ChemComm

Investigation of glycerol incorporation into soraphen A

Alison M. Hill,* Jonathan P. Harris and Alexandros P. Siskos

Department of Chemistry, King's College London, Strand, London, UK WC2R 2LS. E-mail: alison.hill@kcl.ac.uk

Received (in Cambridge, UK) 3rd August 1998, Accepted 25th September 1998

Glycerol has been incorporated mid-chain into the polyketide soraphen A 1 at C-3,4 and C-11,12; the *pro*-(*S*)-hydroxymethyl group of glycerol is lost and one of the hydrogens in the *pro*-(*R*)-hydroxymethyl group is retained at C-11 which excludes hydroxymalonate as the immediate precursor to the vicinal methoxy groups at C-11,12.

In polyketide biosynthesis, both the chain starter and extension units are bound *via* thioester linkages to the polyketide synthase (PKS) where condensation takes place. Subsequent condensations occur with the growing acyl chain remaining enzyme bound, until a chain of the correct length is formed. This is released to give the first enzyme free intermediate which can undergo further enzymically catalysed modifications to yield the final product. Acetate and propionate *per se* are not used as the chain extension units, but are activated by carboxylase enzymes to generate malonyl CoA and methylmalonyl CoA, respectively, which undergo a decarboxylative condensation with the enzyme bound acyl chain to add a C_2 or C_3 unit.[1]

The carbon skeleton of the potent antifungal polyketide metabolite, soraphen A **1**, is derived from a benzoyl CoA starter unit, three acetate and three propionate chain extender units and three methionines (Fig. 1);[2] the vicinal hydroxy groups at carbons 3,4 and 11,12 were not labelled by acetate. Unpublished work from Höfle's laboratory has shown that [2-^{13}C]glycerol was incorporated into **1** at carbons 4 and 12.[3] The incorporation of glycerol mid-chain into polyketide metabolites is highly unusual and has been observed in only two other metabolites: leucomycin[4] and concanomycin.[5] It has been postulated in both cases that glycerol is converted to glycolate (or an activated form such as 2-phosphoglycolate) prior to incorporation into the final metabolite. The incorporation of glycolate into geldanamycin[6] has also been observed and it is postulated to be an intermediate in niddamycin biosynthesis,[7] however, it is possible that for all four polyketides, activation of this C_2 unit by a carboxylase enzyme to generate hydroxymalonyl CoA or methoxymalonyl CoA occurs. Here we report the results of preliminary labelling experiments which establishes that the incorporation of glycerol mid-chain into soraphen A is stereospecific and that hydroxymalonate cannot be the immediate precursor for the C_2 unit incorporated at C-11,12.

When [1,3-$^{13}C_2$]glycerol **2a** was administered to the organism,† enhanced signals in the ^{13}C NMR spectrum were observed in the resulting **1** compared with an unlabelled control sample. The feeding experiment resulted in a complex labelling pattern as glycerol had been extensively metabolised through glycolysis, the Krebs cycle and the shikimate pathway. Consequently, enrichments were observed for all the β-positions of acetate-derived carbons, for all positions which originate from propionate, and the phenylalanine-derived carbons C-17, 2′/6′, and 4′ (Fig. 2). Enrichment of the methionine-derived carbon atoms was also observed due to the metabolism of glycerol to *S*-adenosylmethionine *via* serine. The highest level of incorporation of ^{13}C label into **1**, however, was observed at C-3 and 11 (11–15-fold). The feeding experiment with [2-^{13}C]glycerol also gave rise to a complex labelling pattern which was similar but not identical to that obtained by Höfle.[3] The ^{13}C label was incorporated into C-4 and 12 as well as the carboxylate-derived positions of acetate and the phenylalanine-derived carbons 3′/5′ (data not shown). However, the enrichment levels we observed were much higher (*e.g.* 8–13-fold for C-4 and 12, *cf.* 3-fold) and we also observed enrichments of the carboxylate-derived positions of propionate and the phenylalanine-derived carbon 1′ (data not shown).

