The biosynthesis of pramanicin in *Stagonospora* sp. ATCC 74235: a modified acyltetramic acid †

Paul H. M. Harrison,* Petar A. Duspara, Stephen I. Jenkins, Salima A. Kassam, David K. Liscombe and Donald W. Hughes

Department of Chemistry, McMaster University, 1280 Main Street West, Hamilton, Ontario, L8S 4M1, Canada

Received (in Cambridge, UK) 25th July 2000, Accepted 28th September 2000
First published as an Advance Article on the web 22nd November 2000

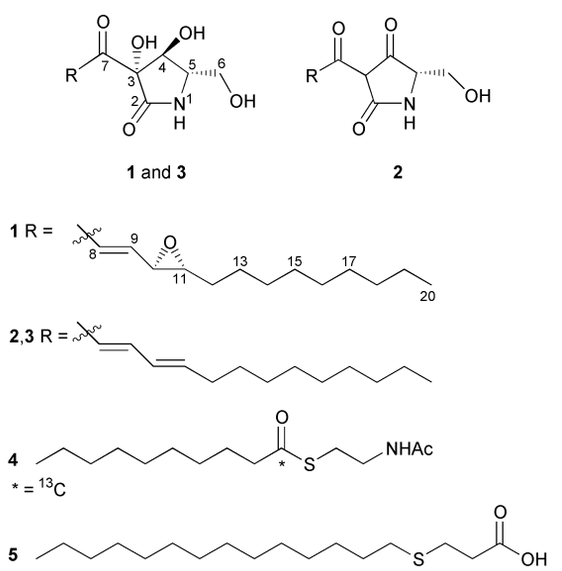
Biosynthetic incorporations of acetate, malonate and serine precursors which had been isotopically labelled with ^{2}H, ^{13}C, ^{15}N and ^{18}O into pramanicin **1** in *Stagonospora* sp. ATCC 74235 were demonstrated. Intact incorporation of a starter acetate and six extender malonates generates the acyclic, hydrophobic tail. A further intact acetate, in preference to malonate, and a serine entity which is incorporated only as the L-enantiomer and with the O=C–CH(N)–CH_2 entity intact, provide the pyrrolidone ring and hydroxymethyl group of **1**. The results are fully consistent with a biosynthetic pathway involving an acyltetramic acid (**2**). The olefinic precursor **3** of the epoxide in **1** is described, and is also shown to co-occur in the cultures. The ratio of **1**:**3** can be controlled by addition of precursors.

Introduction

Pramanicin **1** is a natural product first isolated by bioassay-directed fractionation in 1994 by Schwartz *et al.* from an unidentified fungus which was subsequently shown to be a member of the genus *Stagonospora*.[1] The connectivity in the structure was determined by spectroscopic analysis, and the proton and carbon-13 NMR resonances were carefully assigned by one- and two-dimensional NMR techniques. Further, the relative configuration within the pyrrolidone ring was established, and it was shown that the substituents on the epoxide were in the *trans* orientation. However, none of the absolute configurations was established. Pramanicin was shown to exhibit modest antifungal activity against a range of microbes including the human opportunistic pathogen *Cryptococcus neoformans*, the causative agent of cryptococcal meningitis. Notably, pramanicin is active against both the acapsular and capsular forms. Recently we have shown that pramanicin also exhibits intriguing biological effects on vascular endothelial cells, and have suggested that it may act by increasing calcium permeability in the cell.[2]

We have published two preliminary reports on our studies of the biosynthesis of **1** in *Stagonospora* sp. ATCC 74235, which have shown that the carbon skeleton originates from eight acetate units and one intact L-serine entity.[3,4] These results suggest that **1** is formed *via* an acyltetramic acid derivative (*e.g.*, **2**) which is subsequently modified by oxidation at C-3, keto-reduction at C-4, and formation of the epoxide from the olefin at C-10/C-11, in an as-yet-undetermined order, to furnish the natural product. The acyltetramic acids[5] are an intriguing group of natural products that includes, for example, cyclopiazonic acid, and which are formed biosynthetically by condensation of acetate units with an amino acid.[6–14] Incorporation of L-serine with retention of all four bonds to the α-carbon showed that **1** has the corresponding 5*S* absolute configuration, a conclusion also reached by Barrett *et al.* who recently synthesised the enantiomer of **1**, thus also confirming the absolute configuration of the epoxide moiety to be as depicted.[15]

Herein, we describe these earlier results in detail, as well as



further incorporation experiments designed to test this biosynthetic hypothesis. In particular, this postulate has implications for the biogenesis of the oxygen atoms. These new experiments demonstrate an oxygen-labelling pattern which is fully consistent with the intermediacy of an acyltetramic acid, and which excludes some reasonable alternative biosynthetic sequences. Data to differentiate between various mechanisms of pyrrolidone-ring formation are presented and discussed. The isolation and characterisation of co-metabolite **3** is also described.

Results

A. Culture of *Stagonospora* sp. ATCC 74235, and isolation of 1 and 3

Pramanicin **1** was readily and consistently produced by cultures of *Stagonospora* sp. ATCC 74235, essentially under the growth, isolation and purification conditions reported by Schwartz *et al.*[1] We found that reduction of the glucose levels in the medium, from 75 to 40 and even to 10 g dm^{-3}, had essentially

† The IUPAC name for tetramic acid is 2,5-dihydro-4-hydroxypyrrol-2-one.

 DOI: 10.1039/b006007k

no adverse effect on production of **1**, although this modification improved the level of incorporation in biosynthetic experiments (see below). During the final phase of purification, which we found could be conducted conveniently by reversed-phase MPLC on a C_8 column, a small amount of a related natural product (**3**) was frequently isolated, which was slightly more strongly retained than was **1**. Subsequent biosynthetic experiments (see below) using *N*-acetyl-*S*-[1-^{13}C]decanoylcysteamine **4** and the known β-oxidation inhibitor 3-(tetradecylthio)-propanoic acid[16] **5** resulted in alteration of antibiotic production so that smaller amounts of pramanicin were produced, while the co-metabolite level was enhanced. HPLC analysis of aliquots of culture medium from controls, as well as flasks to which were added **4** in ethanol; **5**; or ethanol alone, showed that **4** and **5** each cause an increase in formation of the co-metabolite, while neither completely abolished production of **1**. The combination of **4** and **5** essentially completely inhibited formation of **1** under these conditions, resulting in **3** as the only significant product. This HPLC result was confirmed by preparative incubations, which resulted in the exclusive isolation of **3**.

Preliminary analysis of this compound by proton and carbon-13 NMR immediately suggested structure **3**, a reasonable proposal on biosynthetic grounds. Compound **3** was reported by the Merck group as the product of treatment of pramanicin with potassium selenocyanate,[1] a reagent known to convert epoxides stereospecifically to olefins.[17] However, the spectral data reported for the product of this reaction did not precisely match those for material from our cultures.

The natural material clearly possessed a long aliphatic chain, and the polar pyrrolidone head group exhibited proton resonances in the NMR spectrum at essentially identical shifts, and with similar coupling constants to **1** (Table 1). The NMR signals for the epoxide protons were missing, but there were resonances for four olefinic protons as opposed to the two in pramanicin: a doublet and a doublet of doublets, attributable to H-8 and H-9 respectively, were still present, with $J_{8\text{–}9}$ 15.1 Hz (*cf.* 15.6 Hz in **1**), supporting *trans* (*E*) stereochemistry for this olefin. The remaining two olefinic protons (H-10 and H-11) formed a strongly coupled overlapping multiplet in methanol-d_4, a result that seemed different to that reported by the Merck group. It was thus not immediately possible to assign stereochemistry to this second double bond. Therefore it was feasible that the organism had produced an isomer of **3**, *e.g.* the 10,11-*cis* (*Z*) olefin, perhaps as a shunt metabolite, or that the KSeCN treatment of **1** in the previously reported work had caused some undetected effect above and beyond the expected deoxygenation of the epoxide (*e.g.*, production of *cis* olefin). We thus sought further confirmation of the structure.

One-dimensional proton and carbon-13 spectra were recorded at high resolution in methanol-d_4 (Table 1), as well as in DMSO-d_6; the latter solvent revealed the presence of an NH proton as well as protons of OH groups bonded to a primary, a secondary and a tertiary centre. Next followed proton COSY and inverse-detected one- and multiple-bond proton–carbon correlated spectroscopy; the results of these experiments are summarised in Fig. 1 and Table 1. Although all of the data were completely consistent with structure **3**, these results were still inconclusive with respect to double-bond geometry. Several solvents were evaluated in order to enhance the resolution of the H-10 and H-11 protons and to measure the coupling constant between them. The largest shift difference was found in acetonitrile-d_3, but strong coupling effects even at 500 MHz were still present. Spectral simulation allowed a good fit for this system (Fig. 2), provided that the H-10–H-11 coupling constant was ≈15 Hz, supporting a *trans* geometry at this double bond.

Chemical evidence also favoured structure **3**. In our hands, the selenocyanate reaction of **1** described by the Merck group proved inefficient, but did furnish a small amount of product

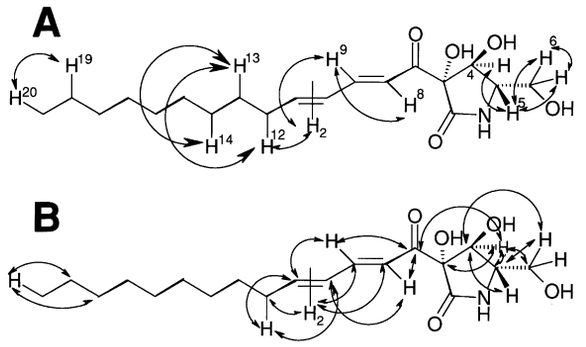


Fig. 1 Summary of results from two-dimensional NMR experiments which support structure **3**: unambiguous correlations were determined from A: the COSY spectrum, and B: the long-range ^{13}C–^{1}H heteronuclear shift-correlated spectrum. One-bond C–H correlations are given in Table 1.

