guided purification procedures. The structure of fumagillin was determined by the physico-chemical properties, detailed ^{1}H and ^{13}C NMR analysis, and mass spectroscopy [19].

2.4. Synthesis of TNP470 and biotinylated fumagillin

TNP470 was synthesized from fumagillin as described previously [20]. Biotinylated fumagillin was synthesized by a coupling reaction via the carboxyl group of fumagillin using an activated biotin reagent (Pierce). The structures of TNP470 and biotinylated fumagillin were determined by their physico-chemical properties, detailed ^{1}H and ^{13}C NMR analysis, and mass spectroscopies. Biotinylated fumagillin was confirmed to be effective in MT-Vpr1 cells although it was weaker than the original.

2.5. Human cell culture and immunological techniques

Cell culture, synchronization and FACS analysis of MT-Vpr1 cells, a HeLa derived cell line with zinc inducible Vpr expression plasmids, and immunological detection of Vpr in the cells were as described [16]. Human embryonic kidney (HEK) 293 cells expressing SV40 large T antigen (293T) were propagated in DMEM medium supplemented with 10% FCS. Human primary monocytes and differentiated macrophages were obtained from peripheral blood mononuclear cells of healthy donors as described [21].

2.6. Preparation of viruses

To generate the single-round replication incompetent luciferase reporter virus stocks (NL-Luc-E$^-$R$^+$ or NL-Luc-E$^-$R$^-$)[2], 293T cells were co-transfected with the proviral DNAs (obtained from Dr. Nathaniel Landau through the AIDS Research and Reference Reagent Program) and plasmids encoding vesicular stomatitis virus envelope protein (pCMV-VSV-G-RSV-Rev). Culture supernatants were harvested at 60 h after the transfection and titrated.

2.7. Infectivity assays

Primary macrophages in 24 well plates were inoculated with VSV-G pseudotyped reporter viruses (NL-Luc-E$^-$R$^+$(VSV-G) or NL-Luc-E$^-$R$^-$(VSV-G); 1.5 ng of $p24^{gag}$ antigen), cultured in the absence or presence of the drug (fumagillin or TNP470) for 6 days, harvested, lysed in luciferase assay substrate (Promega) and assayed for luciferase activities using Wallac ARVO SX 1420 (Perkin–Elmer).

3. Results and discussion

3.1. Isolation of Vpr inhibitors using budding yeast cells

To isolate small molecules that inhibit the activity of Vpr, we have established a screening system using budding yeast cells expressing Vpr. As shown in Fig. 1A, yeast cells with copper-inducible Vpr expression plasmids [22] were embedded in agar plates containing the inducer ($CuSO_4$). Then, paper filters containing extracts of broth from cultured microorganisms (fungi, actinomycetes or bacteria) were put on agar plates, and the plates were incubated at 30 °C for several days. Since Vpr strongly inhibits the growth of yeast cells [22], no significant growth was usually detected even after 4–5 days of incubation. However, very occasionally, significant growth could be detected surrounding the paper filters, indicating that the culture broth extracts on the filters have an activity that antagonizes the action of Vpr (Fig. 1B). As a result of our extensive screening program, we have purified the active compound and identified it as fumagillin (Fig. 1C and D), a compound known to be a potent inhibitor of angiogenesis [11]. Commercially available fumagillin (Sigma) had specific activity similar to that of our purified compound (not shown). The activity of fumagillin could also be detected when a galactose inducible system was used for Vpr expression (not shown), suggesting that this compound reverses the action of Vpr itself rather than the expression of Vpr by copper inducible system (see below).

3.2. Effect of fumagillin and TNP470 on Vpr induced cell cycle arrest in HeLa cells

Next, we examined the ability of fumagillin to antagonize Vpr function in human cells. As described above, one of the characteristic functions of Vpr in human cells is induction of the cell cycle arrest at G2 phase [23,24]. We previously established a HeLa derived cell line (MT-Vpr1) stably transfected with a zinc-inducible Vpr expression vector [16]. In this cell line, induction of Vpr expression arrests cell cycle at G2 phase in more than half of the total cells a day after addition of the inducer (Fig. 2A, Zn). When fumagillin (10 ng/ml) was added before the addition of zinc, the G2 arrested population was

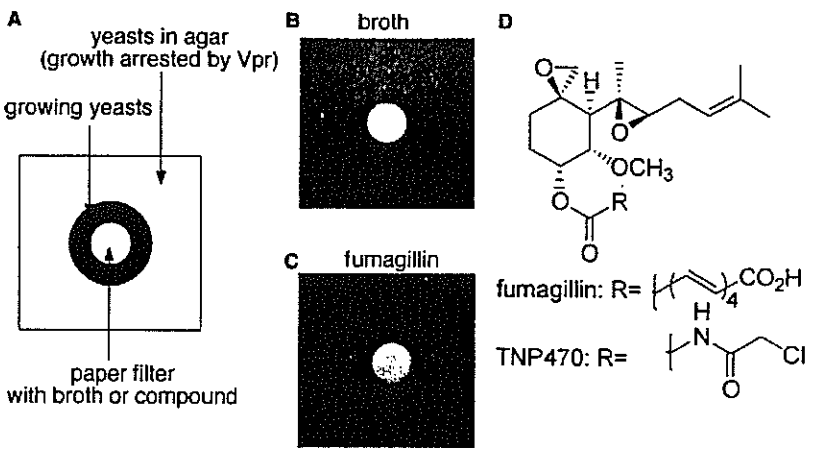

Fig. 1. Screening system to isolate Vpr inhibitors. (A) Schematic presentation of Vpr screening system. Budding yeast cells expressing Vpr were embedded in agar plates containing inducer (copper). Paper filters with broths or compounds to be tested were put on the plates. Only the yeasts surrounding filters that contain Vpr inhibitors were able to grow. (B,C) Growing yeasts surrounding filters containing 10 μl of extract from the culture broth of a fungus with Vpr inhibitory activity (B) or purified fumagillin (C; 2 mg/ml, 10 μl). Plates were incubated for 4 days at 30 °C. (D) Chemical structures of fumagillin and TNP470.

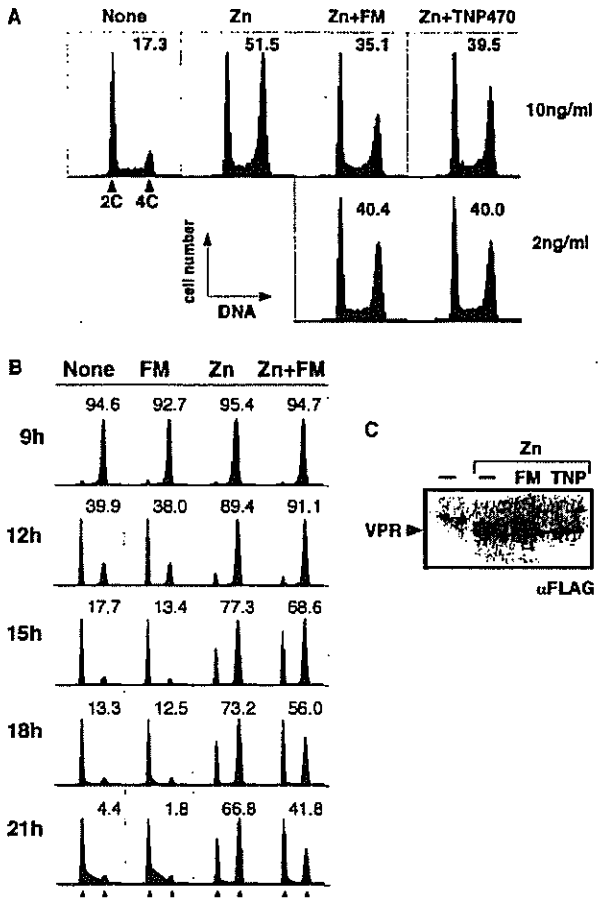

Fig. 2. Fumagillin and TNP470 inhibit Vpr activity in HeLa cells. (A) Thirty minutes before the addition of zinc, fumagillin (FM) or TNP470 was added to MT-Vpr1 cells at the concentrations described. Cells were cultured for a day in the presence or absence of zinc (Zn; 150 μM) and harvested for FACS analysis. Numbers in the figures represent the percentage of cells with 4C DNA contents. (B) Mt-Vpr1 cells were synchronized at G1/S border [16], then released in the presence or absence of zinc (Zn; 150 μM) and/or fumagillin (FM; 10 ng/ml). Zinc and fumagillin were added at 2 and 1 h before the release, respectively. Numbers in the figures represent the percentage of cells with 4C DNA contents. (C) Cells as in (A) were cultured for 6 h and harvested for western analysis to detect FLAG-Vpr expression [16]. Drugs were added at 10 ng/ml.

significantly reduced (Fig. 2A, Zn + FM). Thus, fumagillin can partially inhibit the action of Vpr in mammalian cells as well. The effect of higher doses (100 ng/ml and 1 μg/ml) of fumagillin on the action of Vpr was almost similar to that at 10 ng/ml in this system (not shown).

