JOC*Note*

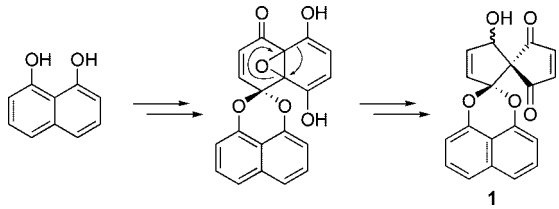
Biosynthesis of *spiro*-Mamakone A, a Structurally Unprecedented Fungal Metabolite

Sonia A. van der Sar,[†,§] Gerhard Lang,[†] Maya I. Mitova,[†] John W. Blunt,[†] Anthony L. J. Cole,[⊥] Nicholas Cummings,[⊥] Gill Ellis,[†] and Murray H. G. Munro[†,*]

Department of Chemistry and School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand.

murray.munro@canterbury.ac.nz

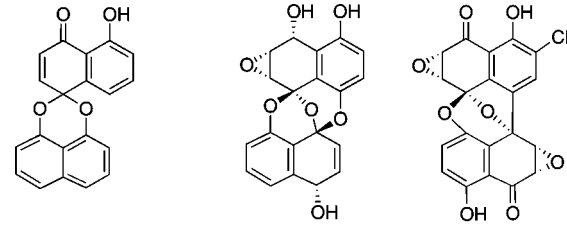
Received July 15, 2008



1

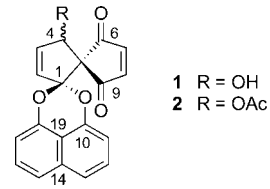
Biosynthetic studies on *spiro*-mamakone A (**1**), a potently cytotoxic and antimicrobial compound from an endophytic fungus isolated from the New Zealand native tree *Knightia excelsa* (rewarewa), confirm the polyketide origins of this unique compound belonging to the spirobisnaphthalene class of compounds. The biosynthesis proceeds via an unprecedented symmetric enedione with the two halves of the molecule being formed from two separate pentaketide units connected by oxidative coupling.

The spirobisnaphthalenes, first isolated 15 years ago,[1] contain two naphthalene-derived C_{10} units bridged through a spiroketal linkage. This class of compounds has been divided into three subclasses: those with two oxygen bridges, for example palmarumycin CP_1,[2] those with three oxygen bridges, as in preussomerin A,[1] and those with two oxygen bridges and one C−C bridge such as spiroxin A.[3] Bode et al. studied the biosynthesis of the spirobisnapthalene cladospirone bisepoxide,[4,5] and proposed that 1,8-dihydroxynaphthalene (DHN) was the probable precursor.



palmarumycin CP_1 preussomerin A spiroxin A

During our investigation into novel bioactive compounds from New Zealand endophytic fungi, we reported[6] the isolation and structure elucidation of a new relative of the spirobisnaphthalenes, *spiro*-mamakone A (**1**), which had been isolated from an endophytic fungus from the New Zealand native tree *Knightia excelsa* (rewarewa). The carbon skeleton of the spiro[4.4]nonadiene portion of the molecule was found to be unprecedented in any naturally occurring compounds. Like those of the related spirobisnaphthalenes, the structure of **1** was immediately suggestive of a polyketide origin; however, the unusual nature of the spirononadiene skeleton prompted an investigation into the underlying biosynthetic pathway leading to *spiro*-mamakone A (**1**).



1 R = OH
2 R = OAc

Results and Discussion. Time-course experiments in liquid culture ascertained optimal times for inoculation with labeled precursor and for harvesting of the culture. Consequently, after 24 h of growth the precursors, [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-acetates, were added to the culture medium, then after a further 5 days of fermentation organic extracts of the cultures were prepared by repeated extraction with EtOAc. The crude extracts were purified with reversed-phase semipreparative HPLC to give the variously labeled *spiro*-mamakones A (**1**).

