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本期导读：

铂类抗肿瘤药物纳米递送系统研究进展

孙飘，丁　杨，周建平

罗米地辛潜在杂质的分离与鉴定

熊磊，闵涛玲，陈昌发，胡海峰

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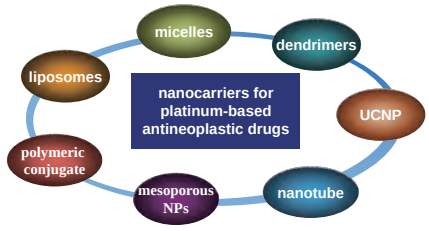

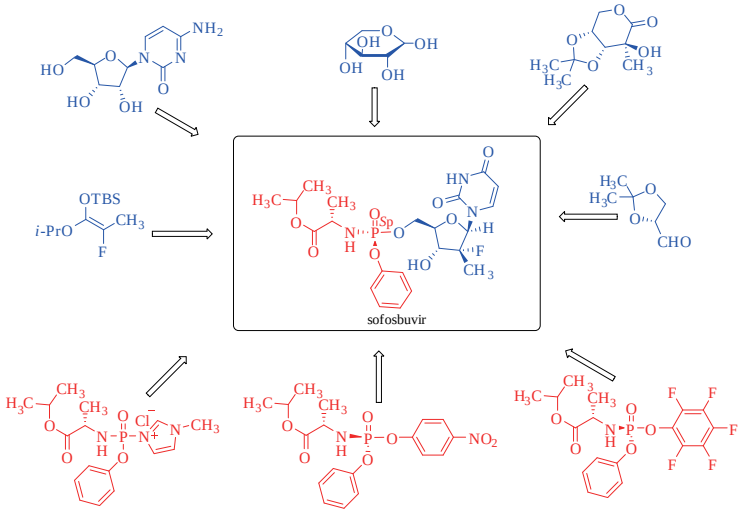

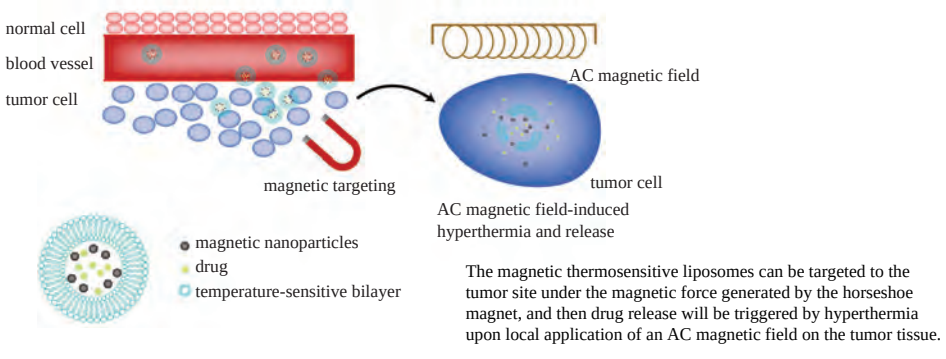

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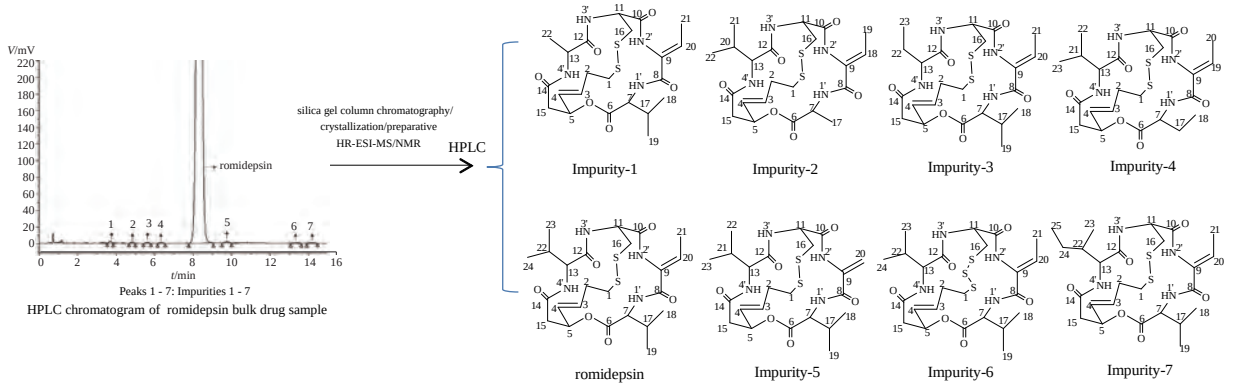

Peaks 1 - 7: Impurities 1 - 7
HPLC chromatogram of romidepsin bulk drug sample

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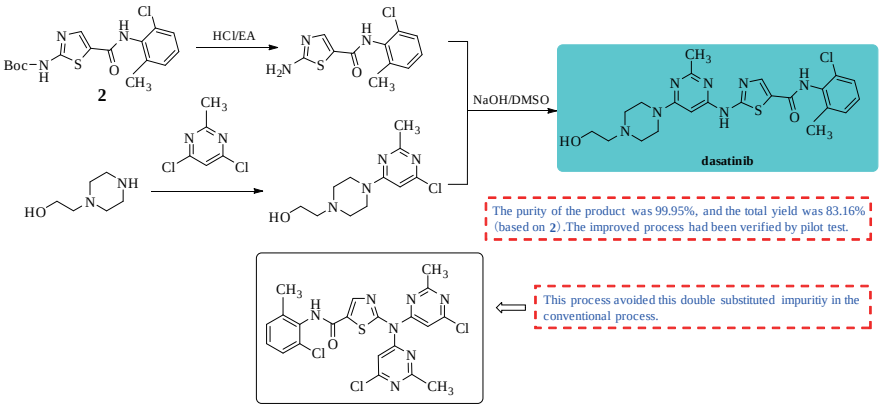

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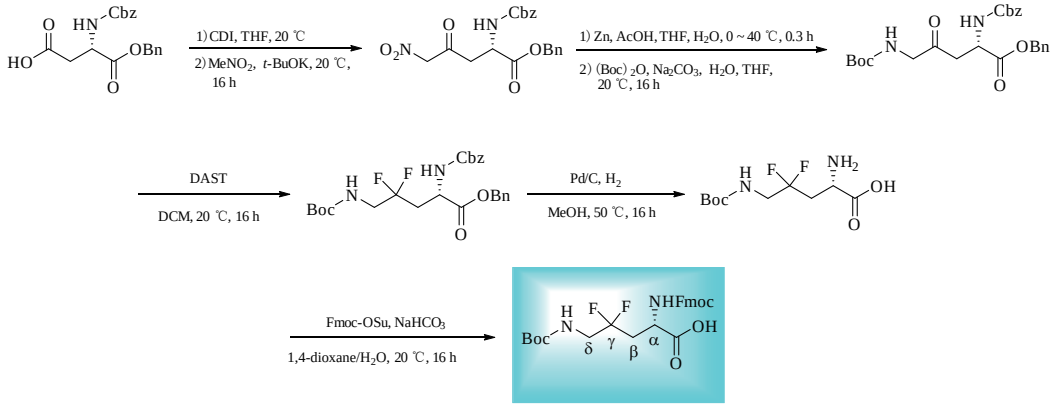

