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Synthesis and biological activities of chaetocin and its derivatives*

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Abstract: Chaetocin, a natural product isolated from fungi of *Chaetomium* species, is a member of the epipolythiodiketopiperazines (ETPs), which have various biological activities, including cytostatic and anticancer activities. Recently, the inhibitory activity toward histone methyltransferases (HMTs) was discovered for chaetocin. We previously reported the first total synthesis of chaetocin and various derivatives. During studies on the structure–activity relationship for HMT inhibition, we found that the enantiomer of chaetocin (*ent*-chaetocin) is a more potent apoptosis inducer than natural chaetocin in human leukemia HL-60 cells. Mechanistic studies showed that *ent*-chaetocin induces apoptosis through the caspase-8/caspase-3 pathway.

Keywords: apoptosis; anticancer activity; antitumor activity; bioactive molecules; chaetocin; histone methyltransferase.

INTRODUCTION

Chaetocin (**1**) is a natural product isolated from the fungus *Chaetomium minutum* [1,2]. It is a member of the epipolythiodiketopiperazine (ETP) class of natural products (Fig. 1), which exhibit various biological activities, such as antitumor and antifungal activities, by interacting with a range of proteins [3,4]. Among them, chaetocin was reported to inhibit histone methyltransferases (HMTs), which catalyze the methylation of histone proteins [5]. Histone methylation plays an important role in the control of chromatin architecture, which in turn is related to epigenetic control. Indeed, HMTs are now thought to be involved in a broad range of biological phenomena [6], so selective inhibitors of HMTs would be useful tools for epigenetic research, as well as candidate therapeutic agents for epigenetic diseases. Recently, chaetocin was reported to induce remodeling of the nuclear architecture in fibroblasts [7]. Considering the importance and potential clinical significance of HMT inhibitors, we set out to synthesize chaetocin, and achieved the first total synthesis [8,9].

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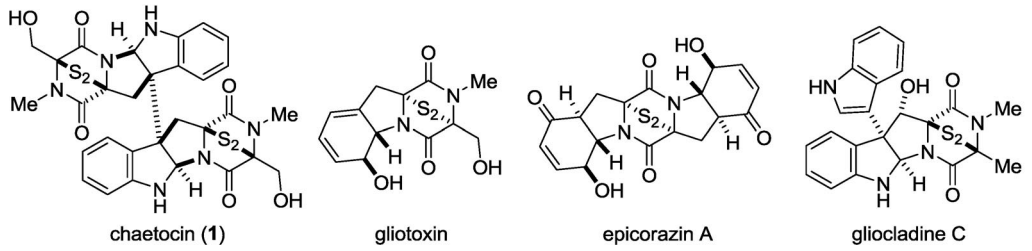


Fig. 1 ETP compounds isolated from various fungal species.

In order to investigate the structure–activity relationship for HMT-inhibitory activity, we also synthesized various derivatives (Fig. 2) [8,9]. During this work, we found that the unnatural enantiomer of chaetocin (*ent*-**1**) showed stronger apoptosis-inducing activity than natural chaetocin, and this activity was not related to HMT inhibition [10]. Here, we review the biological activities of chaetocin and its derivatives.

