Bioorganic & Medicinal Chemistry Letters 19 (2009) 1122–1125

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Biosynthetic studies of spiroleptosphol

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ARTICLE INFO

Article history:
Received 3 November 2008
Revised 5 December 2008
Accepted 26 December 2008
Available online 1 January 2009

Keywords:
Biosynthesis
Labeling experiments
Spiroleptosphol
TCA cycle

ABSTRACT

Studies of the biosynthesis of spiroleptosphol (**1**) revealed that **1** comprised a heptaketide (C1, C5–C10, and C12–C18 moiety), two methyl carbons (C19 and C20) from methionine, and a C_3 unit (C3, C4, and C11 moiety) derived through the TCA cycle.

We recently isolated spiroleptosphol[1] (**1**, Fig. 1) as a cytotoxic substance from the saprophytic ascomycete *Leptosphaeria doliolum* collected from mugwort stems.[2] Our structural studies revealed that **1** is a novel member of the 3-methylidene-2-oxaspiro[4.5]decan-1-one family containing oxaspirol,[3] arthropsolide A,[4] oxaspirodion,[5] paecilospirone,[6] massarigenin A,[7] mycosporulone,[8] 6-epi-5′-hydroxymycosporulone,[9] and rosigenin.[10] These exhibited various biological activities. The stereochemistries of the 3-methylidene-2-oxaspiro[4.5]decan-1-one unit in these compounds are quite diverse.

Camarda et al. proposed the biosynthesis of rosigenin that involves rearrangement of a hydrated isocoumarin derivative,[10] but their mechanism was based only on its structure and not experimental evidence. Ayer et al. discussed the biosynthesis of arthropsadiol based on ^{13}C labeling patterns of the related compounds[11], however, they failed to obtain ^{13}C-labeled arthropsolide A even though it was the main metabolite in regular cultivation. These histories and the unique framework prompted us to study the biosynthesis of the 3-methylidene-2-oxaspiro[4.5]decan-1-one unit employing *L. doliolum*. We found that *L. doliolum* effectively incorporated isotope-labeled acetic acids, methionine, and succinic acid into **1**, which enabled analyses of the labeling pattern to lead the outline of the biosynthetic pathway.

After *L. doliolum* was cultured at 25 °C in regular potato-sucrose medium (1.0 L) on a rotary shaker (110 rpm) for 5 days, ^{13}C-labeled sodium acetate, methionine, sodium propionate, or citric acid (each 30 mg), were added separately. The resulting culture medium was further cultured under identical conditions for 3 days, and the following established chromatographic purification yielded labeled **1** (*ca* 30 mg).

Specific incorporations of ^{13}C singly labeled acetic acid into **1** were first investigated by signal enhancements in ^{1}H-decoupled ^{13}C NMR spectra.[12,13] (Fig. 2). When sodium [1-^{13}C]acetate was added, increments of seven ^{13}C resonances, C1, C6, C8, C10, C13, C15, and C17 were observed at a similar level (0.9–1.3% incorporation) whereas these carbon atoms were not labeled by sodium [2-^{13}C]acetate. An experiment using sodium [2-^{13}C]acetate complementarily enriched ^{13}C ratio at seven carbons (C5, C7, C9, C12, C14, C16, and C18) at a similar level (1.1–1.6% incorporation). We assumed a heptaketide **2** as the biosynthetic intermediate (Scheme 1) because carbons labeled by sodium [1-^{13}C] acetate and sodium [2-^{13}C]acetate were alternately arranged.

Although incorporation levels were lower, C3 and C11 signals were considerably enhanced by sodium [2-^{13}C]acetate (0.7% and 0.3%, respectively). Due to very low increment in the ^{13}C NMR spectra, it was difficult to discuss ^{13}C incorporation for C4 and C20 in

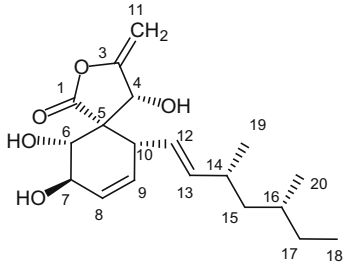

Figure 1. Structure of spiroleptosphol (**1**).

