Pironetin, a Novel Plant Growth Regulator Produced by *Streptomyces* sp. NK10958

III. Biosynthesis

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In our screening of microbial secondary metabolites for plant growth regulators, we found a new compound from the culture broth of *Streptomyces* sp. NK10958 and named it pironetin[1]. The structure of pironetin was determined to be (5*R*,6*R*)-5-ethyl-5,6-dihydro-6-((*E*)-(2*R*,3*S*,4*R*,5*S*)-2-hydroxy-4-methoxy-3,5-dimethyl-7-nonenyl)-2H-pyrane-2-one[2]. In this report, we describe incorporation experiments with ^{13}C-labeled precursors and the biosynthetic origin of the carbon atoms in pironetin. The usefulness of pironetin in agriculture will be reported separately[3].

Materials and Methods

Labeled Compounds

Sodium [1-^{13}C]acetate (99% ^{13}C enriched), sodium [2-^{13}C]acetate (99%), sodium [1,2-$^{13}C_2$]acetate (99%), sodium [1-^{13}C]propionate (99%), sodium [1-^{13}C]butyrate (99%) and L-[*methyl*-^{13}C]methionine (99%) were purchased from Sigma Chemical Co., U.S.A.

Fermentation

A loopfull spores of the strain *Streptomyces* sp. NK10958 was inoculated into 100 ml of a seed medium consisted of glycerin 2%, soy bean meal 2% and NaCl 0.3% (pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask, and cultured at 27°C for 2 days on a rotary shaker (220 rpm). One milliliter of this seed culture was transferred to 100 ml of the production medium consisted of glycerin 4%, soy bean meal 2% and NaCl 0.3% (pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask and cultured at 27°C on a rotary shaker (220 rpm). One milliliter of each ^{13}C-labeled compound solution at a concentration of 30 mg/ml in water was added into a flask on 48 hours after inoculation. The fermentation was continued 48 hours after the feed of ^{13}C-labeled compound.

Isolation

Each fermentation broth (100 ml × 10 flasks) was combined and extracted with 1.0 liter of EtOH. The residual mycelia were filtered and the filtrate was concentrated *in vacuo* to an aqueous solutions. The aqueous solution was extracted three times with 500 ml of ethyl acetate. After washing with 200 ml of saturated NaCl solution, the extract was concentrated to dryness *in vacuo*. The extract was chromatographed on a silica gel (Merck, type 60) and eluted with the mixture of *n*-hexane and acetone (10 : 1). The pironetin containing fractions were collected and concentrated *in vacuo*. The residue was re-chromatographed on a silica gel (Fuji-Davison, Japan, BW-700) and eluted with the mixture of *n*-hexane and acetone (10 : 1). The pironetin containing fractions were collected and concentrated *in vacuo* to give colorless crystals of pironetin.

NMR

^{13}C NMR spectra were recorded on a Bruker AC 300 plus spectrometer. Each ^{13}C-enriched pironetin was dissolved in $CDCl_3$ at the concentration of about 20 mg in a NMR tube. The increment of signal intensities caused by ^{13}C-enrichment were determined from each signal intensity of ^{13}C-enriched pironetins by comparison with the signal intensity of natural pironetin.

Results and Discussion

Relative enrichments and ^{13}C-^{13}C coupling constants based on the ^{13}C NMR spectra of pironetin derived from ^{13}C-labeled precursors are listed in Table 1. Relative enrichments were normalized to peak intensities for the C-19 signals on [1-^{13}C]acetate, [2-^{13}C]acetate, [1-^{13}C]propionate and [1-^{13}C]butyrate-labeled pironetins and for the C-7 signal on L-[*methyl*-^{13}C]methionine-labeled pironetin. In the ^{13}C NMR spectrum of [1-^{13}C]acetate-labeled pironetin, high level of enrichments were observed for C-1, C-3, C-5, C-11, C-13 and C-15. These enrichements correspond to high level of enrichment for C-2, C-4, C-6, C-12, C-14 and C-16 in the ^{13}C NMR spectrum of [2-^{13}C]acetate-labeled pironetin respectively. The ^{13}C-^{13}C coupling of intact doubly-labeled acetate units were observed at these positions. These results indicate the incorporation of six acetates into pironetin.

Low level of enrichements were observed for C-7 and C-9 in [1-^{13}C]acetate-labeled pironetin, and C-7, C-8, C-9, C-10, C-17 and C-18 in [2-^{13}C]acetate-labeled pironetin. The ^{13}C-^{13}C coupling of intact doubly-labeled acetate units could not be observed for these positions. These results suggest that the metabolism of [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]acetates to propionates in TCA cycle or glyoxylate cycle occurred in the fermentations before incorporation to pironetin.

In the ^{13}C NMR spectrum of [1-^{13}C]propionate-labeled pironetin, high level of enrichements were observed for C-7 and C-9. The incorporation experiment of L-[*methyl*-^{13}C]methionine showed the methyl carbons C-17 and C-18 are not derived from methionine. These results indicate that two propionates were incorporated

Table 1. Incorporation of ^{13}C-labeled precursors into pironetin.

