TRICHOSTATIN C, A GLUCOPYRANOSYL HYDROXAMATE

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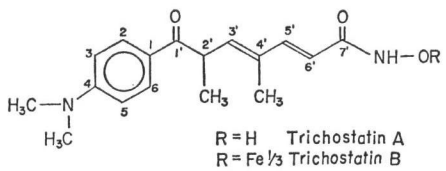
In addition to trichostatins A and B, a new antifungal antibiotic, trichostatin C was isolated. The structure was shown to be a glucoside of trichostatin A by spectroscopic examinations and chemical degradations. Trichostatin C is presumably the first example of a glucopyranosyl hydroxamate from nature.

In the previous paper[1], we reported the isolation of trichostatins active against trichophytons and showed that trichostatin A* is a derivative of a primary hydroxamic acid and trichostatin B is the corresponding ferric chelate. This paper is concerned with the isolation of an additional new antibiotic, trichostatin C, from the metabolites of the same strain, *Streptomyces hygroscopicus* Y-50, and with the structure elucidation of this antibiotic.

Trichostatins A and B were mostly accumulated in mycelia, while trichostatin C was found in the filtrate and extracted with *n*-butanol. The extract was chromatographed on a silica gel column and the active component was finally purified by recrystallization.



Trichostatin C is easily soluble in lower alcohols, soluble in acetone and ethyl acetate, sparingly soluble in chloroform and benzene, and insoluble in water and hydrocarbons. The antibiotic is positive to DRAGENDORFF reagent as is trichostatin A, but negative to ferric chloride. The antifungal activities are summarized in Table 1. The spectrum is similar to those of trichostatins A and B, but trichostatin C is less active against trichophytons.

The UV spectrum of trichostatin C is analogous to that of trichostatin A but trichostatin C is more polar, and its IR spectrum (Fig. 1) exhibits

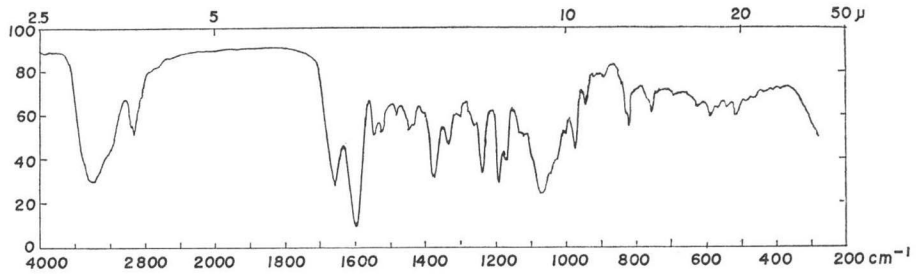
Table 1. Antifungal activities of trichostatin by agar dilution method (mcg/ml).

Test organisms	MIC	MEC
Candida albicans KE-2*	>100	>100
" " M-9*	>100	>100
Saccharomyces cerevisiae IFD 1234*	>100	>100
Pichia farinosa IFO 1163*	>100	>100
Cryptococcus neoformans IFO 1068*	>100	>100
*Trichophyton rubrum***	25.0	12.5
*Trichophyton mentagrophytes***	25.0	12.5
*Trichophyton ferrugineum***	25.0	6.25
*Trichophyton asteroides***	25.0	12.5
*Microsporum gypseum***	50.0	25.0
*Epidermophyton floccosum***	25.0	6.25
*Aspergillus fumigatus***	>100	100.0
*Aspergillus oryzae***	>100	100.0
*Aspergillus niger***	>100	50.0

* 28°C 2 days in SABOURAUD glucose agar medium.
** 28°C 7 days

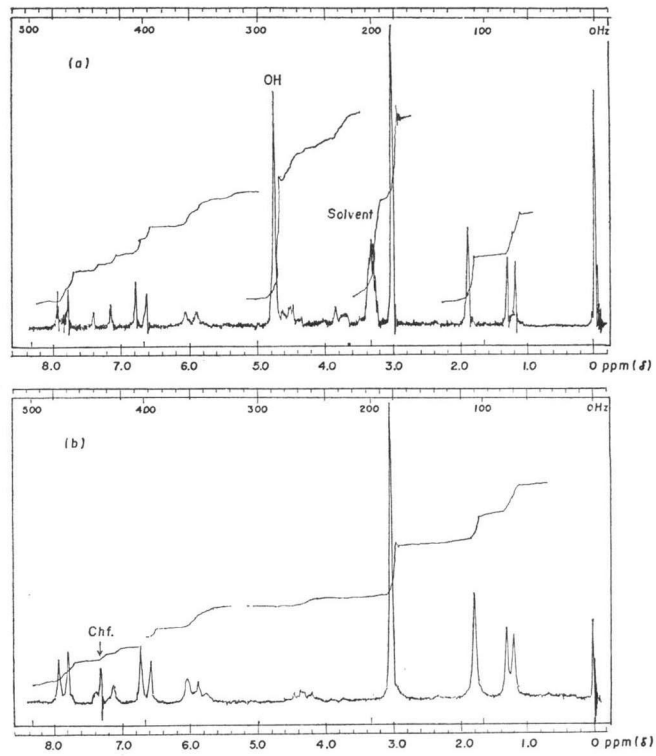
* Trichostatin A had been reported as an amorphous powder but it crystallized in this procedure (see experimental part).