A synthetic analog of fumagillin, TNP470 (AGM-1470; Fig. 1D) is a more potent angiogenesis inhibitor [11]. However, the ability of TNP470 to antagonize Vpr function was similar to or less than that of fumagillin (Fig. 2A, Zn + TNP470). Thus, fumagillin seems to override Vpr-dependent cell cycle arrest in a manner different from that through which it blocks angiogenesis.

In this system, however, if fumagillin has an activity to arrest cell cycle at a phase other than G2 phase, an apparent reduction of G2 arrested cells would be observed. To examine this possibility, the effect of fumagillin on cell cycle progression was examined. After MT-Vpr1 cells were synchronized at G1/S border and released in the presence or absence of fumagillin, progression through S, G2, M and G1 was monitored by FACS analysis (Fig. 2B). In the absence of zinc, cell cycle progression was not affected by fumagillin (None and FM). When Vpr expression was induced by zinc addition, a similar fraction of cells was arrested at G2 at 12 h after the release regardless of the presence of fumagillin. But, in the presence of fumagillin, the fraction of cells arrested at G2 phase was significantly reduced at later time points (Zn and Zn + FM). These results indicate that fumagillin does not affect normal cell cycle progression but reduces the activity of Vpr to arrest the cell cycle. We have also confirmed that neither fumagillin nor TNP470 has any effect on the zinc induced Vpr expression level in MT-Vpr1 cells (Fig. 2C).

3.3. *Vpr inhibits growth of yeast cells independently from MetAP2 pathway*

Fumagillin is known to covalently bind and inhibit a protease, MetAP2 both in human and budding yeast cells [12,13]. However, since there are two reports contradicting each other about the matter whether inhibition of angiogenesis by fumagillin is dependent on the MetAP2 activity or not, the molecular mechanism through which fumagillin inhibits angiogenesis remains to be elucidated [14,15].

We examined whether MetAP2 is on the pathway for Vpr-dependent growth arrest and whether fumagillin blocks the activity of Vpr through the inhibition of MetAP2 or not. In budding yeast, the gene (*MAP2*) that encodes MetAP2 is not essential, because there is a second aminopeptidase, MetAP1, which is insensitive to fumagillin [12,13,18]. Vpr arrested the growth of Δ*map2* strain cells almost as completely as wild type, indicating that MetAP2 is not on the pathway of the Vpr dependent growth arrest (Fig. 3A). The ability of fumagillin to reverse the Vpr dependent arrest in Δ*map2* strain cells was confirmed on paper disk assay as well (data not shown). These results indicate that fumagillin abrogates Vpr function by targeting (a) molecule(s) other than MetAP2. Since the sensitivity to fumagillin and TNP470 is different for Vpr-dependent arrest and inhibition of angiogenesis, the target molecule(s) for these drugs may be different in these two systems.

3.4. *Mechanism of fumagillin to inhibit Vpr function*

Using biotinylated fumagillin, we attempted to detect any covalent or strong binding between Vpr and fumagillin. Biotinylated fumagillin was added to the lysates of MT-Vpr1 cells or yeast cells expressing FLAG-tagged Vpr. After the lysates were separated on SDS–PAGE and transferred on membrane, proteins covalently bound to fumagillin such as MetAP2 were probed with horse radish peroxidase (HRP) labelled streptavidin. Alternatively, proteins associated with biotinylated fumagillin were isolated using streptavidin conjugated agarose beads, and probed with αFLAG antibody to detect FLAG tagged Vpr. In spite of all of these attempts, we were unable to obtain any evidence for the interaction between Vpr and fumagillin (not shown). However, when point-mutations (Q3R, E25K, A30L, W54A, L64A, H71R, R73A, I74R, G75A, C76A, R80A, and R90K) were introduced into Vpr and their sensitivity to fumagillin was examined on paper disk assay, we found that the E25K mutation (the 25th glutamate of Vpr was changed to lysine) makes Vpr significantly resistant to fumagillin (Fig. 3B). Since the E25K Vpr still inhibits growth of yeast cells [25], the mechanism of fumagillin may

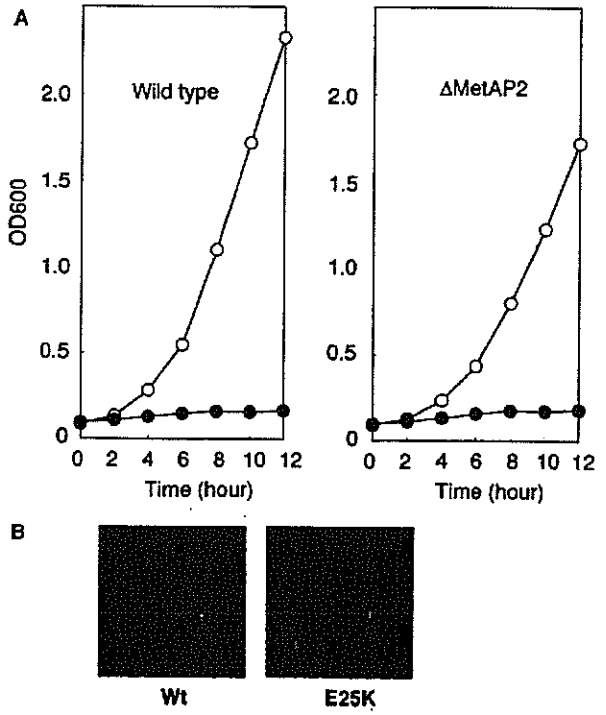

Fig. 3. Mechanism of fumagillin to abrogate the Vpr activity. (A) Vpr inhibits growth of yeast cells independently from MetAP2 activity. Δ*map2* cells (right) or its isogenic control cells (left) were cultured in the presence (closed circle) or absence (open circle) of the Vpr expression. The growth of yeast cells was monitored with the absorbance at 600 nm. (B) E25K mutation makes Vpr resistant to fumagillin. Yeast cells with wild type Vpr (left) or E25K mutated Vpr were embedded in agar plates as in Fig. 1. Paper filters with 20 μg of fumagillin were put on the plates and incubated for 3 days at 30 °C. Photographs were taken with translucent light to increase sensitivity.

be directly on Vpr rather than on a downstream pathway. The precise mechanism through which the E25K mutation renders Vpr resistant to fumagillin is not clear, but it is possible that fumagillin interacts directly (albeit too weakly to detect) with Vpr at residues surrounding E25.

3.5. Inhibition of Vpr-dependent viral gene expression by fumagillin or TNP470

Vpr is required for efficient replication of HIV-1 in non-dividing cells such as macrophages [2–4]. During the HIV-1 life cycle, Vpr functions after entry and reverse transcription, yet prior to, or at the time of, proviral transcription [2]. Thus we examined the effect of fumagillin on the proviral transcription upon the infection using an *env*-deficient HIV-1 vector that allows only a single round of infection. Wild type or frame-shifted Vpr-containing, *env*-deficient HIV-1 reporter vector in which Nef has been replaced by the luciferase gene (NL-Luc-R^+ or NL-Luc-R^-, respectively) [2] was used to infect primary human macrophages (Fig. 4A and B). Luciferase activity, determined 6 days after infection, was about 4 times higher from the Vpr^+ virus than that from the Vpr^- virus, indicating that Vpr is required for efficient expression of virally encoded genes in macrophages [2]. When fumagillin or TNP470 was added at the time of infection, luciferase expression from the Vpr^+ virus but not from the Vpr^- virus was inhibited in a dose-dependent manner. Under these experimental condition,

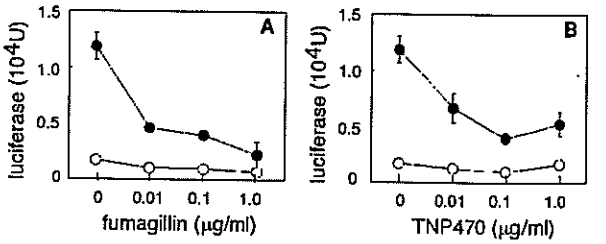

Fig. 4. Fumagillin and TNP470 inhibit Vpr dependent proviral gene expression. (A,B) Macrophages were infected with HIV-1 reporter vector with wild type (closed circle) or truncated (open circle) Vpr and cultured in the presence of fumagillin (A) or TNP470 (B). The proviral gene expression was monitored by the luciferase activity 6 days after the infection using luciferase assay substrate (Promega).

we could not see any sign of toxicity of 1 μg/ml of fumagillin for the macrophages under microscope indicating that the inhibition of viral gene expression in Vpr^+ infected cells is due to the inhibition of Vpr by these drugs rather than to some non-specific toxicity of them. Taken together, our results show that fumagillin or TNP470 suppresses the HIV-1 replication in macrophages through inhibition of Vpr-dependent viral gene expression.

3.6. Concluding remarks

Because it is now evident that Vpr's contribution to the pathogenesis of HIV-1 infection in vivo is crucial, Vpr has been proposed to be an attractive target for developing novel therapeutic strategies for AIDS therapy. Our results show that fumagillin and its derivatives can be used as a new type of AIDS therapeutic drug, which targets Vpr. In this context, it should be noted that fumagillin and TNP470 are already used clinically to treat Kaposi's sarcoma or microsporidiosis in AIDS patients with successful results [26,27], although the effects of these drugs on the viral replication have not been reported. Thus, the day when the fumagillin-derived compounds can be used clinically to prevent HIV-1 replication may come sooner than expected.