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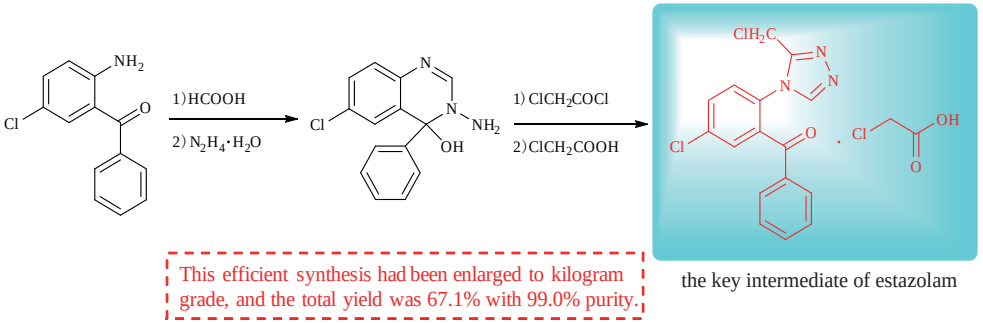
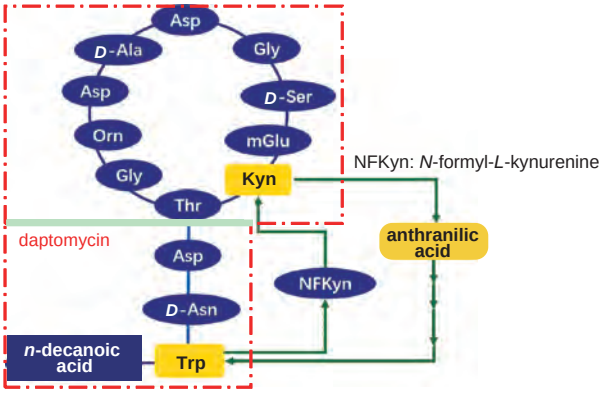
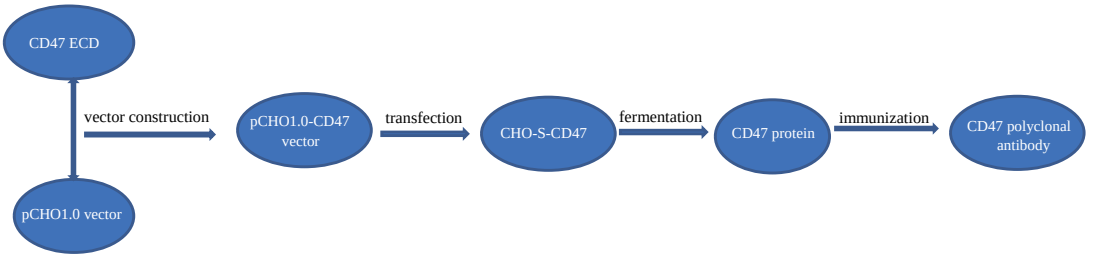
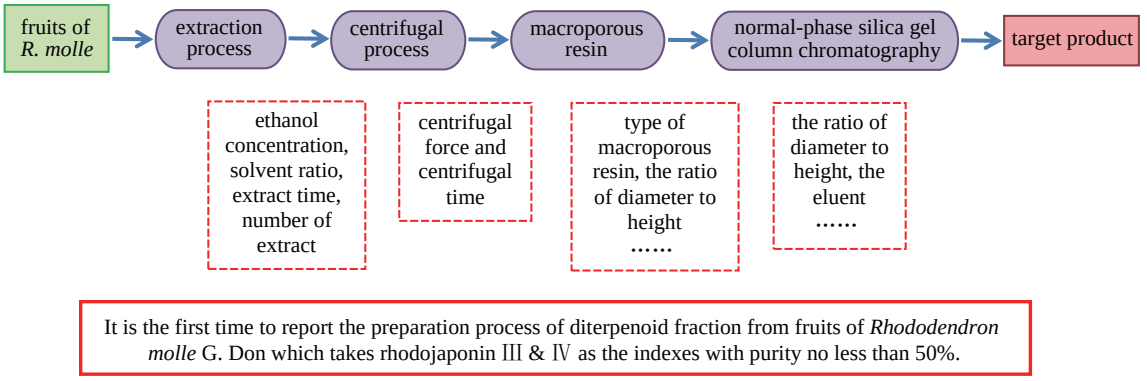


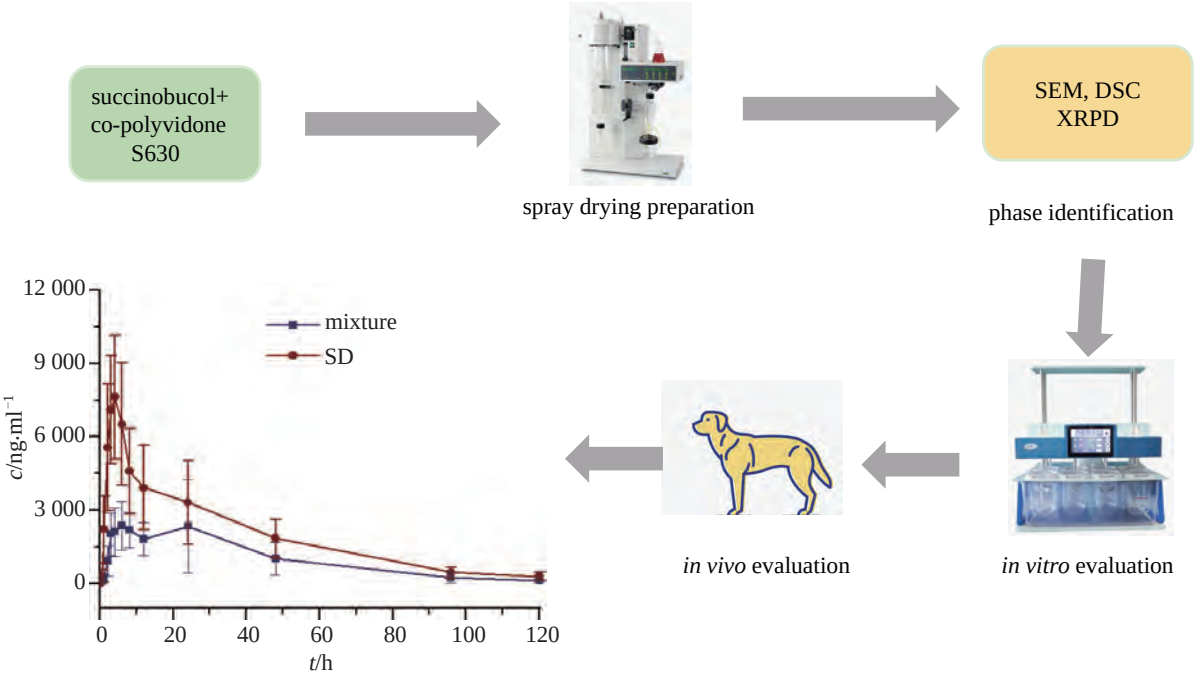
This efficient synthesis had been enlarged to kilogram grade, and the total yield was 67.1% with 99.0% purity.

the key intermediate of estazolam









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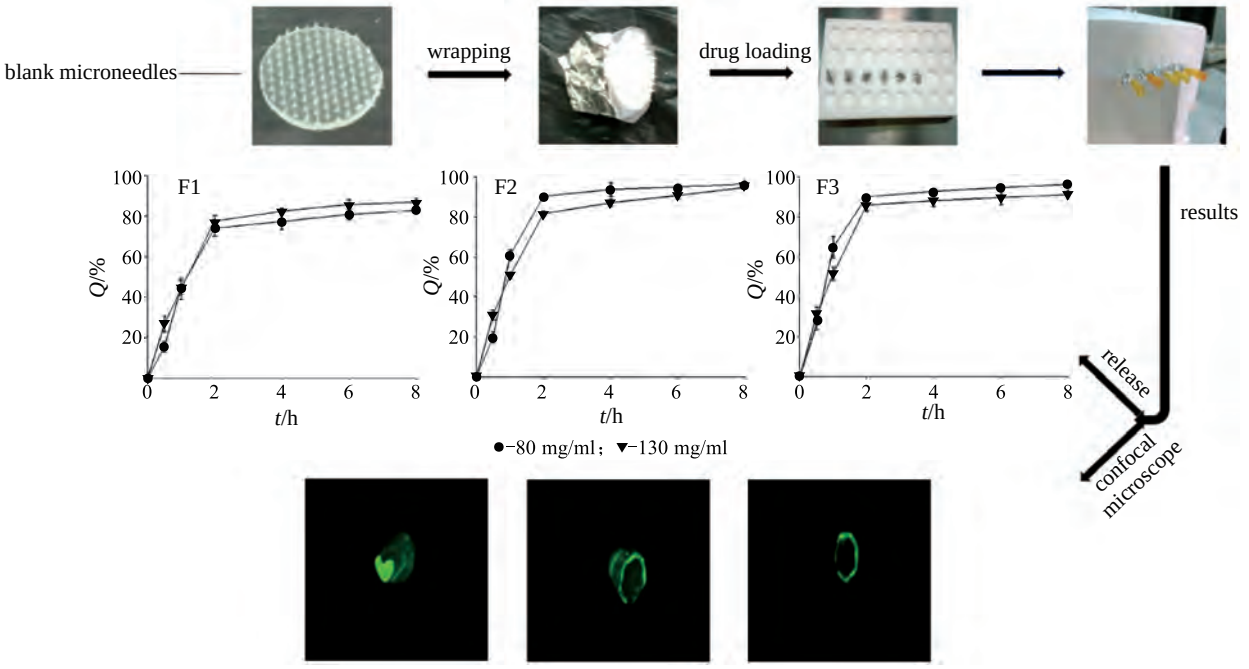

3D Structure Images of the Microneedle by Confocal Microscope

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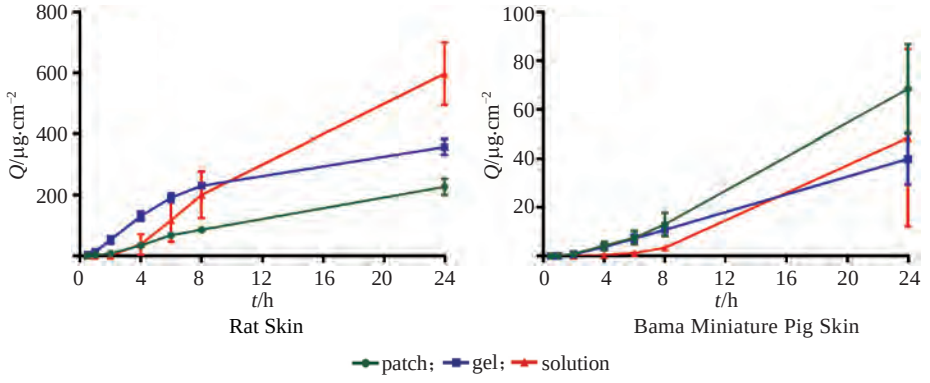