A quantitative measure of incorporation of the [1-^{13}C]- and [2-^{13}C]-acetates was achieved by acetylation of these singly labeled *spiro*-mamakones A (**1**) to form *O*-acetyl-*spiro*-mamakone A (**2**) and comparison against the relative intensities of the equivalent resonances of *O*-acetyl-*spiro*-mamakone A (**2**) derived from nonlabeled *spiro*-mamakone A (**1**) normalized against the intensity of the acetyl carbonyl resonance (see Table 1, and Supporting Information, Figures S1 and S2). The ^{13}C NMR spectra of **1** labeled with [1-^{13}C]- and [2-^{13}C]-acetate indicated, as expected, a regular polyketide pattern for the 1,8-dioxynaphthalene portion of the molecule (Supporting Information, Figure S3). This was shown in the alternating labeling of the carbons of this moiety, where the even numbered carbons (C-10, C-12, C-14, C-16, C-18) were [1-^{13}C]-acetate derived

* To whom correspondence should be addressed. Tel.: +64-3-3642434. Fax: +64-3-3642110.
† Department of Chemistry, University of Canterbury.
§ Current address: Kekulé Institute of Organic Chemistry and Biochemistry, University of Bonn, Bonn, Germany.
⊥ School of Biological Sciences, University of Canterbury.

(1) Weber, H. A.; Baenziger, N. C.; Gloer, J. B. *J. Am. Chem. Soc.* **1990**, *112*, 6718–19.
(2) Krohn, K.; Michel, A.; Floerke, U.; Aust, H.-J.; Draeger, S.; Schulz, B. *Liebigs Ann. Chem.* **1994**, 1093–7.
(3) McDonald, L. A.; Abbanat, D. R.; Barbieri, L. R.; Bernan, V. S.; Discafani, C. M.; Greenstein, M.; Janota, K.; Korshalla, J. D.; Lassota, P.; Tischler, M.; Carter, G. T. *Tetrahedron Lett.* **1999**, *40*, 2489–92.
(4) Bode, H. B.; Wegner, B.; Zeeck, A. *J. Antibiot.* **2000**, *53*, 153–7.
(5) Bode, H. B.; Zeeck, A. *Phytochemistry* **2000**, *54*, 597–601.
(6) van der Sar, S. A.; Blunt, J. W.; Munro, M. H. G. *Org. Lett.* **2006**, *8*, 2059–61.

10.1021/jo801564c CCC: $40.75 © 2008 American Chemical Society
Published on Web 10/08/2008

TABLE 1. ^{13}C NMR Analysis of the ^{13}C-Labeled *spiro*-Mamakone A (1) and *O*-Acetyl-*spiro*-mamakone A (2)

	1		2		
	[1,2-$^{13}C_2$]-Ac[b,c]			enrichment factors	
C#	δ_C[a]	$^1J_{CC}$, Hz	δ_C[a]	[1-^{13}C]-Ac[d]	[2-^{13}C]-Ac[d]
1	111.7	53.7, 44.1	112.3	0.9	1.6
2	129.9	69.9, 53.7	133.2	2.1	0.8
3	142.7	69.9, 43.5	138.2	0.9	1.7
4	79.1	43.5	80.2	2.3	1.0
5	68.9	44.1	65.7	1.7	0.8
6	198.6	47.9, 44.1	197.1	1.3	1.1
7	151.9	47.9	150.9	1.5	1.4
8	152.2	47.5	151.4	1.6	1.3
9	201.1	47.5, 44.1	199.4	1.5	1.4
10	149.4/148.8	75.1, 61.1	148.8/148.5	2.0	0.7
11	111.0/110.6	55.5, 75.1	111.14/111.1	0.8	1.5
12	129.0/128.7	60.4, 55.5	129.0/128.9	3.0	1.0
13	122.0/122.5	ND	122.5/122.6	0.9	1.6
14	136.1	56.5	136.0	2.7	1.00
15	122.5/122.0	ND	122.6/122.5	0.9	1.7
16	128.7/129.0	60.4, 55.4	128.9/129.0	2.0	0.8
17	110.6/111.0	55.4,75.1	111.1/111.14	0.9	1.5
18	148.8 149.4	75.1, 61.1	148.5/148.8	2.2	0.9
19	114.8	61.1	114.8	0.9	1.7
20 C=O			172.9	1.00	1.00
21 Me			20.7	0.8	0.8

[a] Excluding C-14 and C-19, the pairs of carbons (and protons) of the 1,8-dioxynaphthalene portion of the structure (C-10−C-19) are interchangeable. [b] ND not determined due to signal overlap. [c] The incorporation of [1,2-$^{13}C_2$] was estimated to be 1.3% by comparison between the integrals of the natural abundance ^{13}C signal (1.1%) and the integrals of the coupled satellite signals. ^{13}C−^{13}C coupling constants were obtained from the ^{1}H-decoupled ^{13}C NMR spectrum of **1**. [d] Enrichment factors were quantitatively calculated by preparation of the acetylated analogue, *O*-acetyl-*spiro*-mamakone A (**2**), for each labeled sample and peak intensities determined relative to the normalized intensity of the acetate carbonyl signal at 172.9 ppm from natural abundance *O*-acetyl-*spiro*-mamakone A (**2**).

and the odd numbered carbons (C-l1, C-13, C-15, C-17, C-19) were [2-^{13}C]-acetate derived.