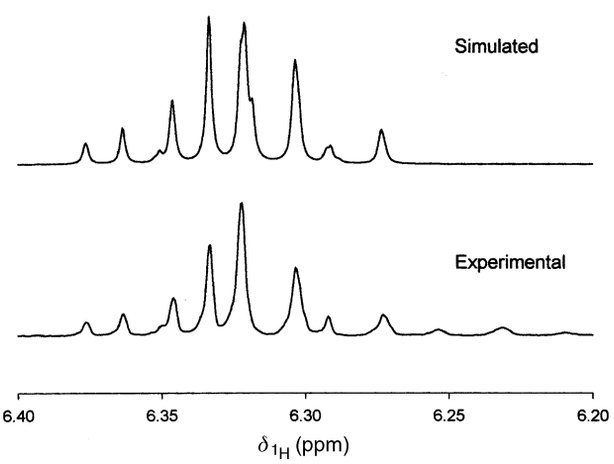


Fig. 2 Experimental and calculated proton NMR spectra of natural product **3** for H-10 and H-11. The parameters for the calculated spectrum are $\Delta\delta$(10–11) 0.041 ppm and (in Hertz): $J_{12a\text{-}12b}$ −13; $J_{11\text{-}12a}$ = $J_{11\text{-}12b}$ = 7.0; $J_{10\text{-}11}$ 15.1; $J_{11\text{-}9}$ −1; $J_{10\text{-}9}$ 10. Trace impurities are seen in the experimental spectrum.

with identical retention time on HPLC to **3** produced by fermentation. In contrast, we found that treatment of pramanicin with NaI and toluene-*p*-sulfonic acid according to the procedure of Baruah *et al.* which is also known to retain the configuration of the epoxide substituents,[18] provided **3** in good yield (72%). This material was identical in all respects (HPLC, proton and carbon-13 NMR, MS) with the fermentation product. These experiments established the structure of the co-metabolite to be **3**; however, we remain unable to explain the reported results with potassium selenocyanate.

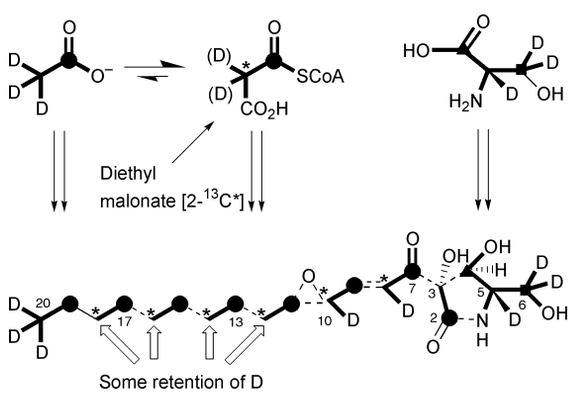
B. Labelling of acetate-derived sites

Preliminary experiments investigated the incorporation of sodium [1-^{13}C]acetate into pramanicin in cultures of *Stagonospora* sp. ATCC 74235 essentially under the growth conditions reported by Schwartz *et al.*,[1] with precursor addition on days 5–8 and isolation on day 9. The resulting **1** showed small (≈0.3–0.4% site^{-1}) but significant incorporation of label into C-2 and seven other sites within the acyl side-chain (Scheme 1). In order to improve the specific incorporation rate, a systematic variation of both the time of precursor addition and the glucose concentration was undertaken. These experiments showed that reduction of glucose from 70 to 40 g dm^{-3}, along with earlier initiation of precursor feeding (days 3–6), improved incorporation substantially. The combination of these variations resulted in pramanicin labelled to the extent of 1.7 to 2.3% per site (Table 1). Label was located at each of the sites C-2, C-7, C-9, C-11, C-13 and C-19, which had been unambiguously assigned in the carbon-13 NMR spectrum of **1** (Scheme 1).

Table 1 Carbon-13 chemical shifts for pramanicin **1** and dienone **3**; proton NMR spectral data for **3**; and incorporations for precursors into **1**

C #[a]	$\delta(^{13}C)$ **1**[b] (ppm)	$\delta(^{13}C)$ **3**[b] (ppm)	$\delta(^{1}H)$ **3**[b] (ppm)	Mult **3**[c] (*J* in Hz)	Fed[d]	Incorp(s)[e]
7	197.91	198.70			A	2.3
2	174.93	175.26			A	1.9
9	145.13	145.53	7.27	ddm (15,10)	A	2.2
8	127.88	124.25	6.72	d (15)	B	2.2
3	88.10	88.07			B	1.2
4	78.99	79.01	4.14	d (7)	C; D	5.2; 8[f]
11	62.90	148.58[i]	6.2–6.3[j]	m	A	2.3
6	62.12	62.20	3.80, 3.55	dd (11,2.5), dd (11,5.5)	D; E	8[f]; 7.5[g]
5	60.33	60.39	3.51	ddd (7,5.5,2.5)	D	8[f]
10	57.78	130.48[i]	6.2–6.3[j]	m	B	2.4
12	33.02	34.20	2.19–2.22	m	B	2.0[h]
18	33.02[k]	33.02	1.29	m		
14–17	30.61	30.63	1.29	m	A; B	1.5[h]; 1.2[h]
14–17	30.61	30.53	1.29	m		
14–17	30.48	30.40	1.29	m	B	1.9
14–17	30.39	30.29	1.29	m	A	1.8
13	26.92	29.82	1.44	quintet (7.5)	A	2.1
19	23.69	23.69	1.29	m	A	1.8
20	14.38	14.40	0.89	t (7)	B	1.1

Notes: [a] The numbering system is based on Ref. 1. [b] Determined in CD_3OD. [c] Multiplicity (standard symbols) and coupling constant(s) in Hertz. [d] Precursors for **1**: A: sodium [1-^{13}C]acetate, B: diethyl [2-^{13}C]malonate, C: DL-[1-^{13}C]serine, D: L-[U-^{13}C,^{15}N]serine, E: L-[3-^{13}C,2-^{2}H]serine. [e] Percentage incorporation into **1** is expressed as number of fold increase in the height of the carbon-13 resonance relative to unlabelled **1**, with correction by standardising the two samples using the mean peak height for all unlabelled carbons. [f] Determined by dividing the sum of the coupled peak heights by the height of the uncoupled, natural-abundance peak. [g] Determined by adding enrichments at both the unshifted and isotopically shifted sites. [h] Values averaged over two overlapping carbons. [i] Carbon-13 assignments for C-10 and C-11 derived from the observed labelling pattern. [j] In methanol, these resonances form a complex, strongly coupled system; see text for assignments in acetonitrile. [k] Assignment by analogy with compound **3**.



Scheme 1 Overview of results of biosynthetic incorporation experiments with labelled acetate and serine samples into pramanicin **1**

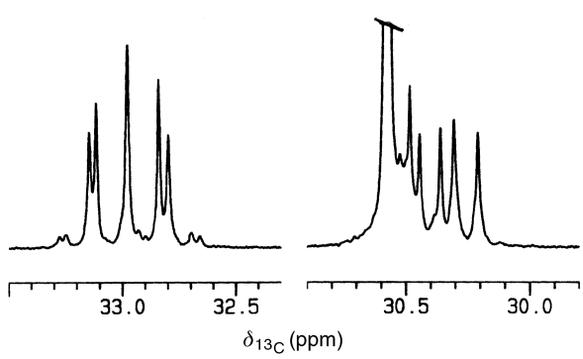


Fig. 3 Sections of the carbon-13 NMR spectrum of pramanicin **1** derived from sodium [1,2-$^{13}C_2$]acetate. Left side: the resonance at δ_C 33, corresponding to C-12 and C-18, exhibits a central, unenriched peak surrounded by two doublets with different coupling constants, corresponding to intact incorporation at both of the different sites. The small peaks at the extreme left and right are due to molecules which contain interunit couplings from adjacent acetate units which are both enriched; this is due to the pulsed feeding protocol. The level of incorporation is 10% of that for signals from intraunit coupling only. Right side: the resonances at δ_C 30.3–30.6, corresponding to carbons from C-14 to C-18, form a complex pattern due to strong coupling. The base peak at δ_C 30.6 is strongly enhanced, despite the absence of single label at any other site, and this must thus be due to suppression of the outside line of the satellites. In addition, natural-abundance peaks at δ_C 30.5 and 30.4 are each surrounded by weakly coupled doublets (see text).

Within the region δ_C 30.3–33, where the five as-yet-unassigned nuclei C-14 to C-18 resonate, the resonance at δ_C 30.39 was enhanced to the same extent as those above. The signal at δ_C 30.61, which results from overlap of resonances from two carbon atoms, was enhanced 1.5-fold, indicative that one of these two sites was labelled to the same extent as above while the other site was not labelled. The signal at δ_C 33.02, which again results from two carbon sites, was not enhanced, so neither of these positions was labelled. Together, the results suggest that **1** is labelled at eight positions from sodium [1-^{13}C]acetate.

Sodium [1,2-$^{13}C_2$]acetate was next incorporated into the *Stagonospora* cultures, giving labelled **1**. Natural-abundance singlet carbon resonances corresponding to each of the atoms C-2, C-3 and C-7 to C-19 were surrounded by doublet signals due to intact incorporation of acetate units (Fig. 3). A combination of high-resolution one-dimensional carbon-13 NMR and two-dimensional inverse-detected gradient INADEQUATE spectroscopy was used to demonstrate which pairs of atoms were coupled to each other. The results showed unambiguously that the four adjacent pairs of atoms C-2 and C-3, C-7 and C-8, C-9 and C-10, and C-19 and C-20 were coupled (Scheme 1). Three further coupled pairs of carbon atoms were observed in the INADEQUATE spectrum. The assigned resonance for C-11 was coupled with a one-bond coupling constant of 43.7 Hz to one of the two overlapping signals at δ_C 33 which must therefore be C-12 and thus C-11–C-12 is an intact acetate unit. The unambiguously assigned C-13 resonance (δ_C 26.9) was coupled to one of the signals at δ_C 30.3–30.6 (^{1}J 34.7 Hz), which must therefore include C-14 and thus C-13–C-14 is an intact acetate unit, even though C-14 cannot be unambiguously assigned. The third correlation that was observed in the INADEQUATE spectrum was between the second signal at δ_C 33, which possessed a one-bond coupling constant (34.7 Hz) that was different from the first signal (Fig. 3), and which

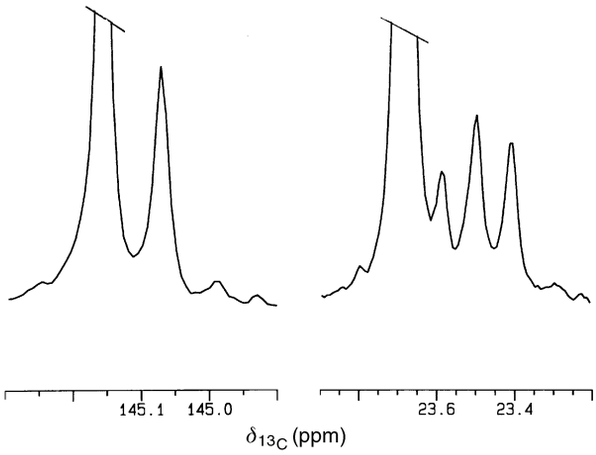


Fig. 4 Sections of the carbon-13 NMR spectrum of pramanicin **1** derived from sodium [1-^{13}C,2-^{2}H$_3$]acetate. Left side: the resonance for C-9 exhibits a peak from a β-deuterium isotope shift, $\Delta\delta$ 0.088 ppm, due to deuterium at C-10. Right side: the resonance for C-19 exhibits three isotopically shifted signals, $\Delta\delta$ 0.088 ppm per D atom, due to the presence of one, two and three deuterium atoms at C-20.

was coupled to one of the remaining signals at δ_C 30.3–30.6; therefore there is another intact acetate unit in the region between C-15 and C-18. No correlation was observed in the INADEQUATE spectrum for the coupling of the two remaining labelled carbons in **1** in this experiment, because these two resonances have very similar chemical shifts (δ_C 30.4–30.6) and are therefore strongly coupled. In this case analysis of the one-dimensional carbon spectrum revealed this strong coupling of the two carbon atoms, confirming the presence of one further intact acetate unit between C-15 and C-18 (Fig. 3). A total of eight intact acetate units was thus found in **1**.