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Enantio- and Diastereoselective Total Synthesis of (+)-Panepophenanthrin, a Ubiquitin-Activating Enzyme Inhibitor, and Biological Properties of Its New Derivatives

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Abstract: The asymmetric total synthesis of (+)-panepophenanthrin, an inhibitor of ubiquitin-activating enzyme (E1), has been accomplished using catalytic asymmetric α aminoxylation of 1,4-cyclohexanedione monoethylene ketal as a key step, followed by several diastereoselective reactions. The biomimetic Diels–Alder reaction of a monomer precursor was found to proceed efficiently in water. The investigation of the biological properties of new derivatives of (+)-panepophenanthrin enabled us to develop new cell-permeable E1 inhibitors, RKTS-80, -81, and -82.

Keywords: asymmetric synthesis · chemical biology · natural products · panepophenanthrin · total synthesis

Introduction

Panepophenanthrin (**1**) is a natural product that inhibits ubiquitin-activating enzyme (E1) and was isolated by Sekizawa and co-workers in 2002 from the mushroom strain *Panus rudis* Fr. IFO8994.[1] As ubiquitin-activating enzyme (E1) plays an important role in the ubiquitin-proteasome pathway (UPP), which regulates a variety of important cellular processes by degradation or processing of target proteins, an inhibitor of ubiquitin-activating enzyme (E1) would be a promising drug candidate for cancers, inflammation, and neurodegenerative disease.[2] Structurally, panepophenanthrin has a complex architecture with a highly substituted tetracyclic skeleton, which contains 11 contiguous stereocenters. Panepophenanthrin belongs to the so-called epoxyquinoid natural-product family, whose members are synthesized by Diels–Alder dimerization of much simpler epoxyquinol monomers.[3] Its synthetically challenging structure along with its important biological activity make panepophenanthrin an attractive synthetic target. In fact, since its isolation in 2002, three groups have already accomplished its total synthesis. Porco and co-workers reported the first asymmetric total synthesis through biomimetic Diels–Alder dimerization of a monomer, synthesized by diisopropyl tartrate mediated asymmetric epoxidation, in which excess amounts (1.6 equiv) of a chiral controller were employed.[4] They clearly explained the reaction mechanism of the Diels–Alder dimerization. Baldwin and co-workers[5] accomplished its total synthesis in racemic form from the known (±)-bromoxone in three steps; enantiomerically pure (−)-bromoxone is known to be prepared by enzymatic resolution,[6] giving the formal total synthesis of the chiral panepophenanthrin. Mehta and co-workers synthesized (+)-panepophenanthrin by using lipase-mediated enzymatic desymmetrization as a key step,[7a] and the (−) isomer was synthesized through lipase-mediated enzymatic resolution by the same group.[7b] Although these are excellent syntheses, no asymmetric catalytic method has been reported. The preparation of chiral (+)-panepophenanthrin and its derivatives in a practical and atom-economical manner is desirable for biological investigations.

Our group has been involved in the chemistry and biology of epoxyquinol dimers such as epoxyquinol A, B, C, and epoxytwinol A, novel angiogenesis inhibitors,[8] epoxyquinol

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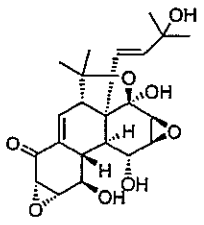
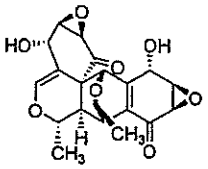

panepophenanthrin (1)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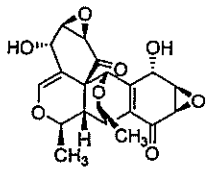
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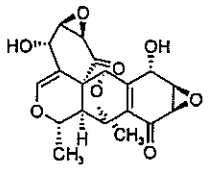
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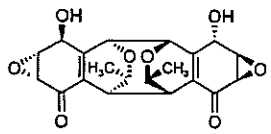
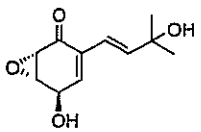
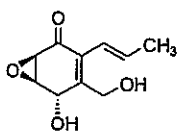
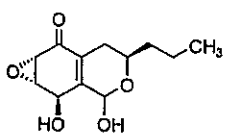
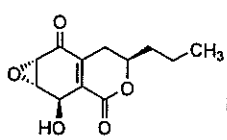
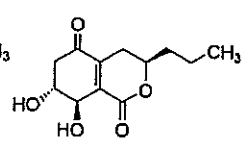

epoxytwinol A


monomer of panepophenanthrin (2)


ECH monomer of epoxyquinols


EI-1941-1


EI-1941-2


EI-1941-3

monomers such as ECH, an inhibitor of FasL-induced apoptosis,[9] and EI-1941-1, -2, and -3, inhibitors of interleukin-1β-converting enzymes.[10] Being interested in its complex structure and important biological activity, we have examined the asymmetric total synthesis of (+)-panepophenanthrin. Although there is a similarity between the monomers of panepophenanthrin and of the epoxyquinols, we have developed a completely different synthetic route from that of our previous synthesis of the epoxyquinols, in which a $HfCl_4$-mediated diastereoselective Diels–Alder reaction of furan[11] and a Diels–Alder reaction of furan with acryloyl chloride as a reactive dienophile, followed by lipase-mediated kinetic resolution, were developed as key steps. The present synthetic route is based on a practical, asymmetric catalytic reaction, which is also completely different from those of the previous three groups.

We have been developing proline-mediated asymmetric catalytic α aminoxylation of carbonyl compounds,[12] which is a powerful method for the synthesis of α-hydroxy carbonyl derivatives. Employing this reaction as a key step and with several diastereoselective transformations, we have accomplished the asymmetric total synthesis of panepophenanthrin, which we disclose herein. On the basis of this established synthetic route, several new derivatives were prepared, and their biological properties were evaluated, which we also discuss.

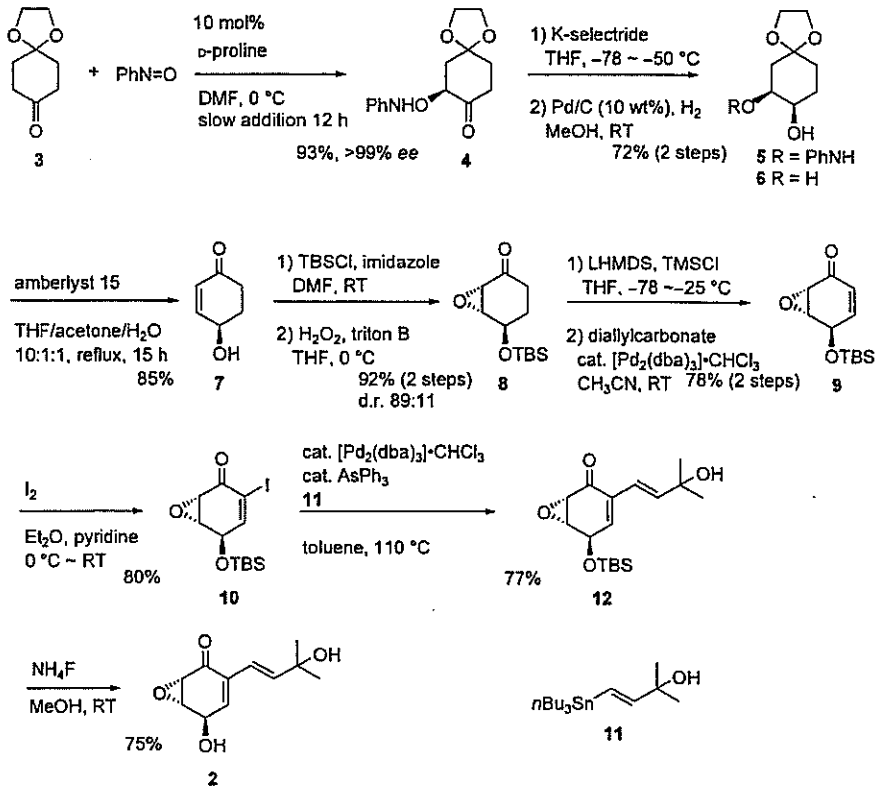
Abstract in Japanese:

1,4-シクロヘキサンジオンモノエチレンケタール (3)に対するプロリンを触媒としたα-アミノオキシ化反応を鍵反応とし、ユビキチン活性化酵素(E1)阻害作用を有する(+)-panepophenanthrin の不斉全合成、および新規類縁化合物の合成を達成した。また、全合成を模倣したモノマー2のディールス・アルダー反応が水中においても速やかに進行することを見いだした。さらに、生物活性評価の結果、細胞膜透過性に優れた新規類縁化合物 RKTS-80 (19)、-81 (20)、-82 (21)を見出した。