The interpretation of the labeling for the nonadiene portion was not straightforward. From the [2-^{13}C]-acetate experiment C-1 and C-3 were labeled (Table 1; enrichment factors of 1.6 and 1.7, respectively), but the labeling of carbons C-6, C-7, C-8, and C-9 had a decreased level of incorporation (Table 1; enrichment factors of 1.1−1.4). From the [1-^{13}C]-acetate experiment C-2, C-4, and C-5 were significantly labeled. Surprisingly, incorporation at C-6−C-9 was also observed, and like that seen from the [2-^{13}C]-acetate experiment, a lower level of incorporation was noted (Table 1). In addition to this unusual labeling pattern, the feeding of the singly labeled precursors established that two of the acetate carbonyl-derived carbons lay adjacent at the C-4 and C-5 positions. This was interpreted as the loss of a carbon derived from the methyl group of acetate from between these two positions.

The results obtained from the experiments with singly labeled precursors were further confirmed by incorporation of [1,2-$^{13}C_2$]-acetate. Coupling constants were determined for all carbons (Table 1 and Supporting Information, Figure S4). In some cases, however, the low signal-to-noise ratios (most notably for the spiroketal and carbonyl carbons) and in addition signal overlap made the *J* value measurements questionable. Due to this complexity, further analysis utilizing an INADEQUATE NMR experiment was carried out (Supporting Information, Figure S5). For the 1,8-dioxynaphthalene portion of the molecule isotopomer effects were observed. In the ^{13}C NMR spectrum the carbon resonances C-10−C-13 and C-15−C-18 each showed two pairs of ^{13}C satellite signals due to the presence of two differently labeled sets of molecules (isotopomers). This was not considered to be coupling between adjacent acetate units due to the low overall incorporation (1.3%) observed. This interpretation was supported by the INADEQUATE NMR spectrum. All the $^1J_{C,C}$ combinations indicative of isotopomers ($^1J_{10,19}$; $^1J_{10,11}$; $^1J_{12,11}$; $^1J_{12,13}$; etc.) were present (Table 1). The near symmetrical nature of the naphthalene subunit was considered the reason why two resolved sets of satellite peaks were not observed for C-14 and C-19 arising as a consequence of the identical or near identical coupling constants. A similar isotopomer effect has previously been reported in the biosynthetic study on the related spirobisnaphthalene, cladospirone bisepoxide.[4]

The isotopomer effect was also observed in the spirononadiene portion of the molecule and led to a complex set of data. Careful analysis of both the INADEQUATE NMR spectrum and the $^1J_{CC}$ coupling constants (Table 1) revealed that intact acetate units did not have the usual alternating array seen in polyketide biosynthesis. Instead C-6−C-9 each appeared to have both an acetate carbonyl AND an acetate methyl origin. C-4 was of acetate carbonyl origin while C-5, also of acetate carbonyl origin, had three different C−C couplings: to C-1, C-6, and C-9. Although the C-5 to C-6/C-9 couplings were of weaker intensity in the INADEQUATE spectrum than the C-5 to C-1 coupling, they were not considered to arise as a consequence of natural abundance as no intensity enhancement was required in processing the spectrum to view these correlations. In addition, no other natural abundance correlation signals were seen at the intensity at which the INDAEQUATE NMR spectrum was viewed. The coupling constants from C-5 to C-1/C-6/C-9 were extracted from the spectrum of the INADEQUATE NMR and each was found to be identical ($J = 44.1$ Hz), thus providing an explanation for the apparent observation of just one pair of satellites for C-5 in the ^{13}C NMR spectrum. A further important observation was the existence of coupling between C-7 and C-8. This coupling was not immediately obvious from either the INADEQUATE or ^{13}C NMR spectra. This was due to the near identity of chemical shifts of these two carbons. In the ^{13}C NMR this generated an irregular satellite

