Biosynthesis of ML-236C and the hypocholesterolemic agents compactin by *Penicillium aurantiogriseum* and lovastatin by *Aspergillus terreus:* determination of the origin of carbon, hydrogen and oxygen atoms by ^{13}C NMR spectrometry and observation of unusual labelling of acetate-derived oxygens by $^{18}O_2$

Kurt Wagschal, Yuko Yoshizawa, David J. Witter, Yaoquan Liu and John C. Vederas *,†

Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

Sodium [1-^{13}C, 2-$^{2}H_3$]-, [2-^{13}C, 2-$^{2}H_3$]- and [1-^{13}C, $^{18}O_2$]-acetate are incorporated in separate experiments into ML-236C 2 and the hypocholesterolemic agent compactin 3 by cultures of *Penicillium aurantiogriseum*, and the regiochemical distribution of ^{2}H, ^{13}C and ^{18}O is determined by ^{13}C NMR spectrometry. In addition, sodium [1-^{13}C, $^{18}O_2$]acetate and $^{18}O_2$ are incorporated into lovastatin (mevinolin) 4 by cultures of *Aspergillus terreus* to re-examine the origin of oxygen atoms. The results show that the main-chain oxygen atoms of 2–4 originate from acetate, and the C-8 oxygen atom of 3 and 4 is derived from molecular oxygen. However, detailed mass spectral analysis shows that significant amounts of aerobic oxygen can be incorporated at sites normally labelled by acetate oxygen, presumably through generation of [^{18}O]acetate by ω-oxidation of fats. On the basis of labelling results, a mechanism is proposed to account for the formation of the bicyclic ring system in these compounds.

Introduction

ML-236C **2** is a close relative of compactin **3** and lovastatin **4** (Scheme 1), which are slow-binding inhibitors of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis in mammals.[1] Since lovastatin (also known as mevinolin, monacolin K and Mevacor) and structurally related compounds are widely prescribed to lower cholesterol levels in humans, there is continuing interest in the chemical synthesis of these drugs and their derivatives.[2–5] Biosynthetically, the carbon backbone of such polyketides and co-occurring metabolites, such as 4a,5-dihydromonacolin L **1** (see below), is assembled from acyl-CoA units *via* a sequential mechanism similar to fatty acid formation. However, polyketide biosynthesis, which has been extensively reviewed,[6–11] differs from fatty acid construction because the oxidation level, choice of extender unit and stereochemistry of the growing polyketide chain can be specifically adjusted after each condensation step to afford a succession of structurally diverse enzyme-bound intermediates. Recent molecular biological studies,[12–19] incorporation of advanced intermediates into complex polyketides,[20–30] and over-expression of functional polyketide synthases (PKS)[12,13] all support this general assembly hypothesis.

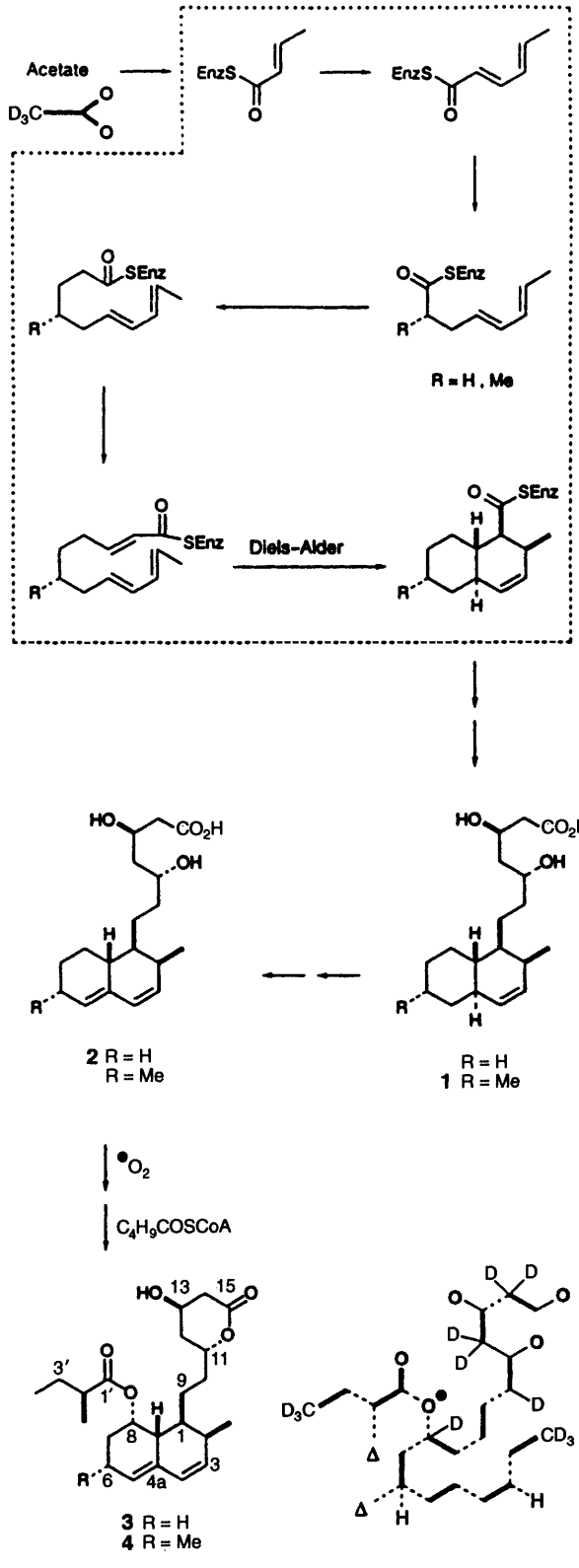
An intriguing feature of the formation of **2–4** based on isotopic labelling studies[31,32] and transformations of post-PKS intermediates,[33–35] is the possible occurrence of an enzyme-catalysed intramolecular Diels–Alder reaction to generate the bicyclic decalin system. Recent work on another decalin-containing system shows that a partially purified oxidizing enzyme can catalyse an *exo*-selective Diels–Alder reaction to form an optically active fungal metabolite, solanapyrone, from an achiral triene precursor, prosolanapyrone II.[36] Model studies on Diels–Alder reactions of analogues of potential intermediates in the lovastatin pathway indicate that enzymatic control is essential to obtain the correct relative, as well as absolute, stereochemistry.[37] However, in contrast to incorporations of *N*-acetylcysteamine (2-acetamidoethanethiol) thiol esters of advanced intermediates in many prokaryotic[20–23,26–29] and some fungal systems,[24–25,30] no intact utilization of di-, tri-, tetra- or hexa-ketide precursors in **2–4** has been reported thus far. As a prelude to further work with cell-free systems and genetically modified organisms, a more detailed examination of the possible oxidation states which can be achieved along the polyketide chain was undertaken. The results, a portion of which have been communicated in preliminary form,[32] require revision of the original conclusions[31] regarding the source of oxygen atoms along the main chain of **4** and show that **2** and **3** are formed analogously. The present study also demonstrates that unexpected and significant levels of aerobic oxygen can be incorporated at sites biosynthetically derived primarily from acetate oxygen, probably due to aerobic ω-oxidation[38] of fatty acids followed by β-oxidation processes.

