PROFESSOR FANG NIE YU (Orcid ID : 0000-0003-4344-7999)

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Minor structural modifications to Pracinostat produce big changes in its biological responses

Rong Jia[a], Pengju Sun[a], Yan Zhang[a], Youjin Ge[a], Niefang Yu[a*]

[a]*Institute of Molecular Design and Drug Discovery, School of Pharmaceutical Sciences, Central South University, Changsha 410013, Hunan, China*

Correspondence

[*]Niefang Yu, Institute of Molecular Design and Drug Discovery, School of Pharmaceutical Sciences, Central South University, Changsha 410013, Hunan, China.

Email: niefang_yu@126.com

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ABSTRACT: A series of compounds similar to Pracinostat that contained benzimidazole ring and *N*-hydroxyacrylamide attached at 5- or 6-position were designed, synthesized and evaluated as HDAC inhibitors. It was interesting to find that the corresponding derivative **1** with *N*-hydroxyacrylamide attached at 5-position was a potent HDAC inhibitor while the others at 6-position were not. This is the first time to demonstrate the position difference plays important role in the HDAC inhibitory activities of the cinnamic hydroxamates.

KEY WORDS

Pracinostat, histone deacetylases, HDAC inhibitors, hydroxamic acids, synthesis

1 | INTRODUCTION

HDACs have emerged as typical effective therapeutic targets for anticancer agents. To date, five HDAC inhibitors have been approved for cancer therapy and many more in different phases of clinical trials for diverse indications [1][2][4]. The hydroxamic acid-based compounds are among those HDAC inhibitors, which received the most widely studies[3]. Among them, Pracinostat **1** is a promising compound, which has been granted Breakthrough Therapy Designation by FDA for the treatment of patients with newly diagnosed acute myelocytic leukemia (AML) who are ≥75 years of age or unfit for intensive chemotherapy[5][6]. Currently, Pracinostat is undergoing phase III clinical trials[5][6][7][8][9].

In pre-clinical animal models, Pracinostat is a potent pan-HDAC inhibitor with favourable pharmacokinetic properties[10][11]. As one of the inventors, the correpsonding author of this article has witnessed the broad and thoroughly structure-activity relationships based on the scaffold of Pracinostat[12][13]. However, no examples were reported in the literature since about the synthesis and bioevaluation of Pracinostat derivatives with *N*-hydroxyacrylamide attached to the 6-position of benzimidazole ring, from our hands or from any other groups (**Figure 1**). Thus, we put ourselves to design, synthsize and compare their differences in biological activities *in vitro*.

2 | EXPERIMENTS AND METHODS

The aim of this study is to first access if similar compounds with only structural postion changes could induce any differences in their enzymatic and cell-based inhibitory activities. Secondly, we would investigate their corresponding signaling pathways (**Figure 2**). To our knowlege, a typical HDAC inhibitor should be able to increase the expression of Ac-H3, Ac-H4, and Ac-α-tubulin, in addition to their corresponding enzymatic and cell-based inhibitory activities.

2.1 | General procedure for the synthesis of target compounds 2a-2p

The synthesis of target compounds 2 were shown in **Scheme 2**. 3-chloro-4-nitrobenzoic acid was selectively reduced by Borane-THF under room temperature and **4** thus obtained in high yield. Compound **6** could be obtained from **4** by managed oxidation and Horner–Wadsworth–Emmons reaction [14]. Amination of ethyl (E)-3-(3-chloro-4-nitrophenyl) acrylate **6** with amine $\mathbf{R^1NH_2}$ under basic conditions gave the intermediates**7a-7h**. Heating a mixture of the **7**, $\mathbf{R^2CHO}$ and $SnCl_2{\cdot}2H_2O$ in AcOH/MeOH (1:9) at 45℃ overnight led to the desired compounds **8a-8p.** Finaly, treatment of the intermediate **8** with excessive Hydroxylamine hydrochloride in sodium methoxide/methanol gave the objective hydroxamates **2** (**scheme 1**).