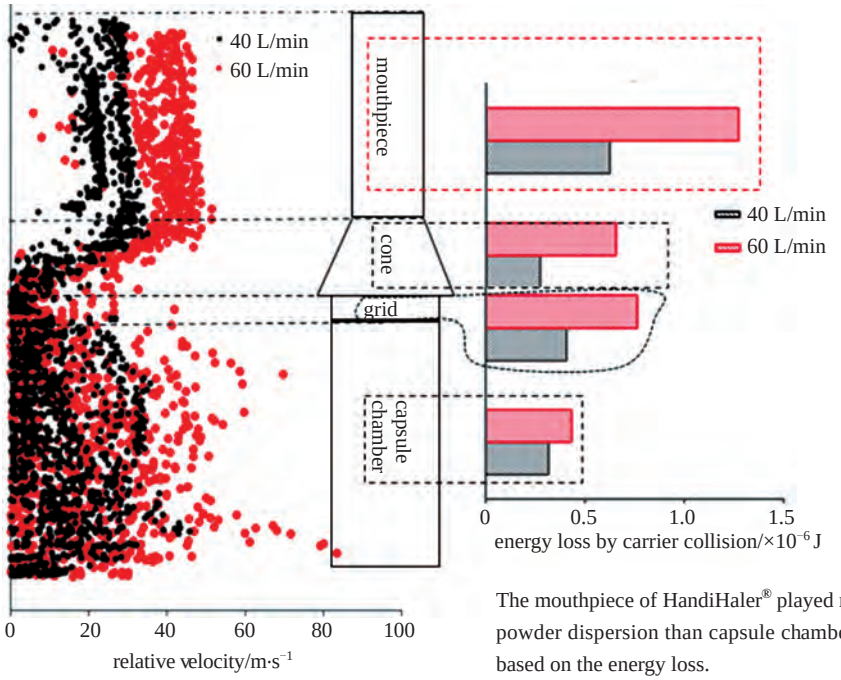

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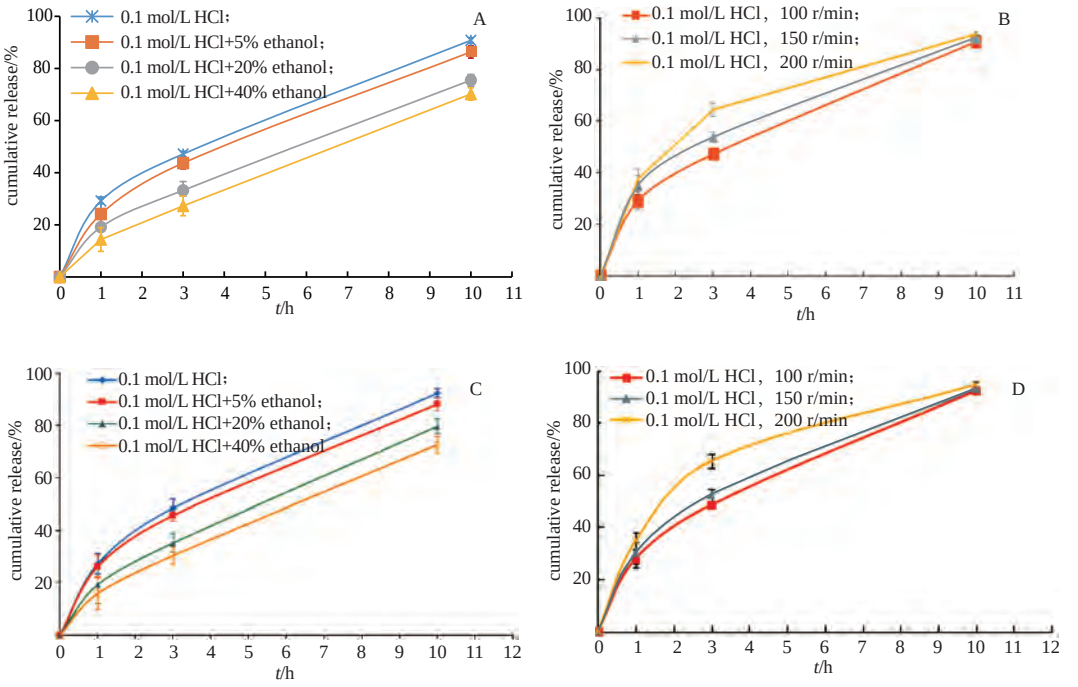
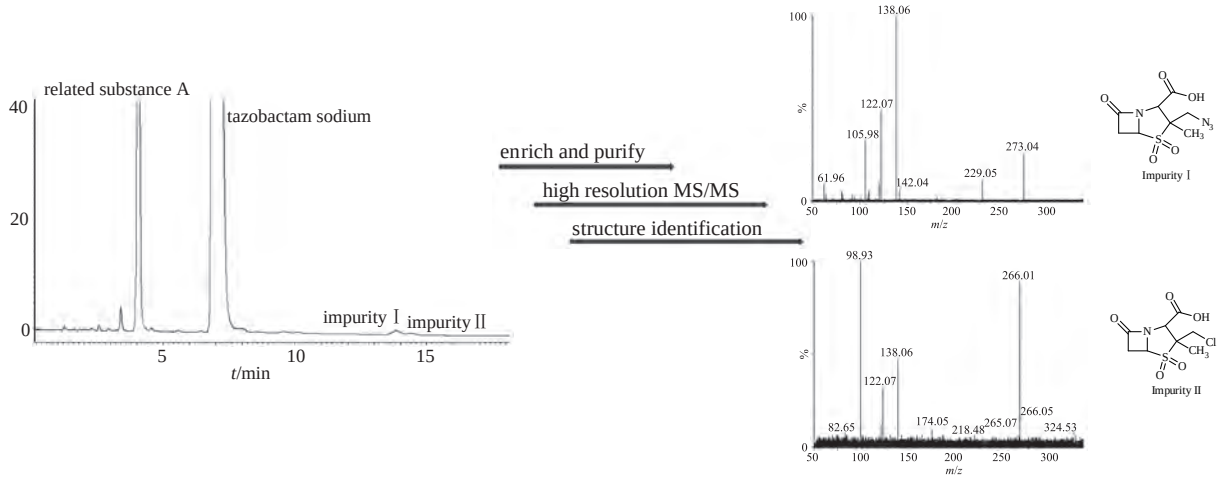
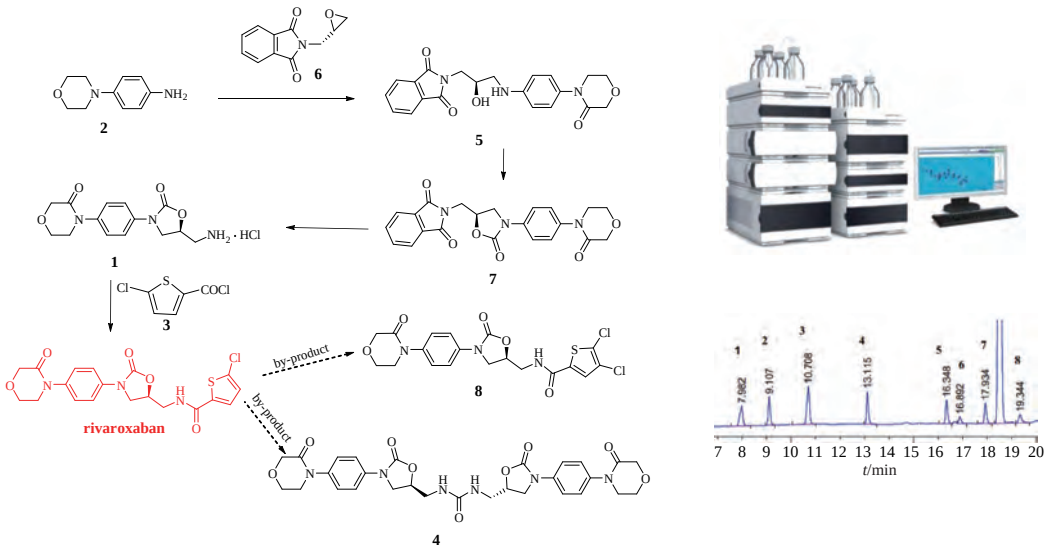
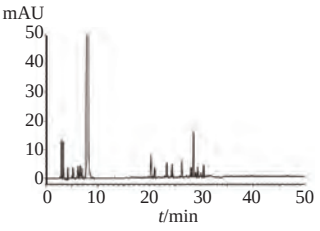


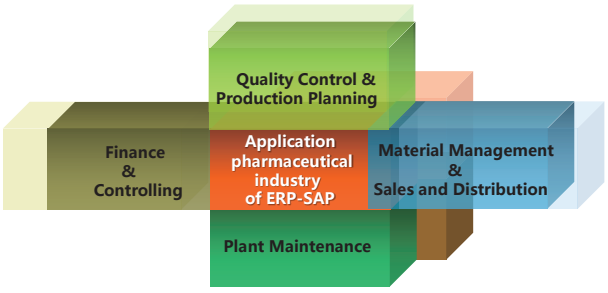
Release Profiles of Metformin Hydrochloride from the Commercial Tablets（A, B）and the Self-made Tablets（C, D）







A new HPLC method was established for simultaneous determination of amoxicillin and its 14 related substances.