Results and discussion

Oxygen-18 labelling studies

A prerequisite for incorporation studies with stable isotopes is the complete determination of ^{1}H and ^{13}C resonances.[39] The full assignments of the ^{1}H and ^{13}C NMR spectra of lovastatin **4** have already been reported.[31] The literature assignments[33] of compactin **3** were confirmed and assignments for the ^{1}H and ^{13}C NMR spectra of ML-236C **2** were made using standard techniques[40] (proton COSY, HMQC and HMBC) (Table 1). An unusual aspect of our early work[31] on formation of **4** by *Aspergillus terreus* ATCC 20542 was failure to detect intact utilization of the carbon–oxygen bonds derived from sodium [1-^{13}C, $^{18}O_2$]acetate into the expected sites at C-11, C-13 and C-15 on the main backbone using ^{18}O-induced isotope shifts in ^{13}C NMR spectra. Only the label at the side chain carbonyl (C-1′) could be clearly seen. In agreement with this observation, incorporation of $^{18}O_2$ into lovastatin using the same strain of *A. terreus*, followed by mass spectral analysis showed that up to four ^{18}O atoms were utilized per molecule of **4**, thereby indicating that aerobic oxygen was biosynthetically introduced at C-8, C-11, C-13 and C-15. However, the decline of

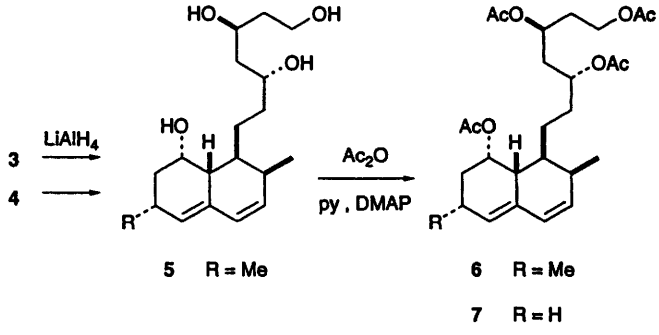
† e-mail: John.Vederas@Ualberta.ca



Scheme 1 Proposed biosynthetic pathway to 4a,5-dihydromonacolin L **1**, ML-236C **2**, compactin **3** and lovastatin **4**. Potential di-, tri-, tetra-, penta- and hexa-ketide intermediates in the assembly process are indicated as enzyme-bound thiol esters (boxed structures) at the oxidation stage attained immediately prior to addition of the next two-carbon unit. The labelling pattern of bonds derived intact from acetate is indicated in the lower right corner.

production of **4** to very low levels precluded analysis of this sample by ^{13}C NMR spectrometry. Since detailed studies by Endo and coworkers[33–35] on late stage post-PKS transformations of lovastatin precursors in *Monascus ruber* had failed to detect any compound less oxidized than 4a,5-dihydromonacolin L **1**, we reinvestigated the oxygen labelling results.

Fortunately, recent subculture and selection from a new strain, *A. terreus* MF 4845, increased yields of **4** to about 200 mg per litre of culture, which permitted re-incorporation of sodium [1-^{13}C, $^{18}O_2$]acetate. The ^{13}C NMR spectrum showed a measurable upfield isotope shift ($\Delta\delta$ 0.038 ppm) for the doubly-bonded oxygen at C-1′ as before, but displayed only broadened signals for C-11, C-13 and C-15. Since two bond carbon–carbon couplings due to multiple incorporations of labelled acetates into the same molecule could cause such broadenings, spin-echo experiments were used to separate coupled and uncoupled resonances.[41] This further resolved the C-1′ isotope-shifted peak, thereby indicating that multiple doubly-labelled acetate units had in fact been incorporated. However, possible isotope-shifted resonances were still not clearly visible at C-11, C-13 and C-15 under these conditions. The magnitudes of ^{18}O isotope shifts depend on structure, and attachment of an electron-withdrawing group (*e.g.* acetyl) increases the separation of the ^{13}C–^{16}O and ^{13}C–^{18}O signals.[39,42] Thus, in order to detect any unresolved signals, labelled **4** was reduced with lithium aluminum hydride to tetraol **5**, which was then converted to the corresponding tetraacetate **6** with acetic anhydride (Scheme 2).



Scheme 2 Conversion of compactin **3** and lovastatin **4** to corresponding tetraacetates **6** and **7**

The ^{13}C NMR spectrum of **6** displayed clear isotope shifts for C-11 ($\Delta\delta$ 0.037 ppm), C-13 ($\Delta\delta$ 0.036 ppm) and C-15 ($\Delta\delta$ 0.030 ppm), thereby demonstrating that the bonds to oxygen at these carbons originate from acetate [Fig. 1(*a*)]. The small size of the ^{18}O-shifted peak relative to the ^{13}C–^{16}O peak is due to the considerable exchange of ^{18}O that occurs relative to the amount of ^{13}C incorporated (*ca.* 1% enrichment per site; twofold signal enhancement). In an attempt to suppress this, the sodium [1-^{13}C, $^{18}O_2$]acetate was converted to its *N*-acetylcysteamine thiol ester ($Me^{13}C^{18}OSCH_2CH_2NHCOMe$) **8** since it has previously been shown that such derivatization aids loading of advanced intermediates into PKS systems.[20–30] However, the incorporation of ^{13}C label was only slightly higher than with the corresponding sodium acetate and no significant reduction of ^{18}O exchange was observable. This suggests that the thiol ester is hydrolysed to free acetate prior to use by the fungal PKS enzyme(s), and is in accord with failure to achieve intact utilization of any advanced di-, tri-, tetra- or hexa-ketide intermediates in **4** by cultures of *A. terreus*.[37]

To re-examine the aerobic oxygen labelling pattern, *A. terreus* MF 4845 was grown in an atmosphere containing $^{18}O_2$ gas as previously described.[31] In the present experiment, a sufficient quantity of labelled **4** was obtained to examine by ^1H-decoupled ^{13}C NMR spectrometry, and this sample showed a single detectable isotope shift at C-8 ($\Delta\delta$ 0.034 ppm), initially indicating that only the oxygen at that site is aerobically derived, in apparent contradiction to our earlier result of four aerobic oxygens per molecule of **4**.[31] However, detailed examination of the chemical ionization mass spectrum (ammonia) of this lovastatin sample showed that although most labelled