The synthetic route for the Pracinostat is shown in **Scheme 2**, which was prepared from ready available 4-Chloro-3-nitrocinnamic acid, **9**.

2.2 | Molecular docking

The crystal structure of human HDAC homolog (PDB code: 1C3R) was retrieved from the Protein Data Bank. All target compounds were docked in the active sites by MOE. Both HDAC homolog and ligand were structurally optimized prior to the actual docking simulation. Prior to the docking, the receptor was pre-treated according to the standard procedure provided by MOE and energy minimized using the Amber-99 forcefield.

The standard protocol implemented in MOE was performed in docking calculations. The binding modes were analyzed by 2D and 3D tool as well as *SiteView* of the MOE software.

2.3 | Biology

2.3.1 | HDAC inhibitory assay

HDAC inhibition were detected using the Amplite™ Fluorimetric HDAC Activity Assay Kit (Green Fluorescence , AAT Bioquest®, Inc), Hela nuclear extract (BioVision) and HDAC6 (BPS bioscience) following the manufacturer's protocol. The assay was performed in a volume of 25 µl at 37℃ in 384-well white plates. The final components of the assay ingredients were 10 µl enzyme solution, 2.5 µl test compounds, and 12.5 µl HDAC Green™ Substrate. The compounds were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 10 mM, and diluted in assay buffer before use. The highest concentration of Pracinostat is 10 µM and the highest concentration of others targeted compounds is 20 µM. The experiment was carried out in triplicate for all investigated IC_{50} values. The IC_{50} values were calculated by Prism Graphpad Prism v.5 software.

2.3.2 | Cell proliferation assay

The antiproliferative activity of synthesized compounds **2a-2p** was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Four human cancer cell lines (HCT-116, A549, MCF-7 and SW-1990) were treated for 72 h with various concentration of the isolated compounds. All cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were kept at 37°C in a humidified incubator with 5% carbon dioxide. All compounds were dissolved in DMSO to make a stock solution at 10 mM and diluted with medium at different concentrations before use. Cells plated in 96-well plates were treated with Pracinostat or Pracinostat derivatives for 72 h at 37°C, and then incubated with MTT for 4 h. After MTT removal, 150 µL DMSO was added into each well and the absorption values were measured at 490 nm using a Cytation5 imaging reader (Bio Tek Instruments. Inc. USA). The IC_{50} values were obtained by Prism Graphpad Prism v.5 software.

2.3.3 | Western blotting

HCT-116 cells were exposed to different concentrations of compound 2a (0.33 µM and 10 µM) and the control group was treated with Pracinostat at a concentration of 0.33 µM, both of them were treated for 24 h. Total protein was extracted, and Western blotting was performed as normally described. The treated cells were collected, RIPA buffer (Solarbio) contained a protease inhibitor (Selleck) cocktail ($V_{RIPA\ buffer}$:$V_{protease\ inhibitor}$=100:1) was used to lyse the cells on ice for 30 min, followed by centrifugation at 12000 rpm for 15 min at 4℃. The supernatants was collected and protein concentration was quantified by a BCA Protein Assay Kit (Solarbio). 25 µg proteins were fractionated by SDS-PAGE (8-14% gradient gels), electrophoresed and transferred on to a PVDF membrane and blocked with 5% non-fat milk in TBS for at least 1 h. The membranes were incubated with the primary and secondary antibodies and detected using the ChemiDoc XRS+ Gel Imaging System (Bio-Rad, USA). Primary antibodies against human Acetyl-Histone H3 and Acetyl-Histone H4 were obtained from Cell Signaling Technology (Beverly, MA, USA). Acetyl α-tubulin antibodies were purchased from Abbkine, Inc. (California, USA).

