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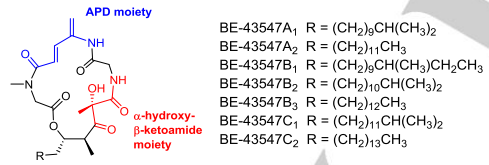
Synthesis and anti-pancreatic cancer stem cell activity of BE-43547A$_2$

Yuanjun Sun,[#] Yahui Ding,[#] Dongmei Li, Ruifei Zhou, Xiuwen Su, Juan Yang, Xiaoqian Guo, Chuanke Chong, Jinghan Wang, Weicheng Zhang, Cuigai Bai, Liang Wang,* and Yue Chen*

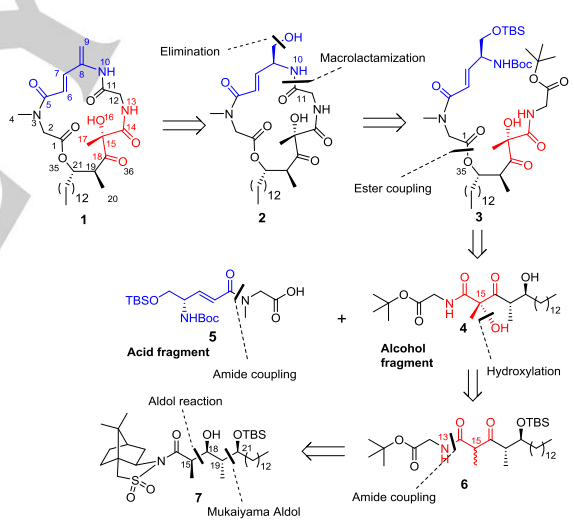
Abstract: The asymmetric total synthesis of cyclic depsipeptide BE-43547A$_2$ was achieved in 15 linear steps on a 350 mg scale in one batch. Our synthesis is featured with highly diastereoselective construction of α-hydroxy-β-ketoamide via α-hydroxylation with a *d.r.* up to 86:1. BE-43547A$_2$ can significantly reduce the percentage of pancreatic cancer stem cells in Panc-1 cells, and dramatically ablate the tumorsphere forming capability of Panc-1 cells. The tumor-initiating assay *in-vivo*, a gold standard for cancer stem cell assays, confirmed BE-43547A$_2$ could abolish the tumorigenesis of Panc-1 cells. The anti-PCSC activity of BE-43547A$_2$ will make this depsipeptide scaffold a start for discovering new PCSC-targeting drug.

Pancreatic cancer, one of the most refractory cancers, has an overall 5-year survival rate of only 1-4%.[1] Pancreatic cancer stem cells (PCSC) may play a key role in the carcinogenesis, metastasis and drug resistance of pancreatic cancer.[2] For example, CD24$^+$CD44$^+$ESA$^+$ pancreatic cancer cells, the commonly recognized PCSC, were able to generate tumors in half of the tested mice with 100 cells injected, which demonstrated 100-fold higher tumorigenic potential than regular pancreatic cancer cells.[3] However, compounds targeting pancreatic cancer stem cells are still very rare. Triptolide, AZD7762 and LDE225 with modest PCSC selectivity, have entered clinical trial against pancreatic cancer.[4] Therefore, new type of compounds that can effectively target PCSC is in urgent need.



Scheme 1. Structure of the BE-43547 family.

