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ISOLATION OF A NOVEL TYROSINE KINASE INHIBITOR, LAVENDUSTIN A, FROM *STREPTOMYCES GRISEOLAVENDUS*

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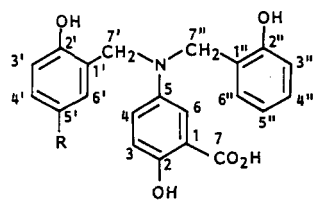
ABSTRACT.—A potent tyrosine kinase inhibitor, lavendustin A [**1**], has been isolated from a butyl acetate extract of *Streptomyces griseolavendus* culture filtrate. It inhibits epidermal growth factor receptor-associated tyrosine kinase with an IC_{50} of 4.4 ng/ml, which is about 50 times more inhibitory than erbstatin. It does not inhibit protein kinase A or C. Its structure, determined by spectral data and total synthesis, is novel, having a tertiary amine in the center with substituted benzyl and phenyl groups. Lavendustin A competes with ATP and is noncompetitive with the peptide. Its structure-activity relationship is discussed.

About 50 oncogenes have been isolated and characterized, and many of them such as src, yes, fgr, abl, erb B, neu (erb B-2), fms, fes, ros, and sis are considered to act through tyrosine kinase activity. Tyrosine kinase inhibitors may be useful not only in the mechanistic study of oncogenes but also in suppression of neoplastic diseases. Erbstatin (1) and genistein (2), both of microbial origin, have been reported as tyrosine kinase inhibitors, but their inhibitory activity is comparatively low, with IC_{50}'s of 0.2–0.6 μg/ml. Therefore, we have screened about 1000 *Streptomyces* culture filtrates for more potent tyrosine kinase inhibitors in a new tyrosine kinase assay system containing an A431 membrane fraction as the enzyme (3,4) and the tridecapeptide RRLIEDAEYAARG as a substrate (5). As a result, we have isolated two compounds of novel structure. We named them lavendustin A [**1**] and lavendustin B [**2**].

RESULTS AND DISCUSSION

For isolation of lavendustin A [**1**], the *Streptomyces griseolavendus* strain MI435-40F6 was cultured in medium containing 2% glycerin, 2% dextrin, 1% soy peptone, 0.3% yeast extract, 0.2% $(NH_4)_2SO_4$, and 0.2% $CaCO_3$ (pH 7.4) for 64 h at 27° on a rotary shaker (180 rpm/min). The broth filtrate (20 liters) was extracted with butyl acetate (20 liters) after adjustment of the pH to 2.4. The extract was concentrated in vacuo to give an oily matter (7.05 g) that was mixed with Si gel (9.0 g) and applied to a Si gel column (120 g). The column was washed with $CHCl_3$-MeOH (100:2); the active compounds were then eluted with $CHCl_3$-MeOH (3:1). The active fractions were concentrated (2.1 g) and charged on a column of Sephadex LH-20 (900 ml), which was developed with



1 R=OH
2 R=H

MeOH. After evaporation of the active fractions, the residue (1.03 g) was further purified twice by hplc with 15% MeCN. The active fractions were collected and extracted with butyl acetate (equal volume, pH 3.0 or 5.0), and the extract was concentrated in vacuo to give a mixture of lavendustins (105.2 mg). The mixture was applied to preparative Si gel tlc [$CHCl_3$-MeOH (2:1)] to obtain lavendustin A (37.0 mg) and lavendustin B (29.7 mg). Lavendustin A: white powder; mp 158–162°; fdms *m/z* $[M]^+$ 381; uv λ max (MeOH) (log ε) 297 (3.71), 280 (3.69), 216 (4.38). Lavendustin B: white powder; mp 140–144°; fdms *m/z* $[M+1]^+$ 366; uv λ max (MeOH) (log ε) 310 (3.32), 275 (3.70), 216 (4.38). They were soluble in MeOH, EtOH, Me_2CO, and DMSO but insoluble in $CHCl_3$, H_2O, and Et_2O. Taxonomic features indicated that the producing strain belonged to *S. griseolavendus*. It has been deposited in the Fermentation Research Institute of Agency of Industrial Science and Technology, Tsukuba, Japan, under the collection number FERM 10285.