3 | RESULTS AND DISCUSSION

All the structures of **2a**-**2p** and key intermediates were characterized by ^{1}H NMR, ^{13}C NMR and ESI-MS and in full agreement with the proposed structures. The spectra were shown in supplemental material.

Docking compound **2a** into the HDAC homology model (PDB code: 1C3R) by MOE software (**Figure 3**), which revealed that the structural moiety of *N*-hydroxyacrylamide of the target compound **2a** interacted with the residues in the active sites of HDAC homology model in the similar way as that of Pracinostat, while the rest of the substituents at postion-1 and postion-2 are quite different. As illustrated in Figure 2A, the *N*-hydroxyacrylamide group of the Pracinostat and 2a has three H-bond interactions with the residues Tyr297, Gly140 and His132 in the ligand-binding pocket, respectively. The length of the hydrogen bonds formed between the *N*-hydroxyacrylamide group of the Pracinostat with the residues Tyr297, Gly140 and His132 are 2.46Å, 2.68Å and 2.55Å, respectively, and the corresponding hydrogen bond energies are -1.4 kcal/mol, -1.1 kcal/mol and -0.9 kcal/mol. Similarly, the length of the hydrogen bonds formed between the *N*-hydroxyacrylamide group of 2a with the residues Tyr297, Gly140 and His132 are 2.81Å, 2.59Å and 2.69Å, respectively, and the corresponding hydrogen bond energies are -1.0 kcal/mol, -2.5 kcal/mol and -3.3 kcal/mol. Notably, the substituent in the 1-position of benzimidazole ring of Pracinostat forms a hydrogen bond with the residue Tyr91 (N−H separation = 2.62 Å, hydrogen bond energies = -0.7 kcal/mol), whereas the compound 2a does not establish this interaction.

The enzyme inhibition assay results showed that all the prepared compounds (**2a**–**2p**) exhibited significantly weaker Pan-HDAC inhibitory activities than Pracinostat (**Table 1**). Specifically, the IC_{50} values of the prepared compounds tested against HDAC6 varied from 0.97 μM to 2.18 μM, higher than that of Pracinostat (IC_{50} = 0.10 μM).

The compounds **2a**-**2p** were evaluated for their cellular potency against HCT-116, MCF-7, A549 and SW1990 cell lines in comparison with Pracinostat. Consistent with the results of the HDAC enzyme activity , all the target compounds showed poorer anti-proliferative activities than Pracinostat, as listed in **Table 2**. In all cell lines tested, Pracinostat showed potent anti-proliferative activities with IC_{50} values ranging from 0.32 μM to 0.52 μM. Compounds **2a-2p,** showed weak inhibitory activity against SW1990 and MCF-7 while seemed almost lost activity against A549 and HCT-116 cells.

Table 2 Anti-proliferative activities of the synthetic compounds

Compound **2a** were chosen for further investigation in comparison with Pracinostat. The acetylation of Histone 3 (Ac-H3), Histone 4 (Ac-H4), and α-tubulin (Ac-α-tubulin) were measured in HCT-116. The experiments (**Figure 4**) demonstrated that compound **2a** did not increased the acetylation level of histone H3, histone H4, as well as α-tubulin. The results corresponded to the enzymatic and cell-based assays.

4 | CONCLUSIONS

In summary, we have demonstated that the attached postion of *N*-hydroxyacrylamide of Pracinostat is vital important for the HDAC inhibitory actvities. The *N*-hydroxyacrylamide attached to the postion-5, related compound such as Pracinostat is a potent HDAC inhibitor with actylation levels increased significantly for H3, H4, and α-tubulin. However, all these acitivities dramatically decreased even lost when *N*-hydroxyacrylamide was attached to the position-6 of the benzamidazole ring. These results were also consisted with the cell-based

inhibitor activities. Docking results reveals that the hydrogen bond interaction between amino unit of the side chain at position 1 (Figure 1) and the hydroxyl group of the tyrosine (Tyr91) plays an important role in HDAC inhibition by Pracinostat. Thus, minor structural modifications might produce big changes in its biological responses and we hope this observation might be useful in the arena of drug hunting.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

FIGURE LEGENDS

Figure 1. The structure of Pracinostat and compound **2a.**

Figure 2. Experimental Flow Diagram. General experimental procedures were applied in this study.