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doi:10.1016/j.bmcl.2008.12.099

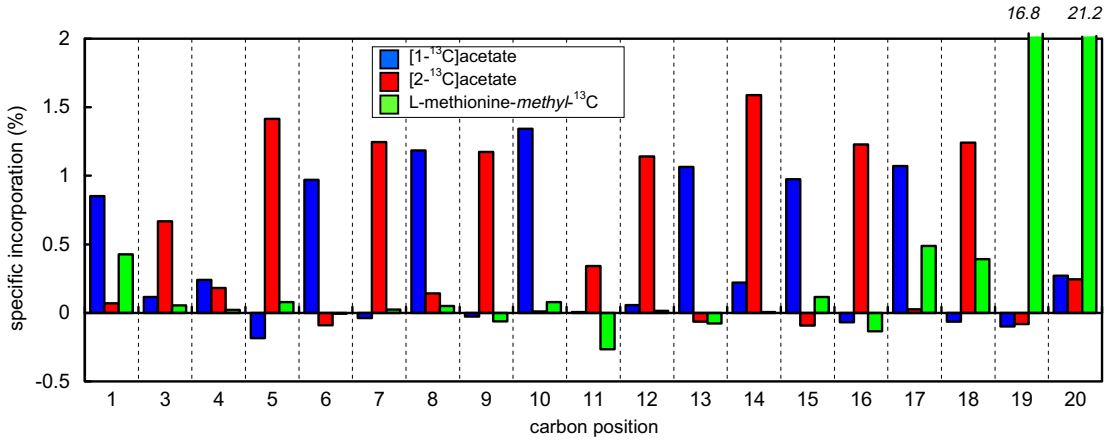
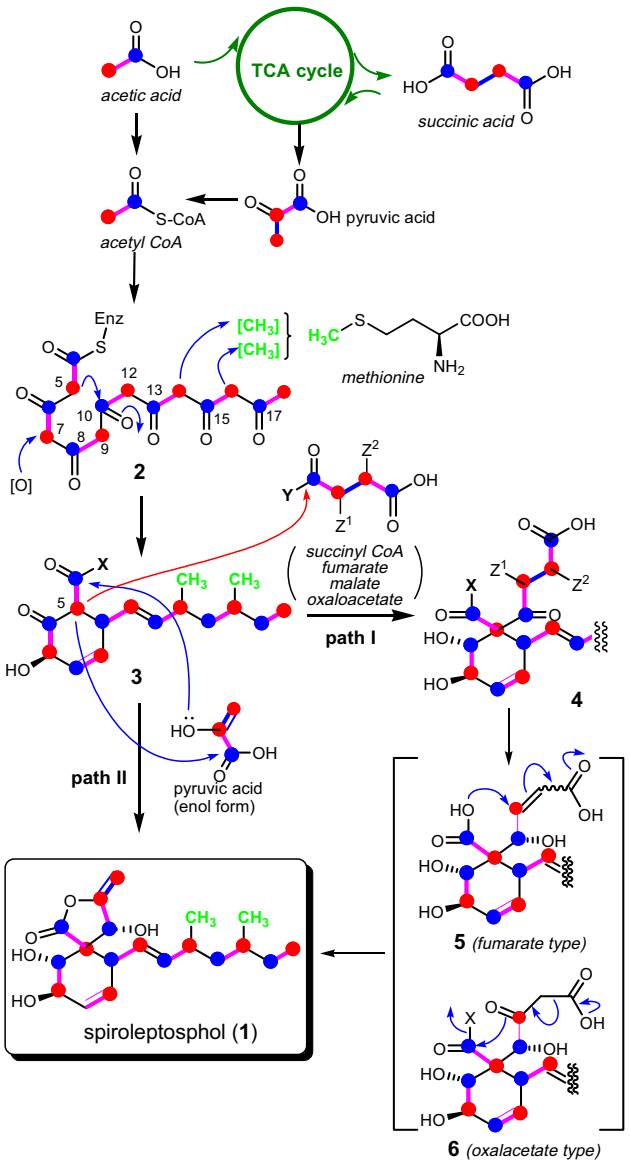


Figure 2. Specific incorporation of ^{13}C isotopes by preparing **1** with sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, and L-methionine-*methyl*-^{13}C $\text{specificincorporation}(\%) = \left(\frac{\text{intensity}_{\text{labeled}}}{\text{intensity}_{\text{natural}}} - 1\right) \times 1.1$.



