Andrastins A ~ C, New Protein Farnesyltransferase Inhibitors
Produced by *Penicillium* sp. FO-3929

II. Structure Elucidation and Biosynthesis

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The structures of new protein farnesyltransferase inhibitors, andrastins A ~ C, were elucidated. The cyclopentane ring of andrastins exhibited keto-enol tautomerism, which made the structure hard to elucidate. Therefore, the structure of andrastin A was elucidated by INADEQUATE and ^{13}C-^{13}C couplings using ^{13}C-labeled andrastin A. The absolute configuration of the *p*-bromobenzoyl derivative of andrastin A was elucidated by X-ray crystallographic analysis and its skeleton was shown to be *ent*-5α,14β-androstane. The biosynthesis of andrastin A was also studied by the incorporation of ^{13}C-labeled acetates. Though the andrastins had a common androstane skeleton, they were biosynthesized from a sesquiterpene and a tetraketide.

In the course of screening for inhibitors of protein farnesyltransferase, we have found a series of new compounds, andrastins A, B, and C (1 ~ 3, Fig. 1), from the cultured broth of *Penicillium* sp. FO-3929[1,2]. From their physico-chemical properties, their structures were shown to be similar. In this paper, the structure elucidation and biosynthesis of 1 ~ 3 are described.

Structure Elucidation of Andrastin A (1)

Chemical shifts in the ^1H and ^{13}C NMR of 1 ~ 3 are shown in Tables 1 and 2, respectively. The HMQC experiments revealed the connectivity of each proton and carbon.

HR-FAB-MS of 1 revealed its molecular formula, $C_{28}H_{38}O_7$[2]. Compound 1 showed 8 methyl, 4 methylene, 5 methine, and 11 quaternary carbon signals in the

Fig. 1. Structures of andrastins A, B, and C (1 ~ 3).

	R
Andrastin A (1)	CHO
Andrastin B (2)	CH₂OH
Andrastin C (3)	CH₃

DEPT spectra. Three partial structures, $-CH_2-CH_2-CH-O-$, $-CH-CH_2-CH_2-$, and $-CH-CH=$, were deduced by the ^1H-^1H COSY. The HMBC experiment revealed that they were connected to form rings A, B, and C as shown in Fig. 2. The arrangements of one acetyl (C-18 and 19), five methyls (C-20, 21, 22, 24, and 25), one aldehyde (C-23), and one quaternary carbon (C-17) were also deduced by the HMBC experiment (Fig. 2). But the remaining atoms, $C_5H_7O_4$, could not be assigned by the HMBC.

Because 1 was assumed to be synthesized *via* a mevalonate pathway from its partial structure, we carried out the incorporation experiment with $[1,2-^{13}C_2]$acetate to enrich the ^{13}C signals in order to detect ^{13}C-^{13}C couplings. Sodium $[1,2-^{13}C_2]$acetate (1 mg/ml) was added to the 40-hours cultured broth and the broth was incubated for a further 60 hours. From 1 liter of the cultured broth, 80 mg of labeled 1 was obtained. The 2D-INADEQUATE of the labeled 1 confirmed the bonds of rings A, B, and C except the bonds of C-8 (δ 42.8)/C-14 (δ 68.6)/C-13 (δ 57.8) as shown in Fig. 3. Moreover, the cross peaks of C-15 (δ 187.4)/C-16 (δ 114.5), C-14/C-26 (δ 171.8), and C-16/C-28 (δ 6.1) were observed. Then the ^{13}C-^{13}C spin decoupling experiments were conducted for the unidentified carbons (Fig. 4). Irradiation of C-15 and C-17 (δ 200.4) simplified the C-16 signal and irradiation of C-14 simplified the C-15 signal. These results revealed a cyclopentane ring (ring D). The long-range

Table 1. ^{1}H NMR (270 MHz) data of 1~3 in CD$_3$OD.

