Biosynthesis of Depudecin, a Metabolite of *Nimbya scirpicola*

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Feeding experiments of labeled acetates to *Nimbya scirpicola* proved the carbon origin of the straight-chain polyketide, deputecin. Differential hydrogen exchange of the 2H label originating from 3H labeled acetate along the polyketide chain occurred. In particular, the deuterium of an epoxide methine at C-3 was lost to a substantial extent in the formation of deputecin.

**Key words:** phytotoxin; *Nimbya scirpicola*; deputecin; biosynthesis; polyketide

Depudecin (I) was first isolated from a culture filtrate of the soil fungus, *Alternaria brassicicola*, by Matsumoto et al., in 1992 and shown to be a metabolite which reverts the rounding phenotype of NIH3T3 fibroblasts transformed with v-ras and v-src oncogenes to the flattened phenotype of the non-transformed parental cells.1,2 We have subsequently encountered the same metabolite during our screening of microbial fermentations for phytotoxins.3 The producing fungus was the phytopathogenic fungus, *Nimbya scirpicola*, which had been isolated from the paddy field weed, *Eleocharis kuroguwai*.4

The deputecin structure includes two hydroxyl groups and two epoxides, and all carbons bear hydrogens. Such a structure may have an advantage in studying the origin of the oxygens and hydrogens in polyketide biosynthesis. In fatty acid biosynthesis, differential hydrogen exchange during the fatty acid synthetase reaction of baker’s yeast and *Brevibacterium ammoniagenes* has been studied by Saito et al.5 They reported that differential hydrogen exchange occurred in the enzymatic fatty acid elongation process, and that the extent of the exchange was directly proportional to the distance from the biosynthetic starter unit. Differential hydrogen exchange has also been reported in the biosynthesis of polyketides, e.g., cytochalasins,6 scytalone,7 and rugulosin.8 But these only showed some of the positions originating from the methyls of deuterated acetate that lost deuterium.

Our present interest in the biosynthesis of deputecin is to demonstrate the hexaketide origin of carbons, and to define whether the hydrogens at C-1, C-3, C-5, C-7, C-9 and C-11 originating from methyl carbons were retained or lost in a proportion correlated to the position in a presumed polyketide intermediate by using variously 1H- and 13C-labeled acetates ([1,-13]C, [2,-13]C, [1, 2, 13C2], [2-H2], and [2-2H, 2-13C]-acetates).

**Materials and Methods**

*General.* Stable isotopes were purchased from Aldrich Chemical Company (Table 1), yeast extracts and the Czapek-Dox medium from Difco, and methanol from Wako Pure Chemical Industries. TLC was performed on aluminium sheets pre-coated with a 60 F254 silica gel layer at a thickness of 0.2 mm (Merck), and column chromatography was carried out with a Diaion HP-20 instrument (Nippon Rensui Co.). Fermentation was carried out in a 500-ml Erlemeyer flask with Fiberm (Tokyo Rikakikai Co.) cellulose forming the immobilized carrier. NMR spectra were measured with a Bruker DAX-500 instrument. Chemical shift values are expressed as δ values, and J values in Hz, with tetramethylsilane (TMS), CDCl3, and CD2CN as internal standards.

*Culture.* Spores of *Nimbya scirpicola* frozen at −85°C were applied to a V8 juice agar plate and grown under a 12 hr light and 12 hr dark schedule for 5 days. Leaving the spores on the agar for more than 10 days or subculturing of the organism resulted in loss of the ability to produce deputecin.

*Fermentation.* A piece of agar bearing the spore-formed culture (1 cm3) was used to inoculate a 500-ml Erlemeyer flask containing the Czapek-Dox medium (100 ml) fortified with 0.1% yeast extract and an immobilized carrier (20 ml). The flask was shaken at 23°C on a rotary shaker (100 rpm). Four

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days after inoculation, sodium $^{13}$C- and/or $^2$H-labeled acetates (1.0 mmol) were each added by injecting a sterile solution. After a further 4 days of culture, the culture broth from each feeding experiment was filtered, and the filtrate was chromatographed stepwise on Diaion HP-20 (2 x 12 cm column) with water, 20%, and 50% methanol. The 50% methanol eluate, which contained pure depudecin, was used for an NMR spectral analysis after evaporation. The effect of sodium acetate on the fermentation of N. scirpicola was determined by the weight of obtained depudecin. At a concentration of more than 2.0 mmol, the production of depudecin was significantly retarded, but at less than 1.0 mmol, no influence was apparent. The labeled compounds and the results of the feeding experiments are listed in Table 1.