Scheme 1. Proposed biosynthetic pathway.

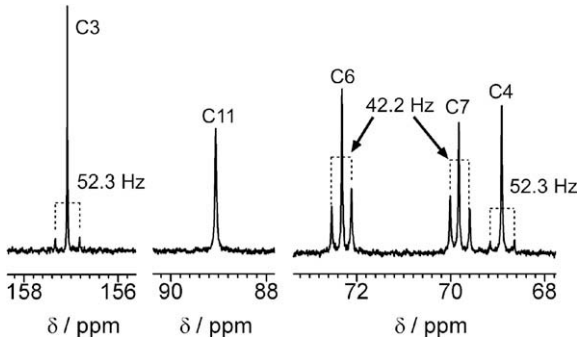


Figure 3. Some signals of the ^{13}C NMR spectrum of 1 prepared with sodium [1,2-$^{13}C_2$]acetate.

experiments with sodium [1-^{13}C]acetate or sodium [2-^{13}C]acetate. At this stage, we could not judge whether these were considerable enhancements or something else caused by experimental errors. Neither of the labeled acetates was incorporated into C19.

The branched side chain implied that propionic acid was the biosynthetic precursor for the C13C14C19 and C15C16C20 units, but ^{13}C-labeled propionate was not incorporated at all. It was found that L-methionine-*methyl*-^{13}C remarkably enhanced the C19 and C20 methyl carbon signals in the ^{13}C NMR spectrum. Incorporation levels for these carbons reached to 17% and 21%, respectively. These data suggested that the C19 and C20 methyl groups were introduced by methylations with methionines.

Feeding experiments with [1,2-$^{13}C_2$]acetate were next performed in order to confirm the distribution of the acetate units.[14] Doubly labeled acetate was successfully introduced as expected to provide labeled **1** in a similar yield as above.

Incorporation of a [1,2-$^{13}C_2$]acetate unit introduced ^{13}C into the two neighboring carbons simultaneously to result flanking satellite signals in the proton decoupled ^{13}C NMR spectrum. For example, the coupling constants of C6 and C7 signals for the labeled isotopomer were both 42.2 Hz (Fig. 3), indicating that the C6 and C7 carbons were derived from the same acetic acid. Further analysis revealed reasonable distribution of all acetate units (Table 1). Signals for C11, C19, and C20 were observed as singlets in this experiment. Notably, signals due to C3 and C4 carbons carried small but distinct satellite peaks. The coupling constants for these satellites were both 52.3 Hz. These results suggested that the acetate unit

Table 1
Coupling constants of **1** labeled with sodium [1,2-^{13}C]acetate

Position	$^{1}J_{CC}$ (Hz)	Position	$^{1}J_{CC}$ (Hz)
C1–C5	51.7	C13–C14	42.4
C6–C7	42.2	C15–C16	35.2
C8–C9	68.0	C17–C18	34.9
C10–C12	42.8	C3–C4	52.3 (low incorporation)

was also introduced into the C3C4. Lower incorporation level for this acetate unit than for other acetate units suggested that the ingredient acetate was utilized indirectly by C3C4 moiety.

Incorporation of TCA metabolites was examined next, because the TCA cycle is responsible for the transformation and regeneration of acetic acid.[15] We presumed that the acetate unit was introduced into the C3C4 positions through the TCA cycle by taking account of relatively lower incorporation than those for other acetate units. Although the TCA cycle derives succinic acid, succinyl-CoA, fumaric acid, malic acid, and oxalacetic acid, incorporation of only [1,2,3,4-$^{13}C_4$]succinic acid was examined due to economic factors. It may not be possible to disclose the direct precursor from them by feeding experiments because of their quick interconversion in the TCA cycle.

