The biosynthesis of pramanicin: intact incorporation of serine and absolute configuration of the antibiotic

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Biosynthetic incorporation of isotopically labelled serines into pramanicin 1 in *Stagonospora* sp. ATCC 74235 shows that L-serine is incorporated as an intact entity with all four bonds to the α-carbon retained; thus 1 is assigned the 5*S* absolute configuration.

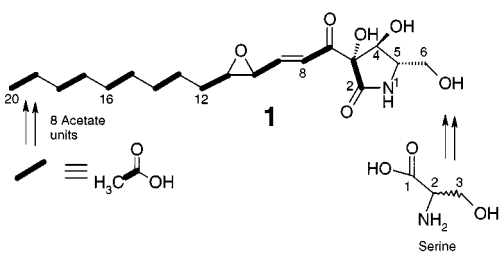
We have recently described the biosynthesis of pramanicin[1] **1** (Scheme 1) in *Stagonospora* sp. ATCC 74235.[2] Eight acetate units combine in the 'head-to-tail' manner typical of fatty acids and polyketides[3] prior to cyclization with the three-carbon precursor corresponding to carbon atoms 4 to 6 in **1**. The resulting putative tetradeca-2,4-dienoyltetramic acid[1] is then presumably further modified to furnish **1**. We demonstrated that incorporation of DL-[1-^{13}C]serine proceeded very efficiently: only C-4 of **1** was labelled, suggesting that serine is the direct amino acid precursor of **1**. However, serine can be converted to a number of metabolites *in vivo*, and we thus sought further evidence for the proposed intact incorporation of a serine entity into **1**. We also wished to establish which enantiomer of serine is utilised in the biosynthetic pathway and to correlate these results with the as yet unknown absolute configuration of pramanicin. Experiments directed toward these issues are described herein.

Cultures of *Stagonospora* sp. ATCC 74235 were grown,† precursors were added, and pramanicin was isolated as described previously.[1,2] Incorporations of L-[2,3,3-$^{2}H_3$]serine, L-[1,2,3-$^{13}C_3$,^{15}N]serine, and the separated D- and L-enantiomers of [2-^{2}H,3-^{13}C]serine‡ furnished samples of pramanicin **2–5**, respectively (Scheme 2).

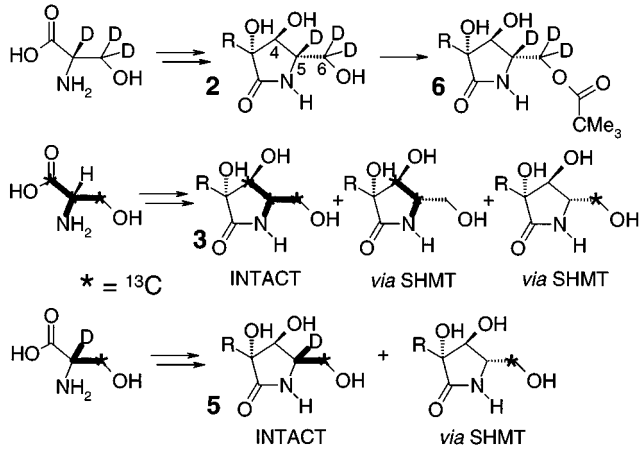
Pramanicin **2** derived from L-[2,3,3-$^{2}H_3$]serine exhibits two resonances in the ^{2}H{^{1}H} NMR spectrum in MeOH. One signal corresponds in chemical shift to one of the two diastereotopic protons at C-6 (δ 3.79), which is well separated from other resonances in the proton NMR spectrum. The other deuterium signal, somewhat larger than the first, correlates with the proton chemical shift of the other C-6 proton and to that of H-5; these two resonances are close in proton chemical shift (δ 3.55 and 3.49, respectively), and the breadth of the signals in the deuterium NMR spectrum precluded their resolution. These resonances remained unresolved in acetone, but the spectrum in DMSO, where the difference in shift between the two protons is larger,[2] did exhibit a clear shoulder, most notably when the sample was heated to 85 °C to reduce viscosity and enhance ^{2}H relaxation. Nonetheless, we sought to clarify this result, and thus **2** was converted to the mono-pivaloyl derivative **6** (pivaloyl chloride, TEA, 70 °C, 30 min, Scheme 2). In the proton NMR spectrum of **6**, the two C-6 protons are shifted downfield by 0.58 and 0.51 ppm, relative to **1**, while H-5 gives a unique resonance which is shifted downfield by only 0.13 ppm. The deuterium NMR spectrum of **6** exhibited three resonances, corresponding to the two H-6 (δ 4.30 and 4.00), and the H-5 (δ 3.55) resonances [Fig. 1(A)].§ However, the D-5 signal intensity was significantly weaker than either of the D-6 resonances, and integration showed that only about 0.5 deuterons were present at this site.