The structures of lavendustins A [**1**] and B [**2**] were determined by nmr spectroscopic analysis, elemental analysis, and total synthesis. The molecular formula of lavendustin A was deduced to be $C_{21}H_{19}NO_6$ by mass spectrum and elemental analysis [found C 62.05, H 5.24, N 3.43; calcd for $C_{21}H_{19}NO_6 \cdot 3/2H_2O$, C 61.76, H 5.43, N 3.43%]. The ^{1}H-nmr spectrum of lavendustin A (Table 1) showed two singlet methylenes at δ 4.49 and 4.55 ppm. There were ten aromatic protons, and they were distributed to two 1,2,5-trisubstituted and one 1,2-disubstituted phenyl rings from the ^{1}H-^{1}H COSY spectrum and the coupling patterns. From the ^{1}H-^{13}C long-range correlation (HMBC) spectrum, the methylene groups were connected to a trisubstituted and a disubstituted phenyl to form two benzyls. These two methylene protons at δ 4.49 and 4.55 ppm showed long-range coupling with the carbons of each phenyl ring (H-7′ with C-1′, C-2′, and C-6′; H-7″ with C-1″, C-2″, and C-6″), and with the quarternary carbon at δ 136.0 ppm of the third phenyl ring, thus suggesting that the tertiary amine nitrogen combined two benzyl groups and a phenyl ring. The substituents on these benzyl groups were indicated to be hydroxy groups because of the chemical shifts of ^{13}C nmr (C-2′ = 150.0 ppm, C-5′ = 151.3 ppm, and C-2″ = 157.3 ppm, Table 2). The presence of carboxylic acid was suggested by the ^{13}C-nmr shift (174.2 ppm, s) and the broad peak (3,700–2,200 and 1,600 cm^{-1}) of ir. The proposed structure was supported by acetylation and esterification. The physico-chemical properties

TABLE 1. ^{1}H-nmr Spectra (400 MHz, CD_3OD) of Lavendustin A [**1**] and Lavendustin B [**2**].[a]

Proton	Compound	
	1	**2**
H-3	6.68 (1H, d, J = 9.2 Hz)	6.59 (1H, d, J = 9.0 Hz)
H-4	7.16 (1H, dd, J = 9.2 Hz, J = 3.2 Hz)	7.04 (1H, dd, J = 9.0 Hz, J = 3.2 Hz)
H-6	7.68 (1H, d, J = 3.2 Hz)	7.72 (1H, d, J = 3.2 Hz)
H-3′	6.63 (1H, d, J = 9.0 Hz)	6.73 (2H, dd, J = 8.0 Hz, J = 1.6 Hz)
H-4′	6.56 (1H, dd, J = 9.0 Hz, J = 3.4 Hz)	7.04 (2H, dt, J = 2.0 Hz, J = 8.0 Hz)
H-5′	—	6.68 (2H, dt, J = 1.6 Hz, J = 8.0 Hz)
H-6′	6.52 (1H, d, J = 3.4 Hz)	7.02 (2H, dd, J = 8.0 Hz, J = 2.0 Hz)
H-7′	4.49 (2H, s)	4.34 (4H, s)
H-3″	6.78 (1H, dd, J = 8.0 Hz, J = 1.6 Hz)	
H-4″	7.10 (1H, dt, J = 2.0 Hz, J = 8.0 Hz)	
H-5″	6.72 (1H, dt, J = 1.6 Hz, J = 8.0 Hz)	
H-6″	7.03 (1H, dd, J = 8.0 Hz, J = 2.0 Hz)	
H-7″	4.55 (2H, s)	

[a]Chemical shifts in ppm downfield from TMS.

TABLE 2. ^{13}C-nmr Spectra (100 MHz, CD_3OD) of Lavendustin A [1] and Lavendustin B [2].[a]

Carbon	Compound	
	1	2
C-1	118.7 (s)	119.8 (s)
C-2	160.6 (s)	159.1 (s)
C-3	118.2 (d)	117.3 (d)
C-4	127.2 (d)	128.2 (d)
C-5	136.0 (s)	140.2 (s)
C-6	123.2 (d)	123.9 (d)
C-7	174.2 (s)	ND[b]
C-1′	122.4 (s)	124.2 (s)
C-2′	150.0 (s)	157.6 (s)
C-3′	116.9 (d)	116.3 (d)
C-4′	117.2 (d)	129.7 (d)
C-5′	151.3 (s)	120.4 (d)
C-6′	117.9 (d)	131.1 (d)
C-7′	58.1 (t)	57.0 (t)
C-1″	121.8 (s)	
C-2″	157.3 (s)	
C-3″	116.2 (d)	
C-4″	130.8 (d)	
C-5″	120.7 (d)	
C-6″	131.5 (d)	
C-7″	58.1 (t)	

[a]Chemical shifts in ppm downfield from TMS.
[b]ND = not determined.

of lavendustin B resembled those of lavendustin A. The mass spectrum of lavendustin B indicated the lack of an oxygen atom from lavendustin A. The ^{1}H- and ^{13}C-nmr spectra of lavendustin B showed the presence of one 1,2,5-trisubstituted and two 1,2-disubstituted phenyl rings, in contrast with lavendustin A. These differences were due to the lack of a hydroxy group at C-5′.

