A NEW ANTIFUNGAL ANTIBIOTIC, TRICHOSTATIN*

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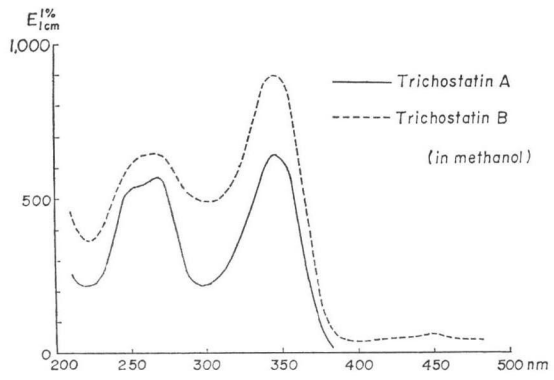
A new antibiotic, trichostatin, was isolated from the metabolites of strains of *Streptomyces hygroscopicus*. It is active against trichophytons and some fungi. The structure was determined to be a derivative of a primary hydroxamic acid by chemical and spectroscopic evidences.

In the course of the screening of antifungal antibiotics, we found that a few strains of *Streptomyces hygroscopicus* produce an antifungal antibiotic, though the yield was generally low and variable. In this paper, the isolation and structural elucidation of this antibiotic are described.

The antifungal principle was extracted with organic solvents from the fermentation broth and separated from other inactive products by chromatography on silica gel. The assay organism used was *Aspergillus niger*. The active fraction positive to DRAGENDORFF reagent was finally divided into two active components by column chromatography on polyamide.

Fig. 1. UV spectra of trichostatins



The main component, trichostatin A, is a colorless amorphous powder easily soluble in most organic solvents. The minor component, trichostatin B, was crystallized from alcohols as dark reddish purple prisms. It is easily soluble in chloroform but sparingly soluble in lower alcohols and ethyl acetate.

As shown in Fig. 1, both compounds exhibit similar UV spectra, but trichostatin B has a broad absorption in the visible region in accordance with its color. The two compounds show partial interconversion on silica gel, and the similar structures of the two are presumed also by the resemblance of their IR spectra (Fig. 2).

The antifungal activities of trichostatins A and B are summarized in Table 1.

The molecular formula of trichostatin A was determined to be $C_{17}H_{22}N_2O_3$ from elementary analysis and molecular weight determination by osmometry. The antibiotic shows acidic nature though it has two nitrogen atoms. The NMR spectrum of trichostatin A (Fig. 3) exhibits the presence of aromatic protons of A_2B_2 type, an aromatic N-dimethyl group, a methyl group on an olefinic carbon and a secondary methyl group. Additional olefinic proton signals and a methine proton signal are likely present, but further informations could not be obtained since

* Formerly called antibiotic A-300. (Japan Kokai 49-14691, 1974)

Fig. 2. IR spectra of trichostatin A (1) and trichostatin B (2) in $CHCl_3$ solution

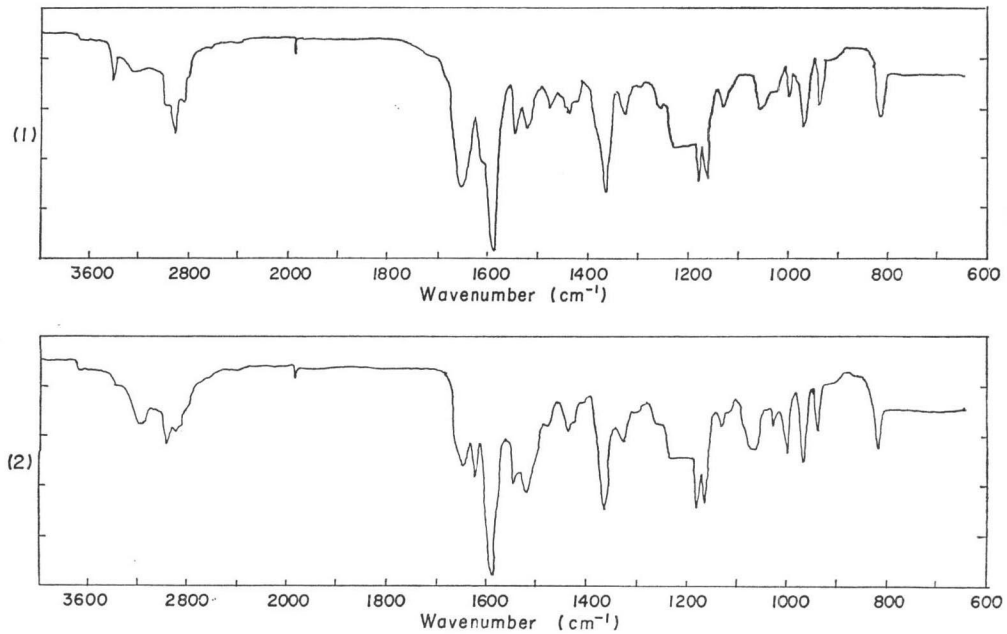


Table 1. Antifungal activities of trichostatins in agar dilution method (mcg/ml)