Figure 3. Pracinostat (purple, stick) and compound **2a** (green, stick) dock into the HDAC model (PDB:1C3R). Hydrogen bond was depicted as black dash line.

Figure 4. Western blot analysis of biochemical markers for apoptosis induction and inhibition by **2a** and Pracinostat in HCT-116 cell line. Cells were treated with **2a** at a concentration of 0.33 μM and 10 μM, respectively, the control group treated with Pracinostat at a concentration of 0.33 μM. Levels of Ac-α-tubulin, Ac-H3 and Ac-H4 were probed by specific antibodies. GAPDH was used as the loading control.

Scheme 1. Reagents and conditions: (a) THF-BH_3 (1 equiv), THF, rt, yield 98%; (b) Dess-Martin periodinane (1.1 equiv), DCM, 0℃, yield 98%; (c) Triethyl phosphonoacetate (1.5 equiv), DBU (0.1 equiv), K_2CO_3 (2 equiv), H_2O (8 equiv),THF, rt, yield 90%-96%; (d) K_2CO_3 (3 equiv), dioxane, 85℃, yield 65%-70%; (e) $SnCl_2{\cdot}2H_2O$ (5 equiv), AcOH/MeOH (1:9), 45℃,yield 47%-55%; (f) $NH_2OH{\cdot}HCl$ (10 equiv)/NaOMe (20 equiv)/MeOH, 0℃ to rt, yield 77%-83%

Scheme 2. Reagents and conditions: (a) H_2SO_4 (1 equiv), CH_3OH, 85 ℃, yield 98%; (b) N,N-diethylethylenediamine (3 equiv), K_2CO_3 (3 equiv), dioxane, 85℃, yield 75%; (c) valeraldehyde (3 equiv), $SnCl_2{\cdot}2H_2O$ (5 equiv), AcOH/MeOH (1:9), 45℃, yield 62%; (d) $NH_2OH{\cdot}HCl$ (10 equiv)/NaOMe (20 equiv)/MeOH, 0℃ to rt, yield 85%

TABLE LEGENDS

Table 1. HDAC inhibitory activities of prepared compounds.

Table 2. Anti-proliferative activities of the synthetic compounds.

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urea/thiourea unit as potent histone deacetylase inhibitors. *Bioorganic & Medicinal Chemistry Letters*. 23, 6432-6435.
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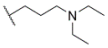
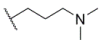
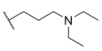
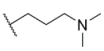
Table 1 HDAC inhibitory activities of prepared compounds

compound	R^1	R^2	Inhibition of HDAC		
			Inhibition at 20 μM (%)	pan-HDAC IC_{50}[a] **(μM)**	HDAC6 IC_{50}[a] **(μM)**
Pracinostat ,1	—	—	98.87	0.14±0.03[b]	0.10±0.03
2a			60.21	2.94±0.58	1.67±0.08
2b			42.84	>20	1.89±0.11
2c			63.45	7.96±0.83	2.18±0.05
2d			27.98	>20	2.06±0.01
2e			65.85	9.62±1.46	1.03±0.04
2f			36.19	>20	1.43±0.10
2g			81.28	2.32±0.29	1.31±0.01
2h			76.29	4.42±0.55	0.97±0.01
2i			59.09	7.53±0.95	1.11±0.02
2j			53.97	5.92±0.82	1.58±0.02
2k			60.33	15.72±0.06	1.30±0.11
2l			55.19	14.83±3.75	1.24±0.08
2m			62.78	10.16±1.05	1.28±0.07
2n			54.77	12.99±1.10	1.46±0.10
2o			79.95	2.57±0.41	1.27±0.01
2p			72.67	3.15±1.84	0.99±0.001