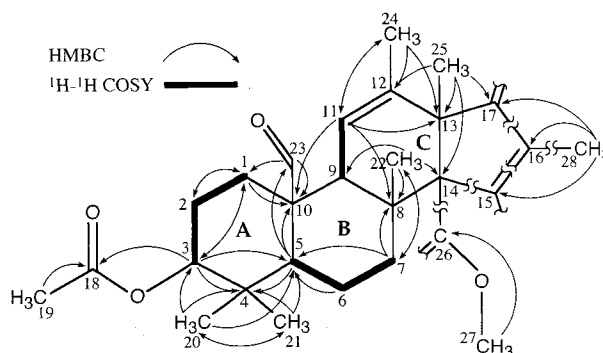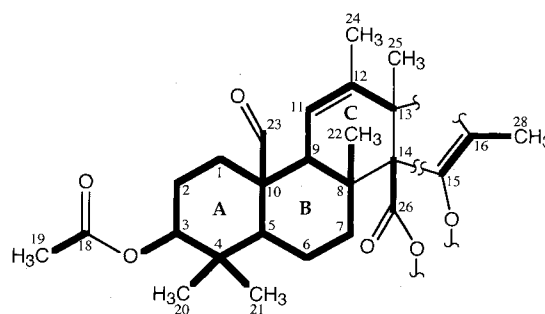| Position	1	2	3
1	0.98 ddd (5.0, 12.4, 13.0), 2.30 ddd (3.3, 3.3, 12.4)	1.01 m, 2.10 m	1.12 m, 1.57 m
2	1.59 m, 2.05 m	1.57 m, 2.26 m	1.59 m, 1.96 m
3	4.62 dd (2.4, 2.4)	4.67 dd (2.6, 2.6)	4.63 dd (2.4, 2.4)
5	1.84 dd (2.4, 15.7)	1.53 m	1.43 m
6	1.70 m, 2.08 m	1.50 m, 2.10 m	1.51 m, 2.04 m
7	2.25 ddd (3.1, 3.1, 12.9), 3.00 ddd (4.0, 12.9, 13.0)	2.10 m, 2.81 m	2.10 m, 2.77 m
9	2.13 br. s	1.90 br. s	1.81 m
11	5.39 br. s	5.71 br. s	5.40 br. s
19	2.05 s	2.04 s	2.03 s
20 (eq)	0.95 s	0.90 s	0.88 s
21 (ax)	0.88 s	0.99 s	0.93 s
22	1.24 s	1.34 s	1.31 s
23	10.18 s	3.77 d (12.2), 3.92 d (12.2)	0.95 s
24	1.75 br. s	1.75 br. s	1.75 br. s
25	1.16 s	1.17 s	1.18 s
27	3.58 s	3.56 s	3.57 s
28	1.59 s	1.60 s	1.60 s

The CD$_3$OD signal (3.31 ppm) was used as a reference. The coupling constants (Hz) are in parentheses.

Table 2. ^{13}C NMR (67.8 MHz) data of 1~3 in CD$_3$OD.

Position	1	2	3
1	29.0 t	30.2 t	34.4 t
2	24.3 t	25.5 t	23.5 t
3	79.0 d	79.9 d	79.6 d
4	38.0 s	37.4 s	37.7 s
5	49.6 d	50.4 d	50.3 d
6	17.9 t	18.4 t	18.8 t
7	33.5 t	34.2 t	34.0 t
8	42.8 s	42.8 s	38.1 s
9	54.8 d	55.1 d	54.4 d
10	53.4 s	43.1 s	43.4 s
11	123.6 d	127.1 d	126.1 d
12	137.0 s	134.2 s	136.4 s
13	57.8 s	58.1 s	58.1 s
14	68.6 s	68.9 s	68.8 s
15	187.4 s	186.8 s	188.0 s
16	114.5 s	114.4 s	114.4 s
17	200.4 s	202.1 s	201.7 s
18	172.2 s	172.5 s	172.5 s
19	21.1 q	21.2 q	21.1 q
20 (eq)	27.1 q	28.4 q	28.2 q
21 (ax)	21.5 q	21.5 q	22.0 q
22	19.76 q	18.0 q	18.1 q
23	206.8 d	62.5 t	17.3 q
24	19.84 q	19.8 q	19.8 q
25	16.0 q	16.0 q	16.1 q
26	171.8 s	172.0 s	172.0 s
27	52.2 q	52.0 q	52.0 q
28	6.4 q	6.4 q	6.3 q

The CD$_3$OD signal (49.0 ppm) was used as a reference.

Fig. 2. Partial structure of 1 elucidated by ^{1}H-^{1}H COSY and HMBC.