	Trichostatin A		Trichostatin B		Trichostatin A diacetate	
	MIC	MAC	MIC	MAC	MIC	MAC
Candida albicans M-9*	>50	>50	>50	>50	>50	>50
*Trichophyton rubrum***	3.13	<0.2	>50	0.2	12.5	0.2
*Trichophyton mentagrophytes***	3.13	<0.2	12.5	<0.025	>50	0.39
*Trichophyton purpureum***	3.13	<0.2	12.5	<0.025	50	0.39
*Epidermophyton floccosum***	>50	0.39	>50	1.56	>50	1.56
*Microsporum gypseum***	>50	0.78	>50	0.78	>50	1.56

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

the spectrum lacks fine structure.

The NMR spectrum of trichostatin B, unexpectedly, does not show any proton signals. Therefore, it was assumed that trichostatin B should include a paramagnetic metal presumably an iron atom, in its molecule. This fact in combination with the acidic nature of trichostatin A led to the assumption that trichostatin should have a hydroxamic acid function.

As expected, the treatment of trichostatin A with ferric chloride in chloroform solution gave a red solution and the IR spectrum of the product was identical with that of trichostatin B. The presence of iron in trichostatin B was confirmed by the elementary analysis showing the molecular formula, $(C_{17}H_{21}N_2O_3)_3Fe$.

The acetylation of trichostatin A with acetic anhydride in pyridine solution gave a diacetate, which was fully acetylated. The IR spectrum shows a carbonyl band characteristic of >N-OAc group at 1805 cm^{-1}. As shown in Fig. 4, the diacetate gave a satisfactory NMR

