# Biosynthetic Studies on a Myxobacterial Antibiotic, Cystothiazole A: Biosynthetic Precursors of the Carbon Skeleton

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Biosynthetic studies on cystothiazole A (**1**), a $\beta$-methoxyacrylate-type antifungal compound from a myxobacterium, were performed by feeding the producing organism, *Cystobacter fuscus*, with stable-isotope-labeled compounds including [2-$^{13}$C]acetate, [1,2-$^{13}C_2$]acetate, [1-$^{13}$C]propionate, L-[1-$^{13}$C]serine, L-[*methyl*-$^{13}$C]methionine, and DL-valine-$d_8$. The polyketide moiety of **1** was found to be derived from acetate and propionate, the bithiazole moiety from L-serine, the *O*-methyl groups from the *S*-methyl group of L-methionine, and the isopropyl moiety from L-valine, which should be the metabolic precursor of isobutyryl-CoA.

Myxobacteria have been increasingly recognized as a valuable source of novel bioactive compounds and have attracted considerable attention in recent years after discovery of promising antitumor agents, epothilones[1]. We previously reported antifungal compounds, the cystothiazoles[2,3], from the myxobacterium *Cystobacter fuscus* strain AJ-13278. Cystothiazole A (**1**), the major and most potent antifungal member of the cystothiazoles, has characteristic bithiazole and $\beta$-methoxyacrylate structures. This kind of structure is known to be a hybrid-type polyketide and is biosynthesized by a combination of polyketide synthases (PKSs) and non-ribosomal peptide synthases (NRPSs)[4]. In recent years the enzymology and molecular genetics of PKSs and NRPSs are attracting great attention in the field of engineering microbial secondary metabolism, such as "combinatorial biosynthesis"[5], because of the potential for developing various diverse chemical structures and important biological activities. Detailed studies on the biosynthesis of hybrid-type polyketides promise to provide the key to engineering biosynthesis of microbial secondary metabolites. The biosynthetic precursors of a related myxobacterial antibiotic, myxothiazol A, was previously investigated by other groups[6,7], and recently, the biosynthetic gene cluster for myxothiazol A encoding PKS and NRPS was reported[8]. Considering these results and structural similarities between **1** and myxothiazol A, **1** should be also biosynthesized *via* a combined system of NRPS and PKS, *i.e.*, the position C-1 to C-6 of **1** is regarded as a polyketide and the bithiazole moiety as a peptide originated from two cysteines (Fig. 1). Hence, we examined feeding experiments using several stable-isotope-labeled precursors. In the present study we describe the details on the determination of the biosynthetic precursors for **1** to clarify the biosynthetic pathway of this unique hybrid-type polyketide.

## Materials and Methods

### General

HPLC was performed on a JASCO high-pressure gradient system with PU-980 pumps and a UV-970 UV/VIS detector. $^{13}$C and $^{1}$H NMR spectra were recorded on a Bruker ARX400 in a $CDCl_3$ solution (99.8% atom enriched). NMR chemical shifts were referenced to the solvent peak of $\delta_H$ 7.26 (residual $CHCl_3$) or $\delta_C$ 77.0 for $CDCl_3$. Mass spectra were recorded on a JMS DX-705L mass spectrometer in the positive FAB mode using *m*-nitrobenzyl alcohol as a matrix. LC/MS was recorded on a

