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NOTES

Trichostatin D, a New Inducer of Phenotypic Reversion in Transformed Cells

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Various oncogenes correlate with tumor phenotypes, which represent transformed morphology, loss of contact inhibition, colony formation in soft agar and tumorigenicity in nude mice. Thus, inducers of phenotypic reversion in oncogene-transformed cells may act as selective antitumor agents without cytotoxicity. Our screening for such substances resulted in the isolation of trichostatin D (Fig. 1), a new member of the trichostatin family[1,2], from an actinomycete identified as *Streptomyces violaceusniger*. This compound induced flat reversion in NIH3T3 murine fibroblasts transformed with human papillomavirus oncogenes (NIH3T3/T-601)[3]. We report here the fermentation, isolation, structure elucidation and biological activities of trichostatin D.

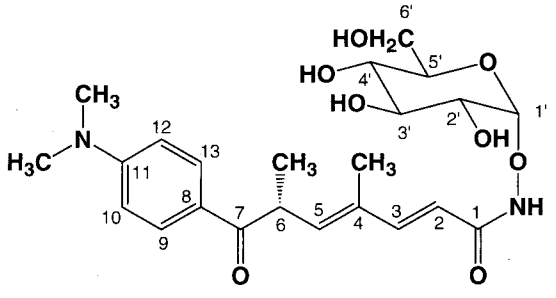
The producing organism was inoculated into 500-ml Erlenmeyer flasks containing 100 ml of a seed-culture medium (soluble starch 1.0%, molasses 1.0%, Polypepton 1.0%, meat extract 1.0%, pH 7.2). The flasks were incubated on a rotary shaker at 27°C for 3 days. A 600-ml portion of the seed culture was transferred into a 50-liter jar fermenter containing 30 liters of a medium consisting of dextrin 3.0%, glucose 0.3%, soybean meal 2.0%, $CaCO_3$ 0.3% and $CoCl_2 \cdot 6H_2O$ 0.001% (pH 7.4). The fermentation was carried out at 27°C for 3 days under agitation of 300 rpm and aeration of 30 liters/minute.

The culture filtrate (30 liters) was applied to a Diaion HP-20 column, which was washed with water and eluted with MeOH. The eluate was evaporated and then partitioned between 1-butanol and water. The organic layer was concentrated and subjected to silica-gel column chromatography with chloroform - MeOH - water (13 : 6 : 1). The active eluate was chromatographed on a silica-gel column with chloroform - MeOH - 29% aqueous ammonia (10 : 5 : 1). Final purification was achieved using HPLC on a YMC-Pack D-ODS-7 column. Development of the column with 35% acetonitrile obtained a single active fraction, which was concentrated to dryness to give a colorless powder of trichostatin D (59 mg).

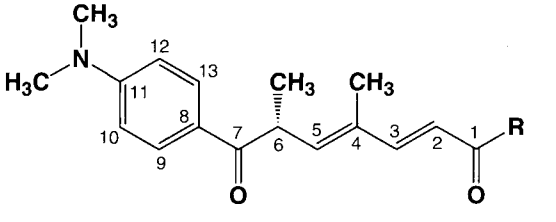
The physico-chemical properties of trichostatin D are summarized in Table 1. The molecular formula was established as $C_{23}H_{32}N_2O_8$ from high-resolution FAB-MS.

The ^1H NMR spectrum of trichostatin D in DMSO-d_6 at room temperature showed broad signals in the alkene and oxyalkane region. Measurement at 75°C sharpened these signals except an olefinic doublet (δ 5.83), which disappeared in turn. The ^{13}C NMR spectrum of trichostatin D exhibited only 22 carbons including four broad olefinic

Fig. 1. Structures of trichostatins A and D, and trichostatic acid.



Trichostatin D



Trichostatin A R = NHOH
Trichostatic acid R = OH

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Table 1. Physico-chemical properties of trichostatin D.

MP	131~135°C
Molecular formula	$C_{23}H_{32}N_2O_8$
FAB-MS (*m/z*)	
Found:	465.2219 $(M+H)^+$
Calcd.:	465.2237
$[\alpha]_D^{21}$ (*c* 0.56, MeOH)	+183°
UV λmax nm (ε)	
MeOH	267 (27,000), 345 (27,000)
MeOH + NaOH	249 (24,000), 340 (27,000)
IR (KBr) νmax cm^{-1}	3400, 1650

Table 2. ^{13}C and ^1H NMR data for trichostatin D.

