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Production of