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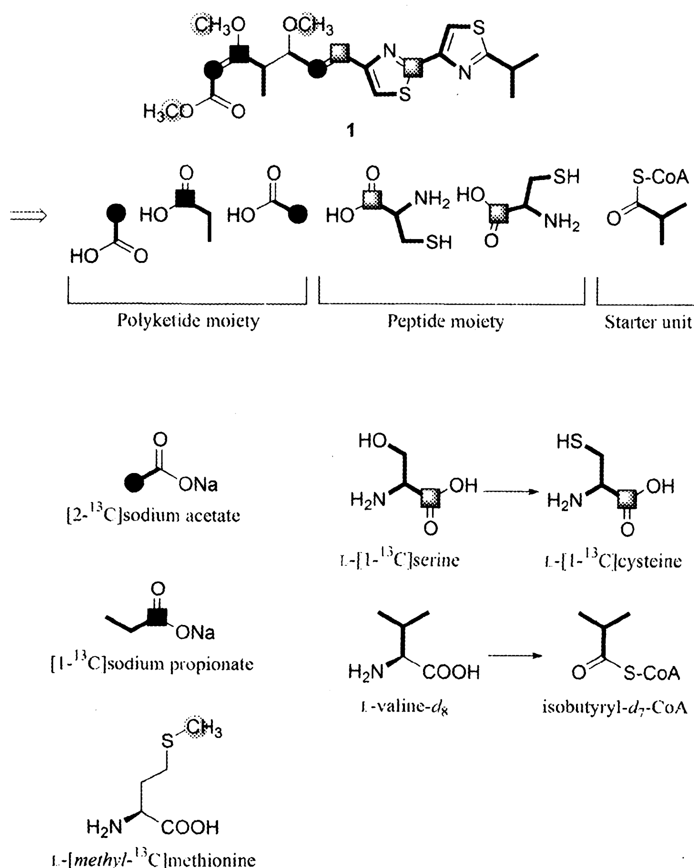
Fig. 1. Structure and putative biosynthetic precursors of cystothiazole A (**1**).




Fisions VG Platform II mass spectrometer in ESI negative mode.

## Chemicals

Stable-isotope-labeled compounds used were sodium [2-$^{13}$C]acetate (99% atom enriched, Cambridge Isotope Laboratories), sodium [1,2-$^{13}C_2$]acetate (99% atom enriched, Aldrich), sodium [1-$^{13}$C]propionate (99% atom enriched, Isotec), L-[1-$^{13}$C]serine (99% atom enriched, Aldrich), L-[*methyl*-$^{13}$C]methionine (99% atom enriched, Aldrich), DL-[2,3,4,4,4,5,5,5-$^2H_8$]valine (abbreviated to DL-valine-$d_8$) (98% atom enriched, Aldrich). 1-Fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) was prepared as described in the literature[9].

## Culture Conditions

The myxobacterium *Cystobacter fuscus* AJ-13278 was obtained from Institute of Life Science, Ajinomoto Co., Inc. The culture stock (in 10% glycerol stored at $-80$°C) of *C. fuscus* AJ-13278 was inoculated onto a Cy medium [Casitone (Difco) 0.3%, yeast extract (Difco) 0.1%, $CaCl_2$-$2H_2O$ 0.1%, Bacto agar (Difco) 1.5%] and cultured at 30°C for 2 days. The plate culture was inoculated into a 300-ml Erlenmeyer flask containing 70 ml of a seed medium consisting of Casitone (Difco) 1%, dried baker's yeast 0.5%, malt extract (Difco) 0.2%, yeast extract (Difco) 0.1%, $MgSO_4$-$7H_2O$ 0.1%, $Mg_3(PO_4)_2$-$8H_2O$ 0.3%, HEPES 1%, and Bacto agar (Difco) 0.05% (pH 7.2 before sterilization). The flask was shaken on a rotary shaker (180 rpm) for 3 days at 28°C. Five milliliters of the seed culture were inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the production medium (total 300~500 ml per each feeding), which was the same as the seed medium except that 2% (w/v) adsorbent resin Sepabeads® SP207 (Nippon Rensui) was added to promote the production of cystothiazole A (**1**). The production culture was carried out on a rotary shaker (180 rpm) at 28°C for 4 days. At 24 and 48 hours after inoculation, 1 ml

of a 0.2 M solution of stable-isotope-labeled precursor in water (filter sterilized) was added independently into each 100 ml culture (final concentration was 4 mM after two additions).

