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Epidithiodiketopiperazine as a pharmacophore for protein lysine methyltransferase G9a inhibitors: Reducing cytotoxicity by structural simplification

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ABSTRACT

Chaetocin (**1**), a structurally complex epidithiodiketopiperazine (ETP) alkaloid produced by *Chaetomium minutum*, is a potent inhibitor of protein lysine methyltransferase G9a, which plays important roles in many biological processes. Here we present our synthetic investigations to identify a simple prototype G9a inhibitor structure based on structure–activity relationship (SAR) studies on chaetocin derivatives. The simple derivative PS-ETP-1 (**14**) was found to be a potent G9a inhibitor with greatly reduced cytotoxicity.

Protein lysine methyltransferases (PKMTs) dynamically regulate gene expression profiles through methylation of lysine residues of histone proteins.[1] In contrast to invariant factors encoded in the DNA sequence, epigenetic gene expression control based on chromatin architecture is reversibly governed by acquired environmental factors.[2] It is becoming clear that PKMTs play critical roles in a range of diseases, and so discovery of potent PKMT inhibitors is attracting increasing attention.[3] G9a inhibitors are of particular interest,[4–7] because G9a is involved in a variety of biological processes, including tumor cell growth,[8,9] gene silencing in ES cells,[10,11] and cocaine-induced neuronal responses.[12] Here we present our studies on the development of epidithiodiketopiperazine (ETP)-type G9a inhibitors as simplified prototype PKMT inhibitors, based on structure–activity relationships (SAR) studies of chaetocin (**1**) derivatives. The cytotoxicity and thioredoxin reductase (TrxR)-inhibitory activity of the newly developed ETPs are also discussed.

Chaetocin (**1**), a naturally occurring product isolated from fungi of *Chaetomium* species, was the first inhibitor to have been reported (Fig. 1).[13,14] Because **1** exhibits various biological activities,[15–18] structural development aimed at increasing its selectivity for PKMTs is important. For example, Imhof and co-workers reported that the cytotoxicity of **1** adversely effected their cellular assays.[13] However, little is known about the structure–activity relationships (SAR) of **1** for PKMT-inhibitory activity,[19] probably because of the structural complexity of **1**. The difficulties in synthesizing **1** arise from (i) the high density of functionalities on the ETP scaffold, and (ii) the dimeric structure connected with a chiral quaternary carbon.[20] In order to address these issues, we recently established a strategy based on early oxidation of the diketopiperazine (DKP) moiety to introduce the disulfide functionalities under Lewis acidic conditions; this approach is critical to avoid β-elimination of the hydroxymethyl group on ETPs. This strategy was the basis of the first total synthesis of **1**.[21–23]

We also reported preliminary SAR studies on **1**, focusing on G9a-inhibitory activity,[21,22] cell-death-inducing activity in human leukemia HL-60 cells,[24] and thioredoxin reductase (TrxR)-inhibitory activity.[25] We found that the disulfide structure in **1** plays an important role in the G9a-inhibitory activity by comparison of **1** with *S*-deficient chaetocins **2**, including both enantiomers.[21,22] On the other hand, an enantiomer of chaetocin, *ent*-**1**, inhibited G9a as potently as **1**,[21,22] while it displayed more potent apoptosis-inducing activity than **1** in HL-60 cells.[24] These results