However, the position of the hydroxy group at the phenyl could be either ortho or para from the carboxylic group. Therefore, we synthesized both possible structures.

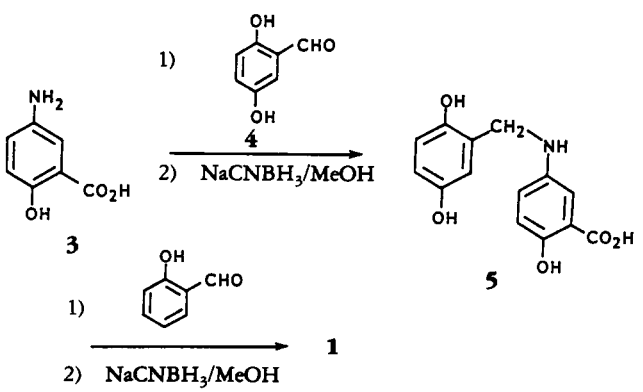


FIGURE 1. Total synthesis of lavendustin A [1].

The total synthesis was accomplished by using successive reductive alkylation of amino-hydroxybenzoic acids and hydroxybenzaldehydes as shown in Figure 1. The synthetic compound **1** gave exactly the same results as the natural product in terms of R_f value, spectral data, and inhibition of tyrosine kinase.

As shown in Table 3, lavendustin A inhibited EGF receptor tyrosine kinase with an IC_{50} of 4.4 ng/ml, which was about 50 times more inhibitory than erbstatin. It did not inhibit protein kinase C or A but weakly inhibited phosphatidylinositol kinase. Lavendustin B showed much weaker activity than lavendustin A. Lavendustin A also inhibited autophosphorylation of the EGF receptor below 10 ng/ml (data not shown).

TABLE 3. Inhibition of Kinases by Lavendustin A [1] and Lavendustin B [2].

Inhibitor	IC_{50} (μg/ml)			
	Tyrosine kinase	A Kinase	C Kinase	PI Kinase[a]
Lavendustin A . . .	0.0044	>100	>100	6.4
Lavendustin B . . .	0.49	—	—	—
Erbstatin	0.20	100	>179	25.0
Staurosporine	0.35	0.0011	<0.01	26.0

[a]Phosphatidylinositol kinase.

The EGF-receptor-associated tyrosine kinase was reported to act in the sequential ordered bi bi mechanism, in which the peptide comes first and ATP second to the enzyme active site (6). Kinetic studies by Lineweaver-Burk plotting indicated that lavendustin A inhibits the tyrosine kinase competitively with ATP (Figure 2A), and non-competitively with the peptide substrate (Figure 2B). Thus, the mechanism of inhibition is different from that of erbstatin, which competes with the peptide, but similar to that of orobol and genistein (7).

Because lavendustin A, having one additional hydroxy group, was much stronger than lavendustin B, we synthesized the 2″,5″-dihydroxy derivative of lavendustin A. However, as shown in Table 4, the inhibitory activity did not increase. Other lavendus-

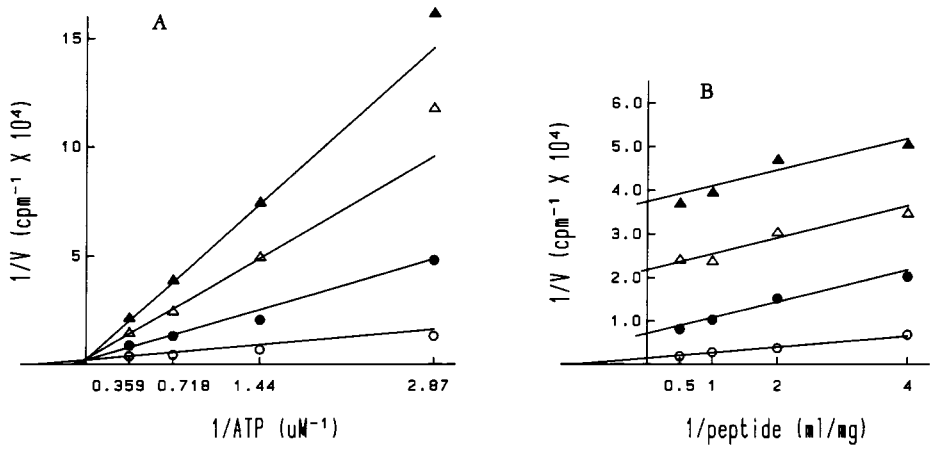
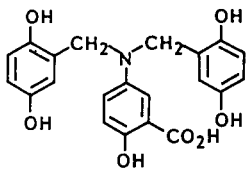
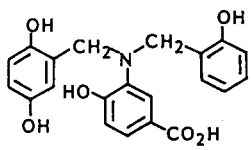
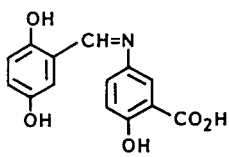
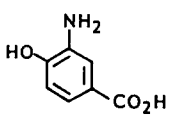