For each resonance for C-3, and C-7 to C-19 in this double-labelled acetate experiment, small doublets of doublets were observed in the 1D-carbon NMR spectrum, surrounding the coupled signals (Fig. 3); these are sites where incorporation of two labelled acetate molecules into two adjacent units has occurred. These satellite signals were missing at the C-2 and C-20 positions, as expected since these positions represent the terminus and start, respectively, of the presumed polyketide chain. The level of labelling for incorporation of two adjacent units was ≈10% of that for labeling of a single unit, for every pair. Such multiple incorporations at adjacent units are a common phenomenon in biosynthetic studies of polyketides such as this, and are the result of the pulsed feeding of acetate.[19]

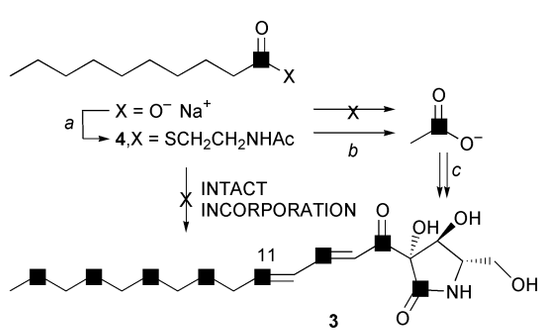
Sodium [1-^{13}C,2-^{2}H$_3$]acetate was incorporated into **1**. Analysis of the carbon-13 NMR spectrum of the derived product showed shifted signals due to the presence of a β-deuterium isotope shift[19,20] at C-9 ($\Delta\delta$ 0.088 ppm), the effect of a single deuterium at C-10; and at C-19, where three signals were observed due to the presence of one, two and three deuterium atoms at C-20 ($\Delta\delta$ 0.088 ppm per deuterium) (Fig. 4 and Scheme 1). No significant isotopically shifted signals were observed at any of the remaining sites which derive from C-1 of acetate. However, this is probably the result of low incorporation of deuterium, signal overlap and/or small isotope shifts,[20] because ^{2}H-NMR of the sample revealed the presence of deuterium in the methylene region and in the olefinic position H-8, as well as in the methyl group as expected. Integration showed that the sum of the levels of deuterium at methylene sites 14, 16 and 18 was less than that at the unique olefinic site H-8.

Next sodium [1-^{13}C,^{18}O$_2$]acetate was prepared by the method of Cane *et al.*.[21] Analysis by both mass spectrometry and high-resolution carbon-13 NMR demonstrated an isotopic incorporation of 99 and 70 atom% ^{13}C and ^{18}O per site, respectively. This material was then used to determine the origin of the oxygen atoms in **1**, using the ^{18}O-induced isotope-shift method.[19] In the high-resolution carbon-13 NMR spectrum of the resulting **1**, isotopically shifted signals were observed at C-2 ($\Delta\delta$ 0.031 ppm) and C-7 ($\Delta\delta$ 0.047 ppm).[19,20] These signals were low in intensity (0.7 and 0.3% incorporation, respectively), while the unshifted signals were enhanced (2.9% incorporation at both sites), implying that considerable exchange of ^{18}O for ^{16}O had occurred within the culture. This exchange is a common phenomenon during biosynthetic studies of polyketides.[19,22]

Diethyl [2-^{13}C]malonate was then incorporated into **1** in cultures of *Stagonospora*. Label was found at each of the sites derived from C-2 of the eight intact acetate units. The extent of incorporation ranged from 1.9 to 2.4% at C-8 and C-10, and by inference at sites C-12 and -18 (based on the average incorporation at the two overlapping resonances at δ_C 33.02) as well as at C-14 and -16 (based on the average incorporation at the two overlapping resonances at δ_C 30.61, and the incorporation at the single, but unassigned, site at δ_C 30.48). By contrast, incorporation levels were lower at C-20 (1.1%) and at C-3 (1.2%). Thus the level of incorporation at the starter acetate unit (C-20) and the terminating unit (C-3) was significantly different from that at the other, malonate-derived extender units (C-8 to C-18).

Since extension of the starter acetate unit by four malonate units is expected to lead to an intermediary decanoyl moiety, sodium [1-^{13}C]decanoate was next tested for incorporation into **1**. Although the resulting **1** was rigorously purified by the standard procedure, the product was contaminated with a small amount of [1-^{13}C]decanoic acid, which co-chromatographs with the target material. The carbon-13 NMR spectrum of this sample showed essentially no incorporation of carbon-13 into **1** (<0.2%), either at the site expected for specific incorporation (C-11) or at any of the sites corresponding to labelling by β-oxidation of decanoate (*i.e.*, those from C-1 of acetate). The recovered decanoic acid was shown to be fully labelled with carbon-13: within the limits of detection, all of the protons at the α-carbon were coupled to carbon-13, and the resonance due to C-α was a doublet without a central, uncoupled singlet.

Labelled decanoic acid was next converted to the *N*-acetylcysteamine derivative **4** in order to improve incorporation, following a standard protocol for conversion of fatty acids to their SNAC thioesters (Scheme 2).[23]



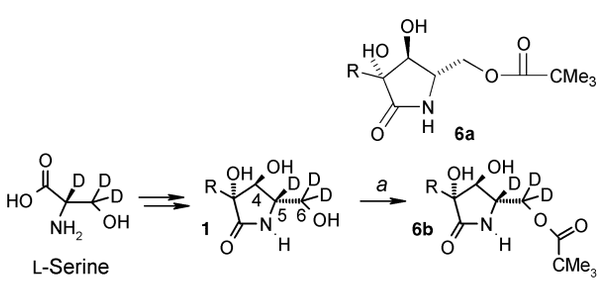
Scheme 2 Synthesis and results of incorporation of *N*-acetyl-*S*-[1-^{13}C]decanoylcysteamine **4** into dienone **3**. *Reagents*: a, (i) EtOC(=O)Cl; (ii) HSCH$_2$CH$_2$NHAc (prepared *in situ*); b, β-oxidation pathway in cells; c, incorporation by normal route.

Incorporation of **4** either alone or in combination with the β-oxidation inhibitor 3-(tetradecylthio)propanoic acid **5** resulted in formation of **3** (see above). In the carbon-13 NMR spectrum of the samples of **1** and **3** (from **4** alone) or of **3** (from the combination), enhanced singlets were observed at each of the sites that had been labelled by C-1 of acetate. The level of incorporation was uniform for each experiment, ranging from 1.0 to 2.7% between experiments. No significant specific incorporation of label was found at C-11 over and above that observed at the other labelled sites, despite attempts to obtain specific incorporation under a range of feeding protocols.

C. Incorporation into the serine entity

Since the results with acetate suggested that the remaining three carbon atoms and the nitrogen of the pyrrolidone ring might originate from serine, the incorporation of this amino acid into pramanicin **1** was investigated. First, label from DL-[1-^{13}C]serine was found to be located exclusively into the C-4 position of **1** with a high level of incorporation (5.2%) (Scheme 1). In order to address the issue of which enantiomer was incorporated, commercial L-[2,3,3-$^{2}H_3$]serine was tested. The resulting sample of **1** was examined by $^{2}H\{^{1}H\}$ NMR spectroscopy in methanol. Two broad resonances were observed, one at δ 3.72 and a slightly more intense signal at δ 3.48. The former could be unambiguously assigned to one of the two diastereotopic deuterons at C-6 (δ_H 3.79, 0.24 ppm from the closest adjacent resonance), while the latter corresponded to the other deuteron at C-6 (δ_H 3.55) and/or deuterium at C-5 (δ_H 3.47). These two signals were not expected to be resolved due to the natural lineshape in the deuterium spectrum ($w_{1/2} \approx 0.1$ ppm). Examination of the proton NMR spectra of pramanicin in a number of solvents showed that the largest shift difference between the H-5 and the H-6 protons occurred in DMSO-d_6. However, when the labelled sample was examined by ^{2}H NMR in DMSO, substantially increased linewidths were observed, probably due to the greater viscosity of DMSO compared with methanol. When the sample was warmed to 85 °C, the thermal reduction in viscosity sharpened the deuterium lines so that the two diastereotopic deuterons at C-6 were well resolved; a shoulder on the more shielded signal was observed, indicative of deuterium at C-5 as well as at both C-6 positions.

To increase the dispersion of the three signals of interest, the pramanicin was derivatised. The trimethylacetyl (pivaloyl) ester, a protective group which is known to selectively protect primary alcohols,[24] was chosen in order to react selectively at C-6, and to provide a substantive shift to the H-6 protons. When treated with pivalic anhydride in Et_3N, pramanicin was converted to the 6-pivaloyl derivative **6a** (56%) (Scheme 3) with



Scheme 3 Incorporation of L-[2,3,3-$^{2}H_3$]serine into pramanicin **1**, and preparation of the 6-pivaloyl derivative **6a**. *Reagent and conditions*: a, pivalic anhydride, TEA, 70 °C, 30 min.

only small amounts of other products, presumably corresponding to reaction at the other alcohol groups, which could be readily removed by chromatography. The proton NMR spectrum of **6a** exhibited all of the resonances expected for pramanicin, except that the two H-6 protons had shifted to δ 4.37 and 4.05, while H-5 was only slightly influenced by the neighbouring pivaloyl group at C-6, shifting to δ 3.62. The material derived from labelled serine was similarly converted to **6b**, and the purified product was examined by deuterium NMR spectroscopy in methanol. Three broad resonances were observed at δ 4.30, 4.00 and 3.55, establishing that deuterium is present at C-5 and at both positions at C-6.‡ Thus, the L-enantiomer of serine is a viable precursor to **1**, although no conclusion can be reached about the role of the D-enantiomer in the pathway at this point.

‡ These spectra have been published in preliminary communications[3,4] and are not reproduced here.