Purification of Labeled Cystothiazole A (1)

At the end of cultivation, the production culture (300~500 ml for each labeling) was centrifuged to give the bacterial cells and the adsorbent resin, which were then extracted with acetone (150~300 ml). The acetone extract was concentrated to 20~50 ml of aqueous suspension, which was extracted 3 times with an equal volume of EtOAc. The EtOAc extracts were combined and concentrated, and the residue was chromatographed on silica gel (Fuji Silysia BW-300, 10~20 g) with hexane - EtOAc (15 : 1, 6 : 1). The fractions eluted from hexane - EtOAc (6 : 1) that indicated intense UV (254 nm) absorption were combined, and then separated by HPLC [Shiseido CAPCELLPAK $C_{18}$ UG120 (10 i.d. ×250 mm), 70% acetonitrile, 2.5 ml/minute, detected at 220 nm] to give a labeled derivative of **1**.

Determination of Absolute Configuration of Valine-$d_8$ from the Culture Broth

DL-Valine-$d_8$ was fed to 100 ml of the production culture as described above. After cultivation, 100 ml of the culture was filtered and the filtrate was passed through a ODS column (Nacarai Tesque Cosmosil75 $C_{18}$-OPN, 20 g). The unadsorbed fraction was separated by HPLC [Shiseido CAPCELLPAK $C_{18}$ UG80 (20 i.d. ×250 mm), 1% acetonitrile in 0.1% TFA, 7.0 ml/minute, detected at 210 nm]. A part (approx. 1/4) of the fraction eluted at 8.2~11.0 minutes was further purified by HPLC [Shiseido CAPCELLPAK $C_{18}$ UG80 (20 i.d. ×250 mm), 1% acetonitrile in 0.1% TFA, 8.0 ml/minute, detected at 210 nm] to give the crude fraction (8.5 mg, Rt=12.4~16 minutes) containing valine-$d_8$.

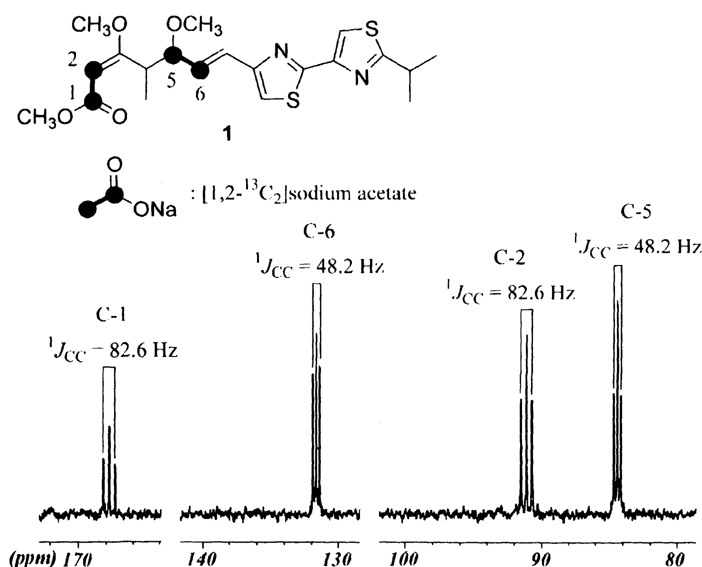
L-FDLA derivatization was performed as described in the literature[9]. A portion (50 $\mu$l) of an aqueous solution of the above valine-$d_8$ fraction (20 mg/ml) or 50 mM DL-valine-$d_8$ (as a standard) was mixed with 20 $\mu$l of 1 M $NaHCO_3$ and 100 $\mu$l of 1% L-FDLA in acetone, and the mixture was incubated for 60 minutes at 37°C. The reaction was quenched by addition of 20 $\mu$l of 1 N HCl and diluted with 810 $\mu$l acetonitrile. A 1-$\mu$l portion of this solution was analyzed by LC/MS. The LC/MS conditions were as follows. A Shiseido CAPCELLPAK $C_{18}$ UG120 column (4.6 i.d. ×150 mm) was used. Aqueous acetonitrile containing 0.1% formic acid was used as a mobile phase (40~70~100% acetonitrile in 0~15~20 minutes linear gradient) at a flow rate of 1.0 ml/minute, the cone voltage and electron multiplier voltage were kept at 30 V and 650 V, respectively, and the ion source at 70°C. A mass range of *m/z* 200~600 was scanned in 1 second.