Table 1 ^{13}C NMR chemical shifts of ML-236C **2** and compactin **3**, and isotope shifts in the ^{13}C NMR spectra of **2**, **3** and **6** (derived from **4**) after labelled acetate incorporation

	δ_C/ppm		^2H-isotope shifts/ppb (type)		^{18}O-isotope shifts/ppb		
Carbon	**2**	**3**	**2**	**3**	**2**	**3**	**6**
1	41.97	36.84	*a*	−43[b]			
2	31.53	30.86	−210(2β), −140(β)	−210(3β), −140(2β), −70(β)			
2-Me	13.91	13.86	*a*	−820(3α), −540(2α)			
3	132.61	132.65	*a*	*c*			
4	128.31	128.19	*c*	*c*			
4a	137.59	133.58	*a*				
5	124.57	123.73	−15[b]	−15[b]			
6	25.52	20.94	*a*	*c*			
7	22.78	26.23	*d*	*c*			
8	27.26	67.49	*a*				
8a	34.64	37.53	+18(β)	+16(β)			
9	24.33	24.04	−88[b]	−91[b]			
10	33.02	32.98	*a*	−400(α)			
11	76.03	76.14	−36(β)	−44(β)	−34	−33	−37
12	36.14	36.20	*a*	−740(2α), −370(α)			
13	62.81	62.78	−120(β)	−110(β)	−17	−15	−36
14	38.66	38.61	*a*	−610(2α), −300(α)			
15	170.53	170.05	+36(β)	+35(β)	−30	−30	−30
1′		176.64		+20(β)		−40	
2′		41.69		−77(α)			
2′-Me		16.94					
3′		26.72		−250(3β), −170(2β), −80(β)			
4′		11.77		−870(3α), −580(2α), −280(α)			

[a] Insufficient **2** obtained from sodium [2-^{13}C, 2-$^2H_3$]acetate fermentation. [b] Complicated by multiple acetate incorporation; see text. [c] No isotope shift detected.

Table 2 Mass spectral data for lovastatin **4** from $^{18}O_2$ fermentations of *A. terreus*

		Intensity (%)				Intensity (%)	
m/z	(**4** from ATCC 20542)[a]	obs[c]	(calc)[d]	*m/z*	(**4** from MF 4845)[b]	obs[c]	(calc)[d]
415	(MH + 10)[a]	1	(0)	432	(MNH_4 + 10)[b]	0	(0)
414		2	(0)	431		0	(0)
413	(MH + 8)	8	(0)	430	(MNH_4 + 8)	0	(0)
412		2	(0)	429		0	(0)
411	(MH + 6)	9	(0)	428	(MNH_4 + 6)	0	(0)
410		3	(0)	427		2	(0)
409	(MH + 4)	12	(0.06)	426	(MNH_4 + 4)	11	(0.06)
408		16	(0.57)	425		14	(0.58)
407	(MH + 2)	64	(4.54)	424	(MNH_4 + 2)	51	(4.64)
406		27	(27.1)	423		28	(27.5)
405	(MH)	100	(100)	422	(MNH_4)	100	(100)

[a] Chemical ionization spectra acquired using isobutane; see ref. 31. [b] Chemical ionization spectra acquired using ammonia; other procedures and conditions as in ref. 31. [c] Observed ion intensities (± 1.5%) for **4** derived for $^{18}O_2$. [d] Calculated nominal ion intensities for unlabelled **4**. Mass spectral values for unlabelled **4** are in agreement within experimental error.

molecules contain a single ^{18}O atom (32% incorporation based on M + 2 peak), a significant number (signal intensity 11% *vs.* 0.06% expected) have two ^{18}O labels (Table 2). In agreement with the unexpectedly high number of ^{18}O labels per molecule in our original report,[31] these results clearly demonstrate that [^{18}O]acetate and/or [^{18}O]malonate must be generated from aerobic oxygen in the biological system for utilization in lovastatin **4**. It appears likely that the shunt pathway responsible for this involves ω-oxidation of fatty acids followed by β-oxidation processes (Scheme 3).[38]

In order to define the origins of the oxygen atoms of compactin **3** and ML-236C **2**, sodium [1-^{13}C, $^{18}O_2$]acetate was fed to producing cultures of *Penicillium aurantiogriseum* ATCC 60762. The compactin **3** produced was directly converted to its corresponding tetraacetate **7** (Scheme 2) in anticipation that the ^{13}C–^{18}O signals would be poorly resolved from the ^{13}C–^{16}O signals. As expected, the ^{13}C NMR spectrum of **7** displayed unambiguous isotope shifts analogous to those observed for **6** at C-11 (Δδ 0.038 ppm), C-13 (Δδ 0.035 ppm) and C-15 (Δδ 0.030 ppm), thereby demonstrating the acetate origin of the oxygen atoms connected to these positions [Fig. 1(*b*)]. Although the complementary experiment involving the generation of **3** from $^{18}O_2$ was not done, the absence of a ^{13}C–^{18}O signal for C-8 in the ^{18}O-acetate enriched sample and the demonstrated aerobic origin of the corresponding oxygen atom in lovastatin **4** strongly suggest that aerobic oxidation occurs at C-8 during biosynthesis of compactin. An unexpected finding was that unlike the isotope-shifted peaks of ^{18}O-acetate enriched lovastatin **4**, the ^{18}O-shifted peaks of the similarly-derived compactin **3** were of comparable size to the ^{13}C–^{16}O signals, indicating little if any exchange. Interestingly, the ^{13}C NMR spectrum of compactin allowed direct observation (*i.e.* without derivatization) of the signals for C-1′ (Δδ 0.040 ppm), C-11 (Δδ 0.033 ppm), C-13 (Δδ 0.015 ppm) and C-15 (Δδ 0.030 ppm). As expected, ^{13}C NMR analysis of ML-236C **2** after incorporation of sodium [1-^{13}C, $^{18}O_2$]acetate also revealed readily apparent (though broadened) ^{13}C–^{18}O signals for C-11 (Δδ 0.034 ppm), C-13 (Δδ 0.017 ppm) and C-15 (Δδ 0.030 ppm) [Fig. 1(*c*)].