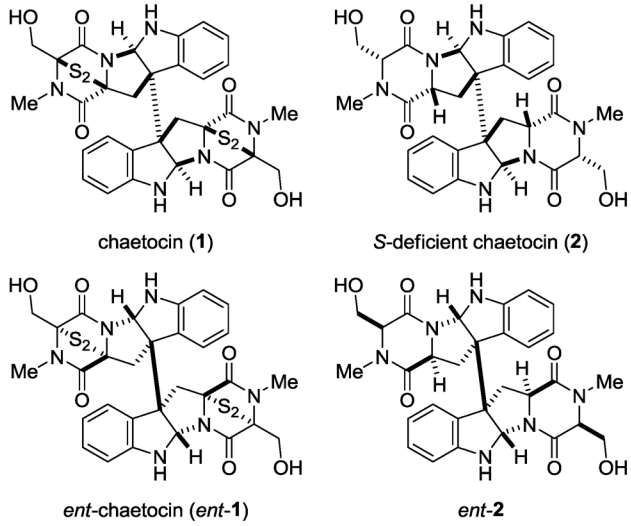
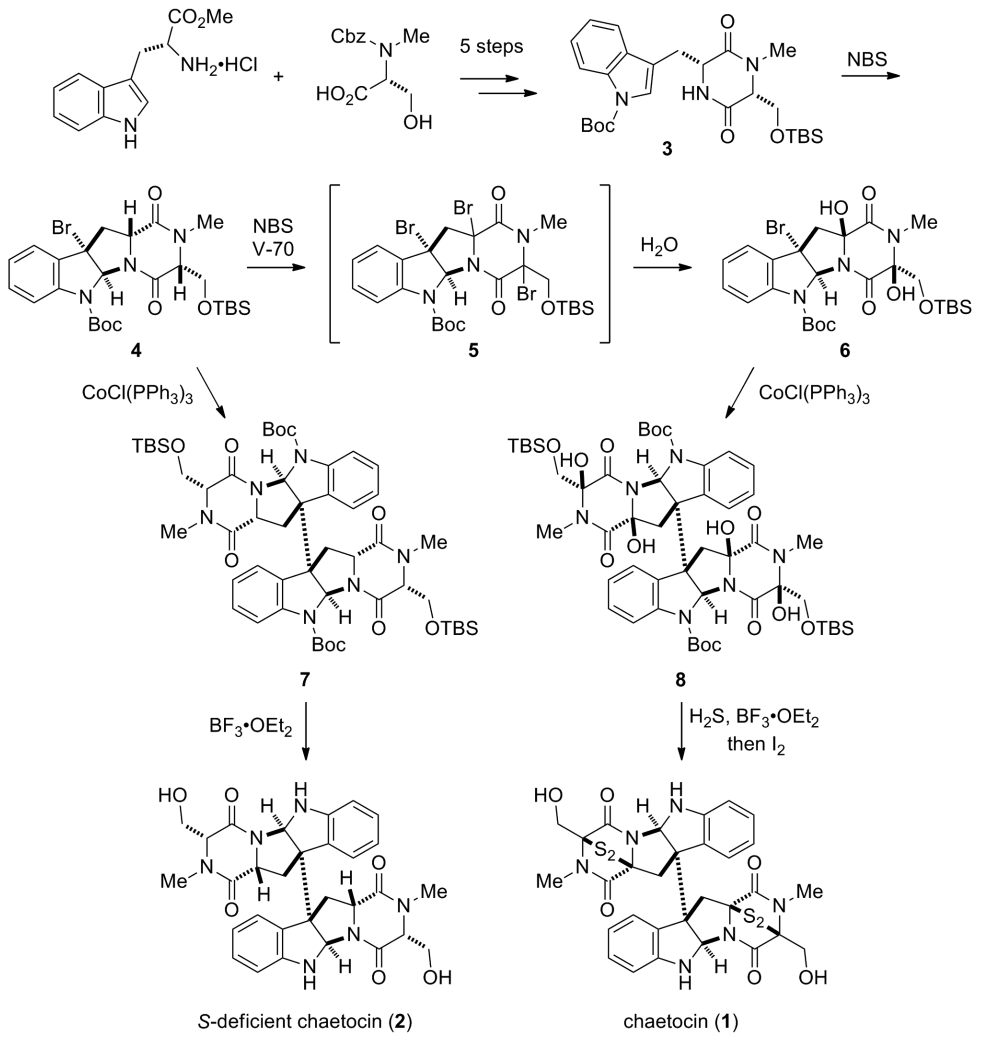


Fig. 2 Structures of chaetocin and its derivatives.