On the other hand, the BE-43547 family, isolated from *Streptomyces sp.*, is composed of cyclic depsipeptides structurally characterized by 4-amido-2,4-pentadienoate (APD) and α-hydroxy-β-ketoamide. Preliminary biological assays of the BE-43547 family indicated its high cytotoxicity against various cancer cell lines.[5] Recently, Poulsen and co-workers made a break-through in designing the first synthetic route to the BE-43547 macrocyclic scaffold.[6] With their chemical synthesis of *ent*-BE-43547A$_1$ and the biosynthesis of the BE-43547 family, they determined the absolute configuration of BE-43547A$_1$~A$_2$ and proposed that the originally reported ^{1}H NMR data for BE-43547A$_1$ and A$_2$ were incorrect.[6] Meanwhile they demonstrated that BE-43457A$_1$ and A$_2$ possess significant hypoxia-selective growth inhibitory activity against human pancreatic cancer Panc-1 cells. We also confirmed hypoxia selectivity of BE-43547A$_2$ against MCF-7 and K562 cell lines (see the supporting information, Table S3). Since intermittent[7] and sustained[8] hypoxia have been reported to induce CSC-like properties resulting in the invasiveness and metastasis of pancreatic cancer cells, we speculated that BE-43457s may target PCSC. For validation of BE-43457s' selectivity towards PCSC, a highly efficient synthetic route to provide sufficient BE-43457s is important. Herein, we report our total synthesis of BE-43547A$_2$ along with its biological activity of selectively ablating PCSC.



Scheme 2. Retrosynthetic analysis of BE-43547A$_2$.

The retrosynthetic analysis is shown in Scheme 1. Mesylation of the primary alcohol **2** followed by elimination was used to introduce APD.[9] Macrolactamization between C11 and N10 could be employed to form **2**. Disconnection of **3** through the C1−O35 bond resulted in the alcohol **4** and acid **5**. The alcohol **4** could be prepared by the hydroxylation of compound **6** at C15.

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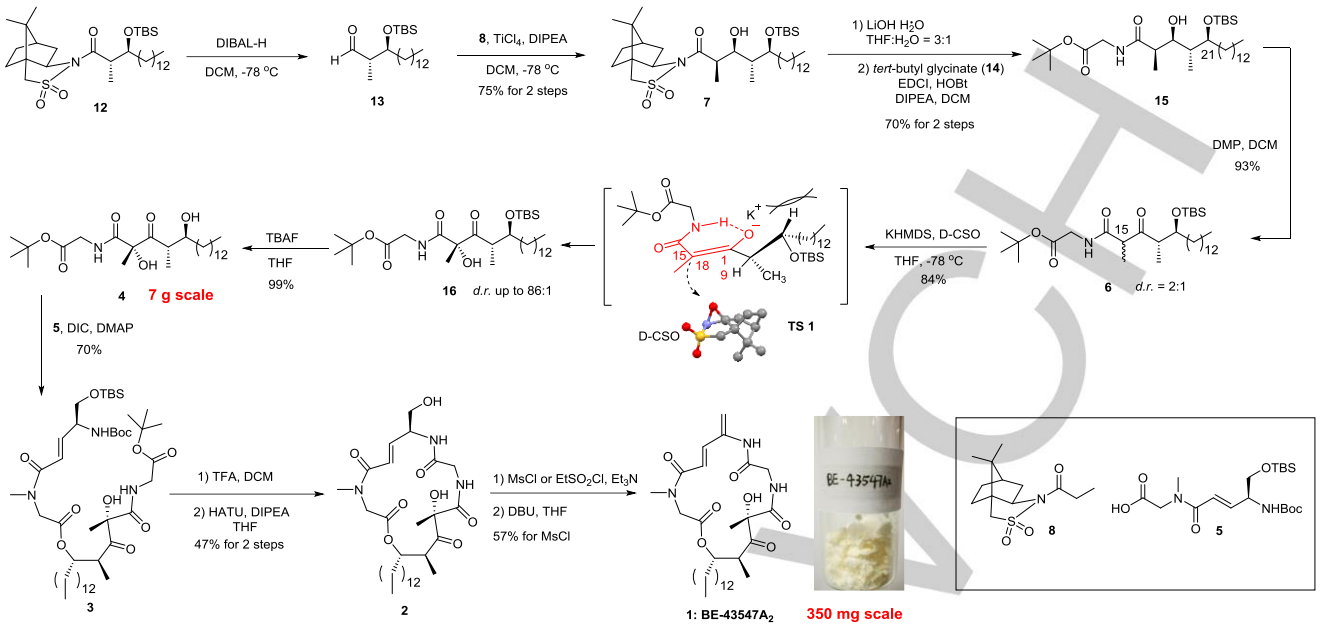
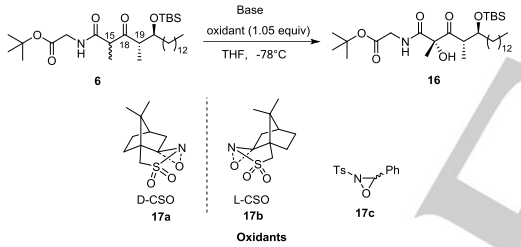