Results and Discussion

Asymmetric Synthesis of (+)-Panepophenanthrin

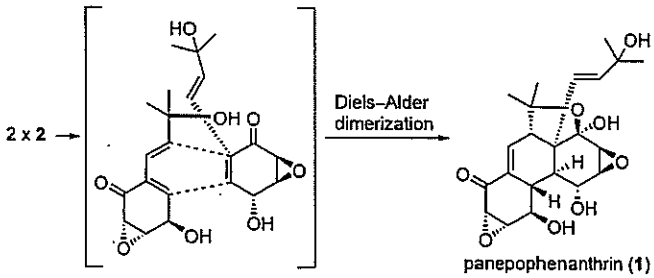
The first reaction in our sequence, α aminoxylation of 1,4-cyclohexanedione monoethylene ketal (**3**) (1.2 equiv) in the presence of D-proline (10 mol%) with slow addition of nitrosobenzene (1.0 equiv) over 24 h proceeded efficiently at 0°C to afford nearly optically pure (*S*)-α-aminoxylated cyclohexanone **4** (>99% *ee*) in 93% yield (Scheme 1). This reaction can be carried out on a large scale to generate 25 g of **4** without compromising yield or enantioselectivity.[12a,b] The (*R*)-α-aminoxylated cyclohexanone, the enantiomer of **4**, has been converted successfully into the fumagillin and ovalicin families by several diastereoselective reactions.[13] The reduction of cyclohexanone **4** with K-selectride proceeded stereoselectively to afford the alcohol *cis*-**5**. In this reduction, the *trans* isomer was not detected. Reductive cleavage of the N–O bond in the presence of Pd/C under a H_2 atmosphere gave the diol *cis*-**6** in 72% yield over two steps. Treatment of **6** with amberlyst in THF/acetone/water at reflux removed the acetal protecting group and resulted in a dehydration reaction to provide 4-hydroxycyclohex-2-enone (**7**) in 85% yield. Enone **7** is an intermediate in the synthesis of (+)-epiepoformin, (+)-epiepoxydon, and (+)-bromoxone by Kitahara and Tachihara.[14] The hydroxy group was protected by using *tert*-butyldimethylchlorosilane and imidazole. Epoxidation with H_2O_2 and triton B by following the protocol of Kitahara and Tachihara[14] gave epoxide **8** stereoselectively. Cyclohexanone **8** was converted into the corresponding cyclohexenone **9** in a two-step procedure in 78% yield:



Scheme 1. Synthesis of the monomer of panepophenanthrin (1). DMF = *N*,*N*-dimethylformamide, TBS = *tert*-butyldimethylsilyl, LHMDS = lithium hexamethyldisilazide, dba = *trans*,*trans*-dibenzylideneacetone.

1) formation of silyl enol ether by reaction of **8** with lithium hexamethyldisilazide and trimethylsilylchloride; 2) treatment of the resultant enol ether with diallylcarbonate in the presence of $[Pd_2(dba)_3]\cdot CHCl_3$ under Tsuji's modified conditions[15] of the Saegusa reaction.[16] Introduction of iodine at C2 of cyclohexenone **9** by reaction with I_2 in a mixture of Et_2O and pyridine[17] gave **10** in 80% yield. Coupling of **10** and vinyl stannane **11** proceeded in the presence of a catalytic amount of $[Pd_2(dba)_3]\cdot CHCl_3$ and $AsPh_3$[18] at 110 °C in toluene to provide the coupled product **12** in 77% yield. Removal of the silyl protecting group by treatment with NH_4F in MeOH afforded monomer **2** in good yield.

It is already known that monomer **2** dimerizes by allowing it to stand at 25 °C in the absence of solvent (24 h, 80% yield) (Scheme 2).[4,5,7] Although panepophenanthrin was synthesized in good yield by this procedure in our hands, these reaction conditions, particularly the absence of solvent, would not be similar to those under which the reaction occurs in living cells. We therefore investigated the dimerization in water, which would be similar to biological conditions. The results are summarized in Figure 1 along with the results obtained with other solvents. Dimerization proceeded efficiently in the absence of solvent as described above, which gave the best result. Whereas the reaction proceeds slowly in MeOH and THF and affords the Diels–Alder product in low yield, the reaction in water is much faster than the reaction in organic solvents. That is, when monomer **2** (1 mg) was dissolved in D_2O (200 μL), the reaction proceeded efficiently to afford panepophenanthrin (**1**) in moderate yield after 33 h. Although Breslow and co-workers showed that some Diels–Alder reactions are faster in water than in organic solvents,[19] the present result, that panepophenanthrin is synthesized in a reasonable yield in water at room temperature, is a piece of evidence to support the supposition that the biosynthesis of panepophenanthrin occurs through a non-enzymatic Diels–Alder reaction in living cells.



Scheme 2. Dimerization of monomer 2 through a Diels–Alder reaction to give panepophenanthrin (**1**).

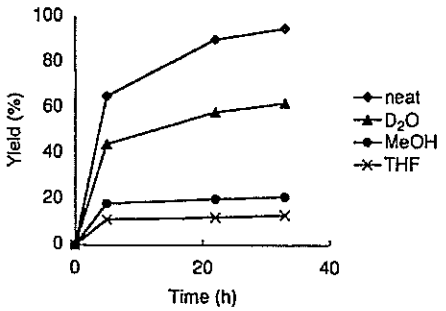
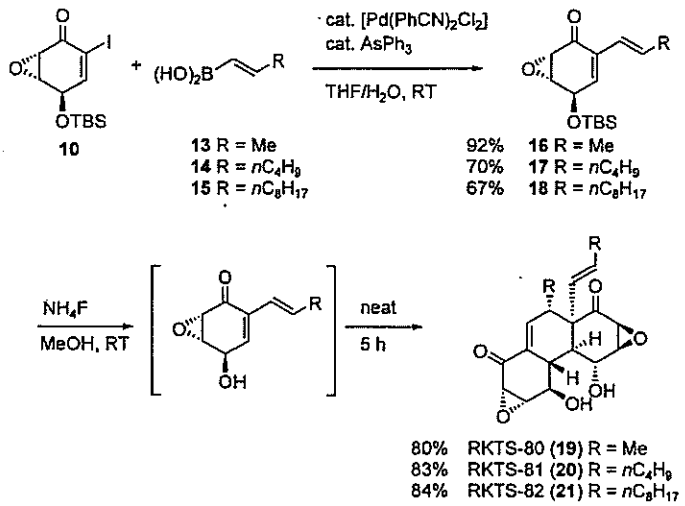


Figure 1. Effect of solvent and time on the yield of the Diels–Alder reaction of 2.

Synthesis of New (+)-Panepophenanthrin Derivatives

With a practical synthetic route to (+)-panepophenanthrin in place, we next investigated the structure–activity relationships of some new derivatives. The effects of the side chain and

ring systems of panepophenanthrin were examined. Monomers with propenyl, hexenyl, and decenyl substituents were synthesized from intermediate **10**. Suzuki coupling of **10** with alkenyl borates **13**, **14**, and **15** proceeded efficiently in the presence of $[Pd(PhCN)_2Cl_2]$ with $AsPh_3$ to provide dienes **16**, **17**, and **18**, respectively, in good yields (Scheme 3). The *tert*-butyldimethylsilyl group was removed



Scheme 3. Synthesis of new panepophenanthrin derivatives RKTS-80, -81, and -82.

by treatment with NH_4F in MeOH to afford the respective alcohols, which dimerized smoothly under neat reaction conditions to give Diels–Alder products RKTS-80 (**19**), RKTS-81 (**20**), and RKTS-82 (**21**), respectively, in good yield as single isomers. Porco and co-workers reported that the *tert*-hydroxy group in the side chain of **2** is not necessary for dimerization, and the same phenomenon was observed in the present derivatives. Once the derivatives were in hand, their biological activity was investigated.

Biological Properties of New (+)-Panepophenanthrin Derivatives

We evaluated the effects of new derivatives RKTS-80 (**19**), -81 (**20**), and -82 (**21**) on E1 activity in vitro. E1 catalyzes the formation of a ubiquitin adenylate intermediate from ubiquitin and ATP, and subsequently the binding of ubiquitin to a cysteine residue in the E1 active site in a thiol ester linkage. E1 activity, therefore, was analyzed by detecting the formation of the E1–ubiquitin intermediate from recombinant E1 and biotinylated ubiquitin in the presence of ATP (Figure 2). The ubiquitylated E1 was observed as the spot at approximately 120 kDa in this assay system. RKTS-80, -81, and -82 inhibited the formation of the E1–ubiquitin intermediate in a dose-dependent manner. The IC_{50} values of RKTS-80, -81, and -82 were 9.4, 3.5, and 90 μM, respectively, quantified by densitometric analysis. Our synthetic (+)-pan-