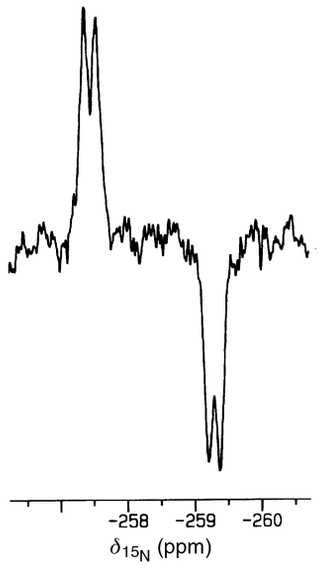
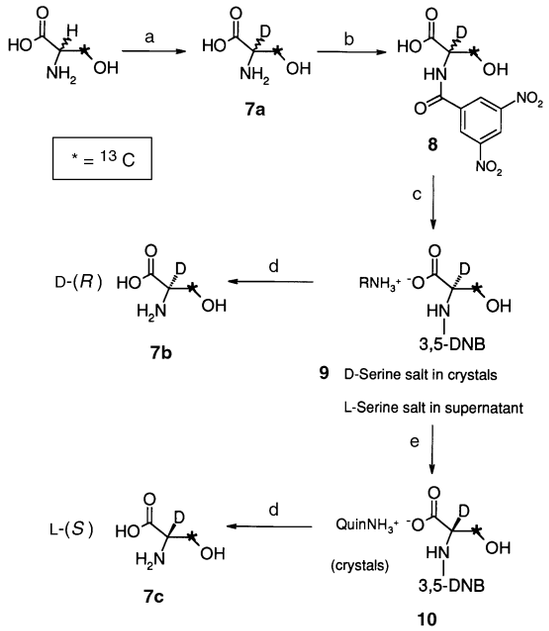


Fig. 5 Section of the nitrogen-15 NMR spectrum of pramanicin **1** derived from [1,2,3-$^{13}C_3$,^{15}N]serine, acquired in the INEPT mode at 50.7 MHz in DMSO-d_6. Coupling to the attached proton is seen in the pair of inverted signals; the smaller splitting is due to the serine-derived attached carbon-13.

With this knowledge, commercial L-[1,2,3-$^{13}C_3$,^{15}N]serine was incorporated into pramanicin. The resulting sample was examined by ^{15}N NMR, using the INEPT procedure to enhance the signal intensity through polarisation transfer from the NH proton.[1] To avoid exchange of the NH position, DMSO-d_6 was used as solvent for this experiment. The spectrum (Fig. 5) shows the presence of a doublet of doublets at δ −256.4 relative to external $MeNO_2$. The chemical shift compares with that reported by Schwartz and co-workers (126.2 ppm relative to 2.9 M $^{15}NH_4Cl$ in 1 M HCl; this converts to −229.1 *via* correlation through the shift of liquid NH_3).[1] Thus, ^{15}N from serine is incorporated into the nitrogen atom of **1**. The observed couplings are due to ($^{1}J_{^{15}N-H}$ 93 Hz) (lit.[1] 92 Hz), and a further one-bond coupling to carbon-13 (10 Hz). Within the limit of detection, all of the nitrogen was coupled to carbon-13, so serine is incorporated with the C–N bond intact. The same C–N coupling was observed in the carbon-13 NMR spectrum of the sample. In this case, the signal at C-5 was observed to be composed of a doublet of triplets (J_d 10 Hz to ^{15}N, and 38 Hz), and a doublet of doublets (J 10 Hz to ^{15}N, and 38 Hz) surrounding the unenhanced natural-abundance signal. At C-4, the natural-abundance singlet was surrounded by a doublet (38 Hz), while the resonance for C-6 was comprised of an enhanced singlet surrounded by a doublet of lower intensity than that at C-4.‡ These results show that **1** derived from the labelled serine is comprised of three isotopomers: the fully labelled [4,5,6-$^{13}C_3$,^{15}N]isotopomer, as well as [4,5-$^{13}C_2$,^{15}N]- and [6-^{13}C]-species.

The incorporation of both D- and L-[2-^{2}H,3-^{13}C]serine was next examined. The separate enantiomers were prepared by exchange of commercial racemic [3-^{13}C]serine (92 atom% ^{13}C), using pyridoxal and aluminium sulfate in D_2O according to the procedure initially developed for [2-^{2}H]serine by Miles and McPhie (Scheme 4).[25] Modifications to this procedure, as described by Townsend *et al.*[26] and later by de Kroon *et al.*[27] for [2-^{3}H,1-^{14}C]serine, were applied to enhance the yield of recovered serine. The resulting racemic [2-^{2}H,3-^{13}C]serine **7a** was resolved by the method of Velluz *et al.*,[28] as modified by Gorissen *et al.*[29] Thus, after conversion of the racemic [2-^{2}H, 3-^{13}C]serine to the 3,5-dinitrobenzoyl (3,5-DNB) amide derivative **8**, the D-enantiomer was preferentially crystallised as the (−)chloramphenicol salt **9**. The free acid was liberated from this salt, and then the 3,5-DNB group was removed with aq. HCl to



Scheme 4 *Reagents and conditions*: a, Pyridoxal, $Al_2(SO_4)_3$, D_2O, pD 9.4; b, 3,5-DNBCl, aq. NaOH; c, D-(−)-*threo*-chloramphenicol (free base), MeOH–EtOAc; d, (i) conc. HCl, (ii) aq. HCl, reflux; e, (i) extract with HCl, (ii) quinidine, EtOH. Overall yields: 14% for **7b** and 15% for **7c**.

give D-[2-^{2}H,3-^{13}C]serine **7b** in 14% overall yield. The material was confirmed to be fully labelled with deuterium, and 92% labelled with carbon-13, by NMR and electrospray MS. Optical rotation confirmed the D configuration, while chiral TLC according to the method of Günther[30] showed the absence of any detectable L-enantiomer. The mother liquor from the chloramphenicol crystallisation (above) was converted to the acid, and the L-enantiomer crystallised as the salt **10** with (+)-quinidine. The salt was acidified, and the 3,5-DNB group deprotected to afford the desired L-[2-^{2}H,3-^{13}C]serine **7c**. This material was also fully labelled with deuterium, but had the opposite optical rotation to that of the D isomer. It migrated as a single spot with higher R_f than the D isomer on the chiral TLC system, in accord with the relative migrations reported in the literature for the enantiomers of serine.[30]

The D- and L-enantiomers of [2-^{2}H,3-^{13}C]serine were each separately mixed with racemic [1-^{13}C]serine as an internal positive control for incorporation into C-4 of pramanicin. The samples of serine were analysed by integration in the proton NMR spectrum of the serine methylene signals that were coupled to carbon-13 at C-3, *vs.* the uncoupled signals. The latter derives from the added racemic [1-^{13}C]serine, plus a small amount of [2-^{2}H]serine which had originated from the commercial starting material, *i.e.* the racemic [3-^{13}C]serine which was purchased contained 8% ^{12}C. The results showed that the sample from the D-enantiomer contained 68% [2-^{2}H,3-^{13}C]-, 6% [2-^{2}H]-, and 13% of each enantiomer of [1-^{13}C]serine, in close agreement to the amounts calculated by mass during mixing of the isotopomers (23% [1-^{13}C]). The corresponding values for the L-enantiomer were 62, 6, 16 and 16%, respectively.

These two samples of serine were separately incorporated into pramanicin **1**, which was then analysed by carbon-13 NMR spectroscopy. For the sample from D-serine, the control site was labelled (1.7% incorporation at C-4) but no enhancement within experimental error, nor an isotopically shifted signal, was observed at C-6. Therefore, only the L-enantiomer of serine is incorporated. For the L-enantiomer of serine, control site C-4 was also enhanced to essentially the same level as in the D-serine experiment (2.1% incorporation), while C-6 was both enhanced (7.6% incorporation) and furnished a signal shifted upfield by 0.074 ppm (2.9% incorporation).‡[19] In this

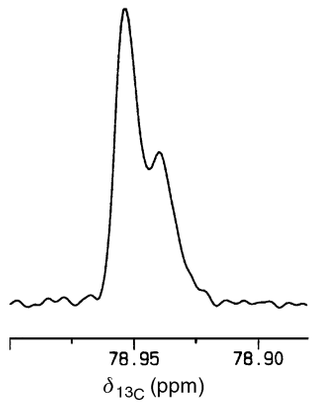
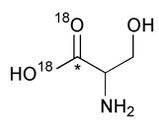


Fig. 6 Partial carbon-13 NMR spectrum of pramanicin **1** derived from [1-^{13}C,$^{18}O_2$]serine, showing the isotopically shifted signal due to intact incorporation of the C–O bond at C-4.

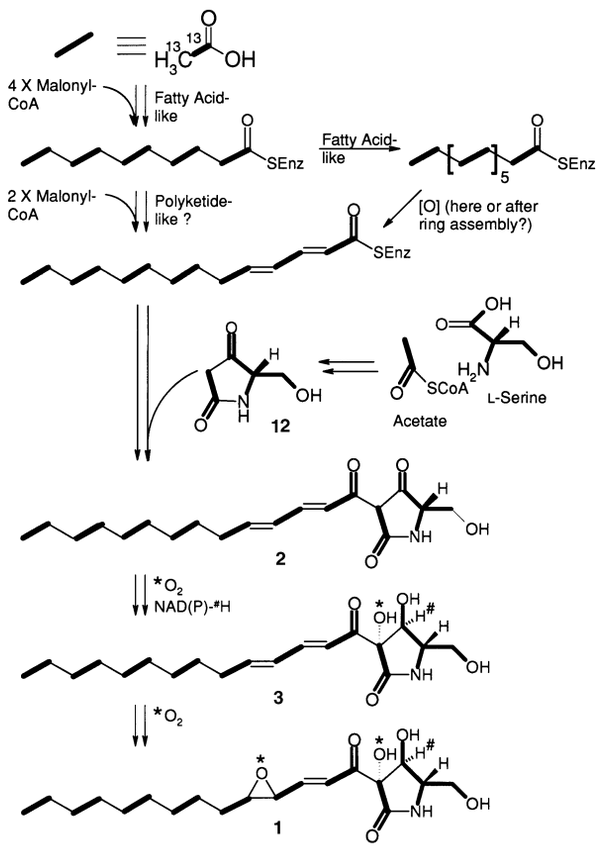


case, the distribution of isotopomers between C-4 and C-6 (26 : 74) reflects the distribution in the L-component of the starting serine (21 : 79) within experimental error. At C-6, the size of the shift for the upfield signal corresponds to that expected for a β-deuterium isotope shift,[20] indicating that an intact ^{13}C–C–^{2}H unit from L-serine was incorporated into **1**. However, some carbon-13 had been incorporated without the accompanying deuterium.