TOTAL SYNTHESIS OF CHAETOCIN AND ITS DERIVATIVES AND THEIR HMT-INHIBITORY ACTIVITY

There have been several synthetic studies of ETPs [11–18], but we were the first to report total synthesis of chaetocin [8,9,19]. The most difficult aspect was to introduce the sulfur functionality in the presence of the hydroxymethyl moieties, which are easily eliminated by base treatment. Thus, we employed an oxidation step using radical bromination-hydrolysis reaction before dimerization. As shown in Scheme 1, we synthesized oxidized diketopiperazine **6** as a dimerization precursor. It was dimerized by Co(I)-mediated radical coupling [11], and finally simultaneous deprotection and sulfur-installation provided chaetocin **1** in only 9 steps [8,9]. The enantiomer *ent*-**1** was similarly synthesized. The *S*-deficient derivatives **2** and *ent*-**2** were also obtained from **7** and its enantiomer.



Scheme 1 Synthesis of chaetocin and *S*-deficient chaetocin.

With chaetocin and its derivatives in hand, we investigated their inhibitory activity toward HMTs. Based on the previous report [20], we examined the inhibition of G9a, one of the HMTs. Chaetocin and its enantiomer were found to show similar activities, while in contrast, sulfur-deficient derivative **2** and its enantiomer *ent*-**2** showed no inhibition of G9a [8]. These results indicated that G9a does not recognize the chirality of chaetocin, whereas the disulfide structure of chaetocin is essential for G9a inhibition. Further studies on the structure–activity relationship of chaetocin derivatives for G9a inhibition are in progress.

INDUCTION OF CELL DEATH BY CHAETOCIN AND ITS DERIVATIVES

In addition to HMT inhibition, ETP compounds were reported to have cytostatic and cytotoxic activities [3,4]. Bible et al. reported selective in vitro and vivo anticancer activity of chaetocin, suggesting that it might be a candidate antimyeloma therapeutic [21]. Imhof et al. reported the HMT-inhibitory activity of chaetocin, but speculated that the cell-death-inducing activity might involve other targets [5].

Therefore, to clarify the relationship between HMT inhibition and cell death induction, we investigated the cyotoxicity of chaetocin derivatives [10].

ent-Chaetocin induced both apoptosis and necrosis of HL-60 cells

Using a human leukemia HL-60 cell assay system [22,23], we examined the cyotoxicity of chaetocin and its derivatives. In the presence or absence of test compounds, cell viability was determined by measuring the fluorescence of alamarBlue, which is reduced to the fluorescent form in live cells. As shown in Fig. 3A, both chaetocin and *ent*-chaetocin equally reduced cell viability at high concentration (30 μM). Interestingly, the viability of *ent*-chaetocin-treated cells showed some recovery in the concentration range from 0.3 to 3 μM, but this was not the case with chaetocin.

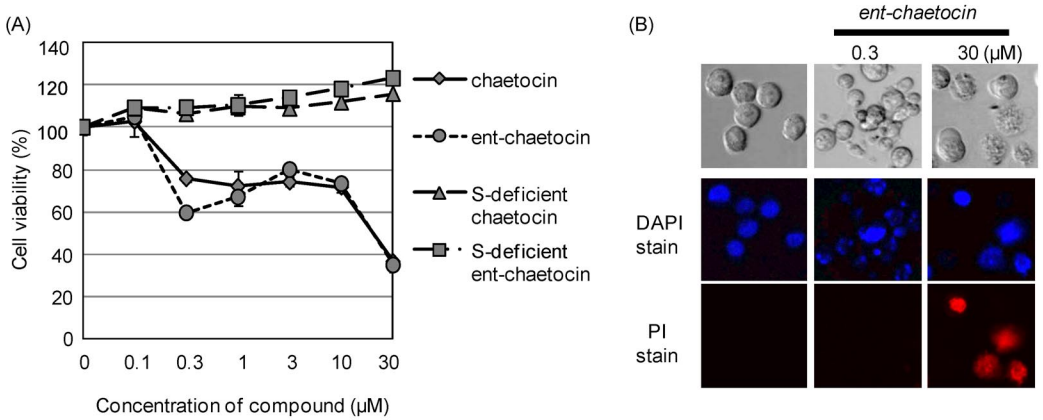


Fig. 3 (A) Effect of chaetocin derivatives on cell viability. HL-60 cells were treated with chaetocin derivatives for 4 h, and cell viability was determined by alamarBlue assay. (B) Morphological changes induced by *ent*-chaetocin. HL-60 cells treated with *ent*-chaetocin for 4 h were stained with DAPI/PI. Nuclear fragmentation (DAPI, blue) as an indicator of apoptosis and PI-stained nuclei (PI, red) as an indicator of necrosis were observed using confocal laser microscopy.