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研究论文

Isolation and Identification of Potential Impurities of Romidepsin

XIONG Lei, MIN Taoling, CHEN Changfa, HU Haifeng*

(*State Key Lab. of New Drug & Pharmaceutical Process, Shanghai Institute of Pharmaceutical Industry, China State Institute of Pharmaceutical Industry, Shanghai 201203*)

ABSTRACT: Romidepsin is an antitumor drug isolated from the fermentation broth of *Chromobacterium violaceum* NO.968. Seven impurities with the concentration range from 0.06% to 0.23% in romidepsin bulk drug were detected by HPLC. These impurities, named as Impurity-1, Impurity-2, Impurity-3, Impurity-4, Impurity-5, Impurity-6 and Impurity-7, were isolated from romidepsin bulk drug by reverse phase preparative HPLC. Their molecular structures were confirmed by 1D (^{1}H, ^{13}C, DEPT-135), 2D (HSQC, HMBC and ^{1}H-^{1}H COSY) NMR spectra and MS data. Compared with the chemical structure of romidepsin, Impurity-1 and Impurity-2 lacked two methyl groups at C-22 and C-17, respectively; Impurity-3, Impurity-4 and Impurity-5 lost one methyl group at C-22, C-17 and C-20, respectively; Impurity-6 contained a trisulfide bond and Impurity-7 possessed an additional methylene group at C-22. All these impurities might be the by-products of the biosynthesis pathway of romidepsin and some key factors in the fermentation process that related to the formation of impurities were studied herein.

Key Words: romidepsin; impurity; isolation; structure elucidation

罗米地辛潜在杂质的分离与鉴定

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（中国医药工业研究总院上海医药工业研究院，创新药物与制药工艺国家重点实验室，上海 201203）

摘要：罗米地辛是 1 种抗肿瘤抗生素，最初是从紫色色杆菌（*Chromobacterium violaceum*）NO.968 中分离得到的。首先，使用 HPLC 法对罗米地辛原料药进行分析，检测到 7 个杂质，含量 0.06%～0.23%。然后，通过反相制备液相获得以上杂质，分别命名为杂质 -1、杂质 -2、杂质 -3、杂质 -4、杂质 -5、杂质 -6 和杂质 -7。最后，通过 1D（^{1}H、^{13}C、DEPT-135）、2D（HSQC、HMBC、^{1}H-^{1}H COSY）核磁数据以及质谱数据确定上述杂质的化学结构。结果表明，杂质 -1 和杂质 -2 分别在 C-22 位和 C-17 位上比罗米地辛相应位置少 2 个甲基。杂质 -3、杂质 -4 和杂质 -5 分别在 C-22、C-17 和 C-20 位较罗米地辛相应位置少 1 个甲基。杂质 -6 含有 1 个三硫键，而杂质 -7 在 C-22 位上比罗米地辛相应位置多 1 个亚甲基。这些杂质可能是罗米地辛生物合成过程中的副产物，同时本研究对发酵过程中产生杂质的关键因素进行了考察。

关键词：罗米地辛；杂质；分离；结构解析

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1 Introduction

Romidepsin is a histone deacetylase inhibitor (HDACi)[1], approved by FDA for the treatment of recurrent T lymphocytic carcinoma[2-3] and peripheral T lymphocytic carcinoma[4]. The latest studies showed that romidepsin could activate the human latent HIV

virus[5], and these findings provided new possibilities for HIV cure.

Romidepsin was first isolated from the fermentation broth of *Chromobacterium violaceum* NO.968[1], and its biosynthetic gene clusters were studied[6-8]. Similar gene clusters were found in other microorganisms such as *Burkholderia* and *Pseudomonas*, and led to the discovery of romidepsin derivatives like thailandepsins[9], spiruchostatins[10], and FR901375[11].

There is no report about the separation and identification of the impurities of romidepsin. In order to meet the drug safety requirements, any individual impurities with amount no less than 0.1% must be identified. Unlike chemically synthesized drugs, romidepsin is derived from microorganisms which means most impurities may be by-products of the biosynthetic pathway of romidepsin and can be detected in the fermentation broth of *C. violaceum* NO.968.

Romidepsin bulk drug was obtained in Section "**2.5**" of this study and the separation process was described in the previous report[1]. Seven impurities in the concentration range of 0.06% - 0.23% were detected in the romidepsin bulk drug by HPLC. In this paper, we reported identification, isolation, structure elucidation of impurities of romidepsin. The conditions for the formation of impurities were studied, and guidance was provided for the development of high quality romidepsin bulk drug.

2 Experimental Method

2.1 Strains

A bacterium strain used in the experiment was *C. violaceum* NO.968 (NO. FERM BP-1968, from IPOD[a])[1,6].

2.2 Materials and Reagents

HPLC grade acetonitrile was purchased from Fisher Scientific International Inc. (Pittsburgh, American). Deionized water was purchased from Wahaha Group Co., Ltd. (Hangzhou, China). AR grade cyclohexane, ethyl acetate, isopropanol, methanol, *L*-cysteine, *L*-arginine, and KH_2PO_4 were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Chemical grade glucose, corn starch, beef extract, casein, mannitol, and corn syrup were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). $CDCl_3$ was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

2.3 High Performance Liquid Chromatography (HPLC) Method

An HPLC method was performed by using a Waters 515 pump with a Waters 2487 detector (Waters Corp., Milford, MA, USA). A Waters Symmetry C_{18} column (3.9 mm×150 mm, 5.0 μm particle size) was employed by maintaining temperature at 30 ℃. The mobile phase consisted of water and acetonitrile at the ratio of 65 ∶ 35 (*v*/*v*). The flow rate was maintained at 1 ml/min with UV detection wavelength of 210 nm. The fermentation broth sample was prepared as follows: 0.5 ml of acetone was added to 0.5 ml of fermentation broth, and the mixture was soaked for 2 h, then centrifuged at 12 000×*g* for 5 min. The injection volume was 5 μl. The impurities and romidepsin were dissolved in acetonitrile, then the solution was filtered through 0.22 μm membrane. The injection volume was 5 μl.

2.4 Mass and NMR Spectroscopies

Mass spectra were recorded on Waters Alliance (2695/2487) Q-Tof micro mass spectrometer (Waters Corp., Milford, MA, USA). NMR spectra were recorded on Bruker AV-400 NMR spectrometer operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR using $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal standard. DEPT spectral editing was used to identify methyl and

methine groups as positive peaks and methylene groups as negative peaks. 2D NMR (HSQC, HMBC and ^{1}H-^{1}H COSY) experiments were also performed using the same instrument and in same solvent for the assignment of the related chemical shift values of impurities.

2.5 Fermentation

The bacterial strain was cultured on nutrient agar (Bio-way technology Co., Shanghai, China) at 30 ℃ for 24 h. The seed from above was inoculated into a 750 ml Erlenmeyer flask containing 100 ml of sterile seed liquid medium containing 20 g/L peptone, 20 g/L glucose, and cultured on a rotary shaker (250 r/min at 30 ℃ for 16 h. Fermentation was performed in a FUS-50L (A) fermentor (National Center of Bio-Engineering & Technology, Shanghai, China) containing 30 L of fermentation culture medium [30 g/L glucose, 10 g/L starch, 20 g/L mannitol, 10 g/L casein, 8 g/L beef extract, 6 g/L KH_2PO_4, 2 g/L cysteine, 4 g/L soybean oil, 1 g/L $(NH_4)_2SO_4$, 2 g/L $CaCO_3$, pH 5.5, namely Medium A]. The fermentor was inoculated with 2% of the seed culture and maintained on a 200 r/min rotary shaker at 25 ℃ for 60 h.

2.6 Isolation and Purification

Romidepsin bulk drug was dissolved in methanol and then used for the isolation of impurities by preparative HPLC. Experiments were performed on the Elite P270 instrument (Elite Analytical Instruments Co., Ltd., Dalian, China) equipped with Elite UV 230+ detector (Elite Analytical Technologies Co., Ltd., Tianjin, China) for separation. The mobile phase consisted of water, acetonitrile and methanol at a volume ratio of 70 ∶ 20 ∶ 10. The flow rate was kept at 40 ml/min at the detection wavelength of 210 nm.

2.7 Formation of Impurities

2.7.1 Impurity-1, Impurity-2, Impurity-3 and Impurity-4

Medium B was prepared by adding $(NH_4)_2CO_3$ instead of $(NH_4)_2SO_4$ in Medium A, and other ingredients were unchanged. Inoculate 2% of the seed culture (Section "**2.5**") into a 250 ml Erlenmeyer flask containing 30 ml of Medium A and Medium B, respectively, and maintain on a 250 r/min rotary shaker at 25 ℃ for 48 h. The fermentation broth was analyzed by HPLC to examine the impact of $(NH_4)_2CO_3$ on the formation of romidepsin and the impurities.