**Preparation of depudecin diacetate.** Depudecin (2.0 mg) in 1.0 ml of dry pyridine was treated with 0.5 ml of Ac$_2$O and kept overnight at room temperature. The mixture was then poured into 3.0 ml of distilled water and extracted with 4.0 ml of ethyl acetate. The ethyl acetate layer was washed twice with a CuSO$_4$ solution to give the crude diacetate (3.0 mg). This crude diacetate was treated by preparative TLC (silica gel) with hexane-ethyl acetate (1:1) to give the depudecin diacetate (2.0 mg). $^1$H-NMR $\delta$ (500 MHz, in CDCl$_3$): 1.29 (3H, d, $J=6.5$ Hz, H-1), 2.07 (3H, s, CH$_3$CO), 2.12 (3H, s, CH$_3$CO), 3.00 (1H, dd, $J=2.1$, 5.7 Hz, H-3), 3.07 (1H, dd, $J=2.1$, 5.9 Hz, H-8), 3.28 (1H, m, H-4), 3.32 (1H, m, H-7), 4.83 (1H, quintet, $J=6.5$ Hz, H-2), 5.17 (1H, br. t, $J=5.9$ Hz, H-9), 5.31 (1H, br. d, $J=10.6$ Hz, H-1 cis), 5.37 (1H, br. d, $J=17.4$ Hz, H-11 trans), 5.69 (2H, m, H-5 and 6), 5.82 (1H, ddd, $J=6.0$, 10.6, 17.3 Hz, H-10).

**Results and Discussion**

N. scirpicola was grown by liquid shake culture on a modified Czapek-Dox medium and was supplemented once with an injection of a sterile solution of sodium $^{13}$C- and/or $^2$H-labeled acetates. Chromatography of the culture filtrate gave labeled depudecins. Each flask containing 100 ml of the medium yielded depudecin in the range of 2.9 to 9.0 mg (Table 1). The value varied so much mainly due to the instability of depudecin production by the fungi.

The proton-noise decoupled (PND) $^{13}$C-NMR spectrum of the [1-$^{13}$C]acetate-derived depudecin showed five enhanced signals, attributable to C-2, -4, -6, -8, and -10, whereas the spectrum of depudecin derived from the [2-$^{13}$C]acetate showed enhanced signals for C-1, -3, -5, -7, -9, and -11 (Table 2). Satisfactory specific incorporation of $^{13}$C was generally obtained for both the [1-$^{13}$C] and [2-$^{13}$C]acetate-derived carbon atoms ranging from 17.8 to 25.6 and from 9.5 to 19.5 atom% present, respectively. The specific incorporation was calculated on the basis of the relative intensity to the acetate methyl carbon referred to as 1.1% of the synthesized depudecin diacetate.

The arrangement of intact acetate units in depudecin was studied by adding [1, 2-$^{13}$C]acetate to cultures of N. scirpicola. In the PND $^{13}$C-NMR spectrum of depudecin derived from the [1, 2-$^{13}$C]
Table 3. Incorporation of Sodium [2-2H] and [2-13C, 2-2H] Acetate into Depudecin (1H-NMR, 77 MHz; PND 13C-NMR, 125 MHz)

<table>
<thead>
<tr>
<th>2H C</th>
<th>3H-NMR*</th>
<th>13C-NMR*</th>
<th>isotope shift/ ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>relative intensity</td>
<td>1J(2-13C) / Hz (multiplicity)</td>
<td>protonated 13C/C</td>
</tr>
<tr>
<td>1</td>
<td>3.70</td>
<td>19.0(t, quin., hept.)</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>(s)</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>0.16</td>
<td>26.4(i)</td>
<td>17.3</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>(s)</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>0.76</td>
<td>24.1(t)</td>
<td>3.5</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>(s)</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>0.94</td>
<td>27.0(i)</td>
<td>2.4</td>
</tr>
<tr>
<td>8</td>
<td>1.00</td>
<td>22.0(i)</td>
<td>2.2</td>
</tr>
<tr>
<td>9</td>
<td>—</td>
<td>(s)</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>0.61</td>
<td>24.3(i)</td>
<td>1.03</td>
</tr>
<tr>
<td>11cis</td>
<td>0.90</td>
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</tr>
<tr>
<td>11trans</td>
<td>0.61</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* For CH3CN of depudecin: contained from a feeding experiment with sodium [2-2H] acetate. Internally referenced to 1H-9 as 1.00.