To investigate this phenomenon more precisely, we examined the morphological changes at 0.3 and 30 μM (Fig. 3B). *Ent*-chatocin was found to induce both apoptosis and necrosis, depending on the concentration. At high concentration, it induced necrosis in the same manner as chaetocin. In contrast, at low concentration, it induced typical apoptosis more potently than chaetocin. In comparison with G9a-inhibitory activity, it appears that the apoptosis-inducing activity involves other targets, which can recognize the chirality of chaetocin. Therefore, we examined the molecular mechanism of apoptosis induced by *ent*-chaetocin.

ent-Chaetocin-induced apoptosis through the caspase-8/caspase-3 pathway

To clarify the mechanism of apoptosis induced by *ent*-chaetocin, we examined the effects of known cell death inhibitors. Z-VAD-fmk, a general caspase inhibitor, inhibited apoptosis induced by *ent*-chaetocin, indicating that caspases are involved in *ent*-chaetocin-induced apoptotic signaling. To confirm this, we examined the activity of caspase-3, an apoptosis-executing caspase, and found that it was activated by *ent*-chaetocin treatment, as expected. Furthermore, we investigated the effects of subtype-selective caspase inhibitors. There are two major pathways for activation of caspase-3: (i) the death-ligand-induced pathway mediated by caspase-8, (ii) the mitochondrial pathway mediated by caspase-9 [24].

Therefore, Z-IETD-fmk (caspase-8 inhibitor) and Z-LEHD-fmk (caspase-9 inhibitor) were tested for inhibitory activity against *ent*-chaetocin-induced caspase-3 activation and apoptosis. We found that Z-IETD-fmk, but not Z-LEHD-fmk, inhibited both caspase-3 activation and apoptosis. In addition, caspase-8 was activated during *ent*-chaetocin-induced apoptosis. These results indicate that the caspase-8/caspase-3 activation pathway predominantly contributes to *ent*-chaetocin-induced apoptosis.

Figure 4 summarizes the major pathways involved in apoptosis and the possible site of action of *ent*-chaetocin. Most anticancer drugs induce apoptosis mainly through the mitochondrial pathway mediated by cytochrome c release and caspase-9 activation, and the mitochondrial pathway is inactivated in various drug-resistant tumor cells [25]. Therefore, *ent*-chatocin may target a novel component involved in activation of the caspase-8 pathway.