[a]The IC_{50} values represent an average of triplicate experiments

[b]IC_{50} values ± SD

Table 2 Anti-proliferative activities of the synthetic compounds

Compound	IC_{50}[a] (μM)		Compound	IC_{50}[a] (μM)	
	A549	HCT-116		A549	HCT-116
Pracinostat, 1	0.41±0.06[b]	0.32±0.05	**Pracinostat, 1**	0.41±0.06	0.32±0.05
2a	78.69±15.10	56.68±2.28	**2i**	62.58±3.78	26.19±5.35
2b	> 100	NT[c]	**2j**	87.23±8.69	9.82±16.69
2c	> 100	> 100	**2k**	97.37±13.38	23.98±1.53
2d	> 100	> 100	**2l**	> 100	33.83±1.76
2e	> 100	78.71±3.04	**2m**	72.75±5.15	26.32±3.90
2f	74.56±5.46	34.87±0.40	**2n**	42.36±11.55	22.84±1.72
2g	82.27±3.96	29.80±1.07	**2o**	16.77±1.87	8.11±0.58
2h	27.53±1.75	11.41±3.72	**2p**	87.76±19.52	> 100
Compound	IC_{50}[a] (μM)		Compound	IC_{50}[a] (μM)	
	MCF-7	SW1990		MCF-7	SW1990
Pracinostat, 1	0.46±0.05	0.52±0.004	**Pracinostat, 1**	0.46±0.05	0.52±0.004
2a	32.43±6.41	27.99±2.88	**2j**	3.62±0.61	10.45±0.23
2g	12.72±1.55	10.91±1.29	**2m**	39.59±4.79	9.36±3.96
2h	8.60±1.17	14.73±5.08	**2n**	19.61±3.43	8.40±0.55
2i	16.39±2.72	6.66±0.79	**2o**	4.59±0.58	3.76±1.68