Fig. 3. Partial structure of 1 elucidated by 2D-INADE-QUATE.

coupling between H$_3$-27 (δ 3.58)/C-26 in the HMBC showed that the methoxy residue was attached to C-26. Two carbonyl like carbons, C-15 (δ 187.4) and C-17 (δ 200.4), were neighbors of olefinic C-16 (δ 114.5) in the ring D, which suggested that either C-15 or C-17 should be an oxy-olefinic carbon. The ^{13}C signals of C-15 and C-17 were broad, suggesting that they might be in an equilibrium between keto-enol tautomers. The tautomerism was confirmed by the formation of two p-bromobenzoyl derivatives of 1 as shown below. Thus the

planar structure of 1 was elucidated.

The relative configuration of 1 was examined by differential NOE experiments. As shown in Fig. 5, the NOEs between H-3 (δ 4.62)/H$_3$-21 (δ 0.88), H-5 (δ 1.84)/H$_3$-20 (δ 0.95), H$_\alpha$-6 (δ 2.08)/H$_3$-22 (δ 1.24),

Fig. 4. Structure of 1 elucidated by ^{13}C-^{13}C spin decoupling experiments.

Nocks indicate irradiated carbons and arrowheads indicate simplified carbons.
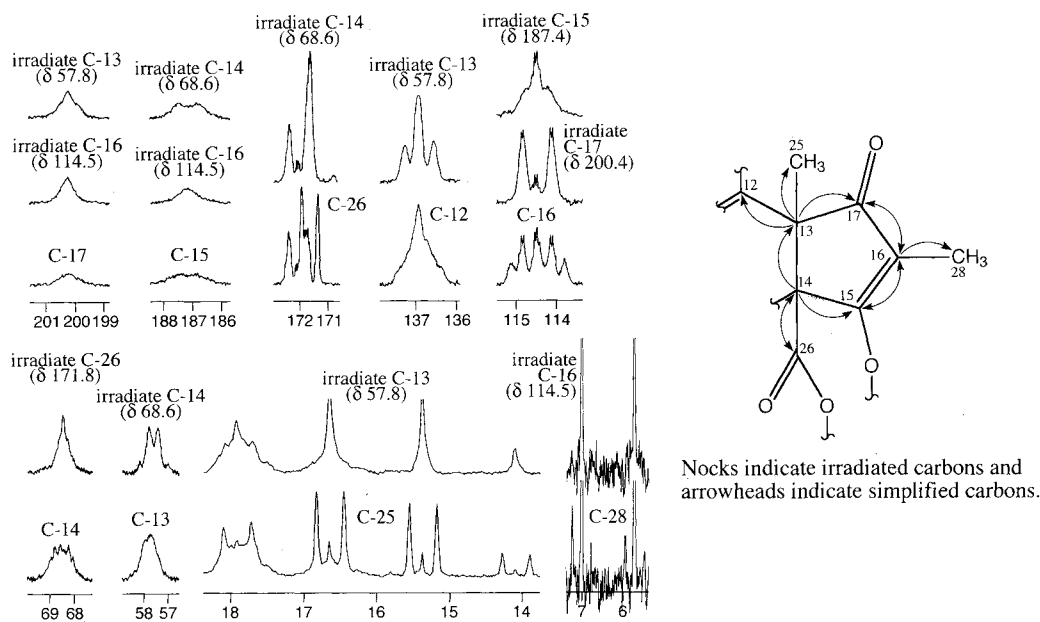
Fig. 5. NOE experiments of 1.
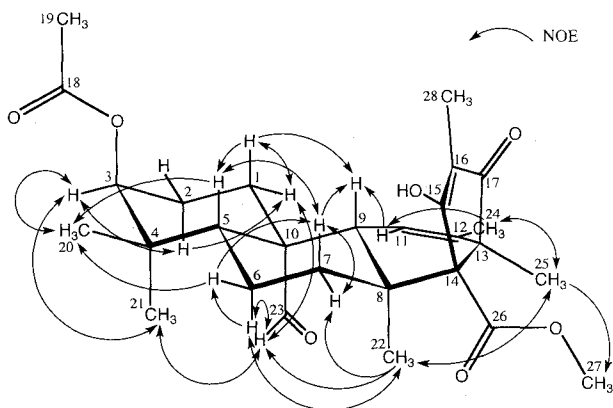
H_α-6/H-23 (δ 10.18), H_3-21/H-23, H_3-22/H-23, H_3-22/H_3-25 (δ 1.16), and H_3-25/H_3-27 (δ 3.58) suggested a 5α,14β-androstane skeleton and a 3α-acetoxy moiety.