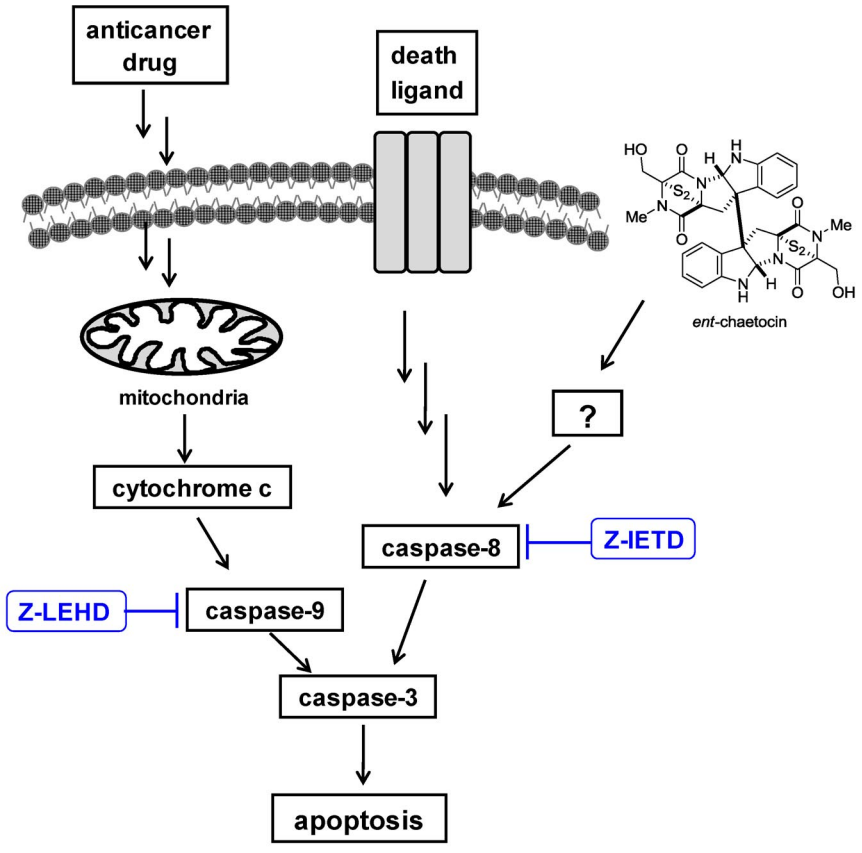


Fig. 4 Apoptosis signaling pathways and effect of *ent*-chaetocin.

Reactive oxygen species (ROS) and cell death

The next step was to identify the direct target of *ent*-chaetocin. In general, disulfide bonds in ETP compounds are easily reduced to dithiol by cellular reductants, such as glutathione or cysteine. Reduced dithiol is reported to be auto-oxidized to disulfide, coupled with the production of reactive oxygen species (ROS), such as superoxide anion or hydrogen peroxide. Redox cycling between disulfide and dithiol increases cellular ROS and induces oxidative stress, leading to cell death [26,27]. The redox-cycle-mediated mechanism need not involve a specific binding protein for ETP compounds. On the other hand, some ETP-binding proteins were also reported to mediate cell death induction [28,29]. The

possible coexistence of two mechanisms, namely, a binding-protein-mediated mechanism and a non-enzymatic redox-cycle-mediated mechanism, makes the mechanistic study of ETP compounds difficult. Considering the chirality-dependency, we speculated that some binding protein(s) would be involved in induction of apoptosis by *ent*-chaetocin. Therefore, we examined the effects of the two enantiomers on cellular redox homeostasis in order to exclude the possibility of non-enzymatic redox cycling, which is expected to be similar for the two enantiomers.

Effects of chaetocin and ent-chaetocin on cellular redox homeostasis

Bible et al. reported that chaetocin showed tumor-selective cytotoxicity by inducing oxidative stress [21]. Therefore, we examined the effects of chaetocin and *ent*-chaetocin on cellular ROS level by using H_2DCFDA, an indicator of ROS, to monitor cellular ROS level in the presence of chaetocin and its derivatives (Fig. 5). But, unexpectedly, chaetocin and *ent*-chaetocin did not increase, but rather reduced, the ROS level. Moreover, *ent*-chaetocin showed this effect more strongly than chaetocin, indicating the involvement of a chirality-sensitive target(s). Although our results were inconsistent with Bible's report, it has recently been reported that chaetocin did not increase cellular ROS in other cell lines [7], indicating that chaetocin's effects on ROS could be different in different cell lines.