2.7.2 Impurity-6

Fermentation was performed in a 250 ml Erlenmeyer flask containing 30 ml of Medium A. The fermentation medium was inoculated with 2% of the seed culture and maintained on a 250 r/min rotary shaker at 25, 28 and 30 ℃, respectively, for 48 h. The fermentation broth was analyzed by HPLC to examine the impact of temperature on the formation of Impurity-6.

3 Results and Discussion

3.1 Detection of Impurities by HPLC

The romidepsin bulk drug was analyzed by HPLC method as described in Section "**2.3**", disclosed the presence of romidepsin (RT=8.33 min, RRT=1.00) along with seven impurities, named as Impurity-1 (RT=3.72 min, RRT=0.45), Impurity-2 (RT=4.86 min, RRT=0.58), Impurity-3 (RT=5.66 min, RRT=0.68), Impurity-4 (RT=6.35 min, RRT=0.76), Impurity-5 (RT=9.77 min, RRT=1.17), Impurity-6 (RT=13.37 min, RRT=1.61) and Impurity-7 (RT=14.17 min, RRT=1.70), respectively. The typical HPLC chromatogram of romidepsin bulk drug was shown in Fig.1A. In this sample, the HPLC purity of romidepsin was 98.59%, and the contents of Impurities 1 - 7 were 0.23%, 0.14%, 0.12%, 0.10%, 0.22%, 0.06% and 0.10%, respectively. The isolated impurities were co-injected with romidepsin to confirm the identity of the impurities based on retention matching (Fig.1B).

3.2 Structure Elucidation

The isolated impurities and romidepsin were subjected to MS and NMR spectroscopic analysis.

Structures of romidepsin and its impurities were shown in Fig.2. The NMR (^{13}C, DEPT and ^{1}H) data of romidepsin and Impurities 1 - 7 were presented in Tab.1 - Tab.4, respectively. The key HMBC and ^{1}H-^{1}H COSY correlations of impurities were described in Fig.3.

3.2.1 Impurity-1

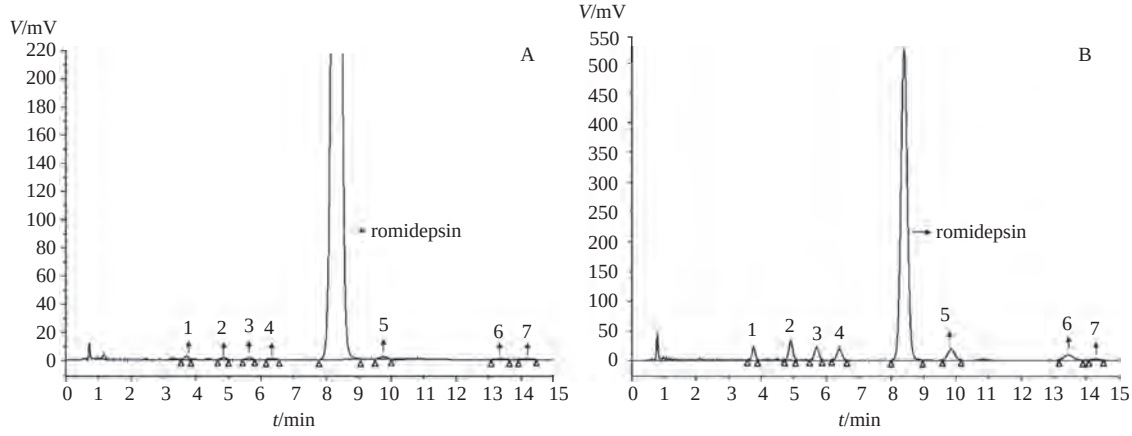
Impurity-1 was obtained as a white amorphous powder. Its molecular formula was determined as $C_{22}H_{32}O_6N_4S_2$ from the $[M+H]^+$ peak at *m/z* 513.184 6 (calculated 513.184 2 for $C_{22}H_{33}O_6N_4S_2$) in HRESIMS spectrum.

The ^{13}C and ^{1}H NMR data (Tab.1 and Tab.2) of Impurity-1 were similar to those of romidepsin. Based on detailed analysis of the above data, it was found two methyl groups (δ_C 19.75, δ_H 1.13 and δ_C 19.51, δ_H 1.11), which connected to C-22 in romidepsin, were absent in Impurity-1. The ^{13}C NMR and DEPT spectra of Impurity-1 indicated the presence of the new methyl carbon signal of C-22 (δ_C 15.8), accompany with the absence of the methine carbon (δ_C 29.2), when compared with that of romidepsin. The HMBC correlation from H_3-22 (δ_H 1.54) to C-13 (δ_C 52.0), together with ^{1}H-^{1}H COSY correlation between H_3-22 (δ_H 1.54) and H-13 (δ_H 4.29 - 4.35) (Fig.3) suggested that one methyl group at C-13 in Impurity-1, compared with the isopropyl group in that of romidepsin. Accordingly, the chemical structure of Impurity-1 was assigned (Fig.2).

3.2.2 Impurity-2

Impurity-2 was obtained as a white amorphous powder. Its molecular formula was determined as $C_{22}H_{32}O_6N_4S_2$ from the $[M+H]^+$ peak at *m/z* 513.184 2 (calculated 513.184 2 for $C_{22}H_{33}O_6N_4S_2$) in HRESIMS spectrum.

The ^{13}C and ^{1}H NMR data (Tab.1 and Tab.2) of Impurity-2 were similar to those of romidepsin except for the absent signals corresponding to two methyl groups (δ_C 18.53, δ_H 1.02 and δ_C 18.41, δ_H 0.99) at C-17. The ^{13}C NMR and DEPT spectra of Impurity-2 showed that the C-17 was a methyl carbon (δ_C 18.0), compared with the methine carbon (δ_C 32.3) in that of romidepsin. The HMBC correlation from H_3-17 (δ_H 1.50) to C-7 (δ_C 49.1) and ^{1}H-^{1}H COSY correlation between H_3-17 (δ_H 1.50) and H-7 (δ_H 4.62 - 4.69) (Fig.3) showing that one methyl group at C-7 in



Peaks 1 - 7: Impurities 1 - 7

Fig.1 HPLC Chromatograms of Romidepsin Bulk Drug Sample (A) and Romidepsin Bulk Drug with Seven Impurities (B)

图 1 罗米地辛原料药 (A) 以及罗米地辛原料药混合 7 个杂质 (B) 的 **HPLC** 图谱

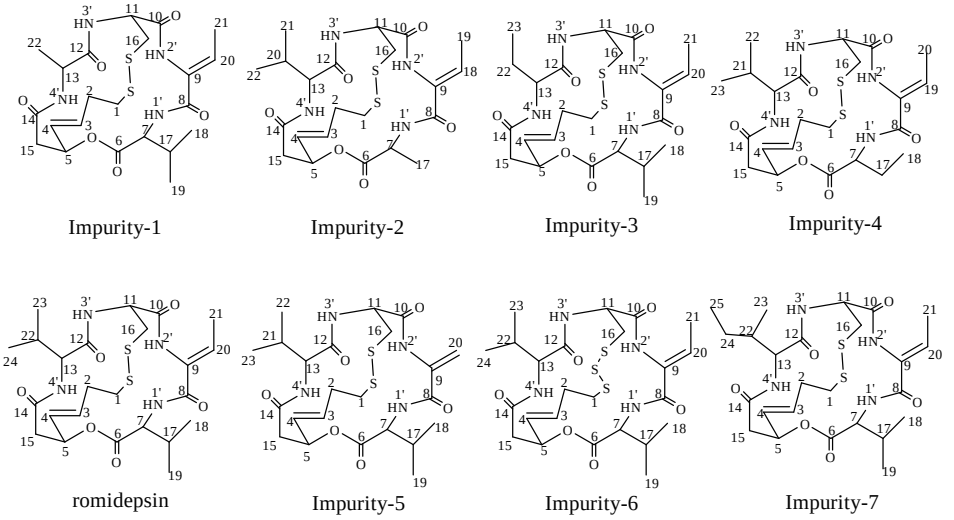


Fig.2 Structures of Romidepsin and Its Impurities

图 2 罗米地辛及其相关杂质的化学结构式

Impurity-1, compared with the isopropyl group in that of romidepsin. Therefore, the structure of Impurity-2 was assigned (Fig.2).

3.2.3 Impurity-3

Impurity-3 was obtained as a white amorphous powder. The molecular formula was determined as $C_{23}H_{34}O_6N_4S_2$ from the $[M+H]^+$ peak at *m/z* 527.200 2 (calculated 527.199 8 for $C_{23}H_{35}O_6N_4S_2$) in HRESIMS spectrum.

The ^{13}C and ^{1}H NMR data (Tab.1 and Tab.2) of Impurity-3 were similar to those of romidepsin with an apparent difference being the absence of a methyl group at C-22. The ^{13}C NMR spectrum of Impurity-3 revealed an additional methylene carbon signal (δ_C 29.2) determined to be C-22, coupled with the disappearance of a methine carbon signal (δ_C 24.0), when compared with that of romidepsin. HMBC correlations (Fig.3) from H_2-22 (δ_H 1.78 - 1.84, 1.96 - 2.06) to C-23 (δ_C 11.3) and from H_3-23 (δ_H 1.11) to C-22 (δ_C 24.0) revealed only one methyl group connected to C-22 in Impurity-3, compared with two methyl groups in that of romidepsin. Based on the above analysis, the structure of Impurity-3 was assigned (Fig.2).