Fig. 3. NMR spectrum of trichostatin A (in $CDCl_3$) at 60 MHz

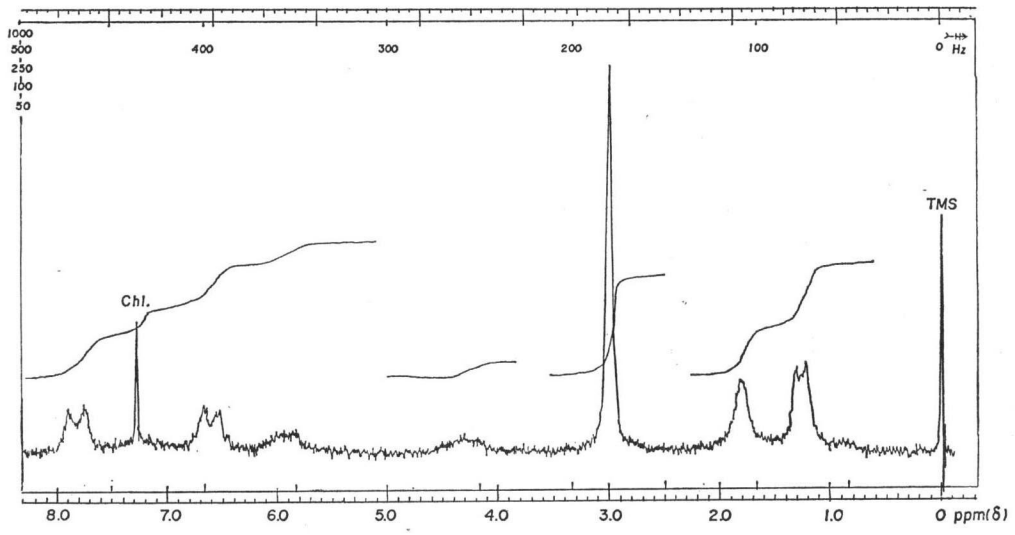
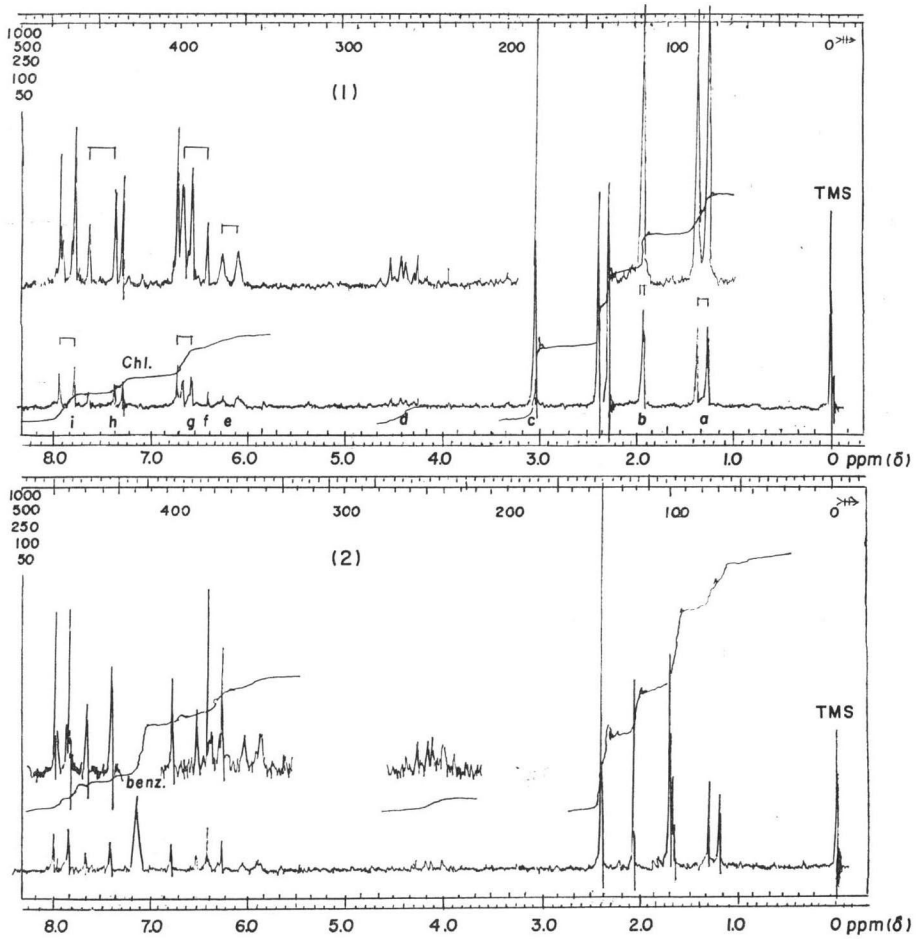
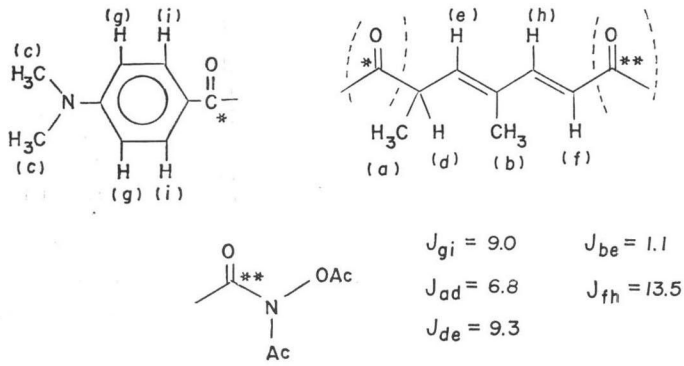


Fig. 4. NMR spectra of trichostatin A diacetate (**1**) in $CDCl_3$, (**2**) in C_6D_6 at 60 MHz



spectrum for the analysis of the signals. Since the spectrum clearly indicates the absence of the signal attributable to CH-OAc proton, trichostatin A has no alcoholic function, and therefore the hydroxamic acid should be primary. From the chemical shifts and the coupling constants, the proton signals could be assigned as below, and this structure fully satisfies the molecular formula of this compound.

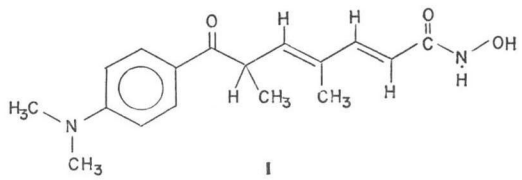


The *p*-dimethylaminobenzoyl part was confirmed by the comparison of the spectrum with that of model compound, *p*-dimethylaminobenzaldehyde. From the comparison of the spectrum of the diacetate with that of trichostatin A, the carbonyl groups, *C=O and **C=O, are presumably represented as benzoyl carbonyl and hydroxamic carbonyl groups, respectively.