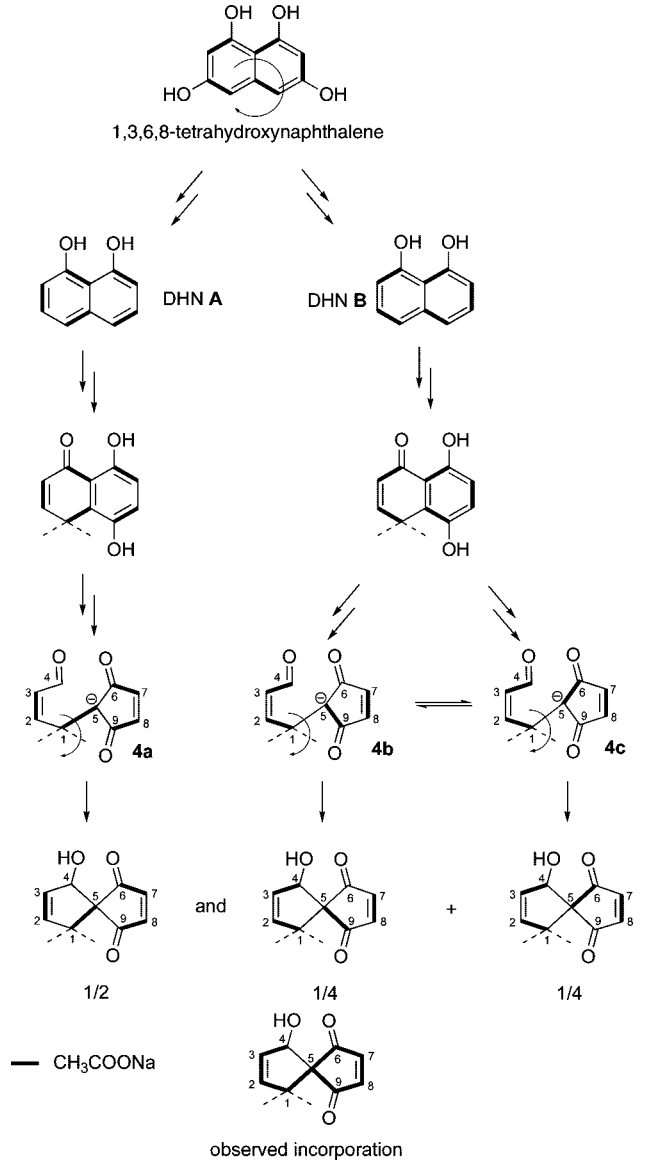


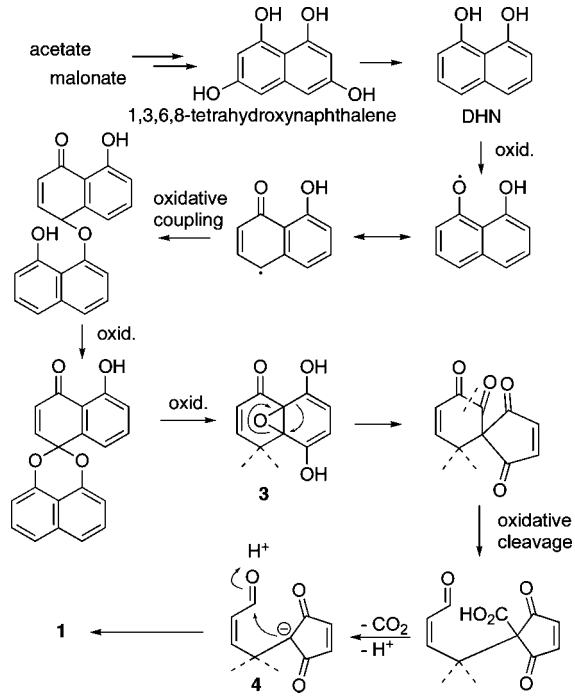
FIGURE 1. The observed enrichment of the spirononadiene portion of *spiro*-mamakone A (**1**) and the proposed origin of the incorporation pattern from 1,3,6,8-tetrahydroxynaphthalene.

pattern (Supporting Information, Figure S6), which was attributed to the "roof effect" resulting from second-order coupling effects.[7]

From the results of the singly labeled feeding studies it is proposed that a carbon of acetate methyl origin in the DHN-derived intermediate is lost during biosynthesis leading ultimately to two carbons of acetate carbonyl origin becoming adjacent in the spirononadiene moiety at positions C-4 and C-5 (Figure 1). In the ^{13}C NMR spectrum of **1** enriched with [1,2-$^{13}C_2$]-acetate the C-4 signal showed a single pair of satellites; the *J* value (43.5 Hz) was indicative of coupling to C-3 (Table 1). The INADEQUATE spectrum confirmed that this was the correct interpretation, thus supporting the initial hypothesis that during the biosynthesis of **1**, an acetate methyl derived carbon is cleaved from between the C-4 and C-5 carbons. On consideration of the structures of some of the known spiro-

(7) Abraham, R. J.; Loftus, P. *Proton and Carbon-13 NMR Spectrosopy: An integrated approach*; Heyden and Son Ltd.: Chichester, UK, 1980.