Fig. 1. IR spectrum of trichostatin C (KBr).



strong absorption bands at 3000~3600 cm^{-1} and 1000~1100 cm^{-1} due to hydroxyl groups. The ^{1}H-NMR spectrum of trichostatin C shows all the CH proton signals corresponding to those of trichostatin A and, in addition, several proton signals assignable as –CH–O protons in 3.0~5.0 ppm region (Fig. 2a, b). These spectral observations are in good agreement with the microanalytical data which suggested the molecular formula, $C_{23}H_{32}O_8N_2$, corresponding to a glycoside of trichostatin A.

Fig. 2. ^{1}H-NMR spectra of trichostatins at 60 M Hz.
(a): Trichostatin C in CD_3OD (b): Trichostatin A in $CDCl_3$.



The ^{13}C-NMR data listed in Table 2 clearly support the above assumption, and from the chemical shifts of the sugar moiety, trichostatin C seems to be an *O*-*β*-glucopyranoside of trichostatin A.

In order to confirm the structure of the sugar moiety, trichostatin C (**I**) was treated with methanolic hydrogen chloride as shown in Chart 1. The water-soluble product, which was isolated in a crystalline form, was identical with methyl α-D-glucopyranoside (**II**).

Since the above methanolysis gave a complex mixture originating from the aglycon, trichostatin C (**I**) was hydrolyzed in aqueous hydrochloric acid. From the lipophilic fraction of the reaction mixture trichostatic acid (**III**) was isolated. The acid, which was also obtained by the hydrolysis of trichostatin A, yielded a methyl ester (**IV**) on standing with diazomethane. The structures of **III** and **IV** were confirmed by ^{1}H-NMR (Fig. 3a, b). Moreover, the acid (**III**) was converted to trichostatin A by treating its acid chloride (**V**) with hydroxylamine. The IR and ^{1}H-NMR spectra of this trichostatin A were identical with those of an authentic sample (in solution), however, its optical purity was degraded due to racemization at C_2' during the acid hydrolysis.

Table 2. ^{13}C Chemical shifts[a] (δ, ppm from TMS) of trichostatin C and its related compounds.

	Trichostatin A	Trichostatin C		Compound VI	Compound VII	Methyl α-D-glucoside	Methyl β-D-glucoside	Methyl tetra-*O*-acetyl-β-D-glucoside
Solvent	$CDCl_3+CD_3OD$	CD_3OD[b]	d_5-Py	d_5-Py	$CDCl_3$	d_5-Py[d]	d_5-Py[d]	$CDCl_3$[d]
1	200.1 (s) (1′)	201.3	198.4					
2	165.7 (s) (7′)	168[c]	163[c]					
3	154.0 (s) (4)	155.6	153.8					
4	145.3 (d) (5′)	147.8	146[c]					
5	140.3 (d) (3′)	142.3	141.3					
6	133.2 (s) (4′)	134.5	133.4					
7	131.0 (d) (2,6)	131.9	131.0		COMe			O-CO-Me
8	131.0 (d)	131.9	131.0		170.3×3			170.5
9	123.7 (s) (1)	124.9	124.8		169.9×2			170.0
10	115.9 (d) (6′)	116.3	116.7		169.4×1			169.3×2
11	111.0 (d) (3,5)	112.0	111.3					
12	111.0 (d)	112.0	111.3					
13		107.9(d)	107.9(1″)	107.4(1)	104.3(1)	101.2(1)	105.4(1)	101.5(1)
14		78.4(d)	79.0(3″)	78.4(3)	72.2(3)	75.3(3)	78.1(3)	72.9(3)
15		77.7(d)	77.8(5″)	78.2(5)	72.2(5)	73.9(5)	78.1(5)	71.8(5)
16		73.2(d)	73.1(2″)	73.5(2)	69.7(2)	73.7(2)	74.8(2)	71.3(2)
17		71.5(d)	71.2(4″)	71.5(4)	68.2(4)	72.0(4)	71.4(4)	68.5(4)
18		62.9(t)	62.5(6″)	62.6(6)	61.4(6)	62.7(6)	62.5(6)	62.3(6)
19	40.9 (d) (2′)	41.9	41.0		N-COMe			O-COMe
20	39.9 (q) (N-Me_2)	40.1	39.6		24.8×2			20.5×4
21	39.9 (q)	40.1	39.6		O-COMe			
22	17.9 (q) (2′-Me)	18.3	18.0		20.5×4			
23	12.6 (q) (4′-Me)	12.8	12.6					