Deuterium labelling studies

In earlier work the utilization of intact acetate-derived carbon–

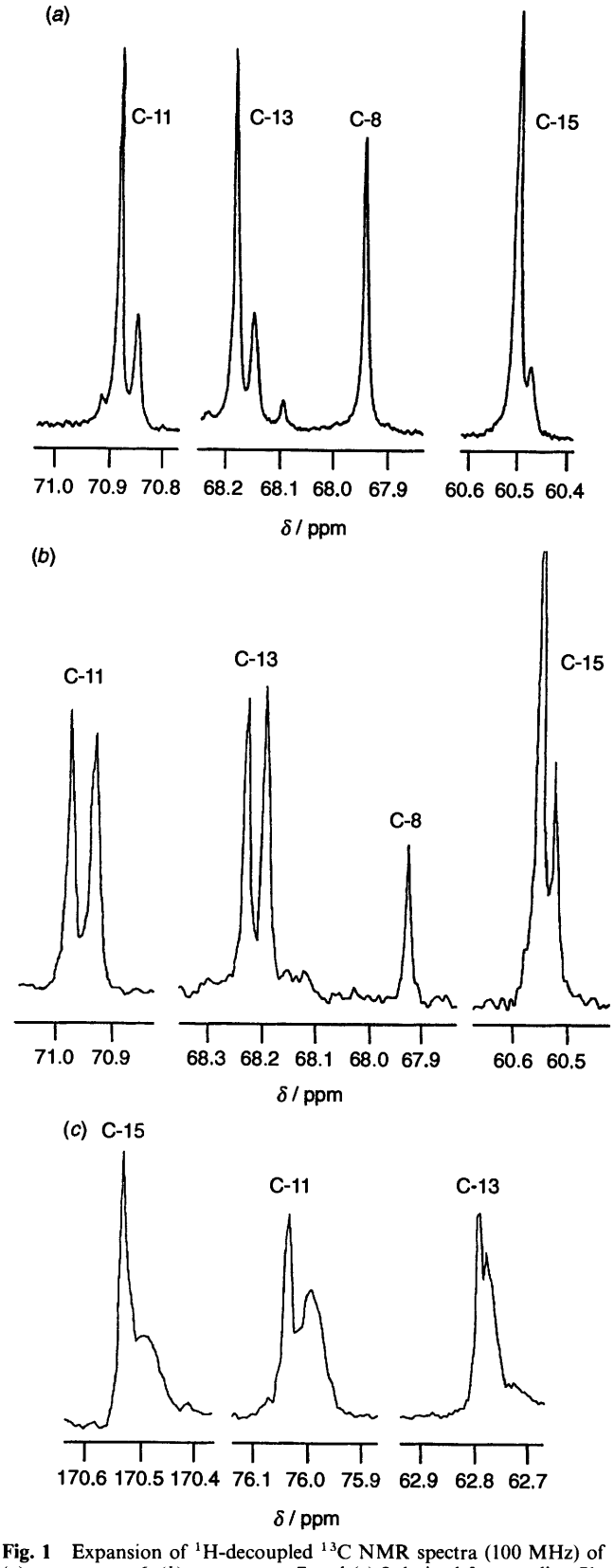
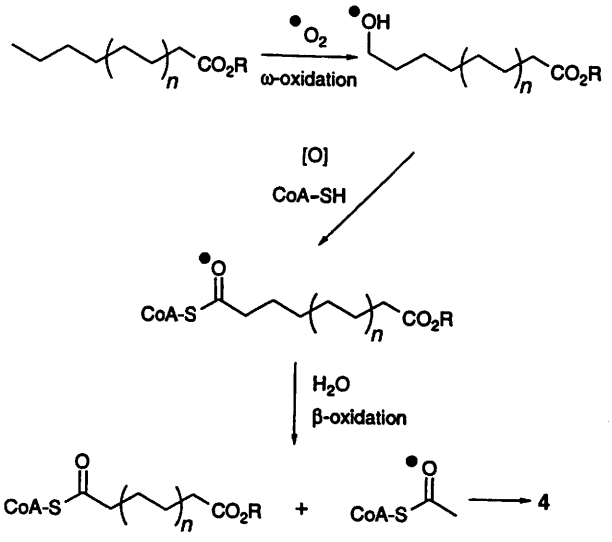


Fig. 1 Expansion of ^{1}H-decoupled ^{13}C NMR spectra (100 MHz) of (*a*) tetraacetate **6**, (*b*) tetraacetate **7** and (*c*) **2** derived from sodium[1-^{13}C, $^{18}O_2$]acetate showing C-11, C-13, C-8 and C-15



Scheme 3 Proposed mechanism of conversion of $^{18}O_2$ to [^{18}O]acetate prior to incorporation into lovastatin **4**

hydrogen bonds in lovastatin **4** by *A. terreus* had been examined, and several unexpected hydrogen losses were observed based on the likely sequence of biosynthetic events.[31] If there is no solvent exchange at intermediate stages, it would be expected that a linear chain polyketide generated from completely deuteriated acetate by a PKS enzyme in unlabelled media would initially possess three deuterium atoms at the starter methyl group, two deuteriums at methylene carbons preceding a keto or hydroxy functionality in the same acetate unit, and only one deuterium at olefinic sites or at methylene carbons preceding a position where the carbonyl of the same acetate unit was completely reduced to CH_2.[8,39] Since ML-236C **2** and compactin **3** are produced by a different organism (*P. aurantiogriseum*) and lack the C-6 methyl group derived from the methyl of *S*-adenosyl methionine, it appeared that comparison of the number of intact acetate-derived carbon–hydrogen bonds could provide valuable clues to common biosynthetic intermediates. Nevertheless, loss of acetate hydrogen must be interpreted with caution because exchange with media (water) during assembly is well-precedented.[43]

In separate experiments sodium [1-^{13}C, 2-$^{2}H_3$]- and [2-^{13}C, 2-$^{2}H_3$]-acetate were incorporated into the carbon skeletons of **2** and **3** using submerged cultures of *P. aurantiogriseum*. The acetate precursors typically gave overall two- to four-fold peak enhancements in the NMR spectra for each labelled carbon. Samples of **2** and **3** derived from [1-^{13}C, 2-$^{2}H_3$]acetate were analysed by deuterium-induced β-isotope shifts[39,44] in ^{1}H-decoupled ^{13}C NMR spectra, whereas direct measurement of α-isotope shifts[39,44] in ^{1}H,^{2}H-decoupled ^{13}C NMR spectra was employed to examine compactin **3** obtained after incorporation of [2-^{13}C, 2-$^{2}H_3$]acetate. The amount (< 3 mg) of ML-236C **2** obtained in the latter experiment was insufficient for reliable analysis of α-isotope shifts. The results (Table 1) clearly demonstrate that **2** and **3** display essentially the same distribution of intact acetate-derived carbon–hydrogen bonds as **4**. Especially surprising is the consistent loss of deuterium from C-3 and C-6. In the case of lovastatin biosynthesis, label loss at C-6 could in principle be attributed to enolization of a carbonyl-containing intermediate to assist the methylation reaction. It is not clear why this should occur at C-6 during formation of **2** or **3**. None of the late stage transformations detected by Endo and co-workers[33–35] account for this hydrogen loss, nor would the hexaketide intermediate immediately prior to the putative enzymatic Diels–Alder reaction[31,32,37] be expected to have lost all label at this position.

