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Microbial Transformation of Trichostatin A to 2,3-Dihydrotrichostatin A

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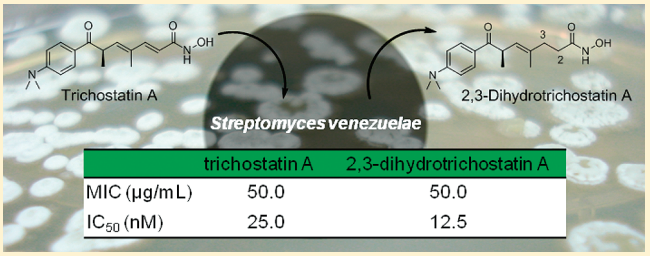
Supporting Information

ABSTRACT: A new reduced hydroxamate, 2,3-dihydrotrichostatin A, was created from trichostatin A by employing a recombinant strain of *Streptomyces venezuelae* as a microbial catalyst. Compared with trichostatin A, 2,3-dihydrotrichostatin A showed similar antifungal activity against *Saccharomyces cerevisiae*, but, interestingly, approximately twice the cytostatic activity against human small-cell lung cancer cells. The production of 2,3-dihydrotrichostatin A via microbial biotransformation demonstrates that the regiospecific and substrate-flexible hydrogenation by *S. venezuelae* provides a new approach for creating natural product analogues with improved bioactive properties.



Trichostatin A, isolated from the soil bacterium *Strpetomyces hygroscopicus*, is a naturally occurring hydroxamate (Figure 1).[1] It was originally discovered as an antifungal antibiotic but is also a histone deacetylase (HDAC) inhibitor with a broad spectrum of epigenetic activities, making it a potential anticancer drug.[2−4] In many cancerous cell lines, overactivation of HDAC results in histone hypoacetylation, and HDAC inhibitors (HDACi) specifically target the HDACs, rendering them functionally inactive. Several HDACi have undergone clinical trials for the treatment of leukemia and solid tumors: vorinostat is an example of a synthetic mimic of trichostatin A approved for the treatment of cutaneous T-cell lymphoma (Figure 1).[5,6] Trichostatin A has previously shown cytostatic activities against various cancer cell lines, such as glioma cells and bladder cancer cells.[7−9] In recent work, trichostatin A treatment of small-cell lung cancer (SCLC) cell lines resulted in decreased cell growth and proliferation.[10] The potential of trichostatin A in cancer therapy has generated interest in producing analogues. In order to modify structurally complex natural products such as trichostatin A and to create improved bioactive agents, biological syntheses can be efficient alternatives to chemical ones. However biotransformed analogues of trichostatin A have not yet been reported.

We recently reported a unique and regiospecific hydrogenation of unsaturated macrolide rings by *Streptomyces venezuelae*, and its application to heterologous macrolides was found to synthesize reduced macrolide antibiotics.[11] The microbial hydrogenation system could recognize the specific neighboring structural elements at each end of the target double bond: a carbonyl and a methyl group (Figure 1). Reported here is an attempt to expand the applicability of this unique biocatalyst to use with a non-macrolide linear polyketide, trichostatin A, in which the above-described structural elements are well conserved. Interestingly, the expected reduced analogue (2,3-dihydrotrichostatin A) resembles the marketed trichostatin A mimic, vorinostat, more closely than it does the natural original, in that the polyketide chain spanning from C1 to C6 is more saturated (Figure 1). The regioselective hydrogenation of this conjugated double bond using traditional synthesis methods is challenging. However, we produced and isolated a previously uncharacterized 2,3-dihydrotrichostatin A from *S. venezuelae* culture supplemented with trichostatin A. Furthermore, its structure was elucidated using HPLC-ESIMS/MS and NMR analyses, and its bioactivity was evaluated against *Saccharomyces cerevisiae* and human SCLC DMS53 cells, respectively.

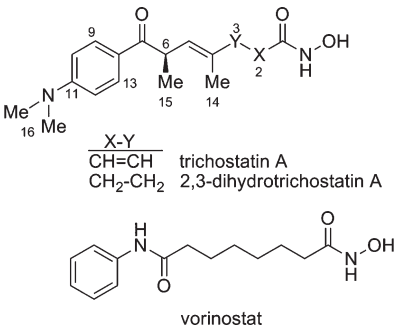


Figure 1. Trichostatin A, its bioconverted analogue 2,3-dihydrotrichostatin A, and its synthetic mimic vorinostat.