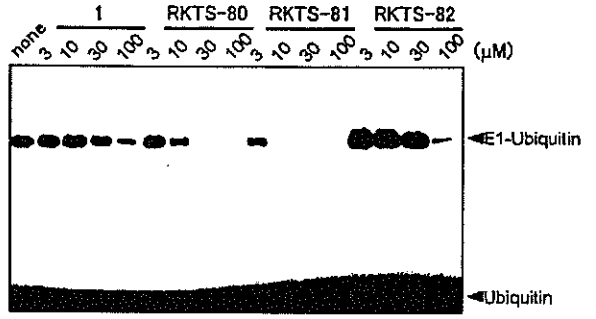


Figure 2. Inhibition of the E1–ubiquitin intermediate formation by (+)-panepophenanthrin (**1**) and new derivatives (RKTS-80, -81, and -82). Recombinant yeast E1, biotinylated ubiquitin, and ATP were incubated in the absence or presence of **1**, RKTS-80, -81, or -82 at various concentrations. The reaction mixture was then subjected to SDS-PAGE, and the biotin moiety was detected by the chemiluminescence method. The bands of E1–ubiquitin and ubiquitin represent the ubiquitylated E1 and the free biotinylated ubiquitin.

epophenanthrin (**1**) also blocked the E1–ubiquitin intermediate with an IC_{50} value of 72 μM. These results indicate that the 2,2-dimethyltetrahydrofuran moiety in **1** is not always necessary to inhibit the formation of the E1–ubiquitin intermediate. We then tested the effects of these compounds on the growth of human breast cancer MCF-7 cells[9,20] (Figure 3). RKTS-80, -81, and -82 blocked cell

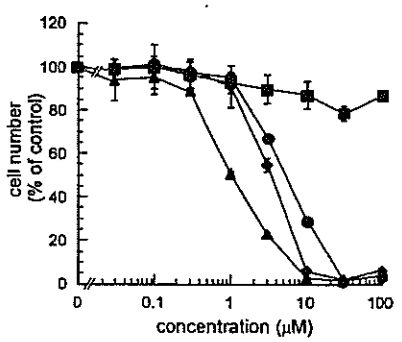


Figure 3. Effects of (+)-panepophenanthrin (**1**) and new derivatives (RKTS-80, -81, and -82) on the cell growth of MCF-7 cells. MCF-7 cells were cultured in RPMI-1640 cells containing 10% fetal bovine serum for 48 h in the presence of **1** (■), RKTS-80 (●), RKTS-81 (▲), or RKTS-82 (◆) at various concentrations at 37°C in a 5% humidified atmosphere. The cell number was evaluated by WST-8.

growth in a dose-dependent manner; IC_{50} values of RKTS-80, -81, and -82 were 5.4, 1.0, and 3.6 μM, respectively. The potency trend in the inhibition of cell growth is different from that in E1 inhibitory activity in vitro. These results might be caused by the difference in membrane permeability as well as the other mechanisms besides E1 inhibition by these compounds. On the other hand, synthetic (+)-panepophenanthrin (**1**) was unable to inhibit cell growth, even at 100 μM, suggesting that **1** might exhibit poor membrane permeability.

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Conclusions

We have completed an enantio- and diastereoselective total synthesis of (+)-panepophenanthrin by the proline-mediated α aminoxylation of 1,4-cyclohexanedione monoethylene ketal followed by stereoselective reactions. Diels–Alder dimerization was found to proceed faster in water than in organic solvent. The investigation of the biological properties of its derivatives in vitro and in vivo showed that the new derivatives RKTS-80, -81, and -82 are effective cell-permeable E1 inhibitors.

Experimental Section

General Methods

All reactions were carried out under an argon atmosphere and monitored by thin-layer chromatography with Merck 60 F_{254} precoated silica gel plates (0.25-mm thickness). Specific optical rotations were measured with a JASCO P-1020 polarimeter. FTIR spectra were recorded on a Horiba FT-720 spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Bruker DPX-400 instrument. High-resolution mass spectral analysis (HRMS) was carried out on a JEOL JMSSX 102A. Preparative thin-layer chromatography was performed with Merck Silica Gel 60 F_{254} and Wakogel B-5F purchased from Wako Pure Chemical Industries, Japan. Flash chromatography was carried out with Silica Gel Merck Art 7734 and silica gel 60N of Kanto Chemical Co. Int., Tokyo, Japan.

4: A solution of nitrosobenzene (13.7 g, 127.9 mmol) in DMF (150 mL) was added through a syringe pump to a solution of 1,4-dioxaspiro-[4.5]decan-8-one (**3**) (20.0 g, 128.1 mmol) and D-proline (1.5 g, 12.7 mmol) in DMF (250 mL) over 24 h at 0 °C, and the mixture was stirred for 30 min at that temperature. The reaction was quenched with pH 7.0 phosphate buffer solution, the organic materials were extracted with ethyl acetate (3×100 mL), the combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo after filtration. Purification by silica-gel column chromatography (hexane/EtOAc=10:1–4:1) gave (*S*)-7-Anilinoxy-1,4-dioxaspiro-[4.5]decan-8-one (**4**) (31.4 g, 119.1 mmol, 93%) as a pearl-yellow solid. $[\alpha]_D^{18}=-78.7$ ($c=1.2$, $CHCl_3$), >99% *ee* (the enantiomeric excess was determined by HPLC with a Chiralcel OD-H column (hexane/2-propanol 10:1), 0.5 mL min^{-1}; major enantiomer $t_r=29.1$ min, minor enantiomer $t_r=26.5$ min); IR (KBr): $\tilde{\nu}=2960$, 2888, 1728, 1602, 1494, 1305, 1122, 1052 cm^{-1}; ^1H NMR ($CDCl_3$) $\delta=1.88$–2.04 (2H, m), 2.16 (1H, t, $J=12.8$ Hz), 2.36–2.46 (2H, m), 2.62 (1H, dt, $J=14.0$, 6.8 Hz), 4.38–4.21 (4H, m), 4.60 (1H, dd, $J=12.9$, 6.5 Hz), 6.87 (2H, d, $J=7.7$ Hz), 6.90 (1H, t, $J=7.2$ Hz), 7.20 ppm (2H, t, $J=7.2$ Hz); ^{13}C NMR ($CDCl_3$): $\delta=$ 34.9, 36.0, 39.7, 64.8, 64.9, 82.7, 107.6, 114.5, 122.2, 128.9, 148.0, 208.6 ppm; HRMS (FAB): calcd for $C_{14}H_{17}NO_4$: 263.1158, found: 263.1172.

6: A solution of K-selectride in THF (1 M, 23.1 mL, 23.1 mmol) was added to a solution of α-aminoxy ketone **4** (3.0 g, 12.0 mmol) in THF (77 mL) at −78 °C, and the reaction temperature was increased to −50 °C over 1.5 h. $NaBO_3$ (10.8 g, 0.0701 mmol) and H_2O (23 mL) were added to the reaction mixture, and stirring was continued for 2 h at room temperature. The two phases were separated, and the aqueous phase was extracted with diethyl ether. The combined organic phase was washed with brine and dried over $MgSO_4$. The organic phase was concentrated in vacuo to give the alcohol **5** (4.6 g), which was used directly in the next reaction without purification. Pd/C (10 wt%; 304 mg, 0.29 mmol) was added to a solution of the crude alcohol **5** MeOH (38 mL). The reaction mixture was stirred under H_2 for 3 h at room temperature. Inorganic materials were removed by filtration through a celite pad, and the filtrate was concentrated in vacuo. The residue was purified by silica-gel column chromatography (hexane/EtOAc=3:1–1:3) to afford (7*S*,8*S*)-1,4-dioxaspiro[4.5]decane-7,8-diol (**6**) (1.7 g, 9.8 mmol, 85%) as a dark-red oil. $[\alpha]_D^{21}=+2.6$ ($c=1.5$, $CHCl_3$); IR (KBr): $\tilde{\nu}=3417$, 2958, 2935, 2888, 1442, 1144, 1101, 1059 cm^{-1}; ^1H NMR ($CDCl_3$): $\delta=1.40$–1.50 (1H, m), 1.60–1.80 (4H, m), 1.80–1.90 (1H, m), 3.20 (1H, s), 3.54 (1H, s), 3.67 (1H, s), 3.79 (1H, s), 3.82–3.88 ppm (4H, m); ^{13}C NMR ($CDCl_3$): $\delta=$ 26.5, 30.1, 37.5, 64.0, 64.2, 69.1, 70.0, 108.6 ppm; HRMS (FAB): calcd for $C_8H_{14}O_4Na$: 197.0785, found: 197.0784.

7: Amberlyst 15 (24.2 mg, 20 wt%) was added to a solution of diol **6** (118 mg, 0.677 mmol) in THF (9.7 mL), H_2O (0.97 mL), and acetone (0.97 mL), and the reaction mixture was stirred at 80 °C for 15 h. The reaction solution was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by silica-gel column chromatography (hexane/EtOAc=1:3) to afford (*R*)-4-hydroxy-2-cyclohexen-1-one (**7**) (64.6 mg, 0.576 mmol, 85%) as a dark red oil. $[\alpha]_D^{30}=+92.3$ ($c=0.7$, $CHCl_3$); IR (KBr): $\tilde{\nu}=3419$, 2954, 2871, 1660, 1205, 1066, 970, 943, 864 cm^{-1}; ^1H NMR ($CDCl_3$): $\delta=1.94$–2.02 (1H, m), 2.30–2.40 (2H, m), 2.45 (1H, s), 2.56 (1H, dt, $J=17.4$, 4.7 Hz), 4.56 (1H, ddd, $J=6.8$, 4.7, 2.2 Hz), 5.94 (1H, d, $J=10.0$ Hz), 6.92 ppm (1H, dt, $J=10.0$, 1.8 Hz); ^{13}C NMR ($CDCl_3$): $\delta=32.4$, 35.3, 66.3, 129.2, 152.9, 198.9 ppm.