Next, the origin of the hydroxylic oxygen at C-4 of pramanicin was investigated. Racemic [1-^{13}C,1-^{18}O]serine **11** was prepared from DL-[1-^{13}C]serine by exchange with $H_2{}^{18}O$–HCl, using the method developed for [1-$^{18}O_2$]serine by Murphy and Clay;[31] however, only 2 equiv. of $H_2{}^{18}O$ and conc. HCl in $H_2{}^{16}O$ was used. After neutralisation, a mixture of serine hydrochloride and NaCl was obtained which was carried forward without further purification for incorporation. The labelled serine contained no organic impurities (by NMR). These modifications conserved isotopes, and ensured a ^{13}C–^{16}O-labelled reference peak in the ^{13}C NMR spectrum of the derived **1**, thus avoiding swamping of the natural abundance peak due to the high level of serine incorporation expected in this experiment.[19] The isotopomer distribution was shown to be approx. 99% ^{13}C and ≈50 : 50 ^{16}O : ^{18}O per site by high-resolution ^{13}C NMR spectroscopy. When pramanicin derived from this material was examined, the high-resolution ^{13}C NMR spectrum showed an enhanced singlet at the shift of C-4 (3.8% incorp.), as well as a signal shifted upfield by $\Delta\delta$ 0.013 ppm[20] due to the presence of an oxygen-18-induced isotope shift[19] (2.2% incorp.) (Fig. 6). Therefore, the results show that a significant proportion of pramanicin molecules are formed from serine with the C–O bond at C-4 of **1** remaining intact from the C=O bond of serine. The remainder have undergone exchange of ^{18}O for ^{16}O.[22]

Discussion

Like many fatty acids[32] and polyketides,[33] the assembly of pramanicin begins with a two-carbon starter unit at C-20 and C-19 (Scheme 5). The methyl 'tail' of acetate provides C-20, while the carboxylate carbon 'head' furnishes C-19, as shown by the labelling experiments with [1-^{13}C]acetate. The C–C bond of acetate remains intact in this process, as demonstrated by the coupling of C-20 to C-19 in pramanicin derived from [1,2-$^{13}C_2$]acetate. In fatty acid biosynthesis, it has long been thought that the starter unit is usually an acyl entity, as opposed to the chain-extension units which are malonate-derived. Recently, the generality of this hypothesis has come into



Scheme 5 Proposed biosynthetic pathways to pramanicin **1**

dispute from work on actinorhodin[34] and tetracenomycin[35] polyketide synthases (PKSs), where malonate is the starter. Leadlay and co-workers also showed that malonate could be decarboxylated by components of two different PKSs to furnish the starter unit *in situ* on the enzyme surface.[36] Discrimination between acetate and malonate units in polyketides has in some cases been made by quantitative labelling experiments, usually using diethyl malonate which acts as a convenient source of malonyl-CoA through hydrolysis and thioesterification *in vivo*.[37] However, malonate may also label all sites equally because the CoA thioesters of acetate and malonate undergo rapid interconversion *in vivo*.[38,39] In the current case, the results with diethyl [2-^{13}C]malonate show that incorporation into the starter, compared with the extender units, is reduced by a factor of two. This effect clearly suggests that malonate must be converted to acetate prior to incorporation into this site. The results compare with the acyltetramic acid erythroskyrine, where a similar low incorporation of malonate into the starter unit was observed.[6] Further, significant retention of three deuterium atoms in **1** derived from [1-^{13}C,2-^{2}H$_3$]acetate shows that acetate can be incorporated without the intervention of malonate into the starter unit in this case, although some molecules may have passed through malonate, as evidenced by the presence of monodeuterio- and dideuterio-isotopomers. Clearly, acetate is an effective starter unit. Again, the acyltetramic acid chaetoglobosin was shown by Tamm and Probst to retain up to three deuterons from C-2 of acetate.[13]

In fatty acid biosynthesis, extension from the starter acetate unit occurs by fatty acid synthase (FAS)-mediated decarboxylative condensation with a malonyl group derived from malonyl-CoA, using the ketosynthase (KS) subunit.[32] After this initial C–C bond-forming reaction, the resulting β-keto group is removed by a sequence of FAS-catalysed steps: the ketone is reduced by a ketoreductase (KR), the resulting alcohol is dehydrated by a dehydratase (DH) to give an α,β-unsaturated acyl chain, and finally conjugate addition of hydride by an enoylreductase (ER) produces a methylene group at the carbon atom derived from C-1 of the starter acetate unit. Long fatty acyl chains are assembled by repetitive cycles of condensation of the ensuing saturated acyl chain with further malonate units (KS) and reduction of the resulting ketone carbon to a methylene group, using KR, DH and ER subunits as above. The condensation of acetate and malonate units thus results in a labelling pattern in fatty acids and polyketides in which acetate units are connected in a 'head-to-tail' manner. PKS's are considered to assemble acetate units in a similar manner, but with the omission of all, some or none of the β-ketone-processing steps (KR, DH and ER), the choice made being dependent upon chain length.[33]

The pattern of labelling and the intact incorporation of acetate into each extender unit in **1** indicate that the acyclic carbon chain in **1** is assembled in a 'head-to-tail' manner: C-11/C-12 and C-13/C-14 are head-to-tail units, while C-15/C-16 and C-17/C-18 are also intact units which are presumably arranged in the same way, although overlap of the ^{13}C NMR resonances precluded unambiguous assignment of the *direction* of incorporation of the latter two acetate moieties. Both labelled acetates and diethyl [2-^{13}C]malonate were found to label each extender unit through to C-11 to an equal extent. Taken together, these results are strongly suggestive that assembly of a fatty acyl entity by an FAS-like enzyme furnishes at least the unfunctionalised C-20 to C-11 saturated chain in **1**, and suggest a decanoyl entity as an obligatory intermediate.

There is precedent in the aflatoxin biosynthetic pathway in *Aspergillus parasiticus* for assembly of the hexanoate starter unit by a short-chain FAS; hexanoate is then extended by a separate PKS which furnishes the polycyclic aromatic core of the natural product.[40] In that case, Townsend *et al.* demonstrated impressive specific incorporations of hexanoate and its *N*-acetylcysteamine (NAC) thioester into the side-chain of averufin, a precursor for aflatoxin B_1.[41] Similarly, octanoate is assembled from acetate by an FAS, then acts as a starter for the PKS in piliformic acid biosynthesis.[42] On the basis of these results, we reasoned that decanoate might similarly be synthesised on a separate FAS enzyme, prior to loading onto a putative PKS for the addition of further acyl units *en route* to **1**. The ability of the PKS to load an acyl thioester which might well originate from a separate enzyme made this system an attractive candidate for incorporation of the corresponding labelled fatty acid or NAC derivative. Thus, we investigated the incorporation of both sodium [1-^{13}C]decanoate and *S*-[1-^{13}C]-decanoyl-NAC **4** into **1**, and/or into **3**, in both the presence and absence of the β-oxidation inhibitor 3-(tetradecylthio)-propanoic acid **5**. The results failed to provide any reproducible evidence for specific incorporation of label into either **1** or **3**; although one experiment did appear to provide a 0.2% specific incorporation into the C-11 site of **3**, we have been unable to duplicate this result. The cells of *Stagonospora* were permeable to putative precursor **4**: sites corresponding to each C-1 of acetate were labelled to the extent of up to 2.7% per site. This non-specific incorporation indicates that the NAC derivative was incorporated exclusively *via* β-oxidation to acetate.[43] No evidence for a pool of free decanoic acid was found, since recovered decanoic acid from the sodium decanoate experiment was still entirely labelled with carbon-13. The failure to observe intact incorporation of **4**, while being inconclusive, may suggest that the FAS creates a chain longer than decanoate, *e.g.*, a fourteen-carbon species such as tetradecanoate. In that case, the obligatory decanoyl moiety would be a transient intermediate on the FAS surface only, and **4** might not be able to load onto the enzyme.

The extension of the presumed decanoyl intermediate occurs by 'head-to-tail' addition of two further intact acetate-derived malonate units (C-10/C-9 and C-8/C-7), as shown by the labelling experiments with acetates and diethyl malonate. We had reasoned that these units may be added by a PKS-like activity, in a manner reminiscent of the biosynthesis of the polyene

class of polyketide antibiotics,[39,44] as opposed to the FAS-like assembly of C-20 to C-11. In this case, the two cycles of malonate decarboxylative addition would each be followed by reduction of the resulting β-keto group by a KR and dehydration of the ensuing alcohol by DH, leading to a tetradeca-2,4-dienoyl entity. The exceptional level of retention of deuterium at C-8 and C-10 over that in the C-18 to C-11 portion of the molecule in the experiment with [1-^{13}C,$^{2}H_3$]acetate supports this idea in that the acetate units in the former region seem to be processed differently from those in the latter. However, the assembly of a saturated tetradecanoyl moiety by a single FAS, followed by dehydrogenation to afford the diene moiety at this or a later stage, a process that is well established in fatty acid β-oxidation,[43] cannot be ruled out. Nonetheless, fatty acids longer than fourteen carbon atoms, such as palmitate, can be excluded from the pathway, since the results with oxygen-18-labelled acetate show retention of the C=O bond at C-7; as well, the origin of the pyrrolidone acetate unit from acetate in preference to malonate supports this conclusion. Incorporation experiments with other NAC derivatives, spurred on by the successful uptake of **4** into the cells of *Stagonospora*, to clarify this issue are currently in progress, and will be reported in due course.

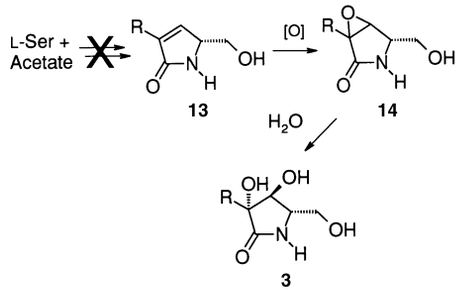
At this point, the assembly of the pyrrolidone head group of **1** begins. The results with labelled acetates show that the C-2 and C-3 atoms, as well as O-2, in **1** are derived from an intact acetate unit which is arranged head-to-tail with respect to C-7. The remaining three carbon atoms, C-4, C-5 and C-6, are derived from the carboxy-, α-, and hydroxymethyl carbon atoms of serine, respectively, as shown by the combined results of the labelling experiments with [1-^{13}C]-, [3-^{13}C,^{2}H]- and [1,2,3-$^{13}C_3$,^{15}N]-serines. This pattern of labelling of the ring in **1** is inconsistent with an origin in glutamate, which we had considered to be a reasonable alternative precursor to N-1 through to C-6. However, the labelling arrangement is identical to that observed in the acyltetramic acid group of metabolites, suggesting that the biosynthetic pathway proceeds through an intermediate such as **2**. All of the members of this class of metabolites that have been investigated biosynthetically possess a tetramic acid ring which is derived from one acetate unit and an amino acid. Thus, the pyrrolidone moieties in erythroskyrine,[6] cyclopiazonic acid,[7] tenuazonic acid,[8] malonomicin,[9,10] streptolydigin,[11] ikarugamycin,[12] the cytochalasans,[13] and aflastatin A[14] are derived from valine, tryptophan, isoleucine, 2,3-diaminopropanoate, methylaspartate, ornithine, tryptophan and alanine, respectively. Tetramic acid intermediates have also been proposed in the biosynthesis of, for example, tenellin[45] and ilicicolin H[46] from phenylalanine, although more recent results have cast this hypothesis into doubt.[47] To the best of our knowledge, pramanicin represents the first example of a (modified) acyltetramic acid which has been shown to be derived from serine, although there are probably other metabolites which are formed similarly such as the interesting bistetramic acid polycephalin C from *Physarum polycephalum* which controls response to light.[48] Serine has been shown to be incorporated into malonomicin,[9] but in this case serves as a precursor for 2,3-diaminopropanoate prior to acyltetramic acid formation.