From the results described above, the relative configuration of 1 is elucidated as shown in Fig. 5.

X-ray Crystallography of 15-(p-Bromobenzoyl)-andrastin A (4)

To confirm the structure and to elucidate the absolute configuration of 1, a p-bromobenzoyl derivative of 1 was synthesized. Of two products formed, 15-(p-bromo-benzoyl)-andrastin A (4) and 17-(p-bromobenzoyl)-andrastin A (5), 4 afforded prismatic crystals from a mixture of CHCl$_3$ and methanol.

The single crystal X-ray crystallographic data for 4

Table 3. Single crystal X-ray crystallographic analysis of 4.

Crystal parameters	
Empirical formula	$C_{35}H_{41}BrO_8$
Formula weight	669.61
Crystal dimensions (mm)	0.2 × 0.4 × 0.3
Crystal system	Orthorhombic
Lattice parameters:	a = 13.817 (6) Å
	b = 23.277 (5) Å
	c = 10.232 (8) Å
	V = 3291 (4) Å^3
Space group	P2$_1$2$_1$2$_1$ (with Z=4)
Density calc (g/cm^3)	1.351
Linear absorption factor (cm^{-1})	21.02
Refinement parameters	
No. of reflections measured	6,096
Nonzero reflections (I>3.00σ)	5,169
R-index Residuals: R^a	0.070
Residuals: R$_w$^{b}	0.075
Goodness of fit indicator^c	6.01

^{a} $\Sigma||F_o|-|F_c||/\Sigma|F_o|$
^{b} $[(\Sigma w(|F_o|-|F_c|)^2/\Sigma wF_o^2]^{1/2}$
^{c} $[(\Sigma w(|F_o|-|F_c|)^2/(No-Nv)]^{1/2}$
   No = number of observations
   Nv = number of variables

are summarized in Table 3. The non-hydrogen atoms were refined anisotropically. The final cycle of full-matrix least-squares refinement was based on 5169 observed reflections and 397 variable parameters and converged with unweighted and weighted agreement factors of R = 0.070, R$_W$ = 0.075. The corresponding R factor for the enantiomer was 0.076 (R$_W$ = 0.080). Thus, the absolute configuration of 4 was concluded to be as shown in Fig. 6. The configuration of 4 was the same as that of 1 suggested by the NOE experiments. The X-ray

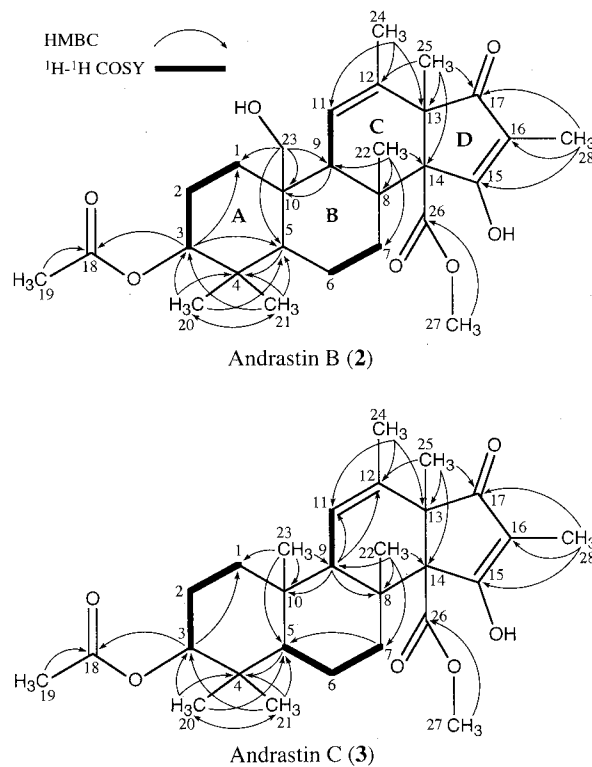Fig. 6. Absolute configuration of 4 elucidated by X-ray crystallography.