These results clearly show that L-serine is a viable precursor to pramanicin: a strong deuterium signal was observed although the precise incorporation could not be measured in this experiment. Further, there is no cryptic oxidation state change at C-3 of serine since both of the diastereotopic deuterons at this position are incorporated. The retention of some deuterium at C-5, derived from H-2 of serine, is most readily explained by direct incorporation of the L-enantiomer. The observed loss of deuterium would arise from an incidental, reversible process which results in net exchange of the α-proton. This solvent exchange could conceivably be accomplished by the action of a serine racemase;[9,10] a transaminase (to interconvert serine and 3-hydroxypyruvate);[9] serine hydroxymethyl transferase (SHMT), which interconverts serine with glycine and methylene-tetrahydrofolate (CH_2-THF);[9,11] or any of several enzymes which catalyse β-elimination or β-replacement reactions on serine or its derivatives.[9] Extensive investigations of amino acid racemases [whether double-base or pyridoxal phosphate (PLP) dependent] have shown that this process invariably occurs with substantial or complete loss of the substrate α-proton.[10] However, the PLP-dependent SHMT catalyses several processes, including racemisation of alanine and the exchange of both the prochiral α-protons of glycine, and thus this enzyme could possibly lead to deuterium-labelled D-serine which could then be incorporated into **1**.

To resolve these issues, we next incorporated L-[1,2,3-$^{13}C_3$,^{15}N]serine. Analysis of the extensive coupling pattern [Fig. 1(B)] in the ^{13}C{^{1}H} NMR spectrum of the derived pramanicin **3** showed the labelling pattern of [4,5,6-$^{13}C_3$,^{15}N]pramanicin, derived from an intact serine molecule, along with the [6-^{13}C]- and [4,5-$^{13}C_2$,^{15}N]-iso-



Scheme 1



Scheme 2

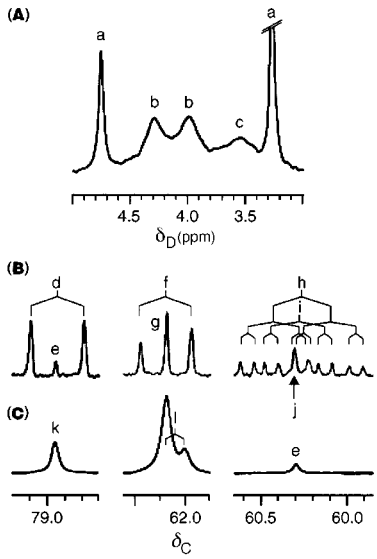


Fig. 1 (A) $^{2}H\{^{1}H\}$ NMR spectrum of 6-pivaloylpramanicin **6** derived from L-[2,3,3-$^{2}H_3$]serine: (a) natural abundance signals from MeOH solvent; (b) diastereotopic deuterons at C-6; (c) 5-D. (B), (C) Partial $^{13}C\{^{1}H\}$ NMR spectra of pramanicin **1** derived from (B) L-[1,2,3-$^{13}C_3$,^{15}N]serine and (C) L-[2-^{2}H,3-^{13}C]serine mixed with DL-[1-^{13}C]serine. Left: C-4; centre: C-6; right: C-5. For complete assignments, see ref. 1,2. Traces in (B) have the same scaling for both axes, as do those in (C), but (B) and (C) have different natural abundance peak heights. (d) $^{1}J_{^{13}C\text{-}4\text{-}^{13}C\text{-}5}$ = 38 Hz; (e) natural abundance signal; (f) $^{1}J_{^{13}C\text{-}5\text{-}^{13}C\text{-}6}$ = 40 Hz; (g) enriched singlet from action of SHMT on labelled CH_2—THF and unlabelled glycine; (h) coupled signals from [4,5,6-$^{13}C_3$,^{15}N]pramanicin from an intact serine unit, $^{1}J_{^{13}C\text{-}4\text{-}^{15}N}$ = 10 Hz; (i) signals for [4,5-$^{13}C_2$,^{15}N]pramanicin from action of SHMT on unlabelled CH_2—THF and labelled glycine; (j) natural abundance signal plus coupled component; (k) enhanced singlet from [1-^{13}C]serine; (l) β-deuterium isotope shift, $\Delta\delta$ 74 ppb.

topomers. The ^{15}N NMR spectrum showed only a coupled signal,¶ so all molecules labelled at C-2 are attached to ^{15}N and all enriched ^{15}N atoms are adjacent to ^{13}C. Thus there is no detectable action of either transaminase or serine β-elimination enzymes *en route* to **1**. However, SHMT is present in this microbial strain, and gives rise to the two non-intact isotopomers *via* cleavage of serine to [^{13}C]CH_2-THF and [$^{13}C_2$,^{15}N]glycine, followed by re-condensation of each fragment with the corresponding unlabelled partner. Nonetheless, the observation of substantial intact incorporation shows that SHMT is not required for the biosynthesis of **1** from serine. The results also exclude biosynthetic mechanisms in which the polyketide component is produced as an amide, and subsequent condensation with an α-keto acid leads to **1**.