The results show that label at carbon-5 is coupled to both C-4 and C-6 in a significant proportion of the molecules of **1** derived from L-[1,2,3-$^{13}C_3$,^{15}N]serine. Therefore the carbon skeleton of serine is incorporated intact into pramanicin. Other labelled molecules possess either a single site of enrichment at C-6, or are labelled at both C-4 and C-5 (and with ^{15}N, see below), but not C-6. Hence there must be an incidental process, which does not have to occur *en route* to **1**, that involves cleavage of the C-2–C-3 bond of serine. The most likely candidate is serine hydroxymethyltransferase (SHMT), which freely interconverts serine with glycine and methylenetetrahydrofolate (Me-THF).[49] Thus the observed results are readily explained by the action of SHMT on [1,2,3-$^{13}C_3$,^{15}N]serine, generating [1,2-$^{13}C_2$,^{15}N]glycine and [^{13}C]Me-THF. These then recombine with unlabelled Me-THF and Gly, respectively, from the cellular pool to generate [1,2-$^{13}C_2$,^{15}N]serine and [3-^{13}C]serine which then incorporate to give the observed isotopomers of **1**.

Not only is serine incorporated with the three carbon atoms intact, but the nitrogen atom derives from serine as well; moreover, serine is incorporated with the C^{α}–N bond intact, as shown by coupling in both the ^{15}N and ^{13}C NMR spectra in the intact isotopomer derived from L-[1,2,3-$^{13}C_3$,^{15}N]serine. This intact incorporation of nitrogen from the amino acid into **1** is again consistent with the acyltetramic acid route: Probst and Tamm observed incorporation of [2-^{15}N]tryptophan into chaetoglobosin.[13] In addition, both illicicolin H and tenellin have been studied using nitrogen-labelled precursors, although the biosynthesis of these metabolites may not involve an acyltetramic acid intermediate.[45–47] The results exclude the action of transaminase on serine as a required component of the pathway to **1**. Further, if serine transaminase is present and active in the *Stagonospora* cells, then it must be acting in an essentially irreversible manner, since all the nitrogen label in **1** was found to be coupled to carbon-13.

The formation of intermediate **2** can be rationalised mechanistically in the same way as has been proposed for other acyltetramic acids; *i.e.*, condensation of the nucleophilic C-2 of acetate or its equivalent with the electrophilic carboxy groups of both the preformed polyketide or fatty acid and the presumably activated serine. The formation of the amide bond by attack of the amino group of serine onto the activated carbonyl group of the ring acetate unit also makes mechanistic sense. These mechanisms have analogy to both polyketide-like chain-extension steps, and to non-ribosomal peptide synthetases (NRPS),[50] but also involve the more unusual polyketide-like C–C bond formation between acetate C-2 and an amino acid carboxy group. Because of the intense interest and activity in both NRPS and PKS which has evolved from genetic work and protein studies on both groups of enzymes, there has been much recent discussion of the similarities between these two classes of enzymes.[50] Further, although many biosynthetic experiments have been conducted on acyltetramic acids (see above), there is a paucity of information on the nature, sequence and timing of the steps involved in this part of the assembly process. For these reasons, it was of interest to investigate further the details of events leading from the assembled fourteen-carbon acyclic chain through to **2**.

First, the proposed mechanism requires that the oxygen atoms at C-2 and C-4 of **2** derive from acetate and the carboxyl oxygen of serine, respectively. The results from incorporation of [1-^{13}C,$^{18}O_2$]acetate and [1-^{13}C,$^{18}O_2$]serine into **1** confirm intact incorporation of these C–O bonds. Thus, if **2** is an intermediate *en route* to **1**, it must also be labelled in accord with the proposed mechanism. The retention of oxygen at C-7, and of deuterium at C-5 and C-6 (below), are also fully consistent with the intermediacy of **2**. Further, the retention of O-4 excludes some alternative pathways. One attractive possibility which can be excluded is the formation of an α-acyl-α,β-unsaturated lactam such as **13** (Scheme 6); epoxidation to give **14**, followed



Scheme 6 A putative route through an epoxide is attractive, but inconsistent with the retention of the C=O bond of serine

by hydrolysis would have accounted for the *trans* stereochemistry of the C-3–C-4 diol. Precedent for such an epoxidised α-acyl-α,β-unsaturated lactam may be found in fusarin C.[51]

In order to elucidate the timing of the C-3–C-7, N-1–C-2 and C-3–C-4 bond-formation events, diethyl [2-^{13}C]malonate was incorporated into **1**. If the C-3–C-7 bond formed first, then it would be expected that C-3 would derive from malonate by a normal, PKS-catalyzed chain extension. If, on the other hand, the N-1–C-2 and/or C-3–C-4 bonds were formed prior to acylation by the side-chain intermediate, then C-3 might originate from acetate. Malonate was in fact found to incorporate into all of the acetate-derived sites, including the starter, acyl-chain extender, and C-3 sites; however, C-3 was labelled to the same extent as the acetate-derived starter unit, and significantly less than the extender units. This effect clearly suggests that the C-2–C-3 unit is derived from acetate. Further, when the results from incorporation of [$^{13}C_2$]acetate are examined in detail, small doublet of doublet signals were observed due to incorporation of multiple, adjacent labelled acetate entities. Carbon resonances from label at C-3 to C-19 are split by both intra- and inter-unit one-bond couplings to generate these signals. The incorporation level for interunit coupling between C-3 and C-7 in **1** was indistinguishable from that of any other pair of interunit-coupled carbons. Since this interunit effect, which has often been observed in acetate incorporations into polyketides, is larger than statistically expected, it has been ascribed to the pulsed feeding of the starting material which results in temporal variations of the levels of labelled precursor within the metabolic pool. Thus, the present result suggests that addition of the C-2–C-3 unit occurs at a time and within a cellular sub-compartment indistinguishable from that of assembly of the C-7-to-C-20 acyl chain. If the pyrrolidone is assembled from acetate and serine prior to acylation, then this intermediate cannot accumulate to a significant extent, because isotopic dilution would occur. While these experiments do not provide an unambiguous answer to the question of the sequence of events for ring assembly, they are fully consistent with and suggestive of a distinct type of process for attachment of the C-2–C-3 unit, perhaps addition of serine to acetate to generate *N*-acetylserine and/or the tetramic acid derivative **14** prior to acylation. Unlike all the results described so far, this experiment stands in contrast to previous observations on acyltetramic acids, where malonate has been widely assumed to be the precursor of the ring acetate unit. On the basis of radiolabelling experiments with malonate, Shibata *et al.*[6] concluded that erythroskyrine was formed from an acetate starter, but that malonate was used in all the extender units including that of the pyrrolidone ring. In malonomycin, Schipper *et al.*, using deuterated precursors, showed that the pyrrolidone is formed by condensation of a preformed 3-oxoadipoyl unit with diaminopropanoate.[10] The current results provide a clear distinction between direct incorporation of malonate and indirect incorporation through acetate, as evidenced by the incorporation rate into the starter unit; this unit was independently shown to be acetate-derived from the [^{13}C,^{2}H$_3$]acetate experiment which therefore acts as a positive internal control for the indirect incorporation rate from malonate. The observation of an essentially identical level of labelling at C-3 as for C-20 thus clearly indicates that the pyrrolidone ring in **1** is formed from acetate. Further investigations to resolve the issue of sequence of bond-forming steps are being undertaken.

Finally on the pathway, transformation of **2** into **3** requires a mechanistically unremarkable reduction at the C-4 keto group, a hydroxylation at the highly acidic C-3 position, a process reminiscent of chemical enolate oxidations with electrophilic oxygen species,[52] and epoxidation of the C-10,C-11 alkene, in an as-yet-undetermined order. Surprisingly, we found that administration of both **4** and **5** enhanced formation of **3** and reduced that of **1**; thus both compounds appear to suppress oxidation of **3** to **1**. The isolation of **3** suggests that the formation of the epoxide from the C-10–C-11 olefin in **3** is the last step in the biosynthetic pathway to pramanicin. This is consistent with the lack of an ^{18}O-induced isotope effect at C-11 when sodium [1-^{13}C,$^{18}O_2$]acetate is incorporated into **1**, a result that suggests, albeit unsurprisingly, that the epoxide oxygen does not derive from acetate. We are not aware of any precedent for the apparent inhibition of an epoxidation reaction, which presumably is promoted by a cytochrome *P*450, by the supposed β-oxidation inhibitor **5**, or by the FAS substrate analogue **4**, although both might be expected to have some competitive inhibitory activity on the cytochrome by virtue of their passing resemblance to the olefinic substrate **3**. Compound **5** has, however, been shown to be processed by both β- and ω-oxidation in the rat,[53] and to induce rat cytochrome *P*4504A1 mRNA levels.[54] Experiments designed to test incorporation of **2** into **3** are also in progress.

The results with deuterated serine samples enable conclusions to be reached about which enantiomer of serine incorporates into pramanicin, as well as the absolute configuration of **1**. The L-enantiomer was incorporated at high levels in the experiments with [2,3,3-^{2}H$_3$]-, [^{13}C$_3$,^{15}N]- and [3-^{13}C,2-^{2}H]-serines. However, the D-enantiomer of [3-^{13}C,2-^{2}H]serine is not a viable precursor, since it was not incorporated despite concurrent incorporation of racemic [1-^{13}C]serine as a positive internal control. For the experiment with [2,3,3-^{2}H$_3$]serine, the results also show significant incorporation of deuterium at both of the diastereotopic C-6 positions. Thus, there is no oxidation state change at C-6. An apparently smaller extent of deuterium labelling was observed at C-5 than at C-6, suggestive of partial exchange and the involvement of an amino acid racemase. On the other hand, this result could be explained by the action of SHMT, which is active in this case (above), and which would generate [^{2}H$_1$]glycine and [^{2}H$_2$]Me-THF. These species would recombine under the action of the freely reversible SHMT with unlabelled serine and Me-THF from the metabolic pools. Since these pools may be unequal in concentration, differing amounts of [2-^{2}H]- and [3-^{2}H$_2$]-serine would be generated, which would lead to different levels of labelling at C-5 *vs.* C-6 of **1**. Since the results of this experiment thus do not demonstrate that all three deuterium atoms from a single molecule of labelled serine incorporate into a single molecule of **1**, it was necessary to show that deuterium was incorporated from C-2 without the intermediacy of SHMT. This result was established by the experiment with L-[3-^{13}C,2-^{2}H]serine; the observation of a β-deuterium isotope shift at C-6 in **1** for approximately 25% of the molecules that are labelled shows that the D-C$^{\alpha}$-^{13}C entity can be incorporated in an intact manner; *i.e.*, without the intervention of SHMT. The enhanced, unshifted signal at C-6 corresponds to the remaining 75% of serine which has been processed by SHMT, or by a serine racemase that, as expected,[55,56] causes loss of the α-deuteron. These results establish that such a racemase is not required in the pathway to **1**. In combination, the results show that the L enantiomer of serine is incorporated into **1** with all four bonds to the α-carbon intact, while the D enantiomer is not incorporated, a result that also argues against the action of a serine racemase. Therefore, **1** must possess the same absolute configuration at C-5 as that in L-serine, and thus has the 5*S* absolute configuration. Since we first published this result as a communication, Barrett *et al.* have reached the same conclusion by synthesis of the enantiomer of pramanicin.[15]