No.	δ_C[a]	δ_H[b] (multiplicity, *J* = Hz)
1	n.d.[c]	
2	116.9	5.83 (d, 15.5)[d]
3	144.0	7.02 (d, 15.5)
4	132.4	
5	140.0	5.88 (d, 9.5)
6	40.4	4.40 (dq, 9.5, 6.5)
7	197.7	
8	123.1	
9, 13	130.4	7.75 (d, 7.9, 2H)
10, 12	110.8	6.67 (d, 7.9, 2H)
11	153.3	
4-Me	12.4	1.85 (s, 3H)
6-Me	17.6	1.12 (d, 6.5, 3H)
N-Me_2	40.4	3.00 (s, 6H)
1'	104.0	4.87 (d, 3.6)
2'	71.5	3.26 (dd, 9.6, 3.6)
3'	73.1	3.42 (t, 9.6)
4'	69.4	3.18 (t, 9.6)
5'	73.6	3.68 (m)
6'	60.2	3.57 (dd, 12.0, 1.8) 3.51 (dd, 12.0, 4.8)

[a] 125 MHz in DMSO-d_6 at 25°C.
[b] 500 MHz in DMSO-d_6 at 75°C.
[c] Not detected.
[d] Data at 25°C.

signals (C-2~C-5).

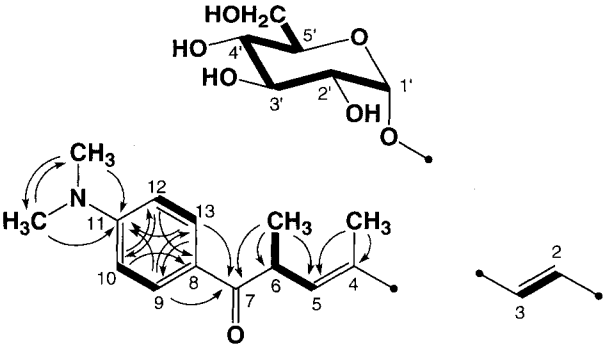
A ^{13}C-^1H COSY experiment established all one-bond ^{13}C-^1H connectivities (Table 2), indicating that the unobserved carbon signal was assigned to a quaternary carbon. The ^1H-^1H COSY spectrum displayed proton spin networks including a *para*-substituted benzene, a *trans* olefin (J=15.5 Hz) and a sugar moiety (Fig. 2).

A heteronuclear multiple-bond correlation (HMBC)[4] experiment revealed ^1H-^{13}C long-range couplings from two equivalent *N*-methyl groups to C-11, from two equivalent

aromatic protons (9-H and 13-H) to C-11 and a ketone carbonyl carbon (C-7), from 6-Me to C-5, C-6 and C-7, and from 4-Me to C-4 and C-5, thereby showing a partial structure terminating in an olefin group as shown in Fig. 2. Splitting carbon shifts for the *trans* olefin (δ 144.0 and 116.9) indicated that this moiety was attached to a carbonyl group due to the unobserved carbon. The two terminal olefins were joined to generate an $\alpha,\beta,\gamma,\delta$-unsaturated carbonyl chromophore based on the UV absorption (λ_{max} 267 nm).

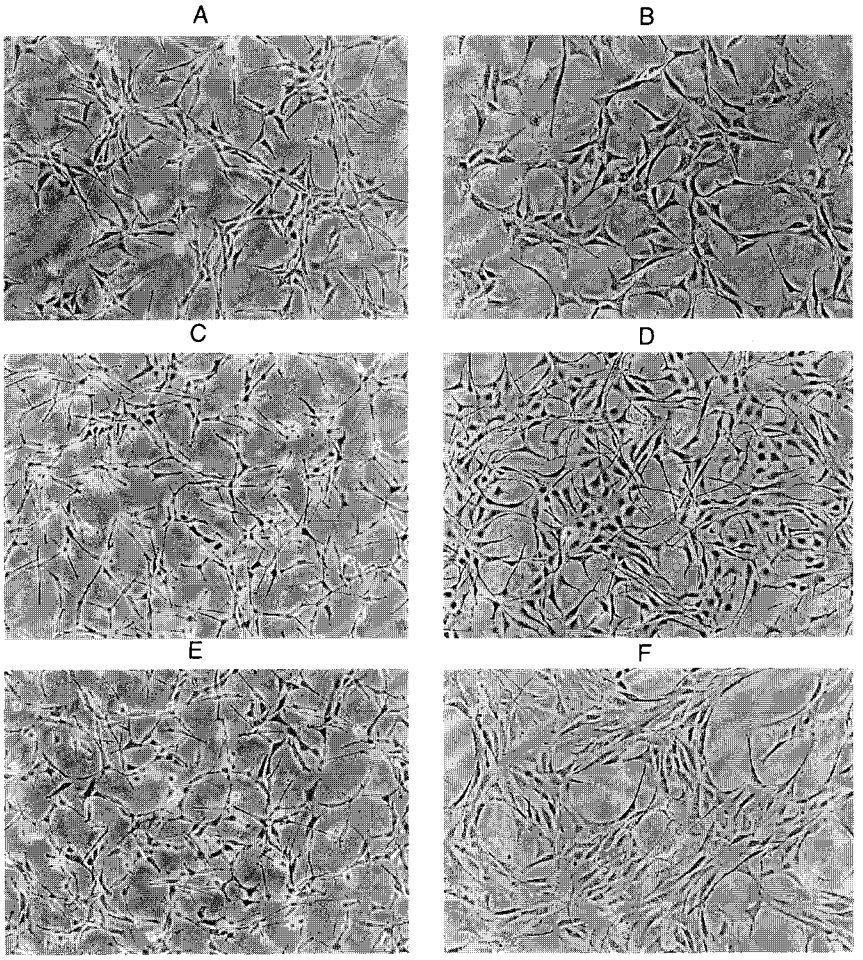
In the sugar moiety, 2′-H to 5′-H displayed large vicinal-coupling constants ($J_{2'\sim3'}=J_{3'\sim4'}=J_{4'\sim5'}=9.6$ Hz) and were required to be in a glucopyranose ring. A small coupling constant (3.6 Hz) on the anomeric proton provided an α configuration for the glucoside. The remaining HN atoms needed to be located between the $\alpha,\beta,\gamma,\delta$-unsaturated carbonyl group and the glucose residue to form a glucopyranosyl hydroxamate.