## Results

The carbon chain of the methyl-branched polyketide portion (C-1 to C-6) of cystothiazole A (**1**) must be derived from acetate-propionate or acetate-methionine precursors through a rather standard polyketide pathway. To elucidate the precursors feeding experiments were performed with stable-isotope labeled compounds. First, a feeding of [2-$^{13}$C]acetate to *C. fuscus* was carried out. The labeled **1** was purified from the cultured cells and an adsorbent resin, which was added to the production culture media, and the $^{13}$C NMR spectra were taken. The $^{13}$C NMR signal intensities were compared with that of natural **1**[2] and are summarized in Table 1. Incorporation of $^{13}$C was found at the C-2 and C-6 position of **1**. In order to obtain more precise information for acetate incorporation, feeding of [1,2-$^{13}C_2$]acetate was next performed in the same way. The doublet signals overlapping with the corresponding singlet, which are due to $^{13}$C-$^{13}$C direct coupling, were observed in the $^{13}$C NMR spectrum as a result of incorporating intact double-labeled acetate molecules into C-1~C-2 and C-5~C-6 (Fig. 2). Subsequently, feeding of [1-$^{13}$C]propionate was carried out and only C-3 was enriched (Table 1). This indicates that C-3, C-4, and 4-Me originated from propionate. Thus, the biosynthetic precursors of the polyketide moiety (C-1 to C-6) were determined to be acetate and propionate.

In most thiazole-containing natural products, the thiazole rings are known to be formed *via* the condensation of an acyl thioester biosynthetic intermediate and L-cysteine with subsequent heterocyclization and dehydrogenation[10]. Since $^{13}$C-labeled cysteines are not readily available commercially, we used L-[1-$^{13}$C]serine, the immediate precursor of L-cysteine in the amino acid biosynthetic pathway, for the feeding experiments. There are examples of $^{13}$C-labeled serine actually being incorporated into thiazole moieties of peptide antibiotics, *e.g.*, thiostrepton and nosiheptide, as in the case of feeding $^{13}$C-labeled cysteine[10,11]. The $^{13}$C NMR spectrum of **1** labeled with L-[1-$^{13}$C]serine clearly showed that the $^{13}$C incorporation occurred at C-7 and C-10 (Table 1). Consequently, the biosynthetic precursor of the bithiazole moiety turned out to be two molecules of L-cysteine converted from

Fig. 2. Partial $^{13}$C NMR spectra (100 MHz, $CDCl_3$) of cystothiazole A (**1**) labeled by [1,2-$^{13}$C]acetate.




L-serine.

Although the methyl group of methoxy groups is well-known to be derived from methionine, the methyl groups of the isopropyl group of **1** might also be derived from methionine. To clarify the precursor of methyl groups in **1** a feeding experiment with L-[*methyl*-$^{13}$C]methionine was carried out. Peak intensities due to the three methoxy carbons in $^{13}$C NMR of **1** indicated a high efficiency of $^{13}$C incorporation, but those due to the isopropyl group did not (Table 1). Thus, all the three methoxy groups of one ester and two ether groups originated from the *S*-methyl group of L-methionine.

The remaining isopropyl moiety of **1** is thought to be the starter unit of the hybrid-type polyketide biosynthetic pathway. The starter unit of polyketide biosynthesis is usually acyl-CoA, *i.e.*, acetyl-CoA or propionyl-CoA, but no incorporation into **1** was observed when acetate or propionate was fed to *C. fuscus*. Branched-chain acyl-CoAs are often found in the biosynthesis of polyketides and fatty acids as biosynthetic starters. Therefore, we anticipated that isobutyryl-CoA was a candidate for the biosynthetic precursor of the isopropyl moiety of **1**. However, since $^{13}$C-labeled isobutyryl-CoA is difficult to purchase, we used a deuterium-labeled valine, a metabolic precursor of isobutyryl-CoA in the amino acid degradation pathway, as a feeding material. In the feeding experiment, DL-valine-$d_8$ was successfully incorporated into **1**. The $^{1}$H NMR of the labeled **1** indicated that the signal intensities of H-14, 14-Me and H-15 drastically decreased to *ca.* 50% of natural **1** (data not shown), indicating the incorporation of deuteriums into these positions. The $^{13}$C NMR signal intensities of C-14, 14-Me, and C-15 also decreased (data not shown). In the FABMS, the molecular ion and fragment ions that included the isopropyl moiety were observed as splitting signals in the ratio of approx. 2 : 3 for $d_7$-labeled and unlabeled **1**, respectively (Figure 3). These results suggested that DL-valine-$d_8$ was incorporated into the isopropyl moiety of **1**. Because the elimination of $\alpha$-deuterium during the conversion of DL-valine-$d_8$ into isobutyryl-$d_7$-CoA should occur, incorporation of the $\alpha$-position of valine could not be confirmed. However, it would not be reasonable that C-13 of **1** corresponding to $\alpha$-carbon of valine is derived from another precursor.