a) ^{13}C-NMR spectra were measured on a Varian NV-14 FT NMR spectrometer at 15.087 M Hz.
b) At 60°C.
c) Broad signals presumably due to the equilibrium, 
d) Obtained by Dr. TORI and his co-worker.

The water-soluble fraction of the hydrolysis gave a syrupy compound, whose TLC was not identical with that of expected D-glucose. The compound probably contains nitrogen atom and seems to be *O*-β-D-glucopyranosyl hydroxylamine (**VI**) from its ^{13}C-NMR (see Table 2). Acetylation of the product gave a crystalline hexa-acetate (**VII**), whose structure was confirmed by elemental analysis and by ^1H- and ^{13}C-NMR spectra.

This fact evidently rules out the possibility of the glucoside linkage to $C_{7'}$ –O in a form of –C=N–OH, and trichostatin C is therefore shown to have structure **I**.
|
O–glucose

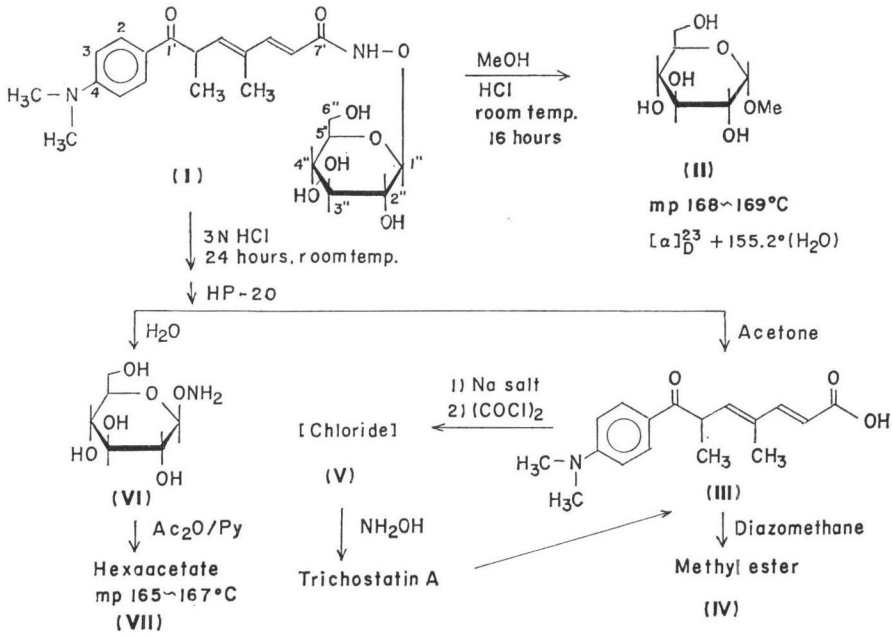
To our knowledge, trichostatin C is the first glycosyl hydroxamate from a natural source.

Experimental

Fermentation

The *Streptomyces* strain Y-50 was inoculated into a 2-liter Erlenmeyer flask containing 800 ml of WAKSMAN medium, which was cultured at 28°C for 48 hours on a rotary shaker (190 r.p.m.). The culture was transferred to a 30-liter jar fermentor containing 20 liters of a medium composed of soluble starch (1.5%), glycerin (0.5%), Polypeptone S (0.5%), corn steep liquor (0.5%) and NaCl (0.3%). Fermentation was carried out at 28°C for 3 days under aeration of 20 liters/minute and agitation of

Chart 1.



400 r.p.m.