Labeled succinic acid was successfully incorporated into **1** to show a characteristic C3 resonance in the proton-decoupled ^{13}C NMR spectrum (Fig. 4). This resonance comprised four types of signals: (i) a central singlet due to the naturally abundant isotopomer; (ii) a doubled-doublet (J = 52.3 and 88.3 Hz) due to the isotopomer in which all of C3, C4, and C11 were simultaneously labeled (**isotopomer A**); (iii) a doublet (J = 52.3 Hz) due to the isotopomer in which C3 and C4 were simultaneously labeled, but C11 was not (**isotopomer B**); and (iv) another doublet (J = 88.3 Hz) due to the isotopomer in which C3 and C11 were simultaneously labeled but C4 was not (**isotopomer C**). Obvious isotope shift ($\Delta\delta$ = 0.025 ppm, 2.5 Hz in the 100 MHz spectrometer) was also observed for **isotopomer A**.[16] Our previous feeding experiment with singly labeled sodium acetate have yielded **isotopomer B,** but **isotopomers A** and **C** were newly discovered in this experiment. **Isotopmer A** utilized three carbons of the ^{13}C-labeled

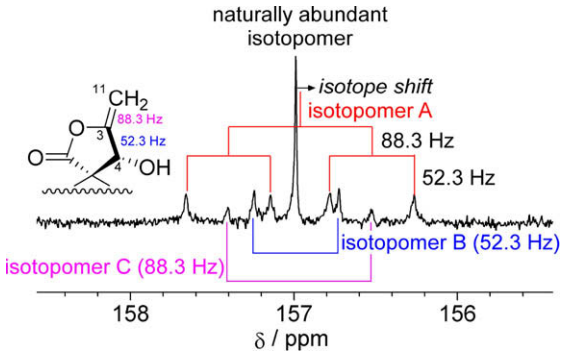


Figure 4. The ^{13}C NMR signal for C3 of the labeled 1 prepared with [1,2,3,4-$^{13}C_4$]succinic acid.

succinic acid, which provide evidence that one of the C4 TCA products (succinyl CoA, succinic acid, fumaric acid, malic acid, or oxalacetic acid) was responsible for the biosynthesis of C11C3C4. Decarboxylation must be involved in this process.

Formation of **isotopomers B** and **C** could be explained by the function of the TCA cycle. Once [1,2,3,4-$^{13}C_4$]succinic acid is introduced in the TCA cycle, it readily combines with *de novo* acetic acid to produce partially labeled citrate[15] (Fig. 5). This labeled citrate is then converted into succinic acid again by the TCA cycle. This process afforded the two isotopomers [1,2-$^{13}C_2$]succinic acid and [1,2,3-$^{13}C_3$]succinic acid in similar amounts because citric acid has a symmetric structure. These succinic acid isotopomers lead **isotopomer B** and **isotopomer C**, respectively .

We would propose pyruvic acid (or its equivalent) as the other candidate precursor because the TCA cycle readily transforms succinic acid into pyruvic acid (or phosphoenolpyruvic acid) via oxaloacetate.[15] The 1,2,3,4-$^{13}C_4$-labeled succinic acid was biologically converted to [1,2,3-$^{13}C_3$]pyruvate which would result **isotopomer A**. Decarboxylation at the C1 position of [1,2,3-$^{13}C_3$]succinic acid would yield [2,3-$^{13}C_2$]pyruvic acid (or its equivalent), which afforded **isotopomer C**. The 1,2-$^{13}C_2$-labeled pyrivic acid (or its equivalent) could be generated by C4 decarboxylation of [1,2-$^{13}C_2$]succinic acid, and the following the similar process produced **isotopomer B**.