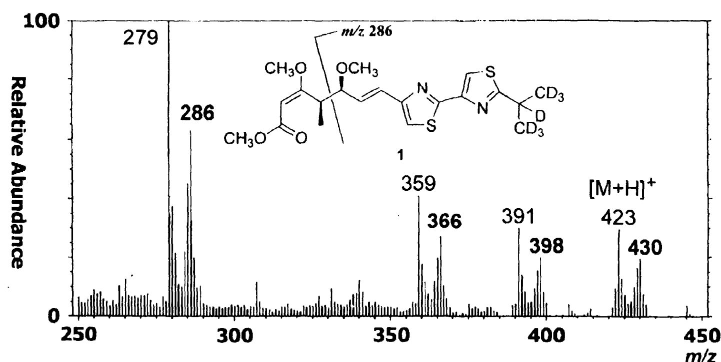
Since we could not conclude that either D- or L-valine-$d_8$ was really incorporated into **1** in the feeding experiment described above, we carried out the next experiment. The unconsumed valine-$d_8$ was recovered from the culture broth and analyzed by "advanced Murfey's method"[9] to determine the absolute configuration of valine-$d_8$ (Figure 4). The mass chromatogram for the recovered valine-$d_8$, which was derivatized with L-FDLA, exhibited a single peak corresponding to the D-valine-$d_8$-L-FDLA standard (Figure 4). This indicated that L-valine was preferentially consumed by *C. fuscus*, and it seemed likely that the isopropyl group of **1** was originated from L-valine.

Table 1. Incorporation of $^{13}$C-labeled precursors into cystothiazole A (**1**).

| Position | $\delta_C$ (ppm) | Relative peak intensities[a] | | | |
|---|---|---|---|---|---|
| | | [2-$^{13}$C]acetate | [1-$^{13}$C]propionate | L-[1-$^{13}$C]serine | L-[*methyl*-$^{13}$C] methionine |
| 1 | 167.7 | 1.2 | 0.9 | 1.0 | 0.9 |
| 1-OMe | 50.8 | 0.9 | 1.0 | 1.5 | **62.1** |
| 2 | 91.1 | **3.3** | 0.9 | 1.0 | 0.6 |
| 3 | 176.7 | 1.1 | **11.1** | 1.0 | 0.9 |
| 3-OMe | 55.5 | 0.9 | 1.0 | 1.5 | **62.8** |
| 4 | 39.8 | 1.3 | 0.9 | 0.9 | 0.8 |
| 4-Me | 14.1 | 1.3 | 1.0 | 1.0 | 1.1 |
| 5 | 84.4 | 1.2 | 1.1 | 1.0 | 1.0 |
| 5-OMe | 57.0 | 0.9 | 1.0 | 1.5 | **59.5** |
| 6 | 131.6 | **3.5** | 0.9 | 0.8 | 1.1 |
| 7 | 125.6 | 0.9 | 1.2 | **15.1** | 1.1 |
| 8 | 154.5 | 0.9 | 1.0 | 0.6 | 1.0 |
| 9 | 114.8 | 1.0 | 1.1 | 0.9 | 1.1 |
| 10 | 162.6 | 1.0 | 1.1 | **14.0** | 1.1 |
| 11 | 148.8 | 1.0 | 1.0 | 0.8 | 1.1 |
| 12 | 114.9 | 0.9 | 1.0 | 0.7 | 1.1 |
| 13 | 178.6 | 0.9 | 1.0 | 0.8 | 0.9 |
| 14 | 33.3 | 0.9 | 1.0 | 1.0 | 1.1 |
| 15.14-Me | 23.1 | 1.0 | 1.0 | 1.0 | 1.1 |

[a] Peak intensities were normalized to the averaged peak intensities of non-labeled carbons (= 1.00).