Scheme 3. Total synthesis of BE-43547A$_2$. DIBAL-H = diisobutylaluminum hydride, EDCI = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, HOBt = 1-hydroxybenzotriazole, DMP = Dess-Martin periodinane, CSO = camphorsulfonyloxaziridine, TBAF = tetra-*n*-butylammonium fluoride, DIC = diisopropyl carbodiimide, HATU = *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene.

Table 1. Optimization of the conditions for the α-hydroxylation of **6**[a]



Entry	base	eq. (base)	oxidant	*d.r.*[b]	Yield[c]
1	LiHMDS	1.0	**17a**	5:1	5%
2	NaHMDS	1.0	**17a**	9:1	35%
3	KHMDS	1.0	**17a**	19~40:1	70~88%
4	KHMDS	0.2	**17a**	68~86:1	71~81%
5	KHMDS	0.1	**17a**	62:1	39%
6	KHMDS	0.2	**17b**	39:1	28%
7	KHMDS	0.2	**17c**	14:1	13%

[a] All reactions were carried out with **6** (0.20 mmol) and CSO (0.21 mmol). [b] *d.r.* was determined by high performance liquid chromatography. [c] Yield was determined by ^{1}H NMR using 1,3,5-trimethoxybenzene as an internal standard.

Synthesis began with the construction of compound **12** (for the synthesis of **12**, see the Supporting Information, Scheme S1). Reduction of **12** with DIBAL-H provided aldehyde **13**, which was then subjected to the aldol reaction[10] with **8** to give **7** in *d.r.* > 20:1. Hydrolysis of **7** followed by amide coupling with **14** provided compound **15**. Oxidation of the alcohol **15** with DMP provided ketone **6** in a 93% yield with *d.r.* = 2:1.

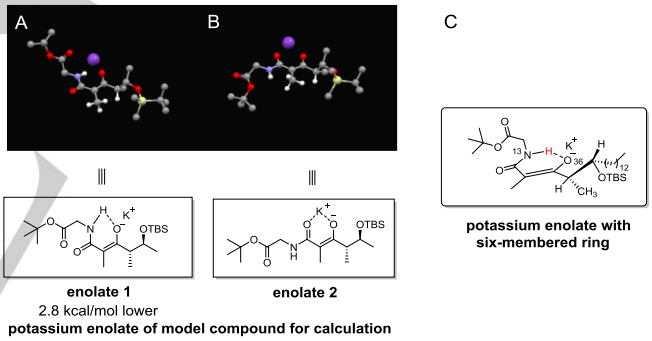
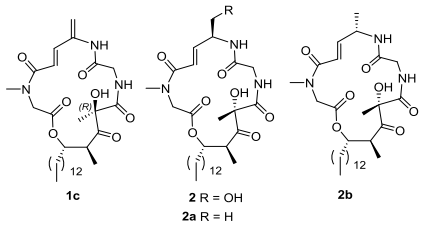


Figure 1. Optimized structures for the intramolecular H-bond involving potassium enolate **1** (A) and potassium enolate **2** (B) of the model compound; proposed potassium enolate with a six-membered ring (C).

Reactions reported for direct α-hydroxylation to construct the acyclic α-hydroxy-β-ketoamide are rare,[11] and we planned to stereoselectively install the OH group at C15 based on the facial bias of the C15-C18-C19 allylic system of enolated **6**. Different conditions were tested (Table 1), and 0.2 eq. of KHMDS as the base and D-CSO[12] as the oxidant were applied as the optimized conditions to provide **16** in a *d.r.* of 68~86:1 (for other conditions, see the Supporting Information, Table S1). Density functional theory calculations indicated that the optimized structure for the intramolecular hydrogen bond involving potassium enolate (Figure 1A) was 2.8 kcal/mol lower in energy than the linear potassium enolate (Figure 1B). These results suggested that the N13-H may be involved in an intramolecular hydrogen bond with O36 to form a six-membered ring, which helped to enhance the facial selectivity in the hydroxylation reaction (Figure 1C).