Conclusions

In summary, pramanicin **1** has been shown to be formed from head-to-tail condensation of a starter acetate unit with six malonate units to provide the acyclic, acyl tail. The pyrrolidone ring is derived from one acetate and L-serine, which was shown to be incorporated with the three carbons, the nitrogen, the carboxy oxygen, the α- and both β-hydrogens as an intact unit. As expected, oxygen at C-1 of both the last unit in the

unsaturated fatty acid side chain, and in the unit within the pyrrolidone ring, is retained. The results suggest the intermediacy of *N*-acetylserine and/or tetramic acid **12** (Scheme 5), which is then acylated by a preassembled fourteen-carbon moiety, leading to acyltetramic acid **2**. Modification by a reduction at C-4 and oxidation at C-3 then ensue. Epoxidation of the 10,11-alkene is probably the last step, since the putative precursor **3** was isolated as a co-metabolite. The observation of retention of all four bonds to the α-carbon of L-serine shows that pramanicin must possess the same absolute configuration at C-5; *i.e.*, 5*S*. Although the results do not yet precisely define the sequence of all steps in this pathway, they allow the exclusion of some of the conceptually reasonable possibilities and provide specific limits on the pathway from simple precursors to **1**. Although decanoyl-NAC **4** was not incorporated, the results show that this type of derivative is available to the cells. Thus the stage is set for further ongoing experiments with advanced precursors, which should shed further light on the pathway to **1**, and presumably by extension to other members of the important acyltetramic acid group of metabolites.

Experimental

General

Stagonospora sp. ATCC 74235 was purchased from the American Type Culture Collection. All media and materials for culture were autoclaved at 121 °C for 20 min. Chemicals were purchased from Aldrich and Sigma and used without further purification. Silica gel was from Silicycle (Montreal). Isotopically labelled precursors were from CIL (Andover, MA). Water was obtained from a Nanopure water-purification system. THF was freshly distilled under nitrogen protection from potassium/benzophenone.

All reactions were performed in flame-dried glassware, under a positive pressure of nitrogen, unless otherwise noted. Air- and moisture-sensitive compounds were transferred by syringe. Concentration of organic solutions was performed on the rotary evaporator (≈25 mmHg), followed by evacuation to constant weight (<0.05 mmHg).

Mps were measured on a Gallenkamp MF370 instrument and are uncorrected. Proton and carbon-13 nuclear magnetic resonance (NMR) spectra were recorded on Bruker AC 200, Bruker AC 300 or Bruker DRX 500 NMR spectrometers. Proton chemical shifts (δ scale) refer to TMS ($SiMe_4$) and those of ^{13}C to $CDCl_3$. Data are given as chemical shift (integral, multiplicity [s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet], coupling constant[s] [*J* in Hz], assignment). Fourier transform (FT) IR spectra were recorded on a Bio-Rad SPC 3200 spectrophotometer or on the tip of the probe of an ASI *in situ* reaction-monitoring system. UV spectra were obtained on a Perkin-Elmer Lambda 9 UV/VIS/NIR spectrophotometer or a Cary 50 machine. Mass spectra were recorded on a Finnigan 4500 mass spectrometer for electron impact (EI) and chemical ionisation (CI) or on a Micromass Quattro LC for electrospray (ES). Optical rotations were measured on a Perkin-Elmer 241MC polarimeter; $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}.

Culture of *Stagonospora*

Freeze-dried cultures were suspended in water, and used to inoculate Petri dishes of potato dextrose agar [prepared from 200 g of potatoes, diced and boiled in water (333 cm^3) for 10 min, filtered through cheesecloth and the filtrate added to agar (Difco, 10 g) and dextrose (13.3 g)]. The plates were incubated in a moist atmosphere at 25 °C for 3 days with a light cycle (40 W fluorescent bulb) of 12 h on, 12 h off. The resulting mycelia were suspended in water and transferred to an unbaffled 500 cm^3 Erlenmeyer flask containing KF seed medium [tomato paste (4 g), corn steep liquor (0.5 g), oat flour (1 g), glucose (1 g), water (99 cm^3) and trace element solution (1 cm^3 of 1 g dm^{-3} $FeSO_4 \cdot 7H_2O$, 1 g dm^{-3} $MnSO_4 \cdot 4H_2O$, 25 mg dm^{-3} $CuCl_2 \cdot 4H_2O$, 100 mg dm^{-3} $CaCl_2$, 56 mg dm^{-3} H_3BO_3, 19 mg dm^{-3} $(NH_4)_2Mo_7O_{24} \cdot 4H_2O$, and 20 mg dm^{-3} $ZnSO_4 \cdot 7H_2O$). The seed flask was incubated at 26 °C in a rotatory incubator-shaker at 240 rpm for 3 days with a light cycle (40 W fluorescent bulb) of 12 h on, 12 h off. Aliquots (2 cm^3) of the resulting suspension of thick grey to black mycelium were used to inoculate twenty-four 500 cm^3 Erlenmeyer flasks containing LCM production medium [corn meal (20 g dm^{-3}), glucose (either 10, 40 or 75 g dm^{-3}), ardamine PH (5 g dm^{-3}), L-leucine (3.5 g dm^{-3}), MES (16.2 g dm^{-3}) and water (100 cm^3) adjusted to pH 6.0 with NaOH]. The production flasks were incubated at 25 °C in a rotatory incubator-shaker at 240 rpm for 7 days with full illumination.

Addition of precursors

Isotopically labelled precursors were added *via* a sterile syringe as aseptic solutions in water, except for diethyl malonate and **4** which were added as ethanol solutions in a volume of 0.2 cm^3 flask^{-1} day^{-1}. Compound **5** (100 mg flask^{-1} day^{-1}) was added as the solid. Additions were made daily in even aliquots over days 3 to 6, except where indicated. The amounts of precursor added to the cultures were: 15 mg flask^{-1} day^{-1} for all serine samples, 50 mg flask^{-1} day^{-1} for optimal incorporation of labelled acetates, 50 mg flask^{-1} day^{-1} for *S*-decanoyl-NAC **4** and 20 mg flask^{-1} day^{-1} for diethyl malonate. The glucose concentration in the medium was: 75 g dm^{-3} for all serine samples, 40 g dm^{-3} for optimal incorporation of labelled acetates, and 10 g dm^{-3} for *S*-decanoyl-NAC **4** and diethyl malonate.

Isolation of pramanicin 1 and dienone 3

Methyl ethyl ketone (100 cm^3 flask^{-1}) was added, and shaking continued in the rotatory incubator-shaker for 1 h. The mixture was centrifuged (8000 rpm, 15 min), and the organic and aqueous layers removed to a separatory funnel. The organic layer was collected, and treated with one volume of hexanes. The aqueous layer was then removed, and the organic layer concentrated *in vacuo*. The resulting extract was purified by flash chromatography on silica gel. After elution with 2% *v*/*v* MeOH–EtOAc, the pramanicin fraction was obtained by elution with 10% *v*/*v* MeOH–EtOAc. This material was concentrated *in vacuo*, dissolved in methanol, and the composition was adjusted to 70 : 30 MeOH–water. The precipitate was removed, and the solution loaded onto a commercial RP-8 extraction column (400 mg). Pramanicin was eluted with 70 : 30 MeOH–water and the eluate concentrated by removing the water as the ethanol azeotrope. The resulting material was dissolved in a minimum volume of 70 : 30 MeOH–water, and applied to a Merck LOBAR RP-8 column which was eluted in 70 : 30 MeOH–water. Fractions containing **1** or the deoxy compound **3** were combined and concentrated by azeotropic distillation with ethanol. For **1**, the spectral data were identical to those reported by Schwartz *et al.*[1] For **3**: mp 135–138 °C; λ_{max} (MeOH)/nm 288.5 (ε/dm^3 mol^{-1} cm^{-1} 26 800); ν_{max}/cm^{-1} 3000–3600br (O–H), 2956, 2923, 2854 (C–H), 1692, 1650 (C=O), 1586, 1009; NMR data are given in Table 1; MS (ES) 353 (M^+).

Dienone 3 from pramanicin 1

To a solution of pramanicin (71.4 mg, 194 μmol) in anhydrous acetonitrile (10 cm^3) were added toluene-*p*-sulfonic acid (107.5 mg) and sodium iodide (141.3 mg). The mixture was stirred for 90 min at room temp., and the solvent was removed. The residue was partitioned between chloroform (5 cm^3) and aq. sodium bicarbonate (1 M; 5 cm^3). After washing of the aqueous layer with two further aliquots of chloroform, the combined organic extracts were dried over sodium sulfate, filtered and concentrated. The residue was purified by chromatography on an RP-8 LOBAR column as above. The desired fractions were

combined and evaporated with ethanol to furnish compound **3** (49.5 mg, 72%).

S-[1-^{13}C]Decanoyl-*N*-acetylcysteamine 4

A solution of [1-^{13}C]decanoic acid (430 mg, 2.5 mmol) in dry THF (50 cm^3) was cooled to 0 °C and triethylamine (0.34 cm^3) then ethyl chloroformate (0.24 cm^3) were added dropwise *via* syringe. The solution was stirred for 1 h at 0 °C. To degassed nanopure water (40 cm^3), through which a slow stream of nitrogen was continuously bubbled, was added KOH (1.4 g) and, after cooling to room temperature, *N*,*S*-diacetylcysteamine (1.21 g). After stirring of the mixture at room temperature for 45 min, the pH was adjusted to 7.8 with conc. HCl. This solution was then added to the above solution of mixed anhydride. After stirring of the mixture for 1 h at 0 °C while the pH was maintained at 8.0 with conc. aq. HCl or KOH solutions, the pH was carefully adjusted to 3 using conc. HCl. Solvent was then removed on the rotary evaporator and the remaining aqueous solution was extracted with diethyl ether (3 × 75 cm^3). The organic layer was washed with 3% aq. HCl, dried over anhydrous sodium sulfate, and concentrated to give crude material as a slightly off-white solid. Flash column chromatography in EtOAc gave product **4** (620 mg, 90%) as a white solid, mp 59–61 °C; ν_{max}/cm^{-1} 3294br (N–H), 2954, 2917, 2850 (C–H), 1702, 1648 (C=O), 1553, 1468, 1360, 1295, 1148, 957, 758; ^{1}H NMR ($CDCl_3$; 200 MHz) δ 6.24 (1H, br s, NH), 3.33 (2H, q, *J* 6.0, CH_2), 2.94 (2H, dt, *J* 6.5, 5.5, CH_2), 2.48 (2H, dt, *J* 6.0, 7.2, CH_2), 1.89 (3H, s, CH_3C=O), 1.57 (2H, m, CH_2), 1.19 (12H, m, 6 × CH_2), 0.80 (3H, t, *J* 6.7, CH_3); ^{13}C NMR ($CDCl_3$; 50 MHz) δ 199.87 (enhanced s), 175.73, 43.94 (d, *J* 46), 39.51, 31.66, 29.19, 29.05 (2C), 28.76 (d, *J* 4), 28.25, 25.49, 22.95, 22.47, 13.91; *m*/*z* (EI) 275 (M + H, weak), 119, 60, 43; *m*/*z* (NH_3 − CI) 275 (M + H).