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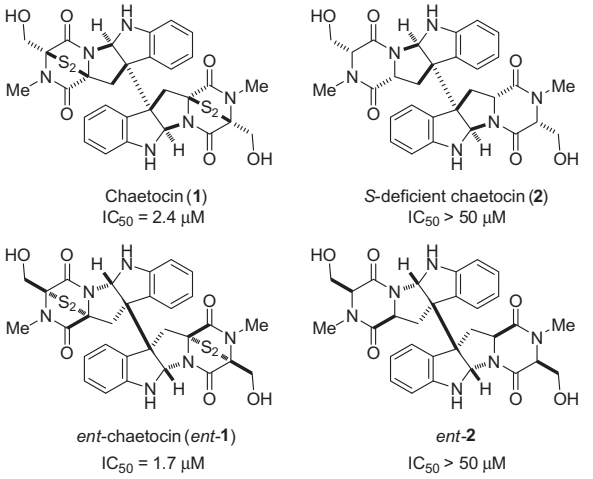
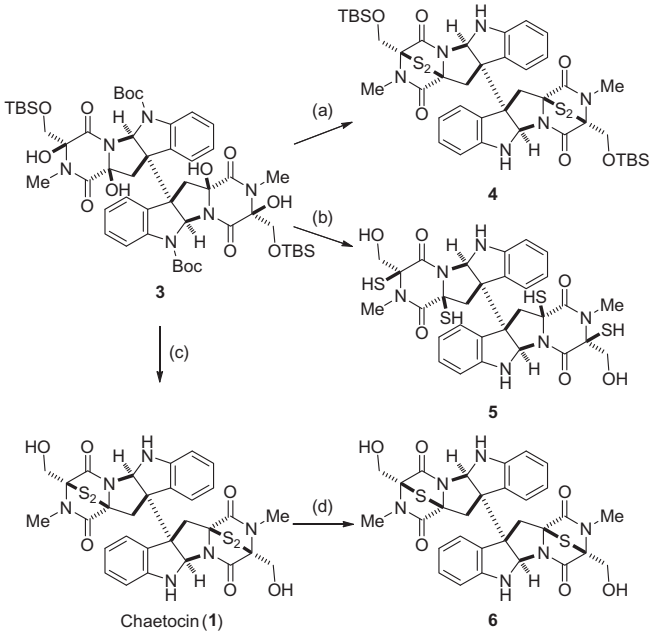


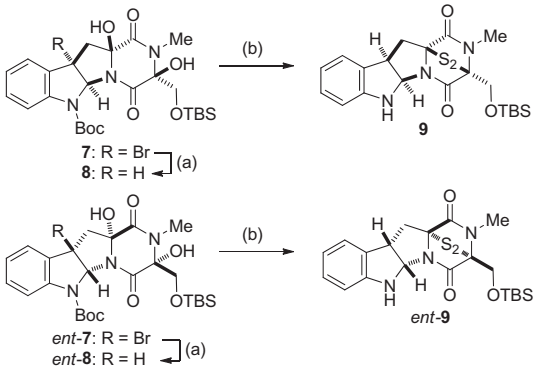
Figure 1. Structures and reported G9a-inhibitory activities[22] of **1**, *ent*-**1**, **2** and *ent*-**2**.

suggested that the cytotoxicity of **1** is not necessarily linked to G9a-inhibitory activity.

Encouraged by these preliminary findings, we planned further SAR studies on **1** in order to identify the essential core structure, including functional groups, of chaetocin (**1**) for G9a inhibition. We initially designed and synthesized TBS-protected chaetocin **4**, reduced chaetocin **5** with four thiol groups, and bridged-monosulfide **6**. The designed compounds were synthesized using the previously reported synthetic intermediate **3** (Scheme 1). In the synthesis of **4** using the procedure with H_2S in the presence of $BF_3{\cdot}OEt_2$, we found that controlling the reaction temperature (−78 to −20 °C) is important to avoid deprotection of the TBS groups on **3**. Subsequent iodine oxidation of the crude mixture promoted S–S formation to give the TBS-protected chaetocin **4**, albeit with low yield. Reduced chaetocin **5** was isolated using a procedure similar to the original one, but without the iodine-mediated



Scheme 1. Reagents and conditions: Synthesis of chaetocin derivatives **4**, **5** and **6**. (a) H_2S, $BF_3{\cdot}OEt_2$, CH_2Cl_2, −78 to −20 °C, I_2, EtOAc (6% in 2 steps); (b) H_2S, $BF_3{\cdot}OEt_2$, CH_2Cl_2, −78 °C to rt (27%); (c) H_2S, $BF_3{\cdot}OEt_2$, CH_2Cl_2, −78 °C to rt, I_2, EtOAc (44% in 2 steps); (d) PPh_3, CH_2Cl_2 (40%).