The remaining sites generally have anticipated levels of deuterium labelling; there is retention of up to three deuterium labels at the starter methyls (methyl at C-2 as well as at the side chain), one deuterium at C-8, and two at C-12 and C-14. Only a single deuterium was detected at C-10, but this may just reflect higher levels of exchange during assembly because the carbon–oxygen bond at C-11 is derived intact from acetate. Analysis of the situation at C-1 and C-2′ is more complicated. A substantial

level of isotope-shifted signal ($\Delta\delta$ 40 ppb, 2:3 ratio unshifted : shifted signal) was detected by ^1H,^2H-decoupled ^{13}C NMR spectrometry at C-1 after incorporation of [2-^{13}C, 2-$^2H_3$]acetate. However, this appears to be at least partly due to a γ-isotope shift caused by adjacent incorporation of another labelled acetate unit bearing three deuteriums at the C-2 methyl group, as demonstrated by an attached proton test (APT) NMR experiment. The α-isotope shift is also of unexpectedly small magnitude. Examination of this site after incorporation of [1-^{13}C, 2-$^2H_3$]acetate shows a significant β-isotope shift at the reporter resonance, C-9, but with a significantly different ratio ($\Delta\delta$ 91 ppb, 88:12 ratio unshifted : shifted signal). Similar effects are seen at C-2′ of **3**. It appears that both C-1 and C-2′ may be labelled by deuterium, but multiple incorporations of labelled acetate units in the same molecule complicate the analysis and obscure the isotope effects at these two sites.

Summary

The current study shows that formation of lovastatin **4** by *A. terreus* and generation of compactin **3** and ML-236C **2** by *P. aurantiogriseum* proceed by very similar pathways. Separate incorporations of sodium [1-^{13}C, $^{18}O_2$]acetate and $^{18}O_2$ gas into **4** demonstrate that intact carbon–oxygen bonds of acetate are retained at all sites except C-8, where the singly-bonded oxygen atom is aerobically introduced. Hence our original suggestion[31] of late stage aerobic oxidation at C-11, C-13 and C-15 is incorrect, and 4a,5-dihydromonacolin L **1** is likely to be the first fully-assembled compound released from the lovastatin PKS enzyme. The initial incorrect proposal[31] was prompted by failure to resolve unexpectedly small ^{18}O-induced isotope shifts in ^{13}C NMR spectra coupled with mass spectral observation of real aerobic oxygen incorporation at those sites. The latter, which has now been verified, clearly arises from an unusual shunt pathway, which probably involves generation of [^{18}O]acetate from fats through ω-oxidation followed by β-oxidation. The current study provides the only example of this phenomenon which we are aware of in an investigation of secondary metabolism. It clearly emphasizes the need for [^{18}O]acetate incorporation experiments to complement labelling studies with $^{18}O_2$ to ensure correct attribution of the biological source of oxygen atoms in fungal metabolites. The present results are in accord with the possible involvement of an enzyme-catalysed Diels–Alder reaction during biosynthesis of the bicyclic skeleton of **2**, **3** and **4**, as outlined in Scheme 1. Further investigations to verify this proposal with cell-free systems are ongoing.

Experimental

General

The instrumentation and procedures used throughout have been previously described.[45] Fermentation procedures were adapted from those published[31] unless otherwise noted. Oxygen-18 gas and sodium [1-^{13}C, 2-$^2H_3$]- and [2-^{13}C, 2-$^2H_3$]-acetate (99% ^{13}C, 98% ^2H) were obtained from Cambridge Isotope Laboratories (CIL) (Andover, MA). Potassium [1-^{13}C, $^{18}O_2$]acetate was prepared *via* hydrolysis of ^{13}C labelled MeCN (99% ^{13}C, from CIL) with 1.1 equiv. of $H_2{}^{18}O$ (98.6% ^{18}O, obtained from Isotek Inc., Miamisburg, OH).[46] The isotopic content of the resultant potassium acetate (78% ^{18}O) was measured by FAB MS in the normal mode using glycerol as a matrix. The ^1H and ^{13}C NMR spectra of **2** and **3** were recorded in $CDCl_3$ and were analysed using standard techniques, such as attached proton test (APT) ^{13}C spectroscopy, COSY, HMQC and HMBC.[40] Analysis of **3** derived from sodium [2-^{13}C, 2-$^2H_3$]acetate incorporations for α-isotope shifted peaks[44] required a triple resonance probe with broad band ^1H and ^2H decoupling and ^{19}F lock (C_6F_6 internal standard).[31,47]

Fermentation of *Aspergillus terreus* (MF 4845) and *Penicillium aurantiogriseum* (ATCC 60762) and isolation of lovastatin 4, compactin 3 and ML-236C 2

Aspergillus terreus MF 4845 (gift from Merck Inc.) was grown and lovastatin (mevinolin) **4** was isolated and purified as previously described.[31] *Penicillium aurantiogriseum* ATCC 60762 (American Type Culture Collection, Rockville, MD, a gift from Dr Horace G. Cutler, Agricultural Research Service, Athens, GA) was grown as described[48] in 500 ml Erlenmeyer flasks with the following modifications. A single twelve to nineteen-day-old cereal–agar slant (per 250 ml: 25 g of Mead Johnson Mixed Cereal Pablum, 5 g of Bacto-Agar, grown at 25 °C in the dark) was used to inoculate a growth medium consisting of 100 ml of sterile media **A**[49] in which Mixed Cereal Pablum was substituted for oat flour. This was incubated on a rotary shaker (220 rpm) for 24 h at 28 °C. Then 10 ml aliquots of the growth media were used to inoculate seven flasks containing 140 ml of production media consisting of 100 mM succinic acid (pH 4.0) MGPB broth (per litre Milli-Q water: 30 g malt extract, 20 g dextrose, 1 g peptone, 11.8 g succinic acid), which were incubated identically to the growth culture for 72 h. Labelled precursors were injected into each flask with 1.0 ml of sterile aqueous labelled sodium or potassium acetate solution (1.0 and 1.2 g in 56 ml, respectively) every 24 h for 8 d. After an additional 24 h, the combined fermentation mixture was extracted and the hydroxy-acid forms of **2** and **3** were lactonized to the corresponding lactones. Concentration *in vacuo* and column chromatography on silica gel eluting with EtOAc–light petroleum (gradient elution: 1:9 to 8:2) afforded a crude mixture of **2** and **3**, which was separated by preparative HPLC (RP-18 silica gel, MeCN–H_2O gradient elution: 40:60 to 55:47, relative retention time of **2**: 28.6 min; of **3**: 35.3 min).

