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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

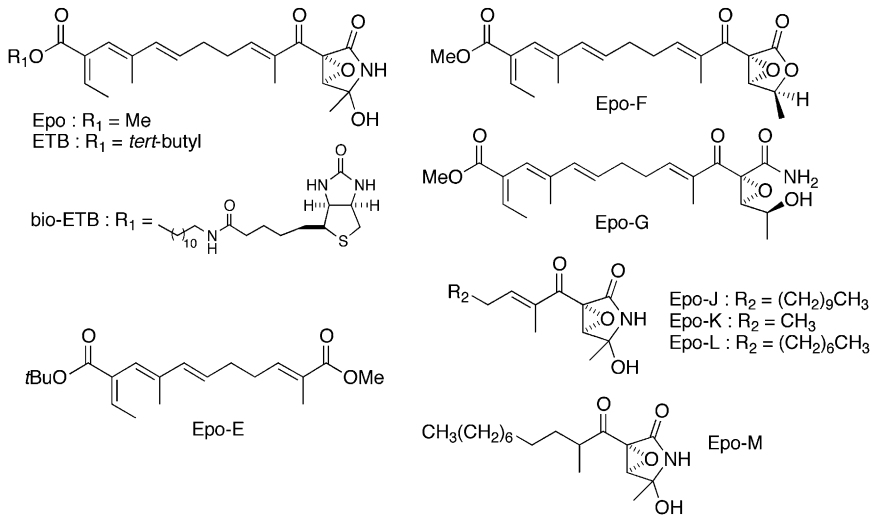
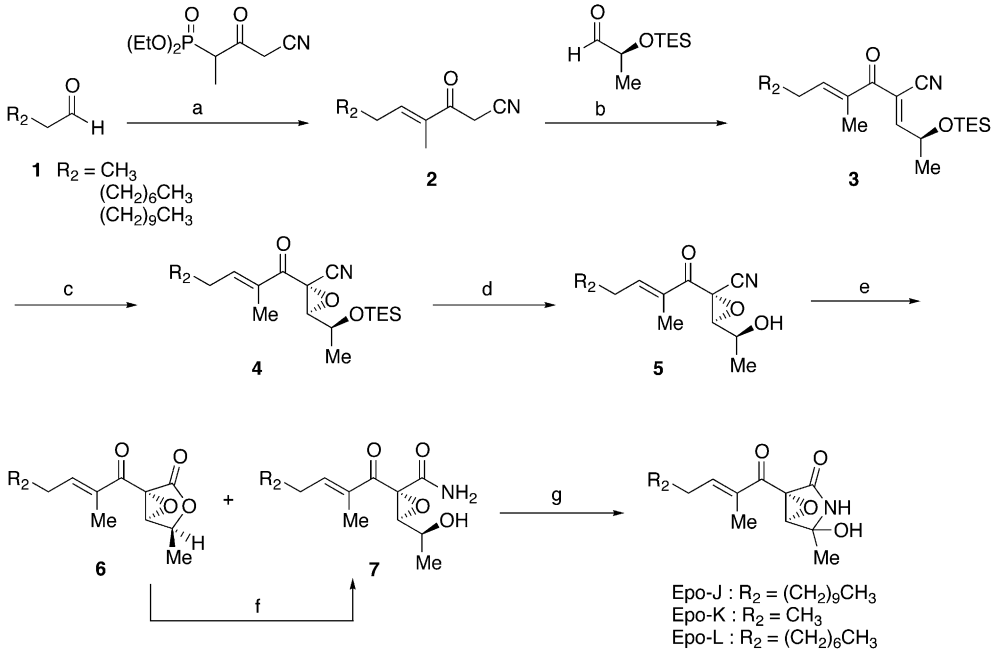


Figure 1. Structures of epolactaene and its derivatives used in this study.



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.

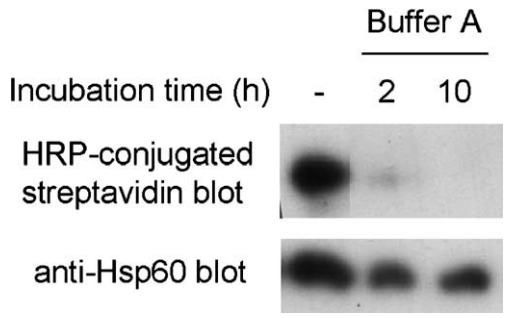


Figure 2. Reversible binding between bio-ETB and Hsp60 protein. Hsp60-His_6 protein (14 μM) in PBS was pretreated with 1.5 equiv bio-ETB. The mixture was then diluted 10-fold in buffer A (8 M guanidium–HCl, 0.5 M Tris(hydroxymethyl)aminomethane–HCl, pH 8.5, 1% dithiothreitol) and incubated for indicated 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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