Fig. 7. Structures of 2 and 3 elucidated by ¹H-¹H COSY and HMBC.

Andrastin B (2)
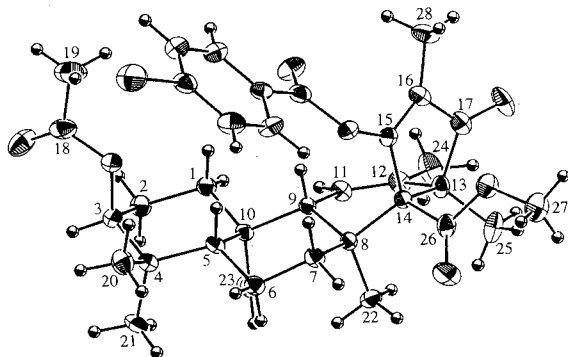
Andrastin C (3)

analysis revealed that the skeleton of 4 is *ent*-5α,14β-androstane.

Structure Elucidation of Andrastins B (2) and C (3)

The molecular formulae of 2 and 3 were elucidated by HR-FAB-MS as $C_{28}H_{40}O_7$ and $C_{28}H_{40}O_6$, respectively. The UV and IR spectra of 2 and 3 were quite similar to those of 1[2]. The ¹H and ¹³C NMR spectra (Tables 1 and 2) also resembled those of 1 except that the proton and carbon signals of C-23 were different. According to the HMBC experiment on 2, the cross peaks from oxymethylene proton (δ 3.77) to C-1 (δ 30.2), C-5 (δ 50.4), C-9 (δ 55.1), and C-10 (δ 43.1) indicated that 2 has an hydroxymethyl residue instead of the aldehyde of 1 (Fig. 7). Similarly, 3 was shown to have a methyl residue at C-10 by the long-range couplings from methyl protons (δ 0.95) to C-1 (δ 34.4), C-5 (δ 50.3), C-9 (δ 54.4), and C-10 (δ 43.3). Though the stereochemistry of 2 and 3 was not studied, the configurations of 2 and 3 were suggested to be the same as that of 1 because the ¹H and ¹³C chemical shifts of 1 ∼ 3 are similar except for positions 10 and 23. Thus, the structures of 2 and 3 were elucidated as shown in Fig. 1.

Recently KOSEMURA *et al.* reported citreohybridonol (6, Fig. 8)[3,4] that was isolated from the mycelium of a hybrid fungi and which had antifeedant and insecticidal activities. The skeleton of 6 and andrastins are the same except for the γ-lactone of 6. Though X-ray analysis of citreohybridone A (7)[5] co-produced with 6 was reported, the absolute configuration was not studied. The citreohybridone group were not sesterterpenes, but were biosynthesized from a sesquiterpene and a tetra-ketide[3,4].

## Biosynthesis of Andrastin A (1)

From the resemblance of the structure of andrastins and 6, we were interested in whether the andrastins were biosynthesized in the same manner as 6. Therefore, we studied the incorporation of ¹³C-labeled acetates into 1. Sodium [2-¹³C]acetate (1 mg/ml) or sodium [1,2-¹³C₂]acetate (0.33 mg/ml) was added to 40 hours cultured broth and the broth was cultured for 60 more hours. To minimize the extraneous couplings due to excess intramolecular labeling, [1,2-¹³C₂]acetate was diluted three times with unlabeled sodium acetate (0.67 mg/ml).

The ¹³C NMR spectrum of 1 labeled with [2-¹³C]-acetate was obtained. The intensity ratios of signals of the labeled 1 to those of unlabeled one were calculated and are shown in Table 4. The positions of enriched carbons of 1 are shown in Fig. 8. The average enrichment ratio was about eight. Though C-18 was enriched over two times, it was suggested to be derived from C-1 of acetate as the neighboring carbon was much enriched.

From the ¹³C NMR spectra of 1 labeled with [1,2-¹³C₂]acetate, the ¹³C-¹³C coupling constants (^1J(C–C)) were analyzed and are shown in Table 4. The acetate arrangement proved by ^1J(C–C) are shown in Fig. 8.