Tab.1 ^{13}C NMR Data of Romidepsin, Impurity-1, Impurity-2 and Impurity-3 in $CDCl_3$ (100 MHz)

表 1 罗米地辛、杂质 -1、杂质 -2 和杂质 -3 的碳谱数据 (100 MHz, $CDCl_3$)

Position[a]	δ_C/DEPT			
	Romidepsin	Impurity-1	Impurity-2	Impurity-3
1	38.2/CH_2	39.1/CH_2	38.1/CH_2	39.1/CH_2
2	30.5/CH_2	31.1/CH_2	30.5/CH_2	31.2/CH_2
3	129.9/CH	130.7/CH	129.8/CH	130.5/CH
4	131.2/CH	131.6/CH	131.3/CH	132.0/CH
5	69.8/CH	69.6/CH	70.0/CH	69.5/CH
6	169.2/qC	169.1/qC	171.9/qC	169.3/qC
7	58.1/CH	58.0/CH	49.1/CH	58.08/CH
8	165.1/qC	165.0/qC	163.9/qC	164.9/qC
9	130.5/qC	130.5/qC	129.9/qC	130.6/qC
10	168.6/qC	168.9/qC	169.1/qC	168.9/qC
11	56.2/CH	56.1/CH	55.8/CH	56.1/CH
12	172.3/qC	173.5/qC	172.5/qC	172.9/qC
13	62.4/CH	52.0/CH	61.9/CH	58.11/CH
14	170.4/qC	169.6/qC	170.1/qC	169.8/qC
15	38.1/CH_2	37.2/CH_2	37.2/CH_2	38.0/CH_2
16	34.1/CH_2	34.0/CH_2	33.6/CH_2	34.2/CH_2
17	32.3/CH	32.4/CH	18.0/CH_3	32.5/CH
18	18.5/CH_3	18.4/CH_3	130.2/CH	18.7/CH_3
19	18.4/CH_3	18.7/CH_3	13.1/CH_3	18.6/CH_3
20	128.6/CH	128.6/CH	29.2/CH	128.9/CH
21	13.2/CH_3	13.0/CH_3	19.5/CH_3	13.2/CH_3
22	29.2/CH	15.8/CH_3	19.6/CH_3	24.0/CH_2
23	19.8/CH_3			11.3/CH_3
24	19.5/CH_3			

[a] Refer chemical structure in Fig.2 for numbering romidepsin and impurities.

3.2.4 Impurity-4

Impurity-4 was obtained as a white amorphous powder. The molecular formula was determined as $C_{23}H_{34}O_6N_4S_2$ from the $[M+H]^+$ peak at *m/z* 527.200 0 (calculated 527.199 8 for $C_{23}H_{35}O_6N_4S_2$) in HRESIMS spectrum.

The structure of Impurity-4 was similar to that of romidepsin, except that one methyl group was absent at C-17 in Impurity-4. The ^{13}C NMR and DEPT spectra (Tab.3) of Impurity-4 indicated that C-17 was a methylene carbon (δ_C 24.9), compared with the methine carbon (δ_C 32.3) in romidepsin. ^1H-^1H COSY correlation (Fig.3) of H_3-18 (δ_H 0.94) with H_2-17 (δ_H 1.90 - 2.00, 2.08 - 2.18) and HMBC correlation (Fig.3) from H_2-17 (δ_H 1.90 - 2.00, 2.08 - 2.18) to C-18 (δ_C 9.1) suggested that one methyl at C-17 in Impurity-4, compared with two methyl groups in that of romidepsin. Based on the spectra data, Impurity-4 was assigned (Fig.2).

3.2.5 Impurity-5

Impurity-5 was obtained as a colorless crystal. The molecular formula was determined as $C_{23}H_{34}O_6N_4S_2$ from the $[M+H]^+$ peak at *m/z* 527.199 7 (calculated 527.199 8 for $C_{23}H_{35}O_6N_4S_2$) in HRESIMS spectrum.

The ^{13}C NMR and ^1H NMR data of Impurity-5 were shown in Tab.3 and Tab.4, respectively. Detail comparison of ^1H and ^{13}C NMR data of Impurity-5 with those of romidepsin revealed the structure similarity, except that a methyl group (δ_C 13.2, δ_H 1.75) at C-20 was absent in Impurity-5. The ^{13}C NMR data of Impurity-5 and romidepsin showed that the unsaturated methine carbon C-20 (δ_C 128.6) in romidepsin was

Tab.2 ^1H NMR Data of Romidepsin, Impurity-1, Impurity-2 and Impurity-3 in $CDCl_3$ (400 MHz)

表 2　罗米地辛、杂质 -1、杂质 -2 和杂质 -3 的氢谱数据 (400 MHz, $CDCl_3$)

Position[a]	δ_H			
	Romidepsin	Impurity-1	Impurity-2	Impurity-3
1	2.95～3.02 (m, 1H), 3.10～3.15 (m, 1H)	2.94～2.98 (m, 1H), 3.17～3.20 (m, 1H)	2.97～3.02 (m, 1H), 3.13～3.16 (m, 1H)	2.92～2.98 (m, 1H), 3.14～3.17 (m, 1H)
2	2.66～2.67 (m, 2H)	2,73 (br s, 2H)	2.65～2.72 (m, 2H)	2.67～2.69 (m, 2H)
3	5.77～5.84 (m, 1H)	5.81～5.88 (m, 1H)	5.78～5.80 (m, 1H)	5.77～5.80 (m, 1H)
4	5.70～5.73 (m, 1H)	5.71～5.75 (m, 1H)	5.76～5.77 (m, 1H)	5.68～5.73 (m, 1H)
5	5.74～5.76 (m, 1H)	5.75～5.78 (m, 1H)	5.68～5.70 (m, 1H)	5.73～5.76 (m, 1H)
7	4.61 (dd, *J*=7.9, 3.7 Hz, 1H)	4.63 (dd, *J*=8.0, 3.6 Hz, 1H)	4.62～4.69 (m, 1H)	4.65 (dd, *J*=8.0, 3.7 Hz, 1H)
11	4.73～4.79 (m, 1H)	4.73～4.79 (m, 1H)	4.77～4.83 (m, 1H)	4.73～4.78 (m, 1H)
13	4.05 (dd, *J*=6.7, 4.4 Hz, 1H)	4.29～4.35 (m, 1H)	4.12 (dd, *J*=6.6, 5.2 Hz, 1H)	4.15～4.19 (m, 1H)
15	2.78～2.80 (m, 2H)	2.77～2.80 (m, 2H)	2.60 (d, *J*=13.4 Hz, 1H), 3.04～3.09 (m, 1H)	2.66～2.67 (m, 1H), 2.80～2.86 (m, 1H)
16	3.17～3.22 (m, 2H)	3.11～3.27 (m, 2H)	3.14～3.18 (m, 1H), 3.26～3.33 (m, 1H)	3.11～3.14 (m, 1H), 3.20～3.26 (m, 1H)
17	2.38～2.46 (m, 1H)	2.39～2.47 (m, 1H)	1.50 (d, *J*=6.8 Hz, 3H)	2.37～2.45 (m, 1H)
18	1.02 (d, *J*= 6.9 Hz, 3H)	1.04 (d, *J*=6.9 Hz, 3H)	6.52～6.57 (m, 1H)	1.02 (d, *J*=6.9 Hz, 3H)
19	0.99 (d, *J*=6.9 Hz, 3H)	1.01 (d, *J*=6.9 Hz, 3H)	1.75 (d, *J*=7.12 Hz, 3H)	0.99 (d, *J*= 6.9 Hz, 3H)
20	6.31 (q, *J*=7.1 Hz, 1H)	6.35 (q, *J*=7.1 Hz, 1H)	2.20～2.29 (m, 1H)	6.33～6.38 (m,1H)
21	1.75 (d, *J*=7.2 Hz, 3H)	1.76 (d, *J*=7.1 Hz, 3H)	1.10 (br s, 3H)	1.74 (d, *J*=7.1 Hz, 3H)
22	2.21-2.29 (m, 1H)	1.54 (d, *J*=7.4 Hz, 3H)	1.12 (br s, 3H)	1.78～1.84 (m, 1H), 1.96～2.06 (m, 1H)
23	1.11 (br s, 3H)			1.11 (t, *J*=7.4 Hz, 3H)
24	1.13 (br s, 3H)			
1'	7.44 (d, *J*=8.0 Hz, 1H)	7.28 (d, *J*=8.2 Hz, 1H)	7.22 (d, *J*=7.1 Hz, 1H)	7.15 (d, *J*=8.0 Hz, 1H)
2'	8.39 (s, 1H)	8.21 (s, 1H)	8.23 (s, 1H)	8.14 (s, 1H)
3'	7.78 (d, *J*=6.6 Hz, 1H)	7.67 (d, *J*=7.1 Hz, 1H)	7.74 (d, *J*=6.8 Hz, 1H)	7.65 (d, *J*=7.0 Hz, 1H)
4'	7.1 (d, *J*=4.5 Hz, 1H)	7.08 (d, *J*=4.3 Hz, 1H)	6.47 (d, *J*=5.3 Hz, 1H)	6.50 (d, *J*=4.6 Hz, 1H)

[a] Refer chemical structure in Fig.2 for numbering romidepsin and impurities.