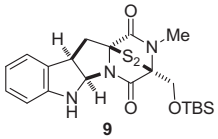
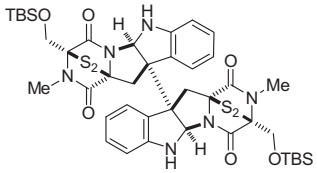
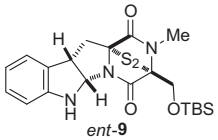
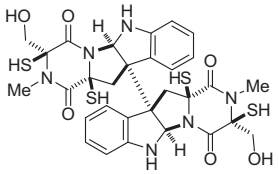
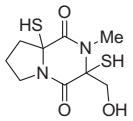
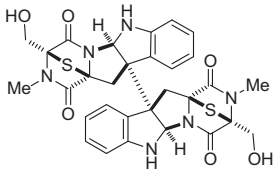
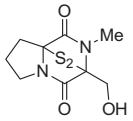
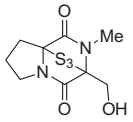
Scheme 2. Reagents and conditions: Synthesis of **9** and *ent*-**9**. (a) *n*-Bu_3SnH, V-70, benzene, rt (quant.); (b) H_2S, $BF_3{\cdot}OEt_2$, CH_2Cl_2, −78 to −20 °C, I_2, EtOAc (**9**: 27% in 2 steps, *ent*-**9**: 34% in 2 steps).

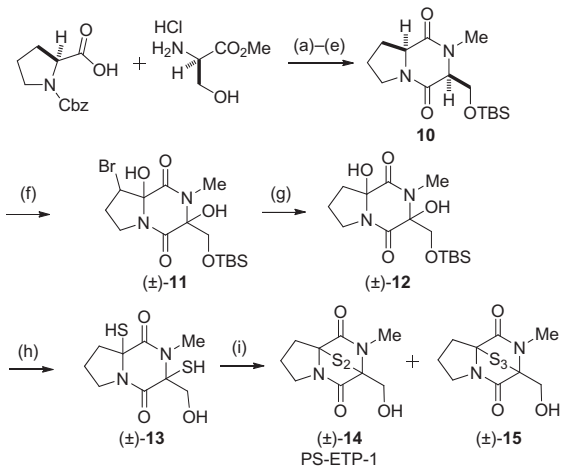
oxidation. The bridged-monosulfide chaetocin **6** was synthesized from chaetocin according to the reported protocol, using triphenylphosphine as a reductant.[26] These modified chaetocin derivatives were tested for G9a-inhibitory activity by using a modified ELISA assay (AlphaScreen™ system, PerkinElmer Inc.). The results are summarized in Table 1. In this new assay system, the IC_{50} value of chaetocin was determined as 7.2 μM, which is slightly higher than the value (IC_{50} = 2.4 μM) previously obtained using the original ELISA assay. A similar tendency was observed with the previous SAR studies using **1**, *ent*-**1**, **2** and *ent*-**2**.[21] The TBS-protected chaetocin **4** showed similar activity (IC_{50} = 7.2 μM) to that of chaetocin (**1**), implying that the hydroxyl groups in **1** are not critical for G9a inhibition. On the other hand, **5** showed less potent inhibitory activity than **1**, and **6** was inactive. These results highlight the importance of the disulfide functionalities in **1** for the G9a-inhibitory activity.