Fig. 2. Partial structures of trichostatin D derived from COSY and HMBC.



Bold lines show proton spin networks and arrows show ^{1}H-^{13}C long-range correlations.

Fig. 3. Flat reversion in transformed cells treated with trichostatin D.



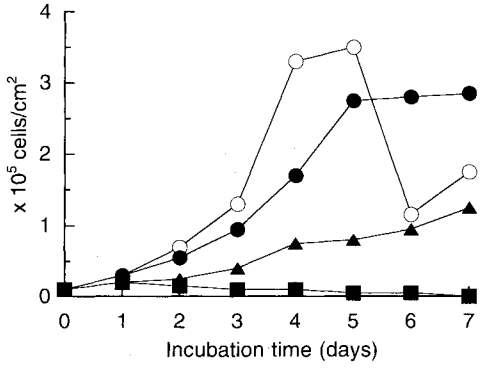
NIH3T3/T-601 (A, B), NIH3T3/*ras* (C, D) and NIH3T3/*hst-1* (E, F) cells at 2.5×10^4 cells/cm^2 were incubated without (A, C, E) or with (B, D, F) 10 ng/ml of trichostatin D for 24 hours.

Table 3. Effect of trichostatin D on the colony formation in soft agar.

Cell line	Trichostatin D (ng/ml)	Colony formation (%)
NIH3T3/T-601	0	47
	10	0
NIH3T3/*ras*	0	26
	10	0
NIH3T3/*hst-1*	0	7.2
	10	0
NIH3T3	0	0
	10	0

Cells (5 x 10^3/dish) were incubated in soft agar with or without trichostatin D for 14 days.

Fig. 4. Effect of trichostatin D on the growth of NIH3T3/T-601 cells.



Cells at 1.25×10^4 cells/cm^2 were incubated without (○) or with 10 ng/ml (●), 50 ng/ml (▲) or 100 ng/ml (■) of trichostatin D. The numbers of viable cells were counted after trypan-blue staining.

The geometrical configuration of C-4 was determined to be *E* from an upfield carbon shift (δ 12.4) for 4-Me. Hydrolysis of trichostatin D with 3 M HCl at 100°C for 2 hours yielded D-glucose and (*R*)-trichostatic acid[5] {$[\alpha]_D^{21}$ +135° (*c* 0.155, MeOH)}. These analyses established the absolute structure of trichostatin D as shown in Fig. 1. Trichostatin D is an α-D-glucopyranoside of trichostatin A, a histone-deacetylase inhibitor produced by *Streptomyces hygroscopicus*[1,6]. The same strain was reported to produce trichostatin C[2], a β-D-glucopyranoside of trichostatin A. However, trichostatin A or C was not detected in the fermentation broth of trichostatin D.

Trichostatin D induced flat reversion in NIH3T3 cells transformed with H-*ras* or *hst*-1 (NIH3T3/*ras*, NIH3T3/*hst-1*)[7] as well as in NIH3T3/T-601 cells (Fig. 3). Growth inhibition was observed in NIH3T3/T-601 cells treated with trichostatin D at the IC_{50} of 33 ng/ml. Trichostatin A showed similar effects on NIH3T3/T-601 cells (IC_{50} 71 ng/ml), although trichostatin C did not inhibit the cell growth at less than 1 μg/ml. Trichostatin D inhibited the colony formation of these transformed cells in soft agar (Table 3). In the presence of 10 ng/ml of trichostatin D, the contact inhibition was observed in NIH3T3/T-601 cells (Fig. 4). These results indicate that trichostatin D can revert the tumor phenotypes of transformed cells. Thus, trichostatin D seems to be a good candidate for selective anticancer agents.

Acknowledgments

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