Deprotection of TBS for **16** by TBAF provided alcohol fragment **4**. With the key fragments **4** and **5** (see the Supporting Information, Scheme S2) in hand, we completed the total synthesis of BE-43547A$_2$. First, fragment **4** was coupled with fragment **5** under DIC and DMAP conditions to give **3** in a 70% yield. All three protective groups in compound **3** were removed in the presence of TFA, and the resulting mixture was subjected to the key ring closing reaction under HATU and DIPEA conditions.[13] Macrolactamization proceeded smoothly to produce 630 mg of compound **2**. Finally, mesylation of **2** followed by elimination afforded 350 mg of BE-43547A$_2$. The spectroscopic data obtained for synthetic **1** were different from those of BE-43547A$_2$ reported in the patent[5], but they were in accordance with the data of biosynthetic BE-43547A$_2$ reported by Poulsen.



Compounds	IC_{50} (μM)
1	0.87 ± 0.11
1c	>20
2	>20
2a	9.59 ± 2.80
2b	>20
Dox	2.49 ± 1.80

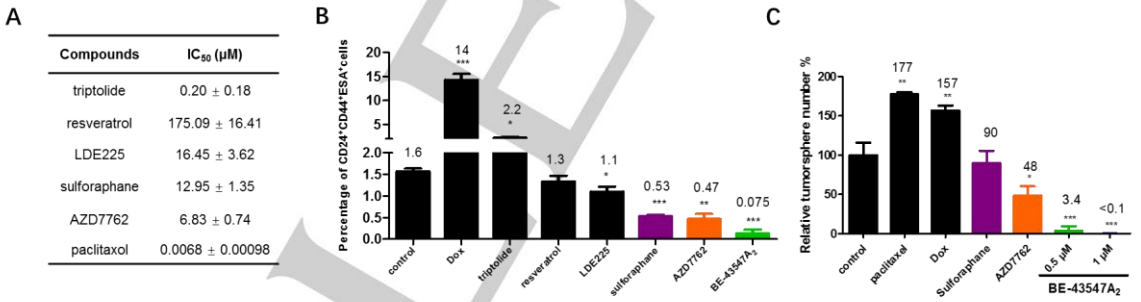
Figure 2. The IC_{50} values of BE-43547A$_2$ and its derivatives against Panc-1 cells.

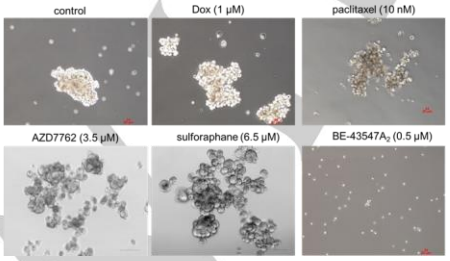
Based on our synthetic route, 4 derivatives (**1c**, **2**, **2a** and **2b**) were easily prepared (see the Supporting Information, Scheme S4) to evaluate the influence of the double bond between C8-C9 and the absolute configuration at C15 (Figure 2). From the results of the MTT assay, BE-43547A$_2$ was the most effective compound compared to the other derivatives. Xenograft zebrafish model experiments using Panc-1 cells further demonstrated that BE-43547A$_2$ has higher anti-pancreatic cancer activities than the compounds **2a** and TBDPS-*ent*-vinylamycin[14] (see the Supporting Information, Figure S4 and S5). The significantly reduced inhibitory activities of **1c**, **2**, **2a** and **2b** implied that both the double bond between C8-C9 and the *S* configuration at C15 are essential to maintain the inhibitory activity against the Panc-1 cell line.