(*R*)-*tert*-Butyldimethylsiloxy-2-cyclohexen-1-one: Imidazole (168 mg, 2.47 mmol) was added to a solution of enone **7** (100 mg, 0.892 mmol) and TBSCl (341 mg, 2.27 mmol) in DMF (1.8 mL) at 0 °C, and the reaction mixture was stirred for 1 h. The reaction was quenched with pH 7.0 phosphate buffer, and organic materials were extracted with EtOAc. The combined organic phases were washed with brine and dried over Na_2SO_4. The organic phase was concentrated in vacuo and purified by silica-gel column chromatography (hexane/EtOAc=30:1) to afford (*R*)-*tert*-butyldimethylsiloxy-2-cyclohexen-1-one (151 mg, 0.663 mmol, 75%) as a pearl-yellow oil. $[\alpha]_D^{30}=+97.9$ ($c=1.2$, $CHCl_3$); IR (KBr): $\tilde{\nu}=2954$, 2858, 1691, 1471, 1383, 1252, 1103, 860, 837 cm^{-1}; ^1H NMR ($CDCl_3$): $\delta=0.01$ (3H, s), 0.02 (3H, s), 0.81 (9H, s), 1.90–2.00 (1H, m), 2.10–2.20 (1H, m), 2.31 (1H, ddd, $J=16.7$, 12.7, 4.5 Hz), 2.53 (1H, dt, $J=16.7$, 4.5 Hz), 4.49 (1H, tt, $J=6.8$, 2.0 Hz), 5.88 (1H, d, $J=10.2$ Hz), 6.79 ppm (1H, dt, $J=$ 10.2, 2.0 Hz); ^{13}C NMR ($CDCl_3$): $\delta=-4.8$, −4.7, 18.0, 25.7, 32.9, 35.4, 66.9, 128.6, 153.9, 198.8 ppm.

8: H_2O_2 (0.88 mL, 7.63 mmol) and triton B (69 μL, 0.15 mmol) were added to a solution of (*R*)-*tert*-butyldimethylsiloxy-2-cyclohexen-1-one (345 mg, 1.52 mmol) in THF (9.5 mL) at 0 °C. The reaction mixture was stirred for 0.5 h at 0 °C and quenched with saturated aqueous NH_4Cl. The aqueous phase was extracted with EtOAc. The combined organic phases were washed with saturated aqueous $NaHCO_3$ and brine and dried over $NaSO_4$. The organic phase was concentrated in vacuo and purified by silica-gel column chromatography (hexane/EtOAc=50:1) to afford (2*S*,3*R*,4*R*)-4-*tert*-butyldimethylsiloxy-2,3-epoxycyclohexan-1-one (**8**) (280 mg, 1.16 mmol, 76%) as a colorless oil. $[\alpha]_D^{30}=-48.5$ ($c=1.0$, $CHCl_3$); IR (KBr): $\tilde{\nu}=2954$, 2858, 1716, 1473, 1362, 1254, 1092, 981, 839, 777 cm^{-1}; ^1H NMR ($CDCl_3$): $\delta=0.03$ (3H, s), 0.04 (3H, s), 0.81 (9H, s), 1.59–1.66 (1H, m), 1.97–2.05 (1H, m), 2.20–2.35 (2H, m), 3.18 (1H, d, $J=3.9$ Hz), 3.38 (1H, t, $J=3.1$ Hz), 4.38 ppm (1H, dd, $J=6.8$, 3.1 Hz); ^{13}C NMR ($CDCl_3$): $\delta=-5.0$, −4.9, 17.9, 25.4, 25.5, 31.5, 54.8, 57.9, 65.1, 204.7 ppm.

9: *n*-Butyllithium (0.8 mL, 1.55 M in hexane) was added to a stirred solution of HMDS (0.31 mL, 1.5 mmol) in THF (4.1 mL) at 0 °C. After 30 min the reaction mixture was cooled to −78 °C, and a solution of epoxide **8** (100 mg, 0.413 mmol) in THF (1.0 mL) was added. TMSCl (0.26 mL, 2.1 mmol) was then added at −78 °C, and the reaction temperature was increased to −25 °C over 1.5 h. Inorganic materials were removed by filtration through a celite pad, and the filtrate was concentrated in vacuo to give the TMS ether (233.0 mg), which was used directly in the next reaction without purification. $[Pd_2(dba_3)]\cdot CHCl_3$ (60.3 mg, 0.0583 mmol) and diallylcarbonate (56 μL, 0.39 mmol) was added to a solution of the TMS ether in MeCN (6.9 mL), and the mixture was stirred for 4 h at room temperature. The reaction mixture was quenched with saturated aqueous $NaHCO_3$, and the organic materials were extracted with $CHCl_3$ (3×5 mL). The combined organic phases were washed with brine and dried over Na_2SO_4. The organic phase was concentrated in vacuo and was purified by silica-gel column chromatography (hexane/EtOAc= 50:1) to afford (2*S*,3*R*,4*R*)-4-*tert*-butyldimethylsiloxy-2,3-epoxy-5-cyclohexen-1-one (**9**) (77.4 mg, 0.322 mmol, 78%) as a colorless oil. $[\alpha]_D^{20}=$

−265 (c=1.1, $CHCl_3$); IR (KBr): $\tilde{\nu}$=2956, 2931, 2858, 1693, 1261, 1092, 839, 806, 779 cm^{-1}; ^1H NMR ($CDCl_3$): δ=0.12 (3H, s), 0.15 (3H, s), 0.89 (9H, s), 3.42–3.42 (1H, m), 3.60–3.62 (1H, m), 4.62–4.63 (1H, m), 5.96 (1H, dt, J=10.5, 1.3 Hz), 6.53 ppm (1H, ddd, J=10.5, 4.5, 2.7 Hz); ^{13}C NMR ($CDCl_3$): δ=−4.7, −4.5, 18.1, 25.6, 53.3, 58.4, 63.6, 126.2, 144.3, 193.2 ppm.

10: A solution of iodine (305 mg, 1.20 mmol) in Et_2O (1.5 mL) and pyridine (1.5 mL) was stirred at 0°C for 20 min in the dark. Enone **9** (144 mg, 0.600 mmol) was added to the reaction mixture at 0°C, and the reaction temperature was raised to room temperature over 2 h. The reaction mixture was quenched with saturated aqueous $Na_2S_2O_3$, and the organic materials were extracted with EtOAc. The combined organic phases were washed with brine and dried over Na_2SO_4. The organic phase was concentrated in vacuo and purified by silica-gel column chromatography (hexane/EtOAc=50:1) to afford (2*S*,3*R*,4*R*)-4-*tert*-butyldimethylsiloxy-2,3-epoxy-6-iodo-5-cyclohexen-1-one (**10**) (178 mg, 0.485 mmol, 80%) as a colorless oil. $[\alpha]_D^{22}$=−105.6 (c=1.2, $CHCl_3$); IR (KBr): $\tilde{\nu}$=2954, 2858, 1697, 1257, 1092, 872, 835, 781 cm^{-1}; ^1H NMR ($CDCl_3$): δ=0.16 (3H, s), 0.18 (3H, s), 0.92 (9H, s), 3.62–3.63 (1H, m), 3.68–3.69 (1H, m), 4.59–4.60 (1H, m), 7.28 ppm (1H, dd, J=5.0, 2.4 Hz); ^{13}C NMR ($CDCl_3$): δ=−4.7, −4.5, 18.1, 25.6, 51.7, 58.2, 66.1, 101.9, 152.6, 187.5 ppm; HRMS (FAB): calcd for $C_{12}H_{20}IO_3Si$: 367.0227, found: 367.0249.

12: $[Pd_2(dba_3)]\cdot CHCl_3$ (8.2 mg, 8.2 μmol), $AsPh_3$ (7.8 mg, 0.026 mmol), and toluene (0.2 mL) were stirred for 20 min at room temperature. A solution of iodoenone **10** (30 mg, 0.08 mmol) and vinyl stannane **11** (37.6 mg, 0.1 mmol) in toluene (0.5 mL) were added to the reaction mixture, which was stirred at 110°C for 5 min in toluene. After cooling, inorganic materials were removed by filtration through a celite pad, the filtrate was concentrated in vacuo, and the residue was purified by silica-gel column chromatography (hexane/EtOAc=5:1) to afford (2*S*,3*R*,4*R*)-4-*tert*-butyldimethylsiloxy-2,3-epoxy-6-(3-hydroxy-3-methylbutenyl)-5-cyclohexen-1-one (**12**) (19.8 mg, 0.0610 mmol) in 77% yield as a colorless oil. Enone **12** is unstable, therefore it was used immediately in the next reaction. ^1H NMR ($CDCl_3$): δ=0.13 (3H, s), 0.16 (3H, s), 0.90 (9H, s), 1.33 (3H, s), 1.35 (3H, s), 3.46 (1H, d, J=3.3 Hz), 3.60–3.65 (1H, m), 4.72 (1H, d, J=4.9 Hz), 6.28 (1H, d, J=16.1 Hz), 6.38 (1H, s), 6.41 ppm (1H, d, J=16.1 Hz).