The above results demonstrated that glycerol was being metabolised *via* glycerate to acetate. Glycerate can also be oxidised to give serine, and subsequently glycine, which is a source of glycolate.[4] Hence, we decided to investigate the possible involvement of glycolate as the C_2 source for the vicinal hydroxy groups at carbons 3,4 and 11,12 by feeding [2-^{13}C]glycine. When [2-^{13}C]glycine was fed to *Sorangium cellulosum*, however, no enhanced signals in the ^{13}C NMR spectrum were observed in the resulting **1** compared with an unlabelled control sample. Labelling studies with [1-^{13}C]glycine and [1-^{13}C]glycolate to investigate leucomycin A biosynthesis resulted in the incorporation of neither precursor into the metabolite, which the authors explained was due to a cell-membrane permeability problem.[4] Hence, it is possible that we have the same problem and that glycolate is still a possible precursor in soraphen A biosynthesis.

To investigate the mechanism of glycerol incorporation into soraphen A further, [$^2H_8$]- and *rac*-, (*R*)- and (*S*)-[1-$^2H_2$]-glycerol‡ were administered to *S. cellulosum* and the resulting **1** analysed by ^{2}H NMR spectroscopy. Deuterium was observed at D-11 and D-14a but it was not incorporated at the same level in all cases (Table 1): the highest percentage incorporation was observed for [$^2H_8$]glycerol and (*R*)-[1-$^2H_2$]glycerol **2b**. The C-3 propionate-derived positions (*i.e.* H-18, 20 and 21) and methionine-derived positions (*i.e.* H-19, 22 and 23) were labelled for the racemic glycerol samples (data not shown) indicating that extensive metabolism had once again occurred.

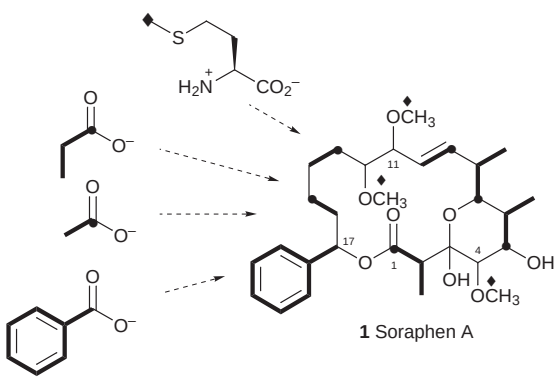

Fig. 1 Biosynthetic origin of soraphen A **1** (ref. 2).

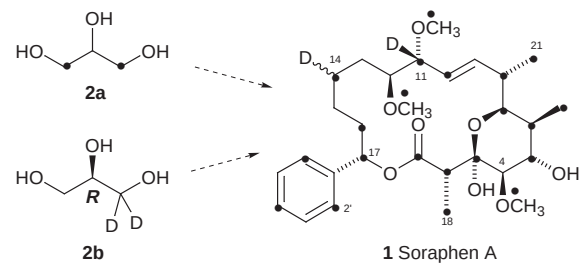

Fig. 2 Incorporation of [1,3-$^{13}C_2$]- and (*R*)-[1-$^2H_2$]-glycerol (**2a** and **2b**) into soraphen A **1**.

Table 1 Incorporation of [$^2H_8$]- and *rac*-, (*R*)- and (*S*)-[1-$^2H_2$]-glycerol into soraphen A **1**

Glycerol	^{2}H Enrichment (%)[a] D-11	D-14
[$^2H_8$]	6.6	2.3
rac-[1-$^2H_2$]	1.5	2.0
(*R*)-[1-$^2H_2$]	5.4	4.3
(*S*)-[1-$^2H_2$]	1.1	0.5

[a] Expressed as % deuterium calculated from natural abundance chloroform signal.