To evaluate the effect of BE-43547A$_2$ on PCSC, we first analyzed the activity of BE-43547A$_2$ against the pancreatic stem cell (with CD24$^+$CD44$^+$ESA$^+$ biomaker) in Panc-1 cells. Some anti-cancer drugs and reported anti-PCSC compounds[4, 15] were used as comparison (Figure 3A). Based on the results at their respective concentration about 2×IC_{50} (Figure 3B), the percentage of stem cells increased by 8.8-fold after Dox treatment, consistent with CSC being resistant to conventional chemotherapy. While both resveratrol[15b] and triptolide (clinical trial, phase II)[4b] resulted in minimal change to the percentage of stem cell. Upon treatment with two reported PCSC-targeting compounds, AZD-7762 (clinical trial, phase Ib)[4c] and LDE-225 (clinical trial, phase I)[4a], 3.4-old and 1.5-fold decrease in the proportion of CD24$^+$CD44$^+$ESA$^+$ stem cells were observed. The same trend was also found in the sulforaphane[15a] treated group (3.0-fold). To our delight, the percentage of CD24$^+$CD44$^+$ESA$^+$ stem cells significantly declined to 0.075% after BE-43547A$_2$ treatment, which was over 21-fold less than that in the untreated control (1.6%), indicative of the PCSC selectivity of BE-43547A$_2$ is significantly superior to that of other reported PCSC-targeting compounds.[4, 15]

A

Compounds	IC_{50} (μM)
triptolide	0.20 ± 0.18
resveratrol	175.09 ± 16.41
LDE225	16.45 ± 3.62
sulforaphane	12.95 ± 1.35
AZD7762	6.83 ± 0.74
paclitaxol	0.0068 ± 0.00098





E

Cell number	Control	Dox (1 μM)	BE-43547A$_2$ (0.25 μM)	BE-43547A$_2$ (1 μM)
5×10^6	6/6	3/3	3/5	0/6
5×10^5	3/6	4/6	0/5	0/6
5×10^4	0/6	0/6	0/5	0/6
TIF	1/0.8×10^6	1/0.5×10^6	1/5.5×10^{6**}	-

Figure 3. BE-43547A$_2$ decreased the percentage of pancreatic cancer stem cell in Panc-1 cells, inhibited tumorsphere formation *in vitro* and reduced the tumor initiation frequency. (A) The IC_{50} value of investigated compounds against Panc-1 cells. (B) Percentage of CD24$^+$CD44$^+$ESA$^+$ cells in Panc-1 cells after treatment with compounds at the concentration about 2×IC_{50}. Dox: 4 μM; triptolide: 0.4 μM; resveratrol: 350 μM; LDE225: 33 μM; sulforaphane: 26 μM; AZD7762: 14 μM; BE-43547A$_2$: 2 μM. (C) Quantitative analysis of tumorsphere formation. Dox, sulforaphane and AZD7762 was used at the concentration about 1/2×IC_{50} (1 μM, 6.5 μM and 3.5 μM respectively). Paclitaxel was used at the concentration of 1.5×IC_{50} (10 nM). (D) Representative images of Panc-1 spheres treated with BE-43547A$_2$, Dox and paclitaxel for 7 days. (E) BE-43547A$_2$ reduced the tumor initiation frequency (TIF) in the tumor initiation assay. (TIF refers to the average

number of cells determined to be required to cause tumor growth in the recipient cohort. The results shown represent the mean ± SD of three independent experiments. * $p < 0.05$ vs control, ** $p < 0.01$ vs control, *** $p < 0.001$ vs control).

PCSC could be enriched in non-adherent spherical clusters of cells which was called tumorsphere. In order to further examine the effect of BE-43547A$_2$ on PCSC, we conducted tumorsphere formation assay (Figure 3C and 3D). With good performance in the $CD24^+CD44^+ESA^+$ biomarker assay, sulforaphane, AZD7762 as well as Dox were chosen as the controls.The results (Figure 3C) indicated that treatment with BE-43547A$_2$ at the concentration of 1/2×IC$_{50}$ induced a significant 29-fold tumorspheres forming reduction compared to the untreated control, which is superior to sulforaphane and AZD7762 (1.1-fold and 2.1-fold of reduction, respectively).