When the L-enantiomer of [2-^{2}H,3-^{13}C]serine was mixed with DL-[1-^{13}C]serine (32 mol%) as internal standard and incorporated into **1**, C-4 and C-6 [18 and 82%, respectively, of total ^{13}C incorporated, Fig. 1(C)] were labelled with carbon-13, and the C-6 resonance exhibited a shifted signal ($\Delta\delta$ 74 ppb, 28% of total C-6 enrichment) due to a β-deuterium isotope shift. The corresponding D-[2-^{2}H,3-^{13}C]serine similarly mixed with DL-[1-^{13}C]serine gave product labelled with carbon-13 only at the control site, C-4.‖ Thus, the D-enantiomer is not a significant precursor for **1**, while the C-3:C-2:H-2 unit of L-serine is partially incorporated in an intact manner into C-6:C-5:H-5 of **1**. The remaining enrichment of the C-6 signal without concomitant incorporation of deuterium can be accounted for by the reversible action of SHMT.

In summary, L-serine is the true biosynthetic precursor for **1**, and is converted with all four atoms which are attached to the α-carbon atom retained. SHMT is not required in the pathway. These results are completely consistent with the proposed route to **1** *via* an acyltetramic acid.[1] The simplest conclusion is that the absolute configuration of pramanicin at C-5 is the same as that of L-serine, *i.e.* 5*S*; the remaining chiral centres in the tetramic acid moiety of **1** are then defined by the work of Schwartz *et al.*,[2] who determined the relative configurations of C-3 to C-5. The absolute configuration of the *trans*-epoxide remains undetermined. There is no evidence for racemisation of L-serine, or epimerisation of other biosynthetic intermediates in the pathway to **1**, which would lead to the opposite configuration of **1**, *i.e.* 5*R*. Although this possibility cannot be rigorously excluded by these or indeed by other whole-cell isotope labelling experiments, it would require the action of an enzyme which alters the configuration yet proceeds with substantial retention of the α-proton; there is little precedent for such an activity.[10] Further work on the pathway to the polyketide moiety of **1**, as well as the X-ray crystal structure of **1**, will be described shortly.

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Notes and references

† *Stagonospora* was cultured in liquid medium LCM (100 ml in 500 ml Erlenmeyer flasks). Labelled serines (12–20 mg per culture flask) were added as sterile solutions in water at 24 h intervals over days 2–6. After 7 days, work-up as previously described gave *ca*. 10 mg of **1** per flask.

‡ The samples of D- and L-[2-^{2}H,3-^{13}C]serine were prepared from DL-[3-^{13}C]serine using the pyridoxal-dependent exchange of the α-proton in D_2O as described for [2-^{2}H]serine by Miles and McPhie (ref. 4), using some of the modifications of de Kroon *et al*. (ref. 5), as well as those of Townsend *et al*. (ref. 6), who prepared [2-^{3}H,1-^{14}C]serines; some minor modifications designed to conserve the yield based on labelled serine were also used. For the same reason, the resolution method of Velluz *et al*. (ref. 7) as modified by Gorissen *et al*. (ref. 8) was used in preference to the multi-step chemical and enzymatic resolution used by the former workers. All new compounds and isotopomers gave satisfactory spectral data; for serine hydrochlorides, $[\alpha]_D^{30}$: D, −9.0 ± 0.5; L, +10.0 ± 0.5 (*c* 1, H_2O).

§ The measured shifts in the deuterium NMR spectrum are 0.005–0.006 ppm upfield of those in the proton spectrum; this can be ascribed to the method of referencing the two spectra. Proton spectra in CD_3OD are referenced to C*H*D$_2$OD at δ 3.30, while the deuterium spectra in CH_3OH are referenced to natural abundance CH_2*D*OH; the observed solvent nuclei thus experience different isotope shifts.

¶ The ^{15}N NMR spectrum was recorded in DMSO-d_6, using polarisation transfer by the INEPT sequence: δ −256.4 relative to CH_3NO_2 at δ 0 (lit.[2] −229.1, converted from a different external standard), dd ($^{1}J_{^{15}N\text{-}^{1}H}$ 93 Hz, lit.[2] 92 Hz, $^{1}J_{^{15}N\text{-}^{13}C}$ 9 Hz).

‖ A small enhancement (0.14%) at C-6 was observed; this may however be accounted for by traces of the L-enantiomer present in the sample (< 2% required).

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