Fluorometabolite biosynthesis: Isotopically labelled glycerol incorporations into the antibiotic nucleocidin in *Streptomyces calvus.*

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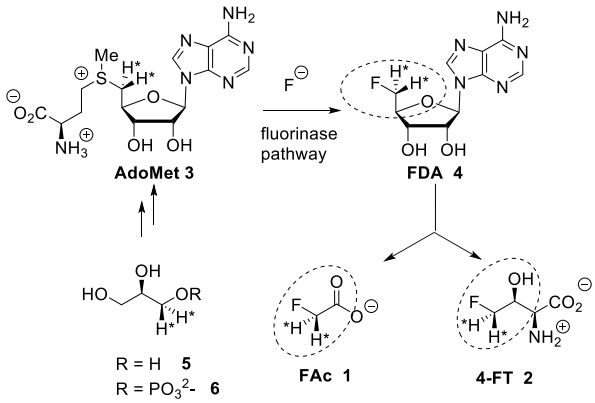
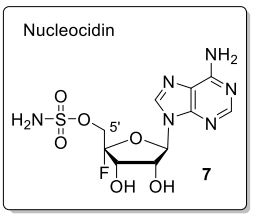
Deuterium and carbon-13 labelled glycerols have been fed to *Streptomyces calvus* fermentations and isotope incorporation into the fluorine containing antibiotic nucleocidin have been evaluated by ^{19}F-NMR. A single deuterium atom was incorporated from [$^2H_5$]- and (*R*)-[$^2H_2$]- glycerol into C-5' of the antibiotic, suggesting that an oxidation occurs at this carbon after ribose ring assembly from glycerol (pentose phosphate pathway), during nucleocidin biosynthesis.





Scheme 1. Fluorinase reaction of AdoMet **3** to give FDA **4** and then the relationship between FDA and FAc **1** and 4-FT **2**. Incorporation of (*R*)-[$^2H_2$]- glycerol determined that both fluoromethyl hydrogens at C5'of FDA **4** are retained in the fluorometabolites.[9] Nucleocidin **7** is a metabolite of *S. calvus*.

Fluorine containing natural products are exceedingly rare in nature.[1] The toxin fluoroacetate (FAc) **1** is the most widely distributed natural product of this class where it has been identified in many plants[2] and a few bacteria.[3,4] The mode of fluoroacetate biosynthesis in plants has not been established, however its biosynthesis in bacteria is known.[5] *Streptomyces cattleya* is a bacterium that co-produces FAc **1** and 4-fluorothreonine (4-FT) **2**.[3] Studies carried out with *S. cattleya* determined that the fluorination enzyme (fluorinase) catalyses a nucleophilic substitution reaction between fluoride ion and *S*-adenosyl-L-methionine (Ado-Met) **3** to generate 5'-fluorodeoxyadenosine (FDA) **4**.[6] This is the only biosynthetic fluorination enzyme known. It has subsequently been identified by gene mining in several other bacteria.[7] FDA **4** is then processed to FAc **1** and 4-FT **2**. A combination of isotopic labelling studies, including isotopically labelled glycerol **5**, and an understanding of the individual steps in the biosynthesis of **1** and **2** have determined that the C5' fluoromethyl and C4' carbon of FDA **4** are converted to FAc **1** and C3 and C4 of 4-FT **2,** the circled regions in Scheme 1.[8] It has also been established that both hydrogens of the fluoromethyl group of FDA **4** are retained in the conversion to the fluoromethyl groups of **1** and **2**.[9]

The antibiotic nucleocidin **7** produced by the actinomycete soil bacterium, *Streptomyces calvus* is another example of the rare fluorometabolites.[10] Its biosynthesis is unknown; however, the presence of the fluorine atom at the C4' position of the ribose ring[11] in **7** is inconsistent with the involvement of the fluorinase found in *S. cattleya.* Full genome sequencing of the nucleocidin producer *S. calvus* ATCC 13382 has not identified a fluorinase gene,[12] thus *S. calvus* may employ a very different process for enzymatic C-F bond formation relative to bacterial FAc **1** production.

Ever since its isolation, from *S. calvus* in 1956,[10] nucleocidin **7** production has proved problematic, with consistently low titres and unpredictable production. Publically deposited strains appear to have lost the ability to produce nucleocidin.[12-14] Some progress has been made to understand the inconsistent production, and it has been observed that a mutation of the *bldA* gene, encoding a Leu-tRNA, is apparent in the publically available strains. Correction of this mutation was recently shown[12] to re-establish production in *S. calvus*. In another study[13] mutation of the *rpoB* gene (rifamycin resistance) led to an apparent increase in nucleocidin production. In this study we use an in-house strain of *S. calvus* T-3018 held by Pfizer. This strain does not have a *bldA* mutation, although production is still fickle with very low titres. Nucleocidin production can however be monitored, even at low production levels, directly from extracts by ^{19}F{^{1}H}-NMR. As a first step towards elucidating the biosynthetic pathway to fluorometabolite **7** we now report the incorporation of various isotopically labelled glycerols **5 a-d**.