[a]The IC_{50} values represent an average of three independent experiments

[b]IC_{50}±SD

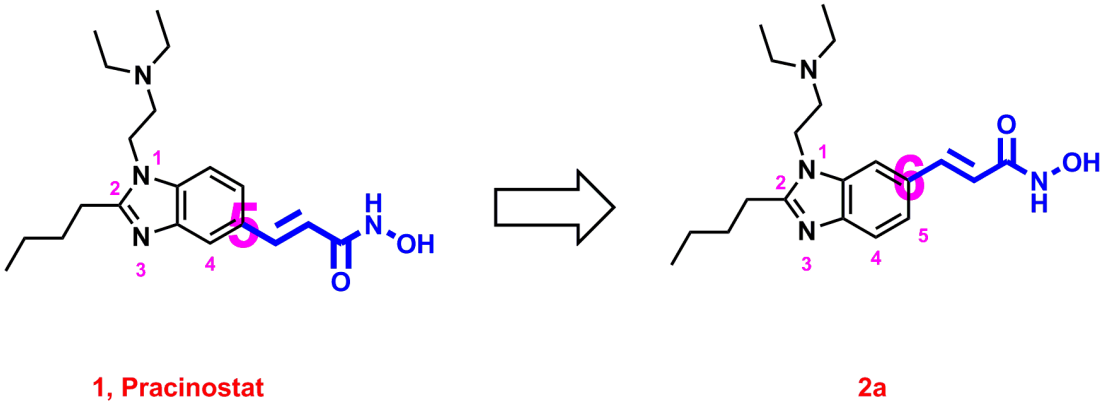


Figure 1. The structure of Pracinostat and compound **2a.**

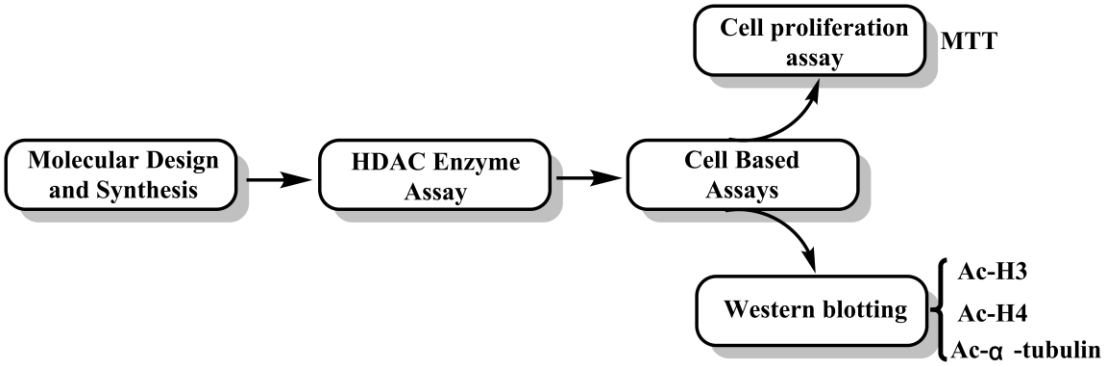


Figure 2. Experimental Flow Diagram. General experimental procedures were applied in this study.

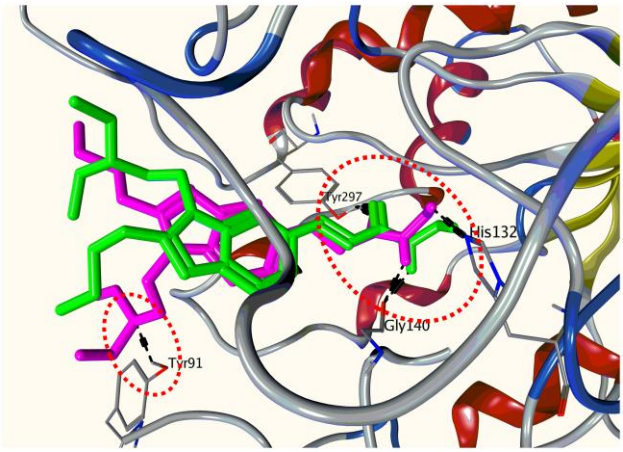


Figure 3. Pracinostat (purple, stick) and compound **2a** (green, stick) dock into the HDAC model (PDB:1C3R). Hydrogen bond was depicted as black dash line.