SCHEME 1. Proposed Biosynthesis of *spiro*-Mamakone A (1)



bisnaphthalenes, specifically palmarumycin CP_1[2] and cladospirone bisepoxide,[4] and the results of the labeling studies on **1**, a possible mechanism for the generation of the spirononadiene system is proposed (Scheme 1). This route involves stepwise oxygenation beginning with the oxidative coupling of two DHN molecules followed by rearrangement of the coupled moiety to a dihydroxynaphthyl epoxide intermediate (**3**). After oxidative cleavage, decarboxylation, and deprotonation steps a symmetric enedione carbanion (**4**) is formed. In a Knoevenagel-type reaction this stabilized carbanion intermediate could attack the aldehyde to generate *spiro*-mamakone A (**1**).

All of the couplings observed in the INADEQUATE NMR spectra are consistent with the proposed biogenesis. However, to account for the complexity of the labeling pattern (shown in Figure 1) it was necessary to invoke both isotopoisomerism and the formation of a subsequent symmetric intermediate. This is the key intermediate necessary to account for the observed interchange of the labels in the C-6−C-9 portion of the spirononadiene, particularly the simultaneous occurrence of the couplings $^1J_{C\text{-}5/C\text{-}9}$ and $^1J_{C\text{-}5/C\text{-}6}$. The isotopomers arise as a consequence of the C_2 symmetry of 1,3,6,8-tetrahydroxynaphthalene, the precursor of DHN. Later in the biosynthetic pathway the symmetric enedione carbanions (**4a**−**c**) are proposed. Structure **4a** represents half of the sample and originated from the DHN isotopomer **A** and displays no incorporation of an intact acetate unit at C-5/C-6 and C-5/C-9. Those molecules (**4b** and **4c**) arising from isotopomer **B** will, however, show intact acetate units at either C-5/C-6 or C-5/C-9 as a consequence of rotation about the C-1/C-5 bond of the proposed symmetric carbanion intermediate. The labeling pattern of the final product then depends on which side of the carbanion attacks the aldehyde carbon. As a consequence one-quarter of the *spiro*-mamakone sample will indicate C-5/C-6 incorporation, another quarter will have C-5/C-9 incorporation, while the remaining half will have no incorporation between C-5 and C-6 or C-9. This partitioning accounts for the observed incorpora-

tions at C-6 to C-9 and why the apparent levels of incorporation were less at these carbons than those observed for the other carbons.

Having a symmetric carbanion such as **4** as an intermediate has another implication. If there were no *re*/*si* facial selectivity in the attack of the carbanion on the formyl group racemic *spiro*-mamakone A (**1**) would be generated. This is an explanation perhaps of the unusual phenomenon of **1** being isolated as a racemate. This is a rare occurrence in biosynthesis due to the usually high stereospecificity of the biosynthetic enzymes. An alternative, and perhaps more rationale explanation is that racemization occurs during isolation and purification of chiral **1** by equilibration between **4** and **1** under nonenzymic conditions.

These labeling experiments provide a picture of the underlying biosynthetic pathway leading to the production of *spiro*-mamakone A (**1**). Like that postulated for cladospirone bisepoxide,[4] our experiments suggest that both the "bottom" and "top" halves of the molecule derive from the same pentaketide precursor via a fungal polyketide synthase, but followed by extensive rearrangement and modification of the "top" half in order to generate *spiro*-mamakone A (**1**) via the symmetric carbanion **4** as intermediate. At this stage, although the direction of the polyketide chain is clear, the starter unit has not been determined. However, the folding pattern appears to be that of a regular fungal polyketide and is supported by the coproduction of mellein,[8] in the growing culture.

Experimental Section

Cultivation of the Unidentified Endophytic Fungus. The fungus was cultured for 24 days on MYE (10 g/L oxoid malt extract, 1 g/L yeast extract, 12 g/L agar) agar plates at 26 °C.[6] A third of the agar plate was taken, sliced into smaller pieces, and used to inoculate the production medium (200 mL) in a flask (1 L) containing MYE broth (10 g/L oxoid malt extract, 1 g/L yeast extract). This was repeated for each experiment. After inoculation the fungus was grown at 26 °C and shaken at 180 rpm for 24 h.