No deuterium was observed at H-4 in **1** from the [$^2H_8$]glycerol feeding experiment, indicating that oxidation at C-2 had taken place before incorporation into H-4. It was not possible to say with certainty that H-12 was not labelled as its signal overlaps with those of H-19 and 23. The incorporation of deuterium at H-11 was 5-fold higher for (*R*)-glycerol compared to its antipode; moreover, incorporation into H-14 was also higher for the (*R*)-enantiomer, demonstrating that this enantiomer was metabolised by the organism in preference to (*S*)-glycerol. Recent experiments conducted with chiral glycerols have shown that (*R*)-glycerol is metabolised in preference to its enantiomer in the biosynthesis of fluoroacetate and 4-fluorothreonine in *Streptomyces cattleya*,[8] and in the biosynthesis of macrophomic acid by the fungus *Macrophoma commelinae*.[9] It is notable that the three different organisms, producing structurally diverse compounds, all metabolise glycerol such that the *pro*-(*R*)-hydroxymethyl group is retained in the metabolite and the *pro*-(*S*)-hydroxymethyl group is lost.

The results from these experiments are consistent with the following observations: the *pro*-(*R*)-hydroxymethyl group is retained and incorporated into **1** at C-3 and 11 [consequently the *pro*-(*S*)-hydroxymethyl group is lost] and C-2 of glycerol is incorporated into C-4 and 12 of **1**. Oxidation of C-2 takes place prior to incorporation into C-4 and possibly also for C-12.

These results raise some interesting questions on how the C_2 unit is incorporated by the soraphen A PKS. Chain extender units, such as malonyl CoA, are attached to the PKS *via* thioester linkages. Clearly, glycerol itself cannot be the immediate precursor in soraphen A biosynthesis. Oxidation of the *pro*-(*S*)-hydroxymethyl group would provide a carboxy group which could be used in the decarboxylative condensation step with the enzyme bound tri- and hepta-ketide intermediates. However, how the *pro*-(*R*)-hydroxymethyl group attaches itself to the PKS is a much more difficult problem. It is possible that glycerol is metabolised to glycolate and then hydroxymalonate (or even methoxymalonate). This mechanism would require oxidation of the *pro*-(*R*)-hydroxymethyl group to a carboxylic acid or coenzyme A thioester, which could then attach to the PKS *via* a thioester linkage. This mechanism is consistent with the ^{13}C labelling experiments, but it would result in complete loss of the deuterium label on the *pro*-(*R*)-hydroxymethyl group, which is inconsistent with the ^{2}H labelling experiments. Hence, while it is possible for the C_2 unit incorporated into C-3,4 of **1** to derive from hydroxy- or methoxy-malonate, retention of the deuterium label at H-11 excludes these precursors as the source for the C_2 unit incorporated at C-11,12. The *pro*-(*R*)-hydroxymethyl group of glycerol must remain as an alcohol (or phosphorylated derivative) or be oxidised to an aldehyde before incorporation into C-11,12; *it cannot be oxidised to a carboxylic acid or coenzyme A thioester*. An alternative, but less likely, explanation is that the *pro*-(*R*)-hydroxymethyl group of glycerol is oxidised to a carbonyl moiety and subsequent reduction, with redelivery of deuterium from a co-factor, results in deuterium enrichment at H-11. Experiments are in progress to further probe the mechanism of glycerol incorporation into soraphen A and to identify a more immediate precursor than glycerol in its biosynthesis. Possible candidates include hydroxypyruvate and glycerate.

Sorangium cellulosum kanamycin resistant wild-type strain M15 was provided by Novartis, Research Triangle Park, North Carolina, USA. We thank Dr T. Schupp (Novartis, Switzerland) for providing Probion S and Mrs J. Hawkes and Mr J. Cobb for NMR support. Deuterium NMR spectra were obtained using the Bruker AMX 400 University of London Intercollegiate Research Service for NMR at King's College. This work was financially supported by funding from King's College, London, The Royal Society, The University of London Central Research Fund and the State Scholarships Foundation (Republic of Greece) (A. P. S.). We thank Dr H. C. Hailes (University College London) for helpful discussions.