Fig. 8.   Labeling pattern of 1 from ¹³C-acetates and postulated biosynthetic pathway of andrastins.
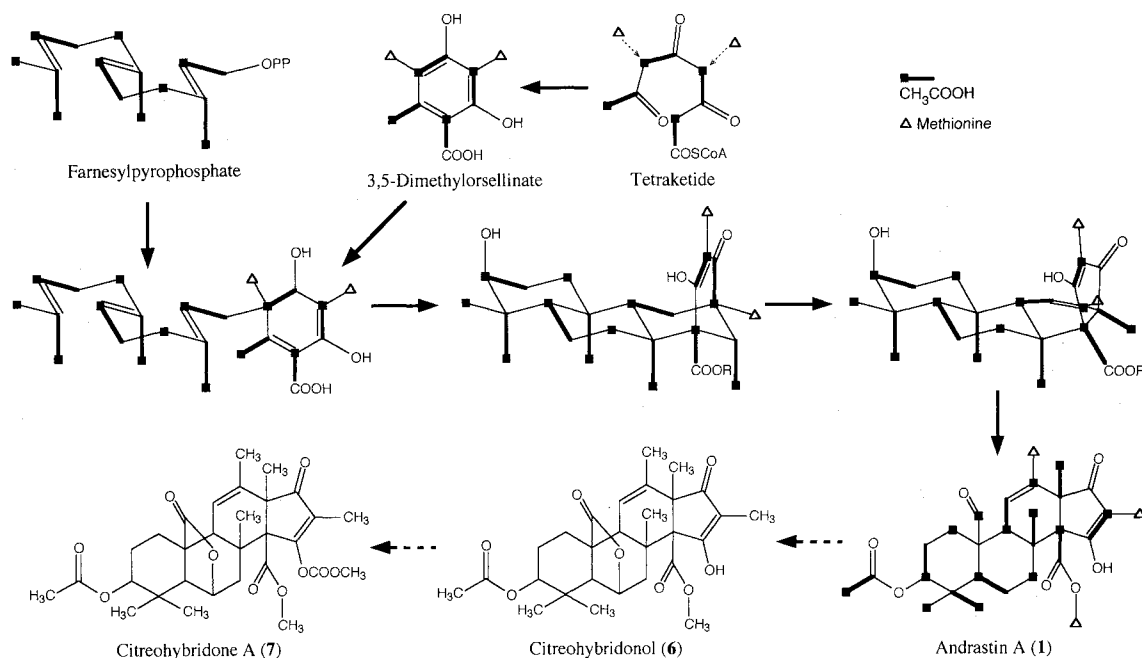
Table 4.   Enrichment ratios and ¹³C-¹³C couplings (¹J(C, C)) of 1 labeled with ¹³C-acetates.

C No.	ppm[a]	[2-¹³C]acetate Enrichment ratio[b]	[1,2-¹³C₂]acetate ¹J(C,C) (Hz)
1	29.0	14.2[c]	
2	24.3	1.0	36.9
3	79.0	7.4[c]	36.9
4	38.0	0.6	35.5
5	49.6	1.9[c]	33.6
6	17.9	0.9	33.6
7	33.5	14.1[c]	
8	42.8	0.8	36.4
9	54.8	8.9[c]	42.0
10	53.4	1.0	37.4
11	123.6	1.0	42.0
12	137.0	8.9[c]	
13	57.8	0.8	38.2
14	68.6	6.0[c]	56.5
15	187.4	ND[d]	73.1
16	114.5	6.4[c]	73.1
17	200.4	ND[d]	
18	172.2	2.3	59.8
19	21.1	8.8[c]	59.8
20 (eq)	27.1	9.6[c]	
21 (ax)	21.6	7.4[c]	35.5
22	19.78	9.2[c]	36.4
23	206.8	12.1[c]	37.4
24	19.85	1.3	
25	16.0	12.5[c]	38.2
26	171.7	0.8	56.5
27	52.2	1.2	
28	6.4	1.2	

[a]   Operated at 100 MHz. The CD₃OD signal (49.0 ppm) was used as a reference.
[b]   Enrichment ratios are relative to the intensity of C-2 signal as 1.0.
[c]   Signals that are suggested to be enriched.
[d]   Signals were too weak to calculate.