Table 2. Nuclear OVERHAUSER effects (increases in integrated signal intensities %) of trichostatin A diacetate (in d_6-benzene at 30°)

Irradiated signal	Observed signal	%
CH_3 (c)	H(g)	26
CH_3 (a)	H(i)	0
CH_3 (a)	H(e)	10
H (d)	H(i)	7
H (d)	H(e)	4
CH_3 (b)	H(e)	0
CH_3 (b)	H(f)	32
H (e)	H(h)	21
H (h)	H(e)	28
H (f)	H(e)	6

The confirmation of the structure was made by the decoupling experiments and the measurement of nuclear OVERHAUSER effects (NOE) (in d_6-benzene, at 100 M Hz) on the diacetate, and the result of the NOE measurement is shown in Table 2. Both experiments agreed with the above assumption and trichostatin A should be shown as (**1**). Accordingly, trichostatin B is corresponding ferric chelate originated from the ferric ion in the fermentation medium.



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Trichostatin has an asymmetric carbon atom and is optically active, but the configuration is yet unknown.

Many hydroxamic acids have been found in natural products including antibiotics, however, only actinonin (antibacterial antibiotic)[1,2] is known as a primary hydroxamic acid. Therefore, trichostatin is the second example of this type from a natural source.

Experimental

Production

The streptomyces strain Y-50 was inoculated into a 500-ml SAKAGUCHI flask containing 100 ml of the medium composed of soluble starch (2.0 %), Soytone (1.5 %), corn steep liquor (0.5 %), glycerin (0.5 %) and NaCl (0.25 %). The medium was incubated at 28°C on a reciprocal shaker for 2 days. The culture was then transferred to the same medium, which was shake-cultured for 4 days in the same manner.

Isolation and purification

The broth (3.9 liters) was filtered and the filtrate was extracted with ethyl acetate to give 155 mg of active fraction (**1**). The mycelium was extracted with acetone and the acetone solution was evaporated *in vacuo*. The residue was extracted with ethyl acetate to give 280 mg of active fraction (**2**). The water layer was filtered and the precipitates were extracted with methanol containing chloroform (methanol 20 %). Evaporation of the solvents gave 390 mg of active fraction (**3**).

Since the three active fractions were essentially composed of the same components, the fractions were combined and chromatographed on a silica gel plate with a solvent system of chloroform-methanol (85:15). The active zone was homogenous in this solvent system and the materials (200 mg) from this zone was chromatographed on a column of 6 g polyamide. Elution with ethyl acetate and the evaporation of the solvent gave 124 mg of trichostatin A as an amorphous powder. UV λ_{max}^{EtOH} nm ($E_{1cm}^{1\%}$): 252 sh (531), 265(582), 341(648). $[\alpha]_D^{20.5}$ +62.8° (±1.1°)(*c* 1.007, EtOH).

Anal. Calcd. for $C_{17}H_{22}O_3N_2$: C, 67.52; H, 7.33; N, 9.27; O, 15.88 %. M.W. 302.36.
Found: C, 67.90; H, 7.65; N, 8.54; O, 16.64 %. M.W. 370.
(osmometry in chloroform)

After elution of trichostatin A, the column was eluted with chloroform to give trichostatin B fraction as a red solution. Evaporation of the solvent and crystallization of the residue from methanol afforded 36 mg of trichostatin B as dark reddish purple prisms, mp 192°C (dec.). UV λ_{max}^{EtOH} nm ($E_{1cm}^{1\%}$): 253 sh (624), 277(651), 341(918), 450(50). CD λ^{chf} nm ([θ]): 376(+16,100); 340(+124,400); 315(0); 300(−33,200); 274(0); 260(+23,900); 248(0); 240(−21,000); 232(0).

Anal. Calcd. for $(C_{17}H_{21}O_3N_2)_3Fe \cdot 3H_2O$: C, 60.41; H, 6.86; N, 8.29; Fe, 5.51.
Found: C, 60.67, H, 6.20; N, 8.35; Fe, 5.51.

Trichostatin B from trichostatin A

A solution of 30 mg trichostatin A in 6 ml chloroform was vigorously shaked with a solution of 100 mg ferric chloride in 3 ml water. The chloroform layer which soon turned to red was separated and evaporated to afford 27 mg of crude product, which was recrystallized from ethanol. The IR spectrum of this product was identical with that of trichostatin B.

Trichostatin A diacetate

To a solution of 40 mg trichostatin A in one ml of pyridine was added one ml of acetic anhydride, and the solution was allowed to stand for 2 hours at room temperature. The solvents were distilled off *in vacuo* and the residue was purified by preparative TLC on Silic AR-7G with a solvent system of chloroform-methanol (95:5). The main zone detected by UV light gave 42 mg of diacetate as an amorphous powder, $[\alpha]_D^{20.5}$ +70.8° (±1.3°) (*c* 0.891, $CHCl_3$). In the IR spectrum, no absorption bands due to NH and OH functions were observed. NMR spectra are shown in Fig. 4.

Acknowledgement

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