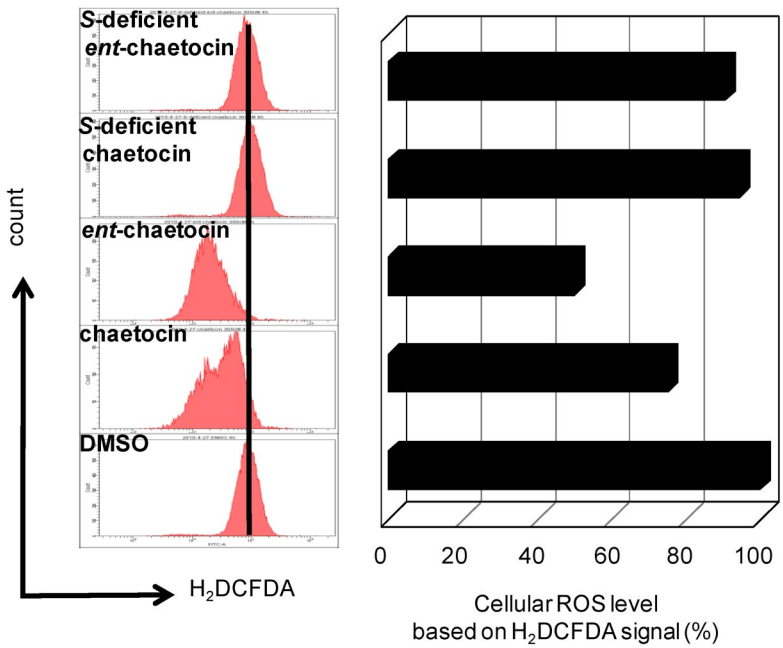


Fig. 5 Effects of chaetocin derivatives (0.3 μM) on cellular ROS level. HL-60 cells treated with *ent*-chaetocin for 4 h were stained with H_2DCFDA (10 μM at final concentration) and analyzed by flow cytometry.

There is a report that gliotoxin (Fig. 1), a well-known ETP compound, reduced cellular ROS via coupling with the thioredoxin (Trx)/thioredoxin reductase (TrxR) system [30]. The Trx/TrxR system is known to scavenge cellular ROS and to protect cells from oxidative stress. The redox cycle of Trx reduces cellular ROS level coupled with oxidation of NADPH. Although it is unclear whether there is a direct interaction between gliotoxin and the Trx/TrxR system, gliotoxin is thought to reduce cellular ROS instead of Trx. Based on this report, we speculated that the same mechanism might be involved in the decrease of cellular ROS level by *ent*-chaetocin. Namely, Trx may reduce *ent*-chaetocin, and the reduced form of *ent*-chaetocin may directly scavenge cellular ROS (Fig. 6). The disulfide form of Trx

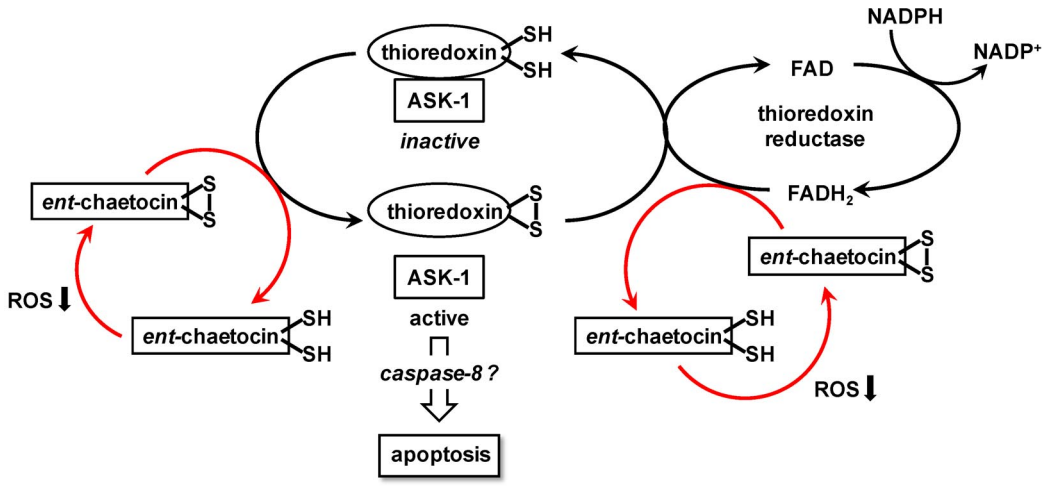


Fig. 6 Possible mechanism of *ent*-chaetocin-induced apoptosis.

is known to activate ASK-1, a ROS-sensitive MAPKKK, which plays an important role upstream of the MAP kinase cascade [31]. *ent*-Chaetocin may oxidize Trx, resulting in activation of ASK-1. ASK-1 is reported to induce apoptosis through both caspase-8-dependent and -independent pathways, though the mechanisms of ASK-1 signaling are unclear [32,33]. Considering the importance of caspase-8 in the *ent*-chaetocin-induced apoptotic signaling [10], the ASK-1/caspase-8 pathway may be involved in apopotosis initiated by *ent*-chaetocin. Thus, *ent*-chaetocin seems likely to interact with some protein(s) related to the Trx/TrxR system.