The results of the incorporation of [1,2-¹³C₂]acetate into 1 were coincident with that of 6[3,4]. Therefore, 1 was suggested to be biosynthesized from a farnesyl pyrophosphate and a tetraketide. Three methyls (C-24, C-27, and C-28) were assumed to be derived from methionines by analogy with the biosynthetic results obtained with 6[3,4]. As there was no study for the incorporation of single labeled acetate into 6, our result confirmed the direction of the acetate arrangement. Furthermore, long-range coupling between C-12 and C-17 was observed in the INADEQUATE spectrum of 1 labeled with [1,2-¹³C₂]acetate, suggesting that the acetate unit of C-12 and C-17 is cleaved by rearrangement. Compound 6 may be biosynthesized from 1 by oxidation and lactonization at C-6 and C-10. A postulated biosynthetic pathway for the andrastins based on the scheme of KOSEMURA et al.[4] is shown in Fig. 8.

Experimental

NMR spectra were obtained with JEOL JNM-EX270 and Valian Unity 400 spectrometers. Mass spectrometry was conducted on a JEOL JMS-AX505 HA spectrometer. Melting points were measured with a Yanaco micro melting point apparatus MP-S3. In the X-ray crystallographic analysis, all measurements were made on a Rigaku AFC-5R diffractometer with graphite monochromated CuKα radiation.

Incorporation of [2-^{13}C]Acetate into 1

The basic methods of production and isolation were the same as described previously[2]. Each 2 ml of the seed culture that was incubated at 27°C for 2 days and was transferred into ten 500-ml Erlenmeyer flasks containing 100 ml of the production medium. After 40 hours fermentation at 27°C, each 100 mg of [2-^{13}C]sodium acetate (Aldrich) solution was added to the flasks. They were cultured for 60 more hours.

The supernatant of the cultured broth (1 liter) was adjusted to pH 3 and extracted with an equal volume of EtOAc. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a brown oil. The oil was applied on a silica gel column (Silica gel 60, 40~63 μm, Merck) and eluted with $CHCl_3$-MeOH. The eluates of $CHCl_3$-MeOH (99:1, 98:2 and 96:4) were concentrated under reduced pressure to give a yellow powder. The powder was further purified by HPLC under the following conditions: column, Senshu pak Pegasil ODS (i.d. 20×250 mm, Senshu Scientific Co., Ltd.); mobile phase, CH_3CN-0.05% H_3PO_4 (3:2~4:1, linear gradient); flow rate, 7 ml/minute; detection, UV 285 nm. Compound 1 was eluted at 18 minutes. The eluate was concentrated to remove CH_3CN and extracted with EtOAc at pH 3 to give a white powder of 1 labeled with [2-^{13}C]acetate (27.9 mg). The ^{13}C NMR spectrum was run at 100 MHz.

Incorporation of [1,2-^{13}C$_2$]Acetate into 1

The method was the same as described for the method for the incorporation of [2-^{13}C]acetate into 1 except the yield of the compounds was different. The measurements of ^{13}C NMR spectra, INADEQUATE, and ^{13}C-^{13}C spin decoupling experiments were obtained at 100 MHz.

Preparation of 15-(p-Bromobenzoyl)-andrastin A (4) and 17-(p-Bromobenzoyl)-andrastin A (5)

To a solution of 1 (10.4 mg, 0.02 mmol) in pyridine (0.2 ml) was added p-bromobenzoylchloride (10.3 mg, 0.04 mmol) and the reaction mixture was stirred at room temperature for 1 hour. The reaction mixture was concentrated and the residue was extracted with EtOAc at pH 3. The organic layer was dried over Na_2SO_4 and concentrated. The resulting residue was purified by preparative TLC (hexane-EtOAc, 4:1) to obtain a mixture of 4 and 5. They were further purified by HPLC under the following conditions: column, Senshu pak Pegasil ODS (i.d. 20×250 mm, Senshu Scientific Co., Ltd.); mobile phase, CH_3CN-0.05% H_3PO_4 (4:1); flow rate, 7 ml/minute; detection, UV 285 nm. Compounds 4 and 5 were eluted at 15 and 17 minutes, respectively. The eluates were concentrated and extracted with EtOAc at pH 3 to yield 3.0 mg of 4 and 4.9 mg of 5. Each compound was recrystallized from a mixture of $CHCl_3$ and MeOH and the crystal of 4 was suitable to apply X-ray analysis.