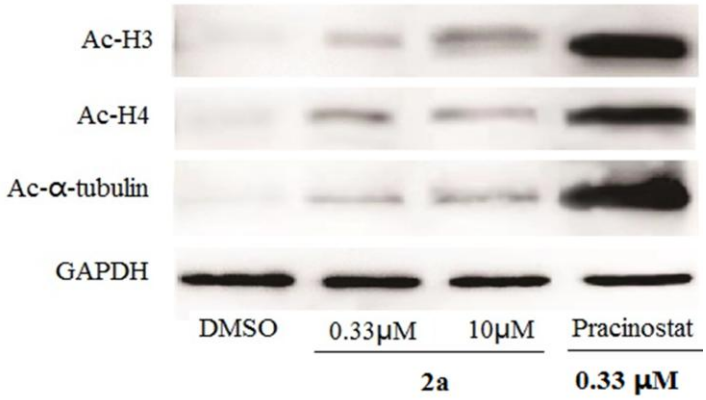
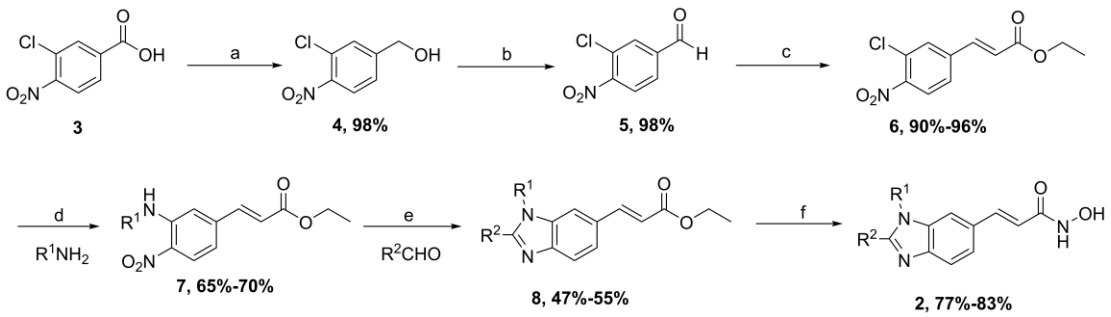
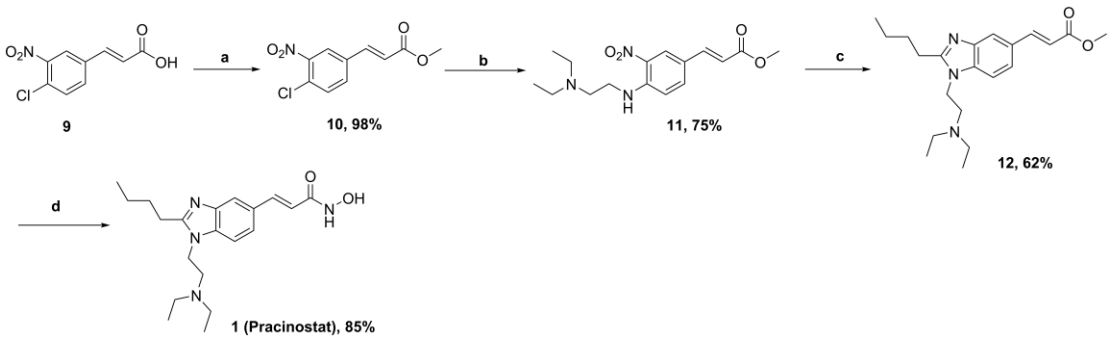


Figure 4. Western blot analysis of biochemical markers for apoptosis induction and inhibition by **2a** and Pracinostat in HCT-116 cell line. Cells were treated with **2a** at a concentration of 0.33 μM and 10 μM, respectively, the control group treated with Pracinostat at a concentration of 0.33 μM. Levels of Ac-α-tubulin, Ac-H3 and Ac-H4 were probed by specific antibodies. GAPDH was used as the loading control.



Scheme 1. Reagents and conditions: (a) THF-BH_3 (1 equiv), THF, rt, yield 98%; (b) Dess-Martin periodinane (1.1 equiv), DCM, 0℃, yield 98%; (c) Triethyl phosphonoacetate (1.5 equiv), DBU (0.1 equiv), K_2CO_3 (2 equiv), H_2O (8 equiv),THF, rt, yield 90%-96%; (d) K_2CO_3 (3 equiv), dioxane, 85℃, yield 65%-70%; (e) $SnCl_2{\cdot}2H_2O$ (5 equiv), AcOH/MeOH (1:9), 45℃,yield 47%-55%; (f) $NH_2OH{\cdot}HCl$ (10 equiv)/NaOMe (20 equiv)/MeOH, 0℃ to rt, yield 77%-83%



Scheme 2. Reagents and conditions: (a) H_2SO_4 (1 equiv), CH_3OH, 85 ℃, yield 98%; (b) N,N-diethylethylenediamine (3 equiv), K_2CO_3 (3 equiv), dioxane, 85℃, yield 75%; (c) valeraldehyde (3 equiv), $SnCl_2{\cdot}2H_2O$ (5 equiv), AcOH/MeOH (1:9), 45℃, yield 62%; (d) $NH_2OH{\cdot}HCl$ (10 equiv)/NaOMe (20 equiv)/MeOH, 0℃ to rt, yield 85%