In order to examine the effect of the dimeric structure of chaetocin (**1**), we next synthesized monomeric ETP derivatives. As shown in Scheme 2, both enantiomers of monomeric derivatives of **1** were synthesized from diketopiperazine **7** and *ent*-**7**, the dimerization precursors for **1** and *ent*-**1**.[21,22] After reduction of the Br group in **7** and *ent*-**7** using *n*-Bu_3SnH and V-70, the resulting **8** and *ent*-**8** were treated with H_2S in the presence of $BF_3{\cdot}OEt_2$ to afford the corresponding **9** and *ent*-**9**. As shown in Table 1, monomeric ETP **9** (IC_{50} = 3.3 μM) showed slightly stronger G9a-inhibitory activity than the parent chaetocin (**1**), indicating that the dimeric ETP structure of **1** is not necessary for G9a inhibition. It is also noteworthy that *ent*-**9** showed weaker activity (IC_{50} = 23.5 μM). These results indicate that the chirality of the ETP framework affects the G9a-inhibitory activity.

Finally, simple ETP derivatives derived from proline and serine, **13**, **14** (PS-ETP-1) and **15**, were synthesized (Scheme 3) and evaluated. First, the DKP **10** was prepared from *N*-Cbz-L-proline and L-serine-methyl ester in 5 steps. Then, to increase the oxidation state of the DKP, **10** was subjected to radical bromination followed by treatment with phosphate buffer. Tribromination and subsequent hydrolysis proceeded to give the racemic diol **11**.[27] After removal of the undesired bromo group in **11** by means of radical reduction, the hydroxyl groups were replaced with thiols to give the corresponding di-thiol **13** as a single diastereomer. Finally, **13** was subjected to I_2-mediated oxidation, and careful separation afforded PS-ETP-1 (**14**) and its trisulfide derivative **15**. As shown in Table 1, the sulfur-bridged derivatives PS-ETP-1 (**14**) and **15** showed similar G9a-inhibitory activity [**14**: IC_{50} = 5.2 μM, **15**: IC_{50} = 5.3 μM]. Dithiol derivative **13** was less potent (IC_{50} = 13.1 μM). These SAR studies are consistent with the results obtained for chaetocin (**1**) and tetra-thiols **5**. Thus, the simple ETP structure appears to be available as a pharmacophore for G9a inhibitors.

Table 1
G9a-inhibitory activity of chaetocin derivatives

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
Chaetocin (**1**)	7.2		
S-Deficient Chaetocin (**2**)	>100		
ent-Chaetocin (*ent*-**1**)	6.4		
ent-**2**	>100	 **9**	3.3
 **4**	7.2	 *ent*-**9**	23.5
 **5**	27.1	 **13**	13.1
 **6**	>100	 **14**	5.2
		 **15**	5.3



Scheme 3. Reagents and conditions: Synthesis of **13**, **14** and **15**. (a) EDC·HCl, HOBt, Et_3N, CH_2Cl_2; (b) TBSCl, imidazole, DMF (94% in 2 steps); (c) H_2, 10% Pd/C, EtOH; (d) NH_4OH aq, MeOH (99%, in 2 steps); (e) MeI, NaH, DMF (91%); (f) NBS, AIBN, CCl_4, reflux, phosphate buffer (pH 7), MeCN (64% in 2 steps); (g) *n*-Bu_3SnH, AIBN, benzene, reflux (83%); (h) H_2S, $BF_3{\cdot}OEt_2$, CH_2Cl_2, −78 °C to rt (**13**: 87%); (i) I_2, EtOAc (**14**: 65% in 2 steps, **15**: 5% in 2 steps).

Reducing the cytotoxicity of ETPs is also an important consideration for the development of selective PKMTs inhibitors. Thus, we next examined the cytotoxicity of these compounds using human leukemia HL-60 cells (Fig. 2).[26] We found that the cytotoxicity of