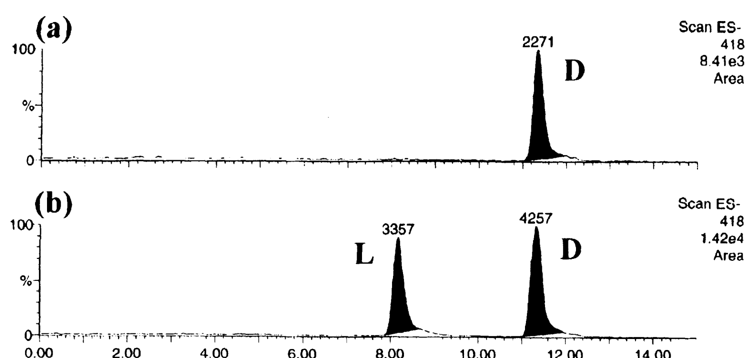
Fig. 3. FABMS (positive) of cystothiazole A (**1**) labeled by DL-valine-$d_8$.




## Discussion

As a result of our feeding experiments, the biosynthetic precursors of cystothiazole A (**1**) are identical with expectation in Figure 1. The polyketide moiety is derived from two acetates and one propionate, and the *O*-methyl carbons are derived from the *S*-methyl carbon of L-methionine. The methyl-branched aliphatic structure would be biosynthesized by type-I PKS[12)], which is commonly found in the biosynthesis of macrolide-type microbial secondary metabolites. The bithiazole and tail-end isopropyl moieties probably originate from L-cysteine and isobutyryl-CoA, respectively, which would be the subsequent metabolites of L-serine and L-valine. The

Fig. 4. LC/MS chromatograms (negative) for valine-$d_8$-L-FDLA derivatives (*m/z* 418).




(a) Valine-$d_8$-L-FDLA derived from the culture broth fed DL-valine-$d_8$. (b) Standard: DL-valine-$d_8$-L-FDLA.

mechanism of the thiazole ring formation by NRPS has been reported for the myxobacterial secondary metabolites, epothilones[13,14] and myxothiazol A[8], the biosynthetic gene clusters of which have been identified. Based on our feeding experiments and the reported biosynthetic genes of myxothiazol A[8], a biosynthetic scheme of **1**, could be drawn as follows: First, isobutyryl-CoA as a specific substrate for the biosynthesis of the cystothiazoles is loaded on NRPS. Two units of cysteine are then condensed, cyclized, and dehydrogenated to form the bithiazole structure. The resulting bithiazole intermediate is transferred to PKS, and followed by sequential condensations of acetate, propionate, and acetate. Modifications such as *O*-methylation and reduction concomitantly occur through the activities of the methyltransferase (MT), $\beta$-ketoacyl reductase (KR), and $\beta$-hydroxyacyl dehydratase (DH) domains of PKS. Finally, the acyl-PKS intermediate is cleaved from PKS by the thioesterase (TE) domain. The mechanism of methyl ester formation is unclear as yet. Polyketides biosynthesized by type-I PKS or PKS/NRPS often form a macrolide structure, or otherwise remain a carboxylic acid after cleavage from PKS. In cystothiazole biosynthesis, however, the final step is probably *O*-methyl transfer to the carboxyl intermediate to form the methyl ester by the MT domain of PKS or another cytoplasmic methyltransferase.

In the present study we outlined the biosynthetic pathway of **1** through feeding experiments. Since it is difficult to elucidate the details of the biosynthetic pathway with feeding experiments alone, investigations in conjunction with other knowledge such as the genome sequence for the biosynthetic gene cluster of **1** need to be addressed. Such the information about biosynthetic genes of bioactive secondary metabolites leads the way to combinatorial biosynthesis, which enables to create structural diversity of secondary metabolites.

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