Thioredoxin reductase inhibition by chaetocin and its derivatives

Recently, Bible et al. identified thioredoxin reductase-1 (TrxR-1), the cytosolic subtype of TrxR, as a novel chaetocin target protein mediating cell death induced by chaetocin [34]. They reported that chaetocin is a competitive substrate for, and inhibitor of, TrxR-1. This is consistent with our hypothesis and suggests that TrxR might be a direct target molecule of *ent*-chaetocin. To test this idea, we next examined the inhibitory activity of *ent*-chaetocin toward TrxR. We monitored the activity of rat TrxR in the presence of chaetocin and its derivatives. As shown in Fig. 7, *ent*-chaetocin showed TrxR-inhibitory activity, as did chaetocin. In contrast, the sulfur-deficient derivatives showed no activity. These results support the hypothesis that inhibition of TrxR is involved in *ent*-chaetocin-induced apoptosis. Since the observed chirality-dependency of TrxR inhibition is weak, it is possible that interaction with other proteins in the Trx/TrxR system, presumably Trx itself, may also contribute to the oxidation of Trx. We are continuing to investigate the effects of *ent*-chaetocin on other proteins related to the Trx/TrxR system.

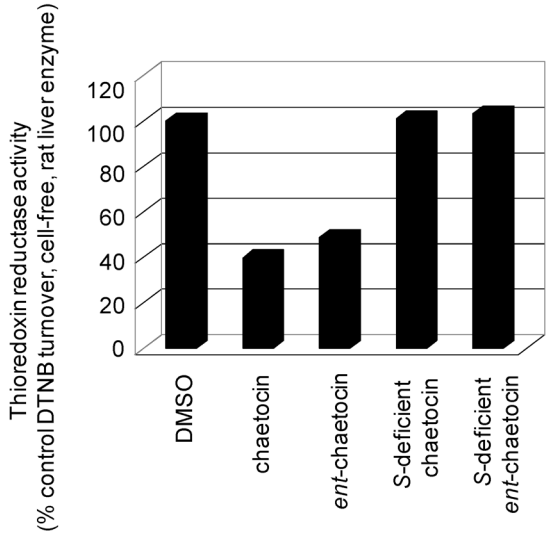


Fig. 7 Inhibition of rat TrxR by chaetocin derivatives. TrxR activity was quantified in the presence of chaetocin derivatives (10 μM) based on the reduction of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB).

CONCLUSION

The ETP compound chaetocin is thought to have various molecular targets in addition to HMT. Therefore, it is important to separate the activities in order to develop potent and selective HMT inhibitors that would be useful tools in epigenetic research. On the other hand, the apoptosis-inducing activity of *ent*-chaetocin is likely to be mediated via the Trx/TrxR-caspase-8 pathway, suggesting that *ent*-chaetocin is a promising lead compound for novel anticancer therapeutics. Structural development aimed at generating more selective HMT inhibitors, as well as apoptosis inducers, is in progress, together with further mechanistic studies.

EXPERIMENTAL SECTION

Flow cytometric analysis of cellular ROS level

HL-60 cells (4×10^5 cells/ml) were suspended in fresh medium in a 24-well plate (1 ml/well). After 2 h incubation, the cells were treated with chaetocin derivatives (0.3 μM) for 4 h and then H_2DCFDA (10 μM at final concentration) was added. After incubation for 30 min, cells were collected and washed with PBS. Then, 20 000 events were recorded by flow cytometry (BD) in the FITC setting log mode.

TrxR Inhibition assay

Cell-free TrxR activity was measured with a Thioredoxin Reductase Assay Kit (Cayman) according to the supplier's protocol. Briefly, chaetocin derivatives (0.5 μl), rat liver TrxR enzyme (10 μl) and diluted assay buffer were plated in a 96-well plate (100 μl/well). The reaction was started by the addition of NADPH (10 μl) and DTNB (10 μl, a substrate of TrxR). TrxR activity in the presence of chaetocin derivatives was quantified based on the change in absorbance at 405 nm within 3 min after DTNB addition.

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