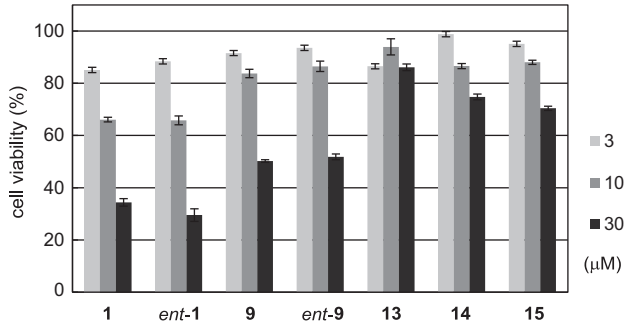


Figure 2. Cytotoxicity of chaetocin derivatives in HL-60 cells. HL-60 cells were treated with chaetocin derivatives for 4 h, and cell viability was determined by alamarBlue assay.[25]

the newly developed simple derivatives **13**–**15** was significantly reduced, compared with that of the parent chaetocin (**1**)/*ent*-**1** or monomeric ETPs **9**/*ent*-**9** at the concentrations we tested.

Thioredoxin reductase (TrxR) was also reported to be a target of chaetocin (**1**), and TrxR-inhibitory activity was suggested to be associated with the cytotoxic effects of **1**.[28] To examine the selectivity between G9a and TrxR, we finally examined the TrxR-inhibitory activity of PS-ETP-1 (**14**) and **15**. Enzymatic reaction of TrxR was quantified in the presence of these compounds (10 μM) based on the reduction of 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) using a Thioredoxin Reductase Assay Kit (Cayman). As shown in Figure 3, PS-ETP-1 (**14**) and **15** showed almost no inhibition of

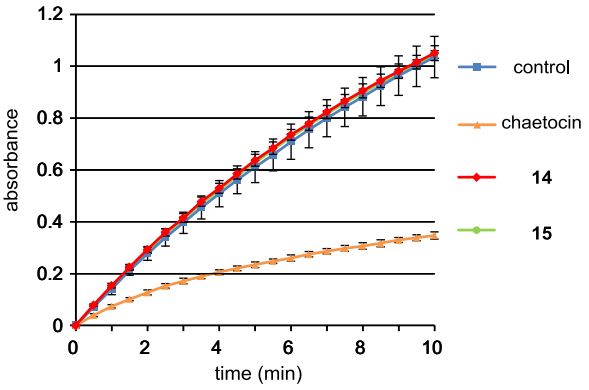


Figure 3. Thioredoxin reductase (TrxR)-inhibitory activity of chaetocin derivatives. TrxR reaction was quantified in the presence of chaetocin derivatives (10 μM) based on the change in absorbance (405 nm).[25]

TrxR, whereas chaetocin (**1**) was inhibitory. Because **1** was reported to be a competitive inhibitor of the natural substrate, thioredoxin (Trx),[28] which has both disulfide bridges and indole structure in the TrxR-binding site,[29] we speculated that elimination of the indole structure from **1** might have contributed to reducing its TrxR-inhibitory activity, perhaps accounting for the decreased cytotoxicity.

In conclusion, we have identified the simple ETP derivative PS-ETP-1 (**14**)[30] as a novel prototype for G9a inhibitors. This finding may also be helpful in the design of other PKMT inhibitors, which are complementary to peptide substrate–competitive inhibitors.[5,6] In addition, the cytotoxicity and TrxR-inhibitory activity of **14** were significantly reduced, compared with those of chaetocin (**1**). Further structural developments of ETPs aimed at constructing enantiomerically distinct environments with improved selectivity, as well as application to cellular assays, are ongoing in our research group.

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30. PS-ETP-1 (**14**): ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.21–2.41 (m, 1H), 3.04 (ddd, J = 8.1, 10.4, 14.4 Hz, 1H), 3.16 (s, 3H), 3.55 (dd, J = 5.7, 9.5 Hz, 1H), 3.62 (m, 1H), 3.91 (ddd, J = 3.4, 8.1, 11.5 Hz, 1H), 4.25 (dd, J = 9.5, 12.6 Hz, 1H), 4.36 (dd, J = 5.7, 12.6 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 23.1, 27.2, 32.8, 46.2, 60.9, 74.7, 76.3, 163.3, 166.6; HRMS (ESI) (m/z) calcd. for $C_9H_{12}N_2O_3S_2Na$ $[M+Na]^+$: 283.0187, found: 283.0182.