1: Excess NH_4F (23.7 mg, 0.641 mmol) was added to a solution of **12** (19.8 mg, 0.0610 mmol) in MeOH (3 mL) at room temperature. The reaction mixture was stirred at room temperature for 12 h and then concentrated in vacuo. The residue was purified by thin-layer chromatography (MeOH/$CHCl_3$ 1:10) to afford monomer **2** (9.3 mg, 0.044 mmol) in 75% yield. The monomer **2** was allowed to stand at room temperature for 33 h and purified thin-layer chromatography (MeOH/$CHCl_3$ 1:10) to afford panepophenanthrin (**1**) (9.2 mg, 0.02 mmol, 95%) as a white solid. Monomer (**2**) ^1H NMR ($CDCl_3$): δ=1.04 (3H, s), 1.05 (3H, s), 3.31 (1H, d, J=3.5 Hz), 3.53–3.55 (1H, m), 4.50 (1H, d, J=5.1 Hz), 6.05 (1H, d, J=16.1 Hz), 6.18 (1H, d, J=16.1 Hz), 6.32 ppm (1H, dd, J=5.1, 2.4 Hz). Panepophenanthrin (**1**): $[\alpha]_D^{24}$=+147.2 (c=0.91, MeOH); lit. $[\alpha]_D^{26}$=+149.8 (c=1.0, MeOH);[11] IR (KBr): $\tilde{\nu}$=2978, 1676, 1597, 1338, 1142, 997 cm^{-1}; ^1H NMR ($CDCl_3$): δ=1.17 (3H, s), 1.20 (3H, s), 1.35 (3H, s), 1.45 (3H, s), 2.03 (1H, br d, J=9.7 Hz), 2.32 (1H, br d, J=10.0 Hz), 3.31 (1H, d, J=4.0 Hz), 3.35 (1H, dd, J=5.0, 1.6 Hz), 3.42 (1H, d, J=4.0 Hz), 3.50 (1H, t, J=3.2 Hz), 3.84 (1H, t, J=3.4 Hz), 4.35 (1H, br s), 4.55 (1H, br s), 5.68 (1H, d, J=16.2 Hz), 5.99 (1H, d, J=16.2 Hz), 6.81 ppm (1H, dd, J=5.0, 3.0 Hz); ^{13}C NMR ($CDCl_3$): δ=26.2, 29.5, 30.3, 32.3, 50.0, 51.2, 55.1, 55.6, 57.1, 57.2, 57.4, 60.7, 66.2, 69.0, 71.8, 79.2, 102.7, 129.3, 138.8, 139.9, 143.0, 196.3 ppm.

16: $[Pd(PhCN)_2Cl_2]$ (7.4 mg, 0.02 mmol) was added to a solution of iodoenone **10** (23.5 mg, 0.0642 mmol), 1-propen-1-ylboronic acid (**13**) (11.0 mg, 0.128 mmol), Ag_2O (23.8 mg, 0.103 mmol), and $AsPh_3$ (11.8 mg, 0.04 mmol) in THF/H_2O (8:1, 1.4 mL), and the reaction mixture was stirred at room temperature for 30 min in the dark. Saturated aqueous NH_4Cl (5 mL) was added to the reaction mixture, which was stirred for 1 h at that temperature. The organic materials were extracted with EtOAc (5 mL). The combined organic phases were washed with brine and dried over Na_2SO_4. The organic phase was concentrated in vacuo and purified by thin-layer chromatography (hexane/EtOAc=30:1) to afford (2*S*,3*R*,4*R*)-6-butenyl-4-*tert*-butyldimethylsiloxy-2,3-epoxy-5-cyclohexen-1-one (**16**) (16.5 mg, 0.0590 mmol, 92%) as a colorless oil. As **16** was unstable, it was used immediately in the next reaction. ^1H NMR ($CDCl_3$): δ=0.12 (3H, s), 0.15 (3H, s), 0.90 (9H, s), 1.79 (3H, d, J=6.4 Hz), 3.42 (1H, d, J=4.0 Hz), 3.62 (1H, s), 4.71 (1H, d, J=4.0 Hz), 6.10 (1H, d, J=16.2 Hz), 6.19–6.26 (1H, m), 6.30–6.40 ppm (1H, m).

19: Excess NH_4F (20.5 mg, 0.554 mmol) was added to a solution of the siloxy monomer **16** (15.5 mg, 0.0553 mmol) in MeOH (3 mL) at room temperature. The reaction mixture was stirred at room temperature for 12 h and then concentrated in vacuo. The residue was purified by thin-layer chromatography (MeOH/$CHCl_3$=1:1) to afford the monomer. The monomer was allowed to stand at room temperature for 5 h to afford RKTS-80 (**19**) (15.0 mg, 0.05 mmol) in 80% yield as a white solid. $[\alpha]_D^{22}$=+83.1 (c=0.1, $CHCl_3$); IR (KBr): $\tilde{\nu}$=3419, 2923, 2854, 1698, 1633, 1455, 1259, 1085 cm^{-1}; ^1H NMR ($CDCl_3$): δ=0.87 (3H, d, J=7.2 Hz), 1.67 (3H, dd, J=6.5, 1.5 Hz), 2.35–2.38 (1H, m), 2.60–2.70 (1H, m), 2.90–3.00 (1H, m), 3.23 (1H, d, J=3.4 Hz), 3.29 (1H, d, J=3.4 Hz), 3.50 (1H, d, J=3.4 Hz), 3.52 (1H, d, J=3.4 Hz), 3.91 (1H, d, J=9.0 Hz), 4.58 (1H, d, J=4.2 Hz), 5.20–5.40 (1H, m), 5.56 (1H, dd, J=16.2, 1.5 Hz), 6.60 ppm (1H, dd, J=4.7, 2.3 Hz); ^{13}C NMR ($CDCl_3$): δ=15.2, 18.4, 29.6, 34.5, 44.8, 53.3, 53.6, 58.1, 61.9, 66.5, 70.6, 76.4, 129.1, 131.0, 131.8, 142.6, 195.4, 203.0 ppm; HRMS (FAB): calcd for $C_{18}H_{21}O_6$: 333.1338, found: 333.1357.

(2*S*,3*R*,4*R*)-4-*tert*-Butyldimethylsiloxy-2,3-epoxy-6-hexenyl-5-cyclohexen-1-one (**17**), (2*S*,3*R*,4*R*)-4-*tert*-butyldimethylsiloxy-6-decenyl-2,3-epoxy-5-cyclohexen-1-one (**18**), RKTS-81 (**20**), and RKTS-82 (**21**) were prepared by the same procedure as that for RKTS-80 (**19**). The physical data for these compounds are detailed below.

17: ^1H NMR ($CDCl_3$): δ=0.13 (3H, s), 0.15 (3H, s), 0.90 (9H, s), 1.21–1.40 (9H, m), 3.51 (1H, d, J=4.4 Hz), 3.62 (1H, s), 4.71 (1H, d, J=4.4 Hz), 6.08 (1H, d, J=16.1 Hz), 6.18–6.32 (1H, m), 6.30–6.37 ppm (1H, m).

20: $[\alpha]_D^{21}$=+25.8 (c=0.2, $CHCl_3$); IR (KBr): $\tilde{\nu}$=3444, 2927, 2857, 1698, 1635, 1465, 1268 cm^{-1}; ^1H NMR ($CDCl_3$): δ=0.80–0.90 (6H, m), 1.20–1.28 (12H, m), 2.47 (1H, dd, J=6.0, 3.8 Hz), 2.72–2.79 (1H, m), 2.80–2.87 (1H, m), 3.29 (1H, d, J=3.3 Hz), 3.33 (1H, d, J=3.3 Hz), 3.53 (1H, d, J=3.3 Hz), 3.56 (1H, d, J=3.3 Hz), 4.03 (1H, d, J=9.2 Hz), 4.75 (1H, d, J=3.8 Hz), 5.30 (1H, td, J=16.4, 6.8 Hz), 5.59 (1H, d, J=16.4 Hz), 6.78 ppm (1H, dd, J=2.8, 2.1 Hz); ^{13}C NMR ($CDCl_3$): δ=13.8, 22.2, 22.6, 29.5, 29.7, 30.4, 31.0, 32.6, 39.9, 44.6, 47.0, 53.3, 53.4, 53.9, 57.5, 61.7, 67.4, 71.6, 130.1, 131.6, 134.8, 142.3, 194.5, 202.4 ppm; HRMS (FAB): calcd for $C_{24}H_{33}O_6$: 417.2277, found: 417.2265.

18: ^1H NMR ($CDCl_3$): δ=0.12 (3H, s), 0.15 (3H, s), 1.00 (9H, s), 1.15–1.30 (17H, m), 3.49 (1H, d, J=4.1 Hz), 3.62 (1H, s), 4.71 (1H, d, J=4.1 Hz), 6.08 (1H, d, J=15.8 Hz), 6.17–6.28 (1H, m), 6.30–6.37 ppm (1H, m).