The most convincing test for cancer stem cells is the limiting dilution tumor-initiating assay (Figure 3E). According to the result, the Dox-treated group (at a concentration of 1 μM) displayed no reduction of the tumor-initiating frequency. In contrast, the BE-43547A$_2$-treated group (at a concentration of 0.25 μM) displayed a 6.8-fold reduction of the tumor-initiating frequency, and no tumor was observed after treatment with 1 μM BE-43547A$_2$. These results confirmed that BE-43547A$_2$ could abolish the capability of tumorigenesis of Panc-1 pancreatic cells *in vivo*.

In conclusion, we have accomplished the asymmetric total synthesis of BE-43547A$_2$ on a 350 mg scale in 15 linear steps. Due to the rare report of the direct α-hydroxylation of acyclic β-ketoamide, our synthesis is featured with highly diastereoselective construction of α-hydroxy-β-ketoamide (*d.r.* up to 86:1). This efficient synthetic sequence can not only provide abundant BE-43547A$_2$, but also resulted in several derivatives for further biological assays. The preliminary structure-activity-relationship study included *in-vitro* assay and *in-vivo* assay with xenograft zebrafish model (see the supporting information, Figure S4 and S5), both experiments indicated that the C8-C9 double bond and 15*S* configuration are essential to maintain the inhibitory activity against the Panc-1 cell line. More importantly, we discovered that BE-43547A$_2$ exhibited superior PCSC selectivity compared to other compounds targeting PCSC. The percentage of $CD24^+CD44^+ESA^+$ stem cells decreased by 21-fold after treatment with 2 μM BE-43547A$_2$. In the tumorsphere forming assay, the number of tumorspheres formed in the BE-43547A$_2$-treated group was around 46-fold less than that in the Dox-treated group. The tumor-initiating assay further confirmed that BE-43547A$_2$ could abolish the capability of tumorigenesis of Panc-1 pancreatic cells. All of the biological assays indicate that BE-43547A$_2$ can selectively ablate pancreatic cancer stem cells. These properties are highly desirable for selecting PCSC drug candidates in preclinical study. The highly productive total synthesis and exciting anti-PCSC activities, along with the unknown mechanism of action, warrant BE-43547A$_2$ as a promising beginning for the discovery of novel PCSC-targeting drugs.

Acknowledgements

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Keywords: BE-43547 • cyclic depsipeptide • cancer stem cells • hydroxylation • pancreatic cancer

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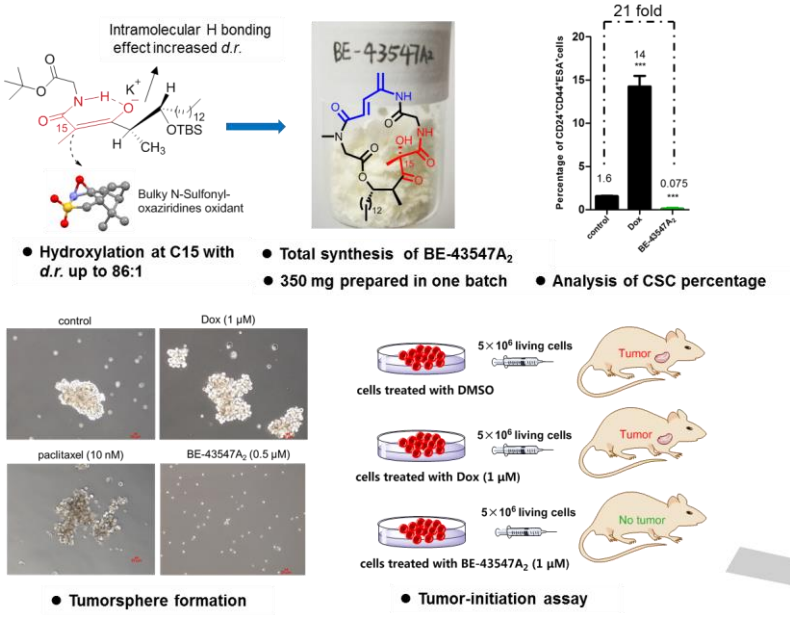

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