For this study we have supplemented fermentation cultures of *S. calvus* with glycerols [2-^{13}C]- **5a**, [$^2H_5$]- **5b**, (*R*)-[$^2H_1$]- **5c** and *(S)*-[$^2H_1$]- **5d**. Despite the low titres, we envisaged that the incorporation of deuterium or carbon-13 isotopes close to the fluorine

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atom of the antibiotic could be reported visually by $^{19}F\{^1H\}$-NMR due to heavy atom induced chemical shifts of the fluorine resonance, and/or ^{13}C-^{19}F-spin–spin coupling in the case of carbon-13 incorporation.

A first experiment explored pulse feeding of glycerol [2-^{13}C]- **5a** to a final concentration of 8mM, in a shake flask fermentation of *S. calvus*. Cultures were worked up after 20 days as previously described.[13] Nucleocidin is excreted from the bacterial cells and thus the cells were spun down in a centrifuge and the supernatant was extracted into butanol. The extract was concentrated and was then analysed by $^{19}F\{^1H\}$-NMR. The resultant spectrum is shown in Figure 1a. It is clear that the fluorine signal for nucleocidin has accompanying satellites due to $^1J_{CF}$ (232Hz) coupling, consistent with a population (~25%) of nucleocidin **7** molecules enriched with carbon-13 at C4'. The satellite signals are off-centre relative to the natural abundance ^{19}F-NMR signal due to a heavy atom α-shift.[8] This incorporation is entirely consistent with pentose phosphate pathway involvement in ribose biosynthesis where carbons C3–C5 of D-ribose derive from the exogenously added glycerol, feeding into the C_3 metabolite pool *via sn*-glycerol-3-phosphate **6**.[15]

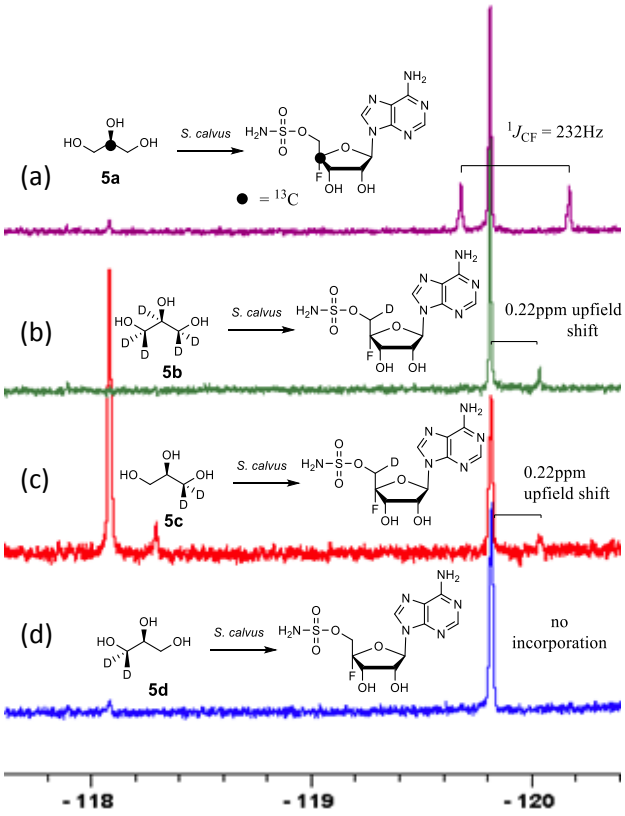
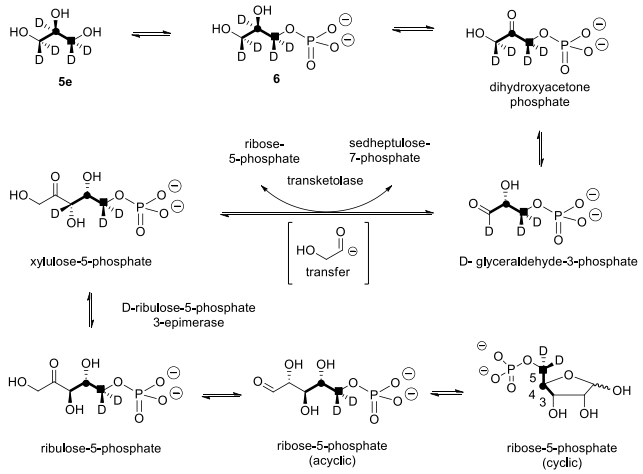


Figure 1. $^{19}F\{^1H\}$NMR of nucleocidin **7** (-119.8ppm) after glycerol **5** feeding experiments to *S. calvus* fermentations. An unknown metabolite (-118.1ppm) is also present in some spectra. (a) [2-^{13}C]-Glycerol **5a**; (b) [$^2H_5$]-glycerol **5b**; (c) (*R*)-[$^2H_2$]-glycerol **5c**; (d) (*S*)-[$^2H_2$]-glycerol **5d**. A pictorial summary of the isotopic labelling outcome is given in each case.