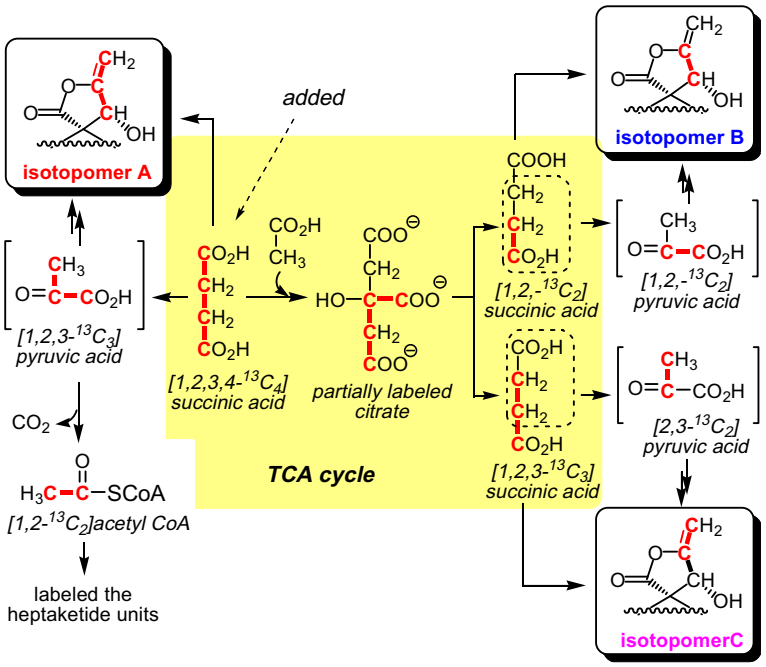


Figure 5. Proposed pathways giving **isotopomers A**, **B**, and **C**.

Signals due to C1, C5–C17 were accompanied by small flanking satellites in the ^{13}C NMR spectrum. These were labeled in a similar level (about 0.5% incorporation) when judged on the basis of their peak intensities. These were assumed to be caused by incorporation of [1,2-$^{13}C_2$]acetate derived from [1,2,3,4-$^{13}C_4$]succinic acid because all the coupling constants in this sample were identical with those of the sample prepared with sodium [1,2-$^{13}C_2$]acetate. It has been well established that the 1,2,3-$^{13}C_3$-labeled pyruvic acid gives [1,2-$^{13}C_2$]acetyl-CoA on oxidative decarboxylation.

These studies revealed the outline of the biosynthetic route of **1** (Scheme 1). Seven acetic acids first constructed heptaketide **2**. Two methyl groups (C19 and C20) were introduced into **2** with methionines. Aldol-type cyclization between C5 and C10 furnished the cyclohexene ring. Further functional transformations (oxidation of C7, C8C9 and C12C13 double-bond formations, and reductions of C15 and C17) afforded **3**. The order of these processes remained unclear. Our experiments indicated that succinic acid was a candidate as the precursor of the C11C3C4 unit (**path I**). Coupling with succinic acid would give **4**. Because the TCA cycle readily converts succinic acid to succinyl CoA, fumaric acid, malic acid, or oxalacetic acid from succinic acid, they should be regarded as equivalents. Cyclization of the γ-exomethylene-γ-lactone ring involving decarboxylation should be the final step in **path I**. We propose two mechanisms for this transformation: (i) 1,4-addition cyclization of fumarate type **5** and successive decarboxyration and (ii) decarboxylative enol formation of oxalacetic acid type **6** and subsequent cyclization of the resulting enol. Our labeling experiments did not provide sufficient information to choose the actual route from these candidates. We proposed pyruvic acid (or its equivalent) was another candidate as the precursor. Pyruvic acid (or its equivalent) derived through the TCA cycle was coupled with C5 of **3** and the subsequent dehydrative cyclization produced **1** (**path II**).

As described, we successfully proposed the outline of the biosynthetic mechanism for **1** on the basis of labeling experiments. This is the first example explaining biosynthetic mechanism of the 2-oxaspiro[4.5]decan-1-one framework found in fungal metabolites based on experimental evidences. Although our labeling experiments with [1,2,3,4-$^{13}C_4$]succinic acid gave complicated signal profile for C3 in the proton-decoupled ^{13}C NMR spectrum, detailed analysis of the signals as well as consideration of the transformation scheme in the TCA cycle enabled assignment of all peaks. These afforded information that succinic acid is responsible for the C11C3C4 unit of **1**.

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