ML-236C 2. $\nu_{max}(CHCl_3)/cm^{-1}$ 3416, 2926, 1709; δ_H(400 MHz, $CDCl_3$, 25 °C, 0.15 M) 0.88 (3 H, d, *J* 7, 2-Me), 1.04 (1 H, 8-H_a), 1.25–1.55 (3 H, m, 1-H, 9-H_a, 10-H_a), 1.55–1.90 (5 H, m, 7-H, 9-H_b, 10-H_b, 12-H_a), 1.90–2.00 (2 H, m, 8-H_b, 12-H_b), 2.00–2.20 (4 H, m, OH, 6-H, 8_a-H), 2.20–2.45 (1 H, m, 2-H), 2.59 (1 H, m, *J* 18, 4, and 2, 14-H_a), 2.71 (1 H, dd, *J* 18 and 5, 14-H_b), 4.36 (1 H, m, *J* 4, 13-H), 4.67 (1 H, m, 11-H), 5.46 (1 H, br s, 5-H), 5.67 (1 H, dd, *J* 10 and 6, 3-H), 5.89 (1 H, d, *J* 10, 4-H) (HRMS: found, 290.1872. Calc. for $C_{18}H_{26}O_3$, 290.1882).

Compactin 3. $\nu_{max}(CHCl_3)/cm^{-1}$ 3440, 2962, 2932, 1725; δ_H(500 MHz, $CDCl_3$, 25 °C, 0.15 M) 0.88 (3 H, t, *J* 8, 4′-H), 0.89 (3 H, d, *J* 7, 2-Me), 1.11 (3 H, d, *J* 7, 2′-Me), 1.30 (1 H, m, 10-H_a), 1.35–1.45 (2 H, m, 3′-H_a, 9-H_a), 1.5 (1 H, m, 9-H_b), 1.60–1.72 (4 H, m, 1-H, 3′-H_b, 7-H_a, 12-H_a), 1.85 (1 H, m, 10-H_b), 1.97 (1 H, m, *J* 15, 12-H_b), 2.07–2.17 (3 H, m, OH, 6-H_a, 7-H_b), 2.28–2.41 (4 H, m, 2-H, 2′-H, 6-H_b, 8_a-H), 2.62 (1 H, m, *J* 18, 4, and 2, 14-H_a), 2.71 (1 H, dd, *J* 18 and 5, 14-H_b), 4.35 (1 H, m, *J* 4, 13-H), 4.62 (1 H, m, 11-H), 5.32 (1 H, br t, *J* 4, 8-H), 5.54 (1 H, br d, *J* 3, 5-H), 5.72 (1 H, dd, *J* 10 and 6, 3-H), 5.96 (1 H, d, *J* 10, 4-H) (HRMS: found, 390.2410. Calc. for $C_{23}H_{34}O_5$, 390.2406).

Incorporation of $^{18}O_2$ into lovastatin 4

The procedure and apparatus previously described[31] was adapted through use of shaker flasks in series rather than a circulating fermentor.[50] The first stage of the fermentation was done as above using a normal atmosphere and sterile media **A**. A series of shaker flasks (125 ml production medium in 500 ml flask) were inoculated with 10 ml aliquots of the growth media and connected to a closed system with a pump operating at a flow rate of 250 ml min^{-1}. To this closed system $^{18}O_2$ gas (50% isotopic purity) was introduced from the first to the seventh day, during which a total of 4.75 litres of O_2 was consumed. The remainder of the fermentation was completed with addition of $^{16}O_2$ gas. On the tenth day the mixture was extracted, and lovastatin (5.4 mg) was isolated as described before. Recrystallization gave 1.3 mg of pure lovastatin for spectral analysis. Chemical ionization mass spectrometry (NH_3) showed an enhancement of M + 2 (32%)

for the sample from the $^{18}O_2$ experiment compared to an unlabelled standard. An increase in the M + 4 peak could be seen, but no significant enhancement of peaks at higher *m/z* could be detected. The ^{1}H-decoupled ^{13}C NMR spectrum ($CDCl_3$, 75 MHz) showed that only the resonance for C-8 (at δ 68.0) had an isotope shift ($\Delta\delta$ 0.034 ppm) due to the incorporation of ^{18}O (proton decoupling, pulse width 14.5 s).

Conversion of lovastatin 4 and compactin 3 to corresponding tetraacetates 6 and 7

A solution of **4**, obtained from sodium [1-^{13}C, $^{18}O_2$]acetate, in dry diethyl ether (8 ml) at 0 °C was treated dropwise with a slurry of $LiAlH_4$ (59 mg) in dry diethyl ether (2 ml). The mixture was stirred and allowed to warm to 20 °C over 3 h. Then 60 μl water, 60 μl aqueous NaOH and 180 μl water were added sequentially. The organic phase was dried ($MgSO_4$) and filtered through Celite. The solvent was evaporated *in vacuo* to give tetraol **5** (67 mg, 80%), which was used without further purification. A solution of this tetraol (47 mg) in dry CH_2Cl_2 (5 ml) was treated with dry pyridine (1 ml), Ac_2O (0.5 ml) and catalytic DMAP at 0 °C. The mixture was stirred under Ar at 20 °C overnight, poured into ice and extracted with EtOAc (3 equal volumes). The combined extracts were washed with 5% $CuSO_4$, water, 5% $NaHCO_3$ and saturated brine. The solution was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was purified by column chromatography on SiO_2 (EtOAc–hexanes, 1:4) to give **6** as an oil (67 mg, 94%), $\nu_{max}(CHCl_3)/cm^{-1}$ 1737, 1243; δ_H(400 MHz, $CDCl_3$, 25 °C) 5.96 (1 H, d, *J* 9.7), 5.75 (1 H, dd, *J* 9.7 and 6.1), 5.55 (1 H, br s), 5.30 (1 H, dd, *J* 5.7 and 3.2), 4.99 (1 H, m), 4.82 (1 H, m), 4.05 (2 H, t, *J* 6.4), 2.41 (1 H, m), 2.32 (1 H, m), 2.19 (1 H, m), 2.02 (6 H, s), 2.01 and 1.98 (each 3 H, s), 1.93–1.78 (6 H, m), 1.73–1.55 (4 H, m), 1.10 (1 H, m), 1.04 (3 H, d, *J* 7.4), 0.88 (3 H, d, *J* 7.0); δ_C(100.6 MHz, $CDCl_3$) 170.90, 170.87, 170.6, 170.4, 133.1, 131.7, 129.6, 128.3, 70.9, 68.2, 67.9, 60.5, 38.2, 37.2, 36.2, 33.1, 32.4, 30.7, 30.6, 27.4, 23.1, 22.6, 21.3, 21.1, 21.05, 20.9, 13.8 (HRMS: found, 492.2725. Calc. for $C_{27}H_{40}O_8$, 492.2723).