Tab.3 ^{13}C NMR Data of Impurity-4, Impurity-5, Impurity-6 and Impurity-7 in $CDCl_3$ (100 MHz)

表 3 杂质 -4、杂质 -5、杂质 -6 和杂质 -7 的碳谱数据（100 MHz, $CDCl_3$）

Position[a]	δ_C/DEPT			
	Impurity-4	Impurity-5	Impurity-6	Impurity-7
1	37.9/CH_2	37.2/CH_2	40.6/CH_2	38.1/CH_2
2	30.4/CH_2	29.2/CH_2	29.0/CH_2	30.2/CH_2
3	129.7/CH	128.9/CH	129.9/CH	128.8/CH
4	131.3/CH	129.9/CH	132.7/CH	130.9/CH
5	69.6/CH	70.2 /CH	70.4/CH	69.9/CH
6	170.3/qC	169.8/qC	169.2/qC	169.1/qC
7	54.0/CH	57.4/CH	58.0/CH	58.2/CH
8	164.1/qC	165.1/qC	164.3/qC	165.1/qC
9	129.9/qC	136.1/qC	129.8/qC	130.5/qC
10	168.8/qC	169.0/qC	169.0/qC	168.7/qC
11	55.8/CH	59.3/CH	50.6/CH	56.7/CH
12	172.2/qC	172.2 /qC	172.5/qC	172.5/qC
13	62.0/CH	62.1/CH	63.0/CH	60.6/CH
14	169.9/qC	171.8/qC	170.2/qC	170.9/qC
15	38.1/CH_2	38.0/CH_2	40.5/CH_2	38.3/CH_2
16	33.8/CH_2	35.4/CH_2	47.8/CH_2	34.5/CH_2
17	24.9/CH_2	31.4/CH	32.3/CH	32.3/CH
18	9.1/CH_3	17.6/CH_3	18.8/CH_3	18.5/CH_3
19	129.8/CH	19.1/CH_3	18.5/CH_3	18.4/CH_3
20	13.1/CH_3	111.3/CH_2	132.0/CH	128.9/CH
21	29.3/CH	29.3/CH	13.4/CH_3	13.3/CH_3
22	19.6/CH_3	18.9/CH_3	29.8/CH	35.4/CH
23	19.6/CH_3	19.3/CH_3	20.1/CH_3	16.1/CH_3
24			19.6/CH_3	26.2/CH_2
25				11.6/CH_3

[a] Refer chemical structure in Fig.2 for numbering romidepsin and impurities.

replaced by an unsaturated methylene carbon (δ_C 111.3) in Impurity-5. Alternatively, the chemical shift value of C-9, which attached in C-20, was at δ_C 136.1 compared with δ_C 130.5 in that of romidepsin. The HMBC correlation from H_2-20 (δ_H 5.66, 5.91) to C-9 (δ_C 136.1) suggested that a methyl group was absent at C-20 in Impurity-5 when compared with that of romidepsin. The chemical structure of Impurity-5 was very similar to that of romidepsin. In fact, the main difference between them was that the vinyl methyl group in romidepsin was disappeared in Impurity-5.

3.2.6 Impurity-6

Impurity-6 was obtained as a colorless crystal. The molecular formula was determined as $C_{24}H_{36}O_6N_4S_3$ from the $[M+Na]^+$ peak at *m/z* 595.170 8 (calculated 595.169 5 for $C_{24}H_{36}O_6N_4S_3Na$) in HRESIMS spectrum, which indicated an additional sulfur atom in the structure of Impurity-6 than that of romidepsin.

By comparison of ^{13}C NMR data (Tab.3) of Impurity-6 with that of romdepsin, it was found that the chemical shift values, C-1, C-2, C-11 and C-16 varied widely. The chemical shift values of C-1, C-2, C-11 and C-16 were δ_C 40.6, δ_C 29.0, δ_C 50.6 and δ_C 47.8, respectively, in Impurity-6 compared with δ_C 38.2, δ_C 30.5, δ_C 56.2 and δ_C 34.1 in that of romidepsin. The extra sulfur atom could affect the chemical shift values of C-1, C-2, C-11 and C-16. By analyzing the structure of romidepsin, it was found that the group between C-1, C-2 and C-11, C-16 was the disulfide bond (-S-S-). Based on the above spectral data, Impurity-6 contained a trisulfide bond (-S-S-S-) (Fig.2) with other parts identical to that romidepsin.

3.2.7 Impurity-7

Impurity-7 was obtained as a colorless crystal. The molecular formula was determined as $C_{25}H_{38}O_6N_4S_2$ from the $[M+H]^+$ peak at *m/z* 555.231 5 (calculated 555.231 1 for $C_{25}H_{39}O_6N_4S_2$) in HRESIMS spectrum.

The ^{13}C and ^1H NMR data (Tab.3 and Tab.4) of Impurity-7 indicated the presence of an additional methylene group (δ_C 26.2, δ_H 1.35 - 1.44, 1.52 - 1.58), when compared with those of romidepsin. HMBC correlations (Fig.3) from H_2-24 (δ_H 1.35 - 1.44, 1.52 - 1.58) to C-22 (δ_C 35.4) and C-25 (δ_C 11.6), together with ^1H-^1H COSY correlations from H_2-24 (δ_H 1.35 - 1.44, 1.52 - 1.58) to H_3-25 (δ_H 0.94 - 0.96) and H-13 (δ_H 4.23) indicated that the additional methylene group was connected between C-22 and C-25. Based on the above data, the structure of Impurity-7 was a romidepsin derivative with an additional methylene group (Fig.2).

3.3 Formation of Impurities

$(NH_4)_2SO_4$ had been used as a nitrogen resource

Tab.4 ^{1}H NMR Data of Impurity-4, Impurity-5, Impurity-6 and Impurity-7 in $CDCl_3$ (400 MHz)

表 4　杂质 -4、杂质 -5、杂质 -6 和杂质 -7 的氢谱数据（400 MHz, $CDCl_3$）

Position[a]	δ_H			
	Impurity-4	Impurity-5	Impurity-6	Impurity-7
1	2.98～3.01 (m, 1H), 3.13～3.16 (m, 1H)	2.97～3.02 (m, 1H), 3.07～3.09 (m, 1H)	2.84～2.87 (m, 1H), 3.23～3.26 (m, 1H)	2.97～3.09 (m, 2H)
2	2.62～2.70 (m, 2H)	2.52～2.56 (m, 1H), 2.64～2.69 (m, 1H,)	2.54～2.57 (m, 1H), 2.69～2.73 (m, 1H)	2.61～2.62 (m, 2H)
3	5.76～5.77 (m, 1H)	5.75～5.79 (m, 1H)	5.72～5.75 (m, 1H)	5.75～5.82 (m, 1H)
4	5.78～5.80 (m, 1H)	5.69～5.73 (m, 1H)	5.81～5.82 (m, 1H)	5.70～5.75 (m, 1H)
5	5.75～5.76 (m, 1H)	5.64 (br s , 1H)	5.66～5.69 (m, 1H)	5.70～5.72 (m, 1H)
7	4.71 (dd, *J*=7.3, 5.1 Hz, 1H)	4.78 (dd, *J*=8.5, 3.4 Hz, 1H)	4.64 (dd, *J*=8.2, 4.1 Hz, 1H)	4.61 (dd, *J*=7.8, 3.6 Hz, 1H)
11	4.80～4.86 (m, 1H)	4.52～4.57 (m, 1H)	5.20～5.23 (m, 1H)	4.77 (q, *J*=7.7 Hz, 1H)
13	4.14 (t, *J*=6.0 Hz, 1H)	3.99 (t, *J*=4.3 Hz, 1H)	3.96～3.99 (m, 1H)	4.23 (t, *J*=5.1 Hz, 1H)
15	2.58 (d, *J*=13.5 Hz, 1H), 2.99～3.01 (m, 1H)	2.76 (d, *J*=13.7 Hz, 1H), 2.85～2.86 (m, 1H)	2.79～2.81 (m, 1H), 2.52～2.54 (m, 1H)	2.76～2.82 (m, 2H)
16	3.17～3.22 (m, 1H), 3.28～3.35 (m, 1H)	2.94～2.97 (m, 1H), 3.00～3.04 (m, 1H)	4.02～4.03 (m, 1H), 2.80～2.83 (m, 1H)	3.17 (d, *J*=8.1 Hz, 2H)
17	1.90～2.00 (m, 1H), 2.08～2.18 (m, 1H)	2.44～2.49 (m, 1H)	2.29～2.37 (m, 1H)	2.35～2.45 (m, 1H)
18	0.94 (t, *J*=7.4 Hz, 3H)	0.95 (d, *J*=6.9 Hz, 3H)	0.97 (d, *J*=6.9 Hz, 3H)	0.97～0.99 (m, 3H)
19	6.54 (q, *J*=7.1 Hz, 1H)	0.99 (d, *J*=6.9 Hz, 3H)	0.94 (d, *J*=6.9 Hz, 3H)	1.01～1.04 (m, 3H)
20	1.76 (d, *J*=7.1 Hz, 3H)	5.66 (s, 1H), 5.91 (s, 1H)	6.61 (q, *J*=7.1 Hz, 1H)	6.33 (q, *J*=7.0 Hz, 1H)
21	2.22～2.30 (m, 1H)	2.20～2.25 (m, 1H)	1.68 (d, *J*=7.2 Hz, 3H)	1.74 (d, *J*=7.2 Hz, 1H)
22	1.13 (d, *J*=1.8 Hz, 3H)	1.08 (d, *J*=3.4 Hz, 3H)	2.14 (br s,1H)	2.06～2.13 (m, 1H)
23	1.14 (d, *J*=1.8 Hz, 3H)	1.06 (d, *J*=3.4 Hz, 3H)	1.06 (d, *J*=6.5 Hz, 3H)	1.08～1.12 (m, 3H)
24			1.08 (d, *J*=6.5 Hz, 3H)	1.35～1.44 (m, 1H), 1.52～1.58 (m, 1H)
25				0.94～0.96 (m, 3H)
1'	7.19 (d, *J*=8.0 Hz, 1H)	7.73 (d, *J*=8.5 Hz, 1H)	7.22 (d, *J*=8.2 Hz, 1H)	7.47 (d, *J*=7.9 Hz, 1H)
2'	8.22 (s, 1H)	7.95 (s, 1H)	7.87 (s, 1H)	8.32 (s, 1H)
3'	7.74 (d, *J*=6.8 Hz, 1H)	7.91 (d, *J*=4.8 Hz, 1H)	7.15 (d, *J*=9.1 Hz, 1H)	7.75 (d, *J*=6.8 Hz, 1H)
4'	6.27 (d, *J*=5.0 Hz, 1H)	7.26-7.27 (m, 1H)	6.50 (d, *J*=4.5 Hz, 1H)	7.19 (d, *J*=4.5 Hz, 1H)