4: HR-FAB-MS, Found m/z 669.2056 (M+H)$^+$, Calcd for $C_{35}H_{42}BrO_8$ 669.2063; mp 149~151°C.

5: HR-FAB-MS, Found m/z 669.2070 (M+H)$^+$, Calcd for $C_{35}H_{42}BrO_8$ 669.2063; mp 243~245°C.

Single Crystal X-Ray Analysis of 4

The colorless prismatic crystal of 4 having approximate dimensions of 0.2×0.4×0.3 mm was mounted on a glass fiber. The data were collected at a temperature of 23±1°C using the ω-2θ scan technique to a maximum 2θ value of 126.4°. Pertinent crystal, data collection, and refinement parameters are summarized in Table 3. Neutral atom scattering factors were taken from CROMER and WABER[6]. Anomalous dispersion effects were included in Fcalc[7]; the values for $\Delta f'$ and $\Delta f''$ were those of CROMER[8]. All calculations were performed using the TEXSAN[9] crystallographic software package of Molecular Structure Corporation. The structure was solved by direct methods[10,11].

References

1) SHIOMI, K.; R. UCHIDA, J. INOKOSHI, H. TANAKA, Y. IWAI & S. ŌMURA: Andrastins A~C, new protein farnesyltransferase inhibitors, produced by *Penicillium* sp. FO-3929. Tetrahedron Lett. 37: 1265~1268, 1996

2) ŌMURA, S.; J. INOKOSHI, R. UCHIDA, K. SHIOMI, R. MASUMA, T. KAWAKUBO, H. TANAKA, Y. IWAI, S. KOSEMURA & S. YAMAMURA: Andrastins A~C, new protein farnesyltransferase inhibitors produced by *Penicillium* sp. FO-3929. I. Producing strain, fermentation, isolation, and biological activities. J. Antibiotics 49: 414~417, 1996

3) KOSEMURA, S.; H. MIYATA, K. MATSUNAGA & S. YAMAMURA: Biosynthesis of citreohybridones, the metabolites of a hybrid strain KO 0031 derived from *Penicillium citreo-viride* B. IFO 6200 and 4692. Tetrahedron Lett. 33: 3883~3886, 1992

4) KOSEMURA, S.; H. MIYATA, S. YAMAMURA, K. ALBONE & T. J. SIMPSON: Biosynthetic studies on citreohybridones, metabolites of a hybrid strain KO 0031 derived from *Penicillium citreo-viride* B. IFO 6200 and 4692. J. Chem. Soc. Perkin Trans. 1 1994: 135~139, 1994

5) KOSEMURA, S.; K. MATSUNAGA, S. YAMAMURA, M. KUBOTA & S. OHBA: The structures of citreohybridone A and B novel sesterterpenoid-type metabolites of a hybrid strain KO 0031 derived from *Penicillium citreo-viride* B. IFO 6200 and 4692. Tetrahedron Lett. 32: 3543~3546, 1991

6) CROMER, D. T. & J. T. WABER: Table 2.2 A. *In*

International Tables for X-ray Crystallography. Vol. IV. *Ed.*, IBERS, J. A. & W. C. HAMILTON, The Kynoch Press, Birmingham, 1974

7) IBERS, J. A. & W. C. HAMILTON: Dispersion corrections and crystal structure refinements. Acta Crystallogr. 17: 781~782, 1964

8) CROMER, D. T.: Table 2.3.1. *In* International Tables for X-ray Crystallography. Vol. IV. *Ed.*, IBERS, J. A. & W. C. HAMILTON, The Kynoch Press, Birmingham, 1974

9) TEXSAN-TEXRAY Structure Analysis Package, Molecular Structure Corporation, 1985

10) GILMORE, C. J.: MITHRIL—an integrated direct methods computer program. J. Appl. Cryst. 17: 42~46, 1984

11) BEURSKENS, P. T.: DIRDIF: Direct methods for difference structures—an automatic procedure for phase extension and refinement of difference structure factors. *In* Technical Report 1984/1. *Ed.*, NIJEMGEN, Crystallography Laboratory, Toernooiveld, 1984