Notes and references

† Cultures of a kanamycin resistant strain of *S. cellulosum* (wild-type strain M15) were used in the incorporation experiments. In a typical experiment, production medium G55 (ref. 11) containing 50 g l^{-1} XAD-1180 resin was inoculated with a 9 day old growth medium G51t (ref. 12) culture and isotopically labelled compounds were pulse-fed in duplicate every 48 h between 10 and 16 days post inoculation. [1,3-$^{13}C_2$]-, [2-^{13}C]- and [$^2H_8$]-glycerol (1 g each) and [1-$^2H_2$]-, (*R*)-[1-$^2H_2$]- and (*S*)-[1-$^2H_2$]-glycerol (0.4–0.58 g each) were added as sterile aqueous solutions. The cultures were harvested on day 19 and the resin extracted with Pr^{i}OH. The crude soraphen A was purified by flash chromatography and preparative reverse phase HPLC (C-18) prior to NMR analysis. Typically 30 mg l^{-1} of soraphen A was obtained.

‡ [1-$^2H_2$]Glycerol was made in four steps from solketal: (i) solketal, $KMnO_4$, NaOH; (ii) CH_2N_2 (60% over 2 steps); (iii) $LiAlD_4$ (95%); (iv) AcOH, H_2O, room temp., 16 h (97%). (*R*)- and (*S*)-[1-$^2H_2$]glycerol were made in two steps from methyl isopropylidine-L-glycerate and methyl isopropylidine-D-glycerate, respectively, in 92% yield as above using a modified version of the protocol previously reported (ref. 8); (*R*)-[1-$^2H_2$]solketal, $[\alpha]_D$ +11.2 (*c* 9.4, MeOH) [lit. +15.3 (neat) (ref. 9)] and (*S*)-[1-$^2H_2$]solketal $[\alpha]_D$ −12.4 (*c* 8.7, MeOH) [lit. −14.9 (neat) (ref. 9)]. All three compounds contained 94% deuterium at C-1.

1 D. O'Hagan, *The Polyketide Metabolites*, 1991, Ellis Horwood, Chichester.
2 A. M. Hill and J. P. Harris, unpublished results.
3 N. Bedorf, B. Böhlendorf, D. Schomberg, K. Gerth, H. Reichenbach and G. Höfle, unpublished results.
4 S. Omura, K. Tsuzuki, A. Nakagawa and G. Lukacs, *J. Antibiot*., 1983, **36**, 611.
5 K. U. Bindseil and A. Zeeck, *Helv. Chim. Acta*, 1993, **76**, 150.
6 A. Haber, R. D. Johnson and K. L. Rinehart Jr, *J. Am Chem. Soc*., 1977, **99**, 3541.
7 S. J. Kakavas, L. Katz and D. Stassi, *J. Bacteriol*., 1997, **179**, 7515.
8 J. Nieschalk, J. T. G. Hamilton, C. D. Murphy, D. B. Harper and D. O'Hagan, *Chem. Commun*., 1997, 799.
9 H. Oikawa, K. Yagi, K. Watanabe, M. Honma and A. Ichihara, *Chem. Commun*., 1997, 97.
10 R. E. Hill, A. Iwanow, B. G. Sayer, W. Wysocka and I. D. Spenser, *J. Biol. Chem*., 1987, **262**, 7463.
11 S. Jaoua, S. Neff and T. Schupp, *Plasmid*, 1992, **28**, 157.
12 T. Schupp, C. Toupet, B. Cluzel, S. Neff, S. Hill, J. J. Beck and J. M. Ligon, *J. Bacteriol.*, 1995, **177**, 3673.

Communication 8/06112B