[a] Refer chemical structure in Fig. 2 for numbering romidepsin and impurities.

in the culture of *C. violaceum* NO.968 to produce romidepsin[12]. However, in this paper, it was found that $(NH_4)_2CO_3$ compared to $(NH_4)_2SO_4$ could significantly inhibited the formation of Impurity-1, Impurity-2, Impurity-3, Impurity-4 and Impurity-5. Impurity-1, Impurity-2, Impurity-3 and Impurity-4 were hardly detected in the HPLC chromatogram of the fermentation broth when $(NH_4)_2CO_3$ (Fig.4B) was used instead of $(NH_4)_2SO_4$ (Fig.4A) in the media, moreover the yield of romidepsin was increased.

The formation of Impurity-6 was regulated by fermentation temperature, when the fermentation temperature was 28 ℃, the yield of Impurity-6 increased by 429.7% and that of romidepsin decreased by 67.7%, when compared with the fermentation temperature at 25 ℃ (Fig.5). Based on the above data, the addition of $(NH_4)_2CO_3$ to the medium and lower fermentation temperature could promote the yield of romidepsin while inhibit the formation of impurities.

However, the generation mechanism of impurities needed further studies and it was speculated that these impurities were by-products in the biosynthesis process of romidepsin. The biosynthetic gene clusters of romidepsin belonged to NRPS-PKS[8]. By analyzing chemical structures of Impurities 1 - 7, except for the Impurity-6, other impurities differ from romidepsin at C-7, C-9 and C-13. The *dep D* is responsible for the assembly of the groups at C-13[6], and *dep E* for

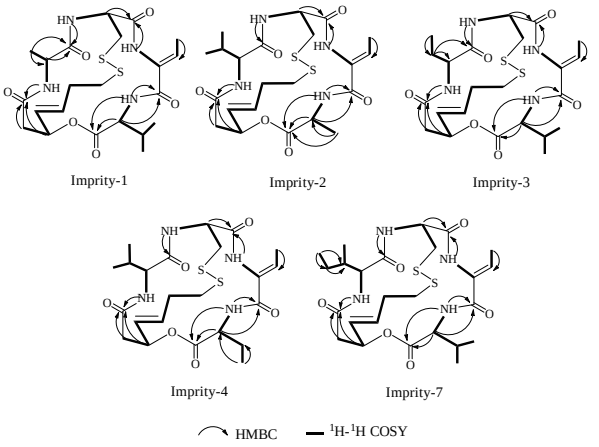


Fig.3 Key HMBC and ^{1}H-^{1}H COSY Correlation of Impurity-1, Impurity-2, Impurity-3, Impurity-4 and Impurity-7

图 3 杂质 -1、杂质 -2、杂质 -3、杂质 -4 和杂质 -7 的关键 HMBC 和 ^{1}H-^{1}H COSY 关联

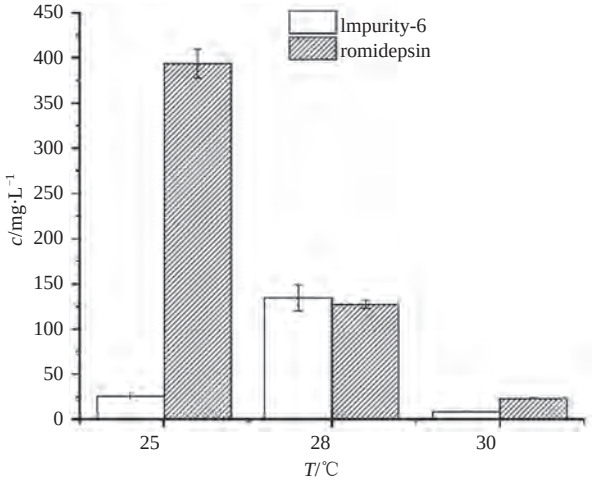


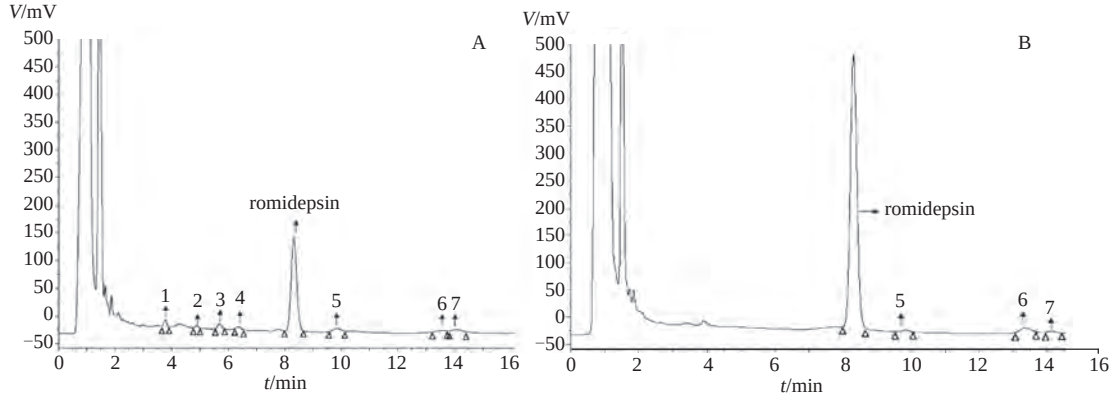
Fig.5 Effect of Fermentation Temperature on the Yields of Impurity-6 and Romidepsin (n=3)

图 5 发酵温度对杂质 -6 和罗米地辛产量的影响

the assembly of groups at C-7 and C-9[6]. There may be some genes that can assemble other groups into the skeleton of romidepsin to form impurities. The formation of impurities was closely related to the strain and the fermentation process. Therefore, in order to inhibit the production of impurities, it was necessary to further study the strain screening and fermentation process.

4 Conclusions

In this study, seven impurities in romidepsin bulk drug were detected and isolated by HPLC. Based on their MS and NMR spectral data, the structures of these impurities were identified. As far as we known, it was the first paper on the detection, structure identification and formation of impurities of romidepsin, which laid the foundation for quality control of romidepsin. Both impurities were structurally related to romidepsin. The



Peaks 1 - 7: Impurities 1 - 7

Fig.4 HPLC Chromatograms of Fermentation Media with Addition of $(NH_4)_2SO_4$ (A), Fermentation Media with Addition of $(NH_4)_2CO_3$ (B)

图 4 添加硫酸铵（A）和碳酸铵（B）的发酵液 HPLC 图谱

NMR data of Impurity-5 and Impurity-7 were reported in the previous report without chemical structure[13]. To the best of our knowledge, this was the first report of the full NMR data assignment of Impurities-5 and Impurity-7. Impurity-6 named chromopeptide A was a known compound produced by a marine derived bacterium *Chromobacterium* sp. HS-13-94[14]. Impurity-1, Impurity-2, Impurity-3 and Impurity-4 were first reported in this paper. These impurities might be the by-products in the biosynthesis pathway of romidepsin. The fermentation medium and temperature could influence the production of impurities, and the fermentation process of low-impurity romidepsin was proposed here.

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