Addition of perdeuterated glycerol **5b** (final conc 8 mM) to a *S. calvus* fermentation led to the incorporation of deuterium (~5%) into nucleocidin **7** as determined by an upfield shift in the resultant $\{^1H\}^{19}F$-NMR spectrum as shown in Figure 1b. The magnitude of this upfield shift at 0.22ppm is consistent with a single vicinal deuterium incorporated *β*- to the fluorine atom. There is a F-C-C-D angular dependence on the magnitude of the shift ranging from 0.15ppm (0°) to 0.35ppm (180°) as determined in classic studies by Lambert and Greifenstein.[16] The observed value is intermediate between these extremes and consistent with a single vicinal C-D (γ-shift) bond approximately at 90° to the C-F bond. This experiment does not however distinguish the location of the incorporation between C3' or C5' of the ribose ring of **7**. To achieve such a distinction, comparative incorporation experiments were explored with the enantiomeric glycerols (*R*)-[$^2H_2$]- **5c** and (*S*)-[$^2H_2$]- **5d**. It is already established that the *pro-R* arm of glycerol delivers C5' of ribose through the pentose phosphate pathway, and that the *pro-S* arm delivers C3' of ribose. Glycerols **5c** and **5d** were prepared synthetically with enantiomeric purities > 95% *e.e.*, as previously described.[9] The resultant $^{19}F\{^1H\}$-NMR spectra from each of these glycerol feeding experiments are shown in Figure 1c and Figure 1d. It is clear that only glycerol (*R*)-[$^2H_2$]- **5c** results in the incorporation (~8%) of deuterium into nucleocidin **7**, and again only a single deuterium atom survives from the double labelled precursor. It follows from the known stereochemistry of glycerol processing through the pentose phosphate pathway, that this deuterium is located at C5'.[15,17]

In this experiment a second fluorometabolite was observed in the $^{19}F\{^1H\}$-NMR spectrum with a signal at -118.1ppm. This metabolite is detected periodically in nucleocidin extracts although its occurrence and level is unpredictable in our experience. Such a fluoro metabolite was also observed in the recent study where nucleocidin production was elicited by reversing the *bldA* mutation.[12] Its structure is unknown although given the similar level of deuterium incorporation from glycerol **5c**, it appears to be metabolically related to nucleocidin either as a biosynthetic precursor, or as a metabolite.

For isomer (*S*)-[$^2H_2$]- **5d** the absence of any deuterium at C3' in **7** is consistent with *sn*-glycerol-3-phosphate **6** being processed to ribulose-5-phosphate *via* xylulose-5-phosphate, and the action of D-ribulose-5-phosphate 3-epimerase, with exchange of the surviving deuterium with bulk solvent.[18] The predicted labelling pattern of ribose-5-phosphate from glycerol is illuystrated in Scheme 2.



Scheme 2. The pentose phosphate pathway showing the fate of glycerol through to ribose-5-phosphate. Glycerol **5e** has a virtual labelling pattern which is a composite of the experimentally administered glycerols **5a-d**, and also highlights the stereochemical fate of the (*pro*-R) hydroxymethyl carbon (■). This pathway predicts incorporation of a C_3 glycerol unit (bold C-C bonds) into C-3-C-5 of ribose-5-phosphate, with the retention of two deuteriums at C-5 and the loss of deuteriums at C-3 and C-4.

The outcome where both **5b** and **5c** glycerols contribute only one deuterium to C5' of nucleocidin can be contrasted with the biosynthesis of FAc, **1** and 4-FT, **2** in *S. cattleya* discussed above and shown in Scheme 1. In those cases two deuterium atoms were incorporated into the fluorometabolites, and by implication into the C5' ribose carbon of FDA **4**.[9a] For nucleocidin only one deuterium is incorporated.

In summary, this study provides the first biosynthetic data on nucleocidin **7** assembly from isotope labelling studies. It is shown that glycerol is incorporated into the ribose ring moiety of nucleocidin, consistent with expectation *via* the pentose phosphate pathway. However, the presence of a single deuterium only at the C5' position of nucleocidin **7** after feeding experiments with glycerols **5b** and **5c** suggests that a hydrogen is lost from this carbon after ribose ring assembly but prior to, or concominant with, fluorine introduction. This observation places a constraint on working hypotheses addressing nucleocidin biosynthesis.

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