General Procedure for Feeding Experiments. Feeding experiments were performed with use of [1-^{13}C]-CH_3COONa, [2-^{13}C]-CH_3COONa, and [1,2-$^{13}C_2$]-CH_3COONa (99% ^{13}C). The precursors were added to the fungal culture 24 h after inoculation of the growth media. Each of the labeled acetates (200 mg) was dissolved in sterile H_2O (10 mL). Under sterile conditions, the precursor solutions were each passed through filters (0.2 μm) into the growing fungal cultures. The final concentration of labeled acetates was 0.95 mg/mL for each experiment. After fermentation for a further 5 days, the mycelium was separated from the culture medium, macerated, and extracted with EtOAc (3 × 100 mL). The culture broth (200 mL) was extracted with EtOAc (3 × 300 mL). The combined EtOAc extracts for each experiment were dried to yield the crude isotopically labeled extract. Purification of *spiro*-mamakone A (**1**) was carried out by semipreparative HPLC (Phenomenex Luna C18, 10 × 250 mm, 5 μm; 45−60% MeCN/$H_2$0; 5 mL/min). The amount of **1** was 18−24 mg in each experiment.

Preparation of *O*-Acetyl-*spiro*-mamakone A (2). Acetylation was carried out by dissolving *spiro*-mamakone A (**1**, 3.0 mg, 9.38 × 10^{-3} mmol) in dry pyridine (200 μL) and acetic anhydride (200 μL, 2.12 mmol) was added. After 15 h the reaction mixture was diluted with distilled H_2O to 10% organic concentration and passed through a C-18 cartridge (Bakerbond SPE, 1 g) equilibrated to 100% H_2O. Using a Vac-Elute system, the cartridge was run to dryness, then a further column volume of H_2O (1 mL) was eluted through the cartridge, followed by MeOH (100%; 1 mL) to give *O*-acetyl-*spiro*-mamakone A (**2**) (2.7 mg, 79.5%) as a pale yellow amorphous solid: $[\alpha]^{20}_D$ 0.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 230 (3.95), 298 (3.72), 314 (3.58), 328 (3.43) nm; IR (KBr disk) ν_{max} 1736,1713, 1605, 1412, 1373, 1258, 1227, 1204, 1134, 1057 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.51 (1H, d, J = 8.3 Hz, Hl5/H13), 7.50 (1H, d, J = 8.3 Hz, H13/H15), 7.41 (2H, t, J = 7.4, 7.4 Hz, H12/H16), 7.18 (1H, d, J = 6.3 Hz, H8), 7.12 (1H, d, J = 6.3 Hz, H7), 6.85 (1H, d, J = 7.3 Hz, H11/H17), 6.83 (1H, d, J = 7.3 Hz, H17/H11), 6.45 (1H, dd, J = 2.0, 5.6 Hz, H3), 6.12 (1H, dd, J = 1.7, 5.8 Hz, H2), 5.81 (1H, br s, H4), 1.97 (3H, s, H21); ^{13}C NMR (see Table 1); EIMS *m*/*z* (%) 362.1 $[M]^+$ (100), 320.1 (37), 291.1 (17), 266.1 (15), 237.1 (8), 197.1 (20), 161.0 (24), 133.0 (17), 114.0 (11); HREIMS *m*/*z* 362.0784 $[M]^+$ (calcd for $C_{21}H_{14}0_6$ 362.0790).

The same experimental procedure was used to prepare *O*-acetyl-*spiro*-mamakones A (**2**) from **1** isolated from the cultures fed with singly labeled acetates (1.5 mg; 100 μL each of pyridine and acetic anhydride).

Acknowledgment. Useful discussions with Professor Tom Simpson, University of Bristol and assistance from B. Clark for MS operations are gratefully acknowledged.

Supporting Information Available: Figures S1−S6 are ^{13}C NMR and 2D-INADEQUATE spectra of labeled and unlabeled *spiro*-mamakone A (**1**) and *O*-acetyl-*spiro*-mamakone A (**2**) samples. This material is available free of charge via the Internet at http://pubs.acs.org.

JO801564C

(8) Abell, C.; Garson, M. J.; Leeper, F. J.; Staunton, J. *J. Chem. Soc., Chem. Commun.* **1982**, 1011–13.