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Table 1. NMR Spectroscopic Data (500 MHz, $CDCl_3$) of 2,3-Dihydrotrichostatin A and Trichostatin A

	2,3-dihydrotrichostatin A		trichostatin A	
position	δ_C, mult	δ_H, mult	δ_C	δ_H
1	172.2, C		166.1	
2	29.8, CH_2	2.26, ddd	117.5	5.27
		2.22, ddd		
3	36.2, CH_2	2.20, m	145.0	7.41
		2.17, ddd		
4	134.1, C		132.8	
5	133.6, CH	5.47, d	140.1	5.68
6	40.9, CH	3.83, dq	40.8	3.84
7	199.6, C		199.4	
8	123.7, C		123.8	
9, 13	130.8, CH	7.78, d	130.8	7.78
10, 12	110.8, CH	6.82, d	110.8	6.83
11	153.6, C		153.6	
14	16.0, CH_3	1.82, s	12.6	2.21
15	17.7, CH_3	0.94, d	17.8	0.93
16	39.9, CH_3	3.05, s	39.9	3.05

A recombinant strain of *S. venezuelae* YJ028,[11] in which the pikromycin polyketide synthase-encoding gene and desosamine biosynthetic genes had been deleted, was incubated with trichostatin A. Supplementation of this non-native hydroxamic acid into the recombinant strain cultivated in 50 mL of SCM media in baffled Erlenmeyer flasks at 30 °C for 2 days, followed by further cultivation for 3 days, led to approximately 18% conversion of trichostatin A into the corresponding reduced trichostatin analogue as the sole metabolite (Supporting Information, Figure S1). In previous in vivo studies using pikromycin as substrate, the conversion rate into its dihydro form was approximately 85%.[11] In an attempt to increase trichostatin A conversion yield, we extended the cultivation time after feeding trichostatin A up to a week. However, no further increased conversion was observed after day 3, as in the case of pikromycin. We also investigated using a cell-free extract made from the YJ028 strain using the same procedure described previously,[11] but there was no significant increase in the conversion yields (<20%). These results suggest that the biocatalytic activity of *S. venezuelae* toward the non-macrolide linear polyketide is limited compared with its natural substrate. The organic extracts obtained from several batch cultures of YJ028 strain fed with trichostatin A were pooled. The bioconverted trichostatin analogue was purified by chromatographic isolation using preparative reversed-phase HPLC with subsequent ESIMS analyses of each fraction (5 mL/fraction) collected. The MS/MS spectrum of trichostatin A at m/z 303, representing its protonated molecular ion, showed fragment ions at m/z 148, 177, and 270, identical to those obtained from a previous report.[12] The MS/MS spectrum of the trichostatin analogue demonstrated two fragment ions at m/z 148 and 177 common to trichostatin A. However, instead of the m/z 270 product ion from trichostatin A, there was a characteristic product ion at m/z 272 (Supporting Information, Figure S2), suggesting the reduction of one of the double bonds existing at either the C2,3 or the C4,5 positions of trichostatin A.

The chemical structure of the biotransformed analogue was further confirmed by ^1H and ^{13}C NMR spectroscopy by comparing its chemical shifts with those of trichostatin A (Table 1 and Supporting Information, Figures S3−S5). The most obvious difference in the ^1H NMR spectra of trichostatin A and its reduced analogue was the absence of signals at 5.27 and 7.41 ppm, typical of olefinic protons (H-2,3) in trichostatin A. The upfield shift of the C-2 and C-3 signals of the parent compound (from 117.5 and 145.0 ppm to 29.8 and 36.2 ppm, respectively) also corroborates the distinction found in the above ^1H NMR data, confirming that trichostatin A was reduced at the C-2,3 double bond.

Table 2. Comparison of MIC and IC_{50} dData of Trichostatin A and Its Analogue 2,3-Dihydrotrichostatin A

	trichostatin A	2,3-dihydrotrichostatin A
MIC (μg/mL)[a]	50.0	50.0
IC_{50} (nM)[a]	25.0	12.5

[a] Antifungal index (MIC) was determined against *S. cerevisiae*, whereas cytostatic index (IC_{50}) was tested against human SCLC DMS53 cell line.

The antifungal and cytostatic activities of trichostatin A as a positive control and 2,3-dihydrotrichostain A were evaluated against *Saccharomyces cerevisiae* and the human SCLC cell line, respectively. The MICs (minimal inhibitory concentrations) of both compounds against the yeast were 50 μg/mL. However, 2,3-dihydrotrichostatin A (IC_{50} ~12.5 nM) was approximately twice as active as trichostatin A (IC_{50} ~25.0 nM) against human SCLC DMS53 cells. This shows that the C2,3-double bond in trichostatin A had a minimal effect on antifungal activity, but enhanced the cytostatic effect on the human SCLC cell line (Table 2 and Supporting Information, Figure S6). A recent study reported that significant suppression of human SCLC cell proliferation was noticed at doses as low as 25 nM of trichostatin A,[10] in agreement with the data of this study. The improved in vitro cytostatic activity of 2,3-dihydrotrichostatin A caused by the removal of the C2,3-double bond prompted the investigation of a three-dimensional docking model of trichostatin A and its analogue using human HDAC7 enzyme, for which crystallographic data are available (PDB ID: 3C10).[13] The root mean squared deviation (rmsd) estimated from computational docking of both ligands into the HDAC7 catalytic site suggests that binding of 2,3-dihydrotrichostatin A to HDAC7 is relatively more stable (rmsd ~0.713 Å) when compared with its parent, trichostatin A (rmsd ~0.737 Å) (Supporting Information, Figure S7).