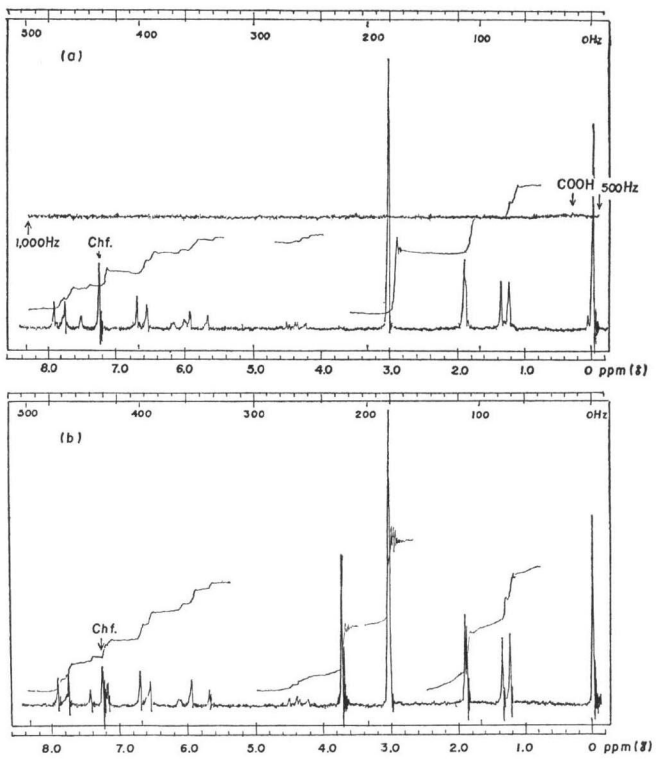
Isolation and purification of trichostatins A and C

About 140 liters of the broth was filtered and the mycelial cake was extracted with 18 liters of acetone. The acetone solution was condensed to about 7 liters *in vacuo* and the residue was extracted with ethyl acetate. Evaporation of the solvent and washing the residue with *n*-hexane gave 5.1 g of crude trichostatin A.

The fermentation filtrate was adsorbed on a column packed with 1.5 liters of HP-20, washed with water and eluted with methanol. The methanol solution was evaporated *in vacuo* and the residue was extracted with *n*-butanol. After washing with water the *n*-butanol solution was evaporated *in vacuo* and the residue was washed with *n*-hexane to yield about 43 g of crude extract which contained trichostatin C.

The crude trichostatin A was chromatographed on a column of 50 g of silica gel using a chloroform - methanol system, and the fractions

Fig. 3. ^{1}H-NMR spectra of trichostatic acid (a) and its methyl ester (b) in $CDCl_3$ at 60 M Hz.



containing trichostatin A were combined. Evaporation of the solution and recrystallization of the residue from ethyl acetate gave 1.6 g of pure trichostatin A, m.p. 150~151°C, which was soluble in lower alcohols, sparingly soluble in chloroform, ethyl acetate, acetone and benzene.

Anal. Calcd. for $C_{17}H_{22}O_8N_2$: C, 67.52; H, 7.33; N, 9.27.
Found: C, 67.28; H, 7.40; N, 9.43.

The trichostatin C-containing mixture was subjected to column chromatography on 100 g of silica gel and eluted with a solvent system of chloroform - methanol (8 : 2). The active fractions were combined and evaporated. The residue was crystallized from ethyl acetate, and the almost pure trichostatin C was further recrystallized from methanol to yield 5.45 g of pure sample as nearly colorless prisms, m.p. 171~173°C, UV $\lambda_{max}^{CH_3OH}$ nm (ε): 268 (14,600), 344 (14,300). $[\alpha]_D^{24}$ +50.5° (±0.9°) (*c* 0.987, CH_3OH).

Anal. Calcd. for $C_{23}H_{32}O_8N_2$: C, 59.47; H, 6.94; N, 6.03.
Found: C, 59.70; H, 6.89; N, 5.78.

Methanolysis of trichostatin C

A solution of 500 mg of trichostatin C in 8 ml of 40% methanolic HCl was allowed to stand at room temperature for 16 hours. The solvent was distilled off *in vacuo* and the residue was dissolved in water. After washing with ethyl acetate and successively with *n*-butanol, the water layer was evaporated to near dryness and the residue was chromatographed on 40 ml of HP-20. The sugar moiety was eluted with water in the first fraction to give 161 mg of a syrup, which was rechromatographed on 15 g of silica gel with a solvent system of chloroform - methanol (8 : 2). The crude product (85 mg) was crystallized from methanol to afford 14 mg of methyl α-D-glucopyranoside (**II**), m.p. 168~169°C, $[\alpha]_D^{23}$ +155.2° (±2.2°) (*c* 0.881, H_2O). The sample was identical with an authentic specimen in all respects.