Carbon		Relative enrichment[a]					Jcc (Hz)
NO	δ (ppm)	[1-^{13}C]-CH_3COONa	[2-^{13}C]-CH_3COONa	[1-^{13}C]-CH_3CH_2COONa	[1-^{13}C]-$CH_3CH_2CH_2COONa$	[Me-^{13}C]-Methionine	[1,2-$^{13}C_2$]-CH_3COONa
1	164.6	3.5**	1.3	1.6	2.4*	1.3	66
2	120.8	1.0	2.7**	1.3	0.5[b]	1.1	66
3	150.6	2.1**	1.2	1.2	25.5**	1.1	38
4	39.1	1.0	2.3**	1.0	0.7[b]	1.1	38
5	77.8	2.7**	1.2	1.2	0.9	1.1	43
6	36.7	1.0	2.6**	0.8[b]	1.0	1.0	43
7	67.4	1.5*	1.8*	23.4**	2.7*	1.0	
8	39.0	1.0	2.0*	0.7[b]	1.0	0.8	
9	91.0	1.5*	1.9*	20.9**	3.2*	0.8	
10	36.1	0.9	1.8*	0.7[b]	1.0	0.8	
11	37.2	2.5**	1.1	1.1	1.5*	1.0	44
12	128.8	1.0	2.7**	1.0	1.3	1.1	44
13	126.8	2.9**	1.2	1.2	1.9*	1.1	43
14	17.9	0.9	2.3**	1.0	1.0	0.9	43
15	20.7	2.1**	1.2	1.2	1.1	1.1	35
16	10.9	1.0	2.4**	1.2	0.8	1.1	35
17	12.1	1.0	2.1*	1.1	0.7	1.0	
18	15.2	1.0	2.0*	1.0	0.7	1.0	
19	61.5	1.0	1.0	1.0	1.0	61.7**	

* Low level of enrichment was observed.

** High level of enrichment was observed.

[a] Relative enrichments were normalized to peak intensities for the C-19 signals on sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, [1-^{13}C]propionate and sodium [1-^{13}C]butyrate-labeled pironetins and for the C-7 signal on L-[*methyl*-^{13}C]methionine-labeled pironetin.

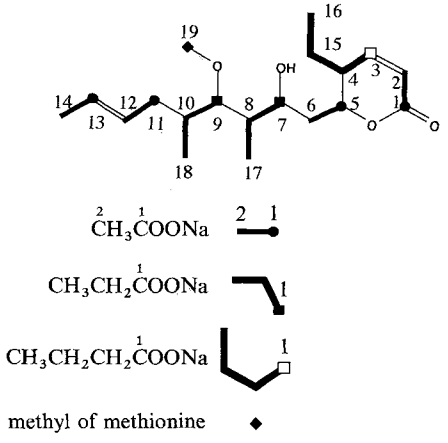
[b] Due to the ^{13}C-^{13}C coupling, the correct values could not be obtained.

directly to pironetin. The incorporation experiment of L-[*methyl*-^{13}C]methionine also showed clearly the methoxyl carbon (C-19) are derived from methyl carbon of methionine. The results mentioned above suggest that two propionate units and one methyl unit of methionine are incorporated into pironetin.

ŌMURA *et al.*[4,5] reported that the C-ethyl group of the aglycon of the 16-membered macrolide antibiotics Leucomycin A_3 and Tylosin is not directly derived from acetate but it is derived from butyrate in their experiments of incorporation of ^{13}C-labeled precursors. SETO *et al.*[6] also reported that the C-ethyl group is derived from butyric acid using the incorporation experiment of [1-^{13}C]-butyrate in their carbon assignment work of a polyether antibiotic Lasalocid. In our experiments the relative enrichements of C-3 and C-4 were weaker than those of C-1 and C-2 or C-5 and C-6 in the [1-^{13}C]- and [2-^{13}C]-acetates incorporation experiments respectively. C-15 and C-16 also showed weaker relative enrichements. This suggested that the acetate units of C-3 to C-4 and C-15 to C-16 are not directly derived from acetate but derived from butyrate.

In order to confirm this hypothesis, the incorporation experiment of sodium [1-^{13}C]-butyrate was investigated. Relative enrichments of pironetin derived from sodium [1-^{13}C]-butyrate are also shown in Table 1. The strong enrichment peak was observed for C-3 as expected. This confirmed that the four carbons (C-3, C-4, C-15 and C-16) of pironetin are derived from butyrate directly. In [1-^{13}C]-butyrate incorporation experiment weak enrichments were observed for C-7 and C-9 and very weak enrichements were observed for C-1, C-11 and C-13. This suggested that butyrate is metabolized to propionate and acetate. Butyrate and propionate are incorporated directly to pironetin in the biosynthesis. Considering our ^{13}C-labeled acetates, propionate and butyrate incorporation experiments, we concluded that pironetin is derived from four acetate units, two propionate units, one butyrate unit and one methyl unit of methionine. The origin of the all carbon atoms of pironetin are summarized in Fig. 1.

Fig. 1. Incorporation of ^{13}C-labeled precursors into pironetin.



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