+ In CD3CN of depudecin: contained from a feeding experiment with sodium [2-13C, 2-2H] acetate.

- Relative peak intensity; integration was measured in the inverse gate proton decoupling spectrum.

0.3-0.7 ppm upfield /1H substitution.

Not detected.

No isolate shifted signals.

Deuterated signals of 11-cis and 11-trans were combined for integration.

acetate, intense 13C-13C coupling signals were observed between C-1 and C-2, between C-3 and C-4, between C-5 and C-6, between C-7 and C-8, and between C-9 and C-10, whereas C-11 was observed as an intense singlet signal, indicating the presence of five intact acetate units, viz., C-1-C-2, C-3-C-4, C-5-C-6, C-7-C-8, and C-9-C-10. These labeling patterns established that the depudecin skeleton was formed by the polyketide pathway and that C-1-C-2 is the starter unit.

The presence of an acetate starter unit in depudecin was unambiguously demonstrated by feeding experiments with [2-13C, 2-2H] acetate. The 13C signals of the 13C-2H species appeared as multiplets shifted ca. 0.3 ppm higher field (isotope effect) than the 13C signal due to the corresponding 13C-1H species in the PND 13C-NMR spectra. A heptet (J = 19.0 Hz) at 18.70 ppm, 0.83 ppm upfield of the normal chemical shift value for C-1, was assigned to a tri-deuterated methyl. These results confirm that C-1 and C-2 came from acetyl CoA. The observed quintet (J = 19.0 Hz) at 18.98 ppm and a triplet (J = 19.0 Hz) at 19.25 ppm correspond to di- and mono-deuterated methyls, respectively. The loss of deuterium atoms and an enriched CH3 singlet suggest considerable exchange of hydrogen from the methyl group in the biosynthesis of depudecin. The other 13C signals with isotope signals were assignable to C-3, C-5, C-7, C-9, and C-11, which were originally from the methyl group of acetates. The intensity of the 2H isotope signals was measured by integrating the 13C-NMR spectrum of the inverse gate proton decoupling method and the ratio of isotopically shifted signals to the singlet which is enriched with 13C, but does not carry deuterium atoms (Table 3).

The signals from C-11 were only two isotopically shifted triplets at 115.83 and 115.88 ppm. No observation of the signal due to the di-deuterated methylene in C-11 may be explained by the possibility that deuterium atoms were exchanged with protons in this position at a higher level, or alternatively that another mechanism participated in biosynthesis other than that based on decarboxylative condensation between polyacetoacetyl CoA and malonyl CoA, with subsequent decarboxylation of the end carbonyl group.

The diverse ratios of protonated carbons to deuterated carbons of 0.5 to 17.3 at the odd-numbered carbons (Table 3) indicates that the loss of the 2H-label varied to an extent along the chain. However, the extent of the exchange did not depend on the distance from the starter unit. This does not agree with the fatty acid biosynthesis elongation process. The rule for hydrogen exchange in the biosynthetic process of depudecin is not clear, but it is probably a fact that hydrogen exchange substantially occurred at the C-3 position (protonated 13C/deuterated 13C = 17.3, Table 3) when compared to the results of the other positions (protonated 13C/deu-
tered $^{13}\text{C} = 0.5-3.5$). These results, together with the specific loss of the $^3\text{H}$ atoms at C-3 (0.16 relative to $^3\text{H}-9$ as 1.00) in incorporating the [2-$^3\text{H}_2$] acetate (Table 3), may mean that the biosynthesis of C-3 involved either comparatively more hydrogen elimination than the biosynthesis of other carbons, or a stereospecific hydrogen elimination process. Although various explanations may be given to rationalize the specific loss of the $^3\text{H}$ atom from C-3, evidence for it has not yet been provided.

No deuterium atom was observed in the carbons from the carbonyl group of the acetate, indicating either no migration of deuterium from the methyl carbons to carbonyl carbons in the presumed polyketide, or that an exchange with the medium must precede such migration.

In an upcoming report, we will address the question of which of the four oxygen atoms was originally from the oxygen of acetic acid or from another source.

References