Hydrolysis of trichostatin C

A solution of 500 mg of trichostatin C in 10 ml of 3 N HCl was allowed to stand at room temperature for 24 hours. The solution was applied to 100 ml of HP-20 column and eluted with 60 ml of water, 400 ml of water, 600 ml of 20% acetone in water and 400 ml of acetone.

The acetone eluate gave 346 mg of a mixture, which was chromatographed on silica gel to separate 114 mg of crude trichostatic acid and 150 mg of recovered trichostatin C. The crude trichostatic acid was dissolved in dilute Na_2CO_3 solution, and the solution was acidified and extracted with ether. The ether solution was dried over Na_2SO_4 and stripped to afford crystalline trichostatic acid (**III**), m.p. 138~140°C. $[\alpha]_D^{23}$ +3.8° (±0.4°) (*c* 1.033, MeOH).

Anal. Calcd. for $C_{17}H_{21}O_8N$: C, 71.06; H, 7.37; N, 4.87.
Found: C, 71.20, H, 7.35; N, 4.72.

The first water eluate gave 75 mg of *O*-β-D-glucopyranosyl hydroxylamine (**VI**) as a syrup.

Acetylation of 50 mg of **VI** with 1 ml of acetic anhydride in 2 ml of pyridine for 16 hours yielded 41 mg of hexaacetate (**VII**), which was recrystallized from ether to give colorless crystals, m.p. 165~167°C. ^{1}H-NMR, δ (ppm): 2.40 (6H) N-Ac_2; 2.13 (3H), 2.06 (3H) and 2.02 (6H), four O-Ac.

Anal. Calcd. for $C_{18}H_{25}O_{12}N$: C, 48.32; H, 5.63; N, 3.13.
Found: C, 48.23; H, 5.45; N, 3.18.

Trichostatic acid (**III**) from trichostatin A

To a suspension of 30 mg trichostatin A in 3 ml of water was added 1 ml of 1.5 N $HClO_4$ and the mixture was allowed to stand at room temperature overnight. The mixture was extracted with ether and the ether layer was shaken with dilute $NaHCO_3$ solution. The $NaHCO_3$ solution was acidified and extracted with ether. The ether layer gave 5 mg of trichostatic acid identical with the above specimen on comparison of IR spectra and TLC.

Methyl ester (**IV**) of trichostatic acid

To a solution of 45 mg trichostatic acid (**III**) in 2 ml of ether was added an excess of ethereal diazomethane, and the solution was kept at room temperature for 30 minutes. After evaporation of the solvent, the residue was dissolved in chloroform and washed with water. The chloroform layer

was dried over Na_2SO_4 and evaporated to give 29 mg of methyl ester (**IV**), which was crystallized from ether. ^{1}H-NMR: see Fig. 3b.

Trichostatin A from trichostatic acid

An ice-cold solution of 174 mg trichostatic acid in 5 ml of methanol was neutralized with a solution of 26.4 mg (1.1 equiv.) of NaOH in 5 ml of methanol, and the mixture was stirred for 10 minutes at room temperature. After addition of 10 ml of benzene the solution was condensed to 5 ml and another 10 ml of benzene was added to the residue, and the solution was evaporated to dryness.

The residual sodium salt was suspended in 15 ml of absolute benzene, and a solution of 80 mg of oxalyl chloride in 1.6 ml benzene was added dropwise to the suspension with stirring. After stirring at room temperature for 1 hour the formation of the acid chloride was complete, but since it was hydroscopic the benzene solution was used in the next reaction without evaporation.

A methanolic solution of free hydroxylamine was prepared from 176 mg of $NH_2OH \cdot HCl$ (4 equiv.) and 115 mg of NaOH in 13 ml of methanol, and to this solution was added the above-mentioned benzene solution of acid chloride at 0°C. The mixture was stirred at room temperature for 2 hours. The solvents were distilled off *in vacuo* and the residue was treated with chloroform and water. The chloroform solution was dried over Na_2SO_4 and evaporated to give crude product, which was fractionated on 15 g of silica gel using a chloroform - methanol system (95 : 5). The fractions which were positive to ferric chloride by spot test were combined and evaporated to afford 195 mg of crude hydroxamic acid. Rechromatography under the same conditions gave 140 mg of trichostatin A (77%), which was identical with an authentic sample by comparison of the IR and NMR spectra and TLC, but the synthesized trichostatin A was essentially optically inactive.

Acknowledgement

The authors are indebted to Dr. K. TORI and his coworker for ^{13}C-NMR measurements and the information on ^{13}C-NMR of glycosides, and to Dr. K. TAWARA for *in vitro* experiments.

Reference

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