6-Pivaloylpramanicin 6a and deuterated sample 6b

To pramanicin (10.4 mg, 28.2 μmol) were added pivalic anhydride (0.02 cm^3) and triethylamine (0.18 cm^3). The mixture was heated to 60 °C for 15 min, cooled, and quenched with a small amount of ice. After removal of volatiles, the gummy residue was dissolved in EtOAc, applied to a flash chromatography column, and eluted with EtOAc. The product was collected in the first few fractions, and concentrated *in vacuo* to give **6a** as a white powder (7.1 mg, 56%); ^{1}H NMR (CD_3OD; 500 MHz) δ 7.06 (1H, dd, *J* 15.6, 1, 8-H), 6.64 (1H, dd, *J* 15.6, 7.0, 9-H), 4.37 (1H, dd, *J* 12.0, 3.0, 6-H^a), 4.17 (1H, d, *J* 7.4, 4-H), 4.05 (1H, dd, *J* 12.0, 4.4, 6-H^b), 3.62 (ddd, *J* 7.4, 4.5, 3.0, 5-H), 3.32 (1H, m, 10-H), 2.92 (1H, m, 11-H), 1.6 (2H, m, 12-H_2), 1.4 (2H, m, 13-H_2), 1.29 (12H, m, 14- to 19-H_2), 1.21 (9H, s, Bu^t), 0.89 (3H, t, *J* 6.6, 20-H_3). Pramanicin derived from DL-[2,3,3-$^2H_3$]serine was similarly converted to the pivaloyl derivative **6b**: ^{2}H NMR (CH_3OH; 76.77 MHz) δ 4.30, 4.00, 3.55.

DL-[2-^{2}H, 3-^{13}C]Serine 7a

DL-[3-^{13}C]Serine (250.5 mg, 2.37 mmol) and $Al_2(SO_4)_3 \cdot 18H_2O$ (57.6 mg) were dissolved in D_2O (3 cm^3); the solution was adjusted to pD 9.6, and lyophilised. Pyridoxal was liberated from the hydrochloride (94 mg) according to the literature procedure,[25] and mixed with the lyophilised serine in D_2O (5 cm^3). The solution was stirred in the dark for 24 h, then adjusted to pH 6.5 with HCl. Ethanol (1.5 vol) was added and the mixture allowed to crystallise overnight at −20 °C. The crystals were collected, washed with EtOH, and dried *in vacuo* over phosphorus pentoxide to give product **7a** (178 mg, 70%), ^{1}H NMR (D_2O; 200 MHz) δ 3.77 (1H, dd, *J* 146, 12, 3-H^a), 3.73 (1H, dd, *J* 146, 12, 3-H^b), 3.62 (0.02H, m, residual 2-H).

3,5-Dinitrobenzoyl-DL-[2-^{2}H,3-^{13}C]serine 8

To a chilled solution of DL-[2-^{2}H,3-^{13}C]serine **7a** (178 mg, 1.66 mmol) in NaOH (1 M; 3 cm^3) was added 3,5-dinitrobenzoyl chloride (230 mg) and the mixture was stirred for 10 min. More NaOH (2 cm^3) and acid chloride (370 mg) were added and the mixture was stirred for 1 h. The precipitate was collected, washed with diethyl ether (2 × 10 cm^3) and dried *in vacuo* to give the product **8** (364 mg, 77%) which was used immediately without further characterisation due to poor solubility.

3,5-Dinitrobenzoyl-D-[2-^{2}H,3-^{13}C]serine chloramphenicol salt 9

Product **8** (364 mg, 1.21 mmol) and chloramphenicol (154.5 mg, 729 μmol, 1.2 eq. with respect to D-enantiomer) were dissolved in methanol (0.35 cm^3) by heating. Ethyl acetate (3.5 cm^3) was added, and crystallisation induced by partial evaporation of methanol and addition of more EtOAc. The crystals were collected, washed with EtOAc, and dried *in vacuo* to give the salt **9** (280 mg, 89% on D-enantiomer), $[\alpha]_D^{28}$ −29.7 (*c* 1.09, H_2O) [lit.,[29] −32.2 (*c* 1, H_2O)]. The filtrate was retained for preparation of the L-enantiomer (see below).

3,5-Dinitrobenzoyl-D-[2-^{2}H,3-^{13}C]serine and D-[2-^{2}H,3-^{13}C]-serine hydrochloride 7b

The chloramphenicol salt **8** (280 mg, 545 μmol) was dissolved in water (4 cm^3) and treated with NaOH (10 M; 0.075 cm^3). Conc. HCl (0.2 cm^3) was added dropwise, and the solution kept until no further tan precipitate appeared. After cooling on ice for 30 min, the precipitate was collected, washed with HCl (5%, 1 cm^3) and dried *in vacuo* over phosphorus pentoxide to give the DNB-serine (127.5 mg, 77%). A mixture of this material (120 mg, 400 μmol), water (0.9 cm^3) and conc. HCl (0.9 cm^3) was heated at reflux for 1 h. The mixture was filtered and the solid was washed successively with HCl (5%; 1 cm^3) and water (1 cm^3). The filtrate was concentrated by azeotropic distillation from ethanol, and the residue triturated twice with acetone, then dried to give the product **7b** (49.8 mg, 87% of hydrochloride), $[\alpha]_D^{28}$ −9.0 (*c* 1, H_2O) [lit.,[29] −12 (*c* 1, HCl)]; ^{1}H NMR (D_2O; 200 MHz) δ 3.82 (1H, dd, *J* 148, 12.5, 3-H^a), 3.76 (1H, dd, *J* 148, 12.5, 3-H^b), 3.62 (m, 0.02H, residual 2-H); ^{13}C NMR (D_2O; 50 MHz) δ 172.4 (s), 60.3 (enhanced s), the C-α signal was not observed; *m*/*z* (NH_3-CI) 108 ([^{13}C,^{2}H]MH^+); *m*/*z* (EI) 108 ([^{13}C,^{2}H]MH^+), 76 ([^{2}H][MH − $^{13}CH_2O]^+$), 75 ([$CD(NH_2)CO_2H]^+$), 62 ([^{13}C,^{2}H]-[MH − $CO_2H]^+$), 58 (unassigned), 44 ([^{13}C,^{2}H][CH_2CH=$NH]^+$); TLC: single spot on CHIRALPLATE in acetone–MeOH–water 10:2:2 with detection by 0.3% ninhydrin and heating.[30]

L-Enriched 3,5-dinitrobenzoyl-[2-^{2}H,3-^{13}C]serine and the quinidine salt 10

The filtrate from the chloramphenicol crystallisation above was concentrated, the resulting oil was suspended in EtOAc (2 cm^3), and the solution was washed successively with HCl (2 M, 1 cm^3), then water; both aqueous layers were further extracted with ethyl acetate. The combined organic layers were dried (Na_2SO_4), filtered, and concentrated *in vacuo* to give a foam (195 mg, 96%) which was then triturated with hexanes, and collected by filtration. This material and quinidine (184 mg, 565 μmol) were heated in ethanol (2.1 cm^3) until they dissolved. After cooling for 90 min, the crystals were collected, washed with ethanol (2 cm^3), and dried (*in vacuo* over phosphorus pentoxide) to give salt **10** (207 mg, 59% on quinidine).

L-3,5-Dinitrobenzoyl-[2-^{2}H,3-^{13}C]serine and L-[2-^{2}H,3-^{13}C]-serine hydrochloride 7c

Salt **10** (207 mg, 331 μmol) was suspended in EtOAc (2 cm^3), and the mixture was washed successively with HCl (2 M; 1 cm^3), then water; both aqueous layers were further extracted with ethyl acetate. The combined organic layers were dried (Na_2SO_4), filtered, and concentrated *in vacuo*. Water (0.8 cm^3) and conc. HCl (0.8 cm^3) were added and the mixture heated at reflux

for 1 h. The mixture was filtered and the solid washed with HCl (5%; 1 cm^3). The filtrate was concentrated by azeotropic distillation from ethanol, and the residue triturated twice with acetone, then dried (*in vacuo* over phosphorus pentoxide) to give the product **7c** (48.7 mg, 100% of hydrochloride), $[\alpha]_D^{28}$ 10.0 (*c* 1, H_2O); TLC: single spot on CHIRALPLATE at lower R_f than the D-enantiomer (above); ^1H NMR, ^{13}C NMR, CI-MS and EI-MS gave results that were identical to those for the D-enantiomer.

DL-[1-^{13}C,$^{18}O_2$]Serine 11

DL-[1-^{13}C]Serine (106 mg), $H_2{}^{18}O$ (0.09 cm^3) and conc. HCl (0.1 cm^3) were sealed into a flame-dried glass tube under a nitrogen atmosphere. The tube was heated at 90 °C in an oil-bath for 5 days. After cooling, the mixture was neutralised with NaOH and lyophilised. The resulting material containing the product and sodium chloride was used without further purification. ^1H NMR (D_2O; 200 MHz) spectrum was indistinguishable from that for DL-[1-^{13}C]serine; ^{13}C NMR (D_2O; 50 MHz) spectrum was as for the starting material but the C=O resonance at δ_C 172.4 was enhanced and exhibited 3 peaks, $\Delta\delta$ 0.027 ppm per ^{18}O; 24% $^{18}O_2$, 48% $^{18}O^{16}O$, 28% $^{16}O_2$, corresponding to 70 atom% ^{18}O per site.

Acknowledgements

Financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC) is gratefully acknowledged. Cambridge Isotopes is thanked for a grant of isotopically labelled compounds. $H_2{}^{18}O$ was a generous gift from Professor J. C. Vederas, University of Alberta. We thank Professor A. D. Bain at McMaster for assistance with the spectral simulation.

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