A similar procedure was used to convert 25 mg of compactin **3** obtained from sodium [1-^{13}C, $^{18}O_2$]acetate to the corresponding tetraacetate **7** in 27% overall yield, δ_H(400 MHz, $CDCl_3$, 25 °C) 5.95 (1 H, d, *J* 9.7), 5.71 (1 H, dd, *J* 9.7 and 6.1), 5.53 (1 H, br s), 5.29 (1 H, br s), 5.00 (1 H, m), 4.82 (1 H, m), 4.05 (2 H, t, *J* 6.8), 2.30 (2 H, m), 2.15 (3 H, m), 2.03 (6 H, s), 2.01 (6 H, s), 1.97–1.75 (3 H, m), 1.75–1.54 (5 H, m), 1.54–1.38 (1 H, m), 1.25–1.10 (1 H, m), 0.85 (3 H, d, *J* 7.0); δ_C(100.6 MHz, $CDCl_3$) 170.97, 170.92, 170.56, 170.42, 133.54, 132.69, 128.14, 123.82, 70.97, 68.22, 67.91, 60.55, 38.24, 37.47, 36.36, 33.11, 30.74, 30.68, 26.19, 22.99, 21.10, 21.08, 21.06, 20.95, 20.87, 13.83 (HRMS: found, 478.2552. Calc. for $C_{26}H_{38}O_8$, 478.2567).

***N*-Acetylcysteamine thiol ester of [1-^{13}C, $^{18}O_2$]acetate 8**

To 0.72 g of sodium [1-^{13}C, $^{18}O_2$]acetate (90% 1-^{13}C, 90–97% ^{18}O) was added 2.74 g of PCl_5 under Ar, and the mixture was heated to 80 °C with gentle stirring. The product, [1-^{13}C, $^{18}O_2$]acetyl chloride, was collected by distillation at 38–52 °C. The acetyl chloride (0.059 g, 88%) was used directly in the next reaction. To a solution of *N*-acetylcysteamine (1.72 g, obtained from partial hydrolysis of diacetylcysteamine) in dry CH_2Cl_2 (5.0 ml) at 0 °C under Ar was added dry pyridine (1.17 ml) and the labelled acetyl chloride. The mixture was stirred for 18 h at 20 °C, and then poured onto ice. The aqueous layer was extracted with EtOAc (2 equal volumes). The combined extracts were washed with 1 M HCl, 5% $NaHCO_3$ and saturated brine. The solution was dried ($MgSO_4$) and the solvent removed *in vacuo*. The product was purified by SiO_2 column chromatography with 50% MeOH in EtOAc as eluent to yield 357 mg (30%) of **8**, $\nu_{max}(CHCl_3)/cm^{-1}$ 3284, 1655, 1623, 1550; δ_H(300 MHz, $CDCl_3$) 6.01 (1 H, br, N*H*), 3.39 (2 H, q, *J* 6.3, NHCH_2), 2.98 (2 H, dt, *J* 4.9 and 6.4, CH_2S), 2.32 (2.7 H, d, *J* 6.2, 0.3 H, s, $^{13}C^{18}OCH_3$), 1.93 (3 H, s); δ_C(75 MHz, $CDCl_3$) 196.2, 170.3, 39.5, 30.5 (d, *J* 47.5), 28.7, 23.1 (HRMS: found, 164.0577. Calc. for $^{12}C_5{}^{13}CH_{11}{}^{16}O^{18}ONS$, 164.0587).

Incorporation of *N*-acetylcysteamine thiol ester 8 into 4

Fermentation was carried out in the same fashion as with sodium [1-^{13}C, $^{18}O_2$]acetate. From the third day to the fifth day, $Me^{13}C^{18}OSCH_2CH_2NHCOMe$ (159 mg in 3 ml of 99% EtOH) was added to the medium, along with the corresponding unlabelled compound (159 mg in 3 ml of 99% EtOH) (each 0.25 ml per flask × 4 flasks × 3 d) every 24 h. On the ninth day, the fermentation mixture was extracted and lovastatin **4** was purified and recrystallized to give 63 mg of product. The mass spectra [both electron impact and chemical ionization (NH_3)] were essentially identical to those of unlabelled standard. The ^{13}C NMR spectrum showed very slightly increased isotopic enrichment compared to sodium [1-^{13}C, $^{18}O_2$]acetate.

Acknowledgements

The authors thank Dr Nancy Fregeau, Dr Thomas T. Nakashima and Mr Glen Bigam for valuable discussions and technical assistance. Cambridge Isotope Laboratories (Andover, MA) generously provided isotopically labelled starting materials through the CIL Research Grant Program. Merck Research Laboratories (Rahway, NJ) donated freeze-dried specimens of *Aspergillus terreus* MF 4845. These investigations were supported by the Natural Sciences and Engineering Research Council of Canada, the Alberta Heritage Foundation for Medical Research and the National Institutes of Health (NIH fellowship F32 GM16207 to K. W.).