EXPERIMENTAL SECTION

General Experimental Procedures. Analyses of trichostatin A (Sigma, St. Louis, MO) and its bioconverted analogue were performed using a Waters/Micromass Quattro micro/MS interface comprising an analytical revered-phased Nova-Pak C_{18} column (Waters, Milford, MA; 150 × 3.9 mm, 4.0 μm) connected directly to a Micromass Quattro micro MS. Chromatographic purification of the analogue of interest was conducted using a preparative HPLC on a Watchers 120 ODS-BP (250 × 10.0 mm, 5.0 μm).[11] NMR samples were prepared by dissolving authentic trichostatin A and purified analogue 2,3-dihydrotrichostain A in 200 μL of $CDCl_3$ and placing the solution in a 5 mm Shigemi advanced NMR microtube (Sigma) matched to the solvent. The ^1H and ^{13}C NMR spectra were acquired using a Varian INOVA 500 spectrometer at 298 K, with chemical shifts reported in ppm using TMS as internal reference. All NMR data processing was performed using Mnova Suite 5.3.2 software.

Biotransformation of Trichostatin A. The mutant strain of *S. venezuelae* YJ028 has been described previously.[11] The organism was

initially streaked for isolation on SPA agar plates (0.1% yeast extract, 0.1% beef extract, 0.2% tryptose, 1.0% glucose, 1.5% agar, and trace amount of $FeSO_4$) and then incubated at 30 °C for 2 days. Isolated colonies were then used to inoculate SCM media (1.5% soluble starch, 2.0% soytone, 0.01% $CaCl_2$, 0.15% yeast extract, and 1.0% MOPS) in baffled Erlenmeyer flasks. Following 2 days of growth at 30 °C on a rotary shaker, the cultures were supplemented with trichostatin A (dissolved in MeOH) at a final concentration of 5 μg/mL and then incubated for an additional 3 days of cultivation. All in vivo experiments were independently carried out in triplicate.

Extraction and Isolation of Bioconverted 2,3-Dihydrotrichostatin A. Whole cultures were extracted and partitioned twice using equal volumes of EtOAc in a 250 mL separatory funnel, after which the organic extracts were then combined and concentrated under vacuum. The dried residues were immediately dissolved in 200 μL of MeOH, and a portion of this solution was subjected to HPLC-ESIMS analysis.[11]

Bioactivity Assays. *S. cerevisiae* ATCC 9763 and the human SCLC DSM53 cell line, which were used respectively as test organisms for antifungal and cytostatic effects, were obtained from American Type Culture Collection (Manassas, VA). The yeast strain was cultured and maintained in Antibiotic 19 medium (Difco, BD Biosciences, San Jose, CA) at 30 °C, whereas human SCLC DMS53 cells were cultured in Waymouth's MB752/1 medium (Invitrogen, San Diego, CA), supplemented with 10% fetal bovine serum (Sigma), penicillin, and streptomycin (Invitrogen).[10] The cell line was incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C. The antifungal activity of the test compounds was determined using the microdilution method of the Clinical and Laboratory Standard Institute (CLSI, formerly NCCLS).[16] Serial 2-fold dilutions were performed using DMSO to give final concentrations between 12.5 and 200 μg/mL, with an aliquot of DMSO (final 0.1%) used as a negative control. The growth of *S. cerevisiae* was monitored at 600 nm using a Labsystems Bioscreen C reader, with MIC determined as the lowest concentration diluted in broth medium that inhibited the growth of the test strain. To evaluate the cytostatic effects, proliferation of DMS53 cells following treatment with the two test compounds was measured using a 3,4-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described.[10] In brief, DMS53 cells were trypsinized and plated in 24-well plates and allowed to adhere overnight. Serial 2-fold dilutions were carried out to give final concentrations between 3.1 and 200 nM, with an aliquot of DMSO used as a control. After treatment for 2 days, media were removed and replaced with the medium containing MTT and incubated for a further 2 h, after which the absorbance was measured at 540 nm. IC_{50} was defined as the lowest concentration at which 50% of the growth of DMS53 cells was inhibited compared with a control cell line grown in the absence of test compound. All assays were performed in at least triplicate.

Protein−Ligand Docking Model Simulations. Recently published crystal complex HDAC7−trichostatin A was used as a template.[13] In silico molecular modeling analyses to predict the interaction of trichostatin ligands with the HDAC catalytic site were performed using AutoDock Vina (version 1.1.1., The Scripps Research Institute, La Jolla, CA).[17]

■ ASSOCIATED CONTENT

Supporting Information. HPLC-ESIMS chromatograms of the biotransformations, ESIMS/MS spectra of trichostatin A and its converted 2,3-dihydrotrichostatin A, ^1H and ^{13}C NMR spectra of 2,3-dihydrotrichostatin A, their antifungal and cytostatic activities against *S. cerevisiae* strain and human SCLC cell line, respectively, and three-dimensional docking modeling of both trichostatins with the HDAC7 catalytical site. This information is available free of charge via the Internet at http://pubs.acs.org.

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