21: $[\alpha]_D^{23}$=+33.8 (c=0.1, $CHCl_3$); IR (KBr): $\tilde{\nu}$=3434, 2925, 2854, 1702, 1465, 1270, 1054 cm^{-1}; ^1H NMR ($CDCl_3$): δ=0.84–0.87 (6H, m), 1.00–1.06 (28H, m), 2.46 (1H, dd, J=6.3, 4.0 Hz), 2.72–2.80 (1H, m), 2.80–2.86 (1H, m), 3.28 (1H, d, J=3.3 Hz), 3.32 (1H, d, J=3.3 Hz), 3.53 (1H, d, J=3.3 Hz), 3.55 (1H, d, J=3.3 Hz), 4.03 (1H, d, J=9.2 Hz), 4.75 (1H, d, J=4.0 Hz), 5.30 (1H, td, J=6.9, 16.3 Hz), 5.58 (1H, d, J=16.3 Hz), 6.78 ppm (1H, dd, J=5.0, 2.1 Hz); ^{13}C NMR ($CDCl_3$): δ=14.1, 22.6, 27.4, 28.9, 29.15, 29.22, 29.3, 29.4, 29.6, 29.7, 30.8, 31.8, 33.0, 40.0, 44.7, 47.1, 53.3, 53.5, 53.9, 57.5, 61.8, 67.5, 71.7, 130.2, 131.6, 134.9, 142.2, 194.5, 202.5 ppm; HRMS (FAB): calcd for $C_{32}H_{49}O_6$: 529.3529, found: 529.3504.

Effect of Solvent and Time on Yield of Diels–Alder Reaction of 2

Three vessels were prepared, in each of which was dissolved monomer **2** (2 mg, 0.01 mmol) in solvent (0.2 mL). The reactions were performed for 5, 22, and 33 h, respectively, at room temperature. Each reaction solution was then concentrated in vacuo, and the NMR spectra were measured in $CDCl_3/CD_3OD$ (10:1). The yield of the dimer was determined by the integral ratio of δ=6.81 (1H, dd, J=5.0, 3.0 Hz) and 6.32 ppm (1H, dd, J=5.1, 2.4 Hz).

Measurement of E1 Activity

The E1 activity was measured on the basis of the formation of the E1–ubiquitin intermediate from E1 and ubiquitin in the presence of ATP. Various concentrations of test compounds were added to 10 μL of the reaction mixture (100 mM tris–HCl pH 9.0, 5 mM $MgCl_2$, 1 mM DTT, 2.5 mM ATP) containing 10 $\mu g mL^{-1}$ of E1 enzyme (BostonBiochem, Boston, MA). After incubation for 15 min at room temperature, 100 ng of biotinylated ubiquitin (BostonBiochem) was added to the reaction mixture, and the resulting mixture was incubated further at 37°C for 15 min. The reaction was terminated by boiling with Laemmli loading buffer. The mixture (10 μL) was loaded on an SDS 7.5% polyacrylamide gel, and electrophoresis was carried out under nonreducing conditions. The proteins were electrically transferred to a PVDF membrane (Millipore, Boston, MA). The membrane was blocked and incubated with streptavidin-conjugated horseradish peroxidase to detect the biotinylated ubiquitin by the enhanced chemiluminescence method (SuperSignal WestPico, Pierce Biotechnology, Rockford, IL). The bands of ubiquitiylated E1 were quantified by Scanning Imager (Molecular Dynamics).

Cell Proliferation Assay in MCF-7 Cells

Human breast cancer MCF-7 cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in an RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum. The cells were seeded at 3×10^3 cells/well in a 96-well plate. After incubating for 18 h at 37°C, various concentrations of test compounds were added, and further incubated for 48 h at 37°C. The cell number was evaluated by the subsequent color reaction. WST-8 solution 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (Nakalai tesque, Kyoto) was added to the medium, and the cells were further incubated for 3 h at 37°C. The absorbance (A_{450}) of each well was measured by using a plate reader (Wallac 1420 multilabel counter) (GE Healthcare Biosciences KK, Tokyo). Cell number (%) was calculated as (experimental absorbance−background absorbance)/(control absorbance−background absorbance)×100.

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Computational Study on the Reaction Mechanism of the Key Thermal [4 + 4] Cycloaddition Reaction in the Biosynthesis of Epoxytwinol A

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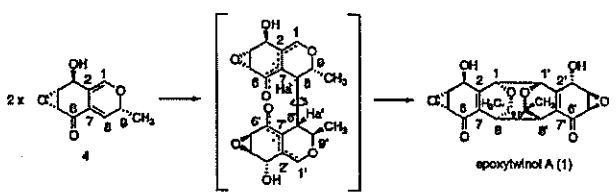
ABSTRACT



The key [4 + 4] cycloaddition in the biosynthesis of epoxytwinol A has been established by theoretical calculations to comprise of three processes. The first step is formation of the C8–C8′ bond generating a biradical intermediate. Next, rotation about the C8–C8′ bond occurs, and finally the C1–C1′ bond is formed. Biradicals stabilized by conjugation and two hydrogen bonds are essential for realization of this rare thermal [4 + 4] cycloaddition.

The [4 + 4] cycloaddition reaction[1] generally proceeds under photoirradiation or in the presence of transition-metal catalysts,[2] and genuine thermal [4 + 4] cycloadditions are predicted to be difficult by the Woodward−Hoffmann rules.[3] However, a few exceptional thermal [4 + 4] cycloaddition reactions are known: Highly reactive *o*-quinodimethane and its derivatives[4] dimerize, while *o*-quinodimethane[5] and corrole[6] react with anthracene or pentacene in a [4 + 4] manner. The dimerization has been proposed to proceed via a biradical mechanism,[4b,c,f] while the mechanism of the latter two reactions has not been investigated in detail.

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We have recently isolated a novel pentaketide dimer and angiogenesis inhibitor epoxytwinol A (**1**)[7] from a fungus which also produces structurally different angiogenesis inhibitors such as epoxyquinols A (**2**)[8] and B (**3**) (Figure 1).[9] The latter are thought to be biosynthesized from a 2*H*-pyran monomer by endo and exo Diels–Alder reaction, respectively, while epoxytwinol A (**1**) would be generated by [4 + 4] cycloaddition reaction of the monomer **4**. Epoxytwinol A (**1**) is found to convert gradually into epoxyquinol B (**3**) at room temperature. Recently, we have synthesized all these compounds by biomimetic pathways from **4**,[10] with epoxytwinol A (**1**) being prepared in the dark, indicating that the [4 + 4] cycloaddition proceeds thermally without photoactivation. Li and Porco have also elegantly synthesized epoxytwinol A (**1**) using alkoxysilanol activation.[10j,11] Epoxytwinol A (**1**) is the first natural product believed to be biosynthesized via the exceptionally rare thermal [4 + 4] cycloaddition, and the mechanism of this reaction is of great interest. In this paper, we disclose the results of our theoretical calculations on this process and on the mechanism of transformation of epoxytwinol A (**1**) to epoxyquinol B (**3**).

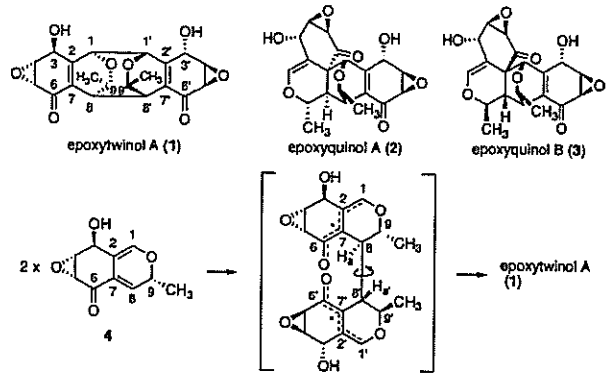

Figure 1. Epoxytwinol A (**1**), epoxyquinols A (**2**) and B (**3**), and the reaction path to epoxytwinol A.

In view of the Woodward–Hoffmann rules,[3] a stepwise rather than concerted mechanism is expected for a thermal [4 + 4] cycloaddition reaction. Indeed, stepwise biradical mechanisms have been proposed for the [4 + 4] dimerization of *o*-quinodimethane and its derivatives.[4b,c,f] The density functional B3LYP treatment with the unrestricted formalism (UB3LYP)[12] provides relatively reasonable results for the energy of biradical or biradicaloid species, and the UB3LYP method[13] has been successfully employed to investigate reaction paths involving biradical species or to compare the energetics for stepwise, biradical reaction paths with those for concerted, closed-shell paths.[14] We therefore employed this method in conjunction with the 6-31G(d) basis set to locate the stationary points along the reaction coordinate for the [4 + 4] cycloaddition reaction.[15]

Unrestricted B3LYP solutions were obtained from a HOMO–LUMO mixed initial guess. For the reactant monomer **4**, and the reaction products **1** through **3**, as well as for the reactant complexes, the eigenvalues of S^2 for UB3LYP calculations were zero, and the total energies were exactly the same as obtained with the corresponding restricted

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