References

1 A. Endo and K. Hasumi, *Nat. Prod. Rep.*, 1993, **28**, 541; A. Endo, *J. Lipid Res.*, 1992, **33**, 1569.
2 D. L. J. Clive, P. L. Wickens and G. V. J. da Silva, *J. Org. Chem.*, 1995, **60**, 5532.
3 L. Novak, G. Hornyanszky, J. Rohaly, P. Kolonits and C. Szantay, *Liebigs Ann. Chem.*, 1995, 1877.
4 G. Solladié, C. Bauder and L. Rossi, *J. Org. Chem.*, 1995, **60**, 7774.
5 J. Schnaubelt and H.-U. Reissig, *Synlett*, 1995, 452.
6 D. A. Hopwood and D. H. Sherman, *Annu. Rev. Genet.*, 1990, **24**, 37.
7 L. Katz and S. Donadio, *Annu. Rev. Microbiol.*, 1993, **47**, 875.
8 D. O'Hagan, *Nat. Prod. Rep.*, 1995, **12**, 1.
9 J. Cortes, K. E. H. Wiesmann, G. A. Roberts, M. J. B. Brown, J. Staunton and P. F. Leadlay, *Science*, 1995, **268**, 1487.
10 C. R. Hutchinson and I. Fujii, *Annu. Rev. Microbiol.*, 1995, **47**, 201.
11 R. McDaniel, S. Ebert-Khosla, D. Hopwood and C. Khosla, *Nature*, 1995, **375**, 549.
12 K. E. H. Wiesmann, J. Cortés, M. J. B. Brown, A. L. Cutter, J. Staunton and P. Leadlay, *Chem. Biol.*, 1995, **2**, 583.
13 R. Pieper, G. Luo, D. E. Cane and C. Khosla, *J. Am. Chem. Soc.*, 1995, **117**, 11373; R. Pieper, G. L. Luo, D. E. Cane and C. Khosla, *Nature*, 1995, **378**, 263; R. Peiper, S. Ebert-Khosla, D. E. Cane and C. Khosla, *Biochemistry*, 1996, **35**, 2054.
14 M. J. B. Brown, J. Cortes, A. L. Cutter, P. F. Leadlay and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1995, 1517.
15 R. McDaniel, C. R. Hutchinson and C. Khosla, *J. Am. Chem. Soc.*, 1995, **117**, 6805.
16 J.-H. Yu and T. J. Leonard, *J. Bacteriol.*, 1995, **177**, 4792.
17 W. P. Revill, M. J. Bibb and D. A. Hopwood, *J. Bacteriol.*, 1995, **177**, 3946.
18 C. M. Kao, G. Luo, L. Katz, D. E. Cane and C. Khosla, *J. Am. Chem. Soc.*, 1995, **117**, 9105.
19 J. Staunton, P. Caffrey, J. F. Aparicio, G. A. Roberts, S. S. Bethell and P. F. Leadlay, *Nat. Struct. Biol.*, 1996, **3**, 188; J. F. Aparicio, I. Molnar, T. Schwecke, A. Konig, S. F. Haydock, L. E. Khaw, J. Staunton and P. F. Leadlay, *Gene*, 1996, **169**, 9.
20 S. Yue, J. S. Duncan, Y. Yamamoto and C. R. Hutchinson, *J. Am. Chem. Soc.*, 1987, **109**, 1253.
21 D. E. Cane and C.-C. Yang, *J. Am. Chem. Soc.*, 1987, **109**, 1255.
22 Z. M. Spavold and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1988, 4.
23 J. Staunton and A. C. Sutkowski, *J. Chem. Soc., Chem. Commun.*, 1991, 1110.

24 Y. Yoshizawa, Z. Li, P. B. Reese and J. C. Vederas, *J. Am. Chem. Soc.*, 1990, **112**, 3212.
25 Z. Li, F. M. Martin and J. C. Vederas, *J. Am. Chem. Soc.*, 1992, **114**, 1531.
26 D. E. Cane, R. H. Lambalot, P. C. Prabhakaran and W. R. Ott, *J. Am. Chem. Soc.*, 1993, **115**, 522.
27 H. Patzelt and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1993, 1258.
28 H. C. Hailes, C. M. Jackson, P. F. Leadlay, S. V. Ley and J. Staunton, *Tetrahedron Lett.*, 1994, **35**, 307.
29 D. E. Cane, W. Tan and W. R. Ott, *J. Am. Chem. Soc.*, 1993, **115**, 527; D. E. Cane and G. Luo, *J. Am. Chem. Soc.*, 1995, **117**, 6633.
30 Y. S. Tsantrizos, F. Zhou, P. Famili and X. S. Yang, *J. Org. Chem.*, 1995, **60**, 6922.
31 R. N. Moore, G. Bigam, J. K. Chan, A. M. Hogg, T. T. Nakashima and J. C. Vederas, *J. Am. Chem. Soc.*, 1985, **107**, 3694.
32 Y. Yoshizawa, D. J. Witter, Y. Liu and J. C. Vederas, *J. Am. Chem. Soc.*, 1994, **116**, 2693.
33 A. Endo, Y. Negishi, T. Iwashita, K. Mizukawa and M. Hirama, *J. Antibiot.*, 1985, **38**, 444.
34 K. Kimura, D. Komagata, S. Murakawa and A. Endo, *J. Antibiot.*, 1990, **43**, 1621.
35 T. Nakamura, D. Komagata, S. Murakawa, K. Sakai and A. Endo, *J. Antibiot.*, 1990, **43**, 1597.
36 H. Oikawa, K. Katayama, Y. Suzuki and A. Ichihara, *J. Chem. Soc., Chem. Commun.*, 1995, 1321.
37 D. J. Witter and J. C. Vederas, *J. Org. Chem.*, 1996, **61**, 2613.
38 R. Rognstad, *Biochem. Arch.*, 1995, **11**, 1.
39 J. C. Vederas, *Nat. Prod. Rep.*, 1987, **4**, 277.
40 *NMR of Macromolecules, a Practical Approach*, ed. G. C. K. Roberts, Oxford University Press, Oxford, 1993.
41 M. P. Lane, T. T. Nakashima and J. C. Vederas, *J. Am. Chem. Soc.*, 1982, **104**, 913.
42 J. C. Vederas, *J. Am. Chem. Soc.*, 1980, **102**, 374.
43 J. B. Spencer and P. M. Jordan, *Biochemistry*, 1992, **31**, 9107.
44 M. J. Garson and J. Staunton, *Chem. Soc. Rev.*, 1979, 539.
45 J. M. Harris, E. A. Bolessa, A. J. Mendonca, S.-C. Feng and J. C. Vederas, *J. Chem. Soc., Perkin Trans. 1*, 1995, 1945.
46 D. E. Cane, T.-C. Liang and H. Hasler, *J. Am. Chem. Soc.*, 1982, **104**, 7274; H. Noguchi, P. H. Harrison, K. Arai, T. T. Nakashima, L. A. Trimble and J. C. Vederas, *J. Am. Chem. Soc.*, 1988, **110**, 2938.
47 B. J. Rawlings, P. B. Reese, S. E. Ramer and J. C. Vederas, *J. Am. Chem. Soc.*, 1989, **111**, 3382.
48 W. A. Bazaraa, Ph.D. Thesis, University of Georgia, 1990 (*Chem. Abstr.*, 1991, **115**, 278034; *Diss. Abstr. Int. B*, 1991, **52**, 592).
49 A. W. Alberts, J. Chen, G. Kuron, V. Hunt, J. Huff and C. Hoffman, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 3957.
50 J. C. Vederas, in *Mycotoxins and Phycotoxins*, ed. Steyn and Vleggaar, Elsevier, Amsterdam, 1986, vol. 97.

Paper 6/02532C
Received 11*th April* 1996
Accepted 2*nd July* 1996