FIGURE 2. Lineweaver-Burk plot of the EGF receptor tyrosine kinase reaction with lavendustin A [1]. (A) Lavendustin A [1] vs. ATP: the inhibitor concentrations are 0 (○), 13 (●), 26 (Δ), and 39 nM (▲). (B) Lavendustin A [1] vs. peptide substrate: the inhibitor concentrations are 0 (○), 26 (●), 52 (Δ), and 79 nM (▲).

TABLE 4. Inhibition of Tyrosine Kinase by Lavendustin-Related Compounds.

Chemicals	Tyrosine kinase IC_{50} (μg/ml)
	0.011
	0.17
	0.76
5	0.012
4	33.0
3	2.7
	>100

tin derivatives and synthetic intermediates were tested for inhibition of tyrosine kinase. The 2,5-dihydroxybenzyl group appears to be essential for the inhibitory activity. Compound **5** (Figure 1) showed potent inhibitory activity, and it may be the core structure responsible for inhibition of tyrosine kinase.

Lavendustin A showed no antibacterial activity. It inhibited the growth of A431 cells (IC_{50}, 26.0 μg/ml), NIH3T3 cells (22.0 μg/ml), RSV-NIH3T3 cells (19.5 μg/ml), P388 leukemia cells (21.5 μg/ml), and L1210 leukemia cells (28.0 μg/ml).

Lavendustin A is a potent and specific inhibitor of tyrosine kinase. Its biological activities are now being studied both in vitro and in vivo. It can be easily synthesized and also may be a suitable starting compound for derivitization.

EXPERIMENTAL

ENZYME ASSAY.—Tyrosine kinase reactions were carried out in a final volume of 60 μl containing HEPES buffer (20 mM, pH 7.2), $MnCl_2$ (1 mM), bovine serum albumin (BSA, 0.125 mg/ml), mouse

EGF (100 ng/ml, Collaborative Research Inc.), the membrane fraction of A431 cells, tridecapeptide (Peptide Institute, Osaka), and [γ-^{32}P]ATP (12 Ci/mmol). The kinase activities were measured by a slight modification of the method described by Carpenter *et al.* (4): Lavendustins were dissolved in DMSO at 10 mg/ml and diluted with H_2O. The EGF receptor was first incubated with EGF and the sample at 0° for 10 min before assay of kinase activity. The kinase reactions were initiated by the addition of the peptide (1 mg/ml) and [γ-^{32}P]ATP (1 μM), and the reaction mixture was incubated for 30 min at 0°. The reactions were terminated by addition of 25 μl of 10% TCA and 6 μl of BSA (10 mg/ml). Precipitated proteins were removed by centrifugation, and 45-μl aliquots of the supernatants were spotted on Whatman P81 phosphocellulose papers (2 cm × 2 cm) that were immediately immersed in 30% HOAc at 25°. The papers were washed three times for 15 min at 15% HOAc, then for 5 min in Me_2CO, and dried. The radioactivity was counted with a liquid scintillation counter. For the kinetic analysis the concentrations of the substrates were altered as indicated. Activities of A kinase (8), C kinase (9), and PI kinase (10) activities were assayed as described in the literature.

TOTAL SYNTHESIS OF LAVENDUSTIN A [**1**].—5-Amino-2-hydroxybenzoic acid (46.3 mg) was added to 2,5-dihydroxybenzaldehyde (42 mg) in 5 ml of MeOH and the mixture incubated at 60° for 15 h to give the precipitate of Schiff base. Sodium cyanoborohydride (20.7 mg) was then added at room temperature to give the crude product **5**. To the reaction mixture was then added 100 ml of a saturated solution of NaCl containing 0.45 mmol of HCl, and the mixture was extracted twice with 100 ml of butyl acetate. The extract was dried with Na_2SO_4 and evaporated to give 53.2 mg of **5**. Next, 37 μl of salicylaldehyde and 18.6 mg of sodium cyanoborohydride in 2.5 ml of MeOH were added to the crude **5** (40.7 mg) and incubated at room temperature for 3.5 h to give **1**. NaCl solution (50 ml) was added as above and the mixture extracted twice with 75 ml of butyl acetate. The extract was dried and evaporated to give 79 mg of the crude product, which was purified by Si gel tlc [$CHCl_3$-MeOH (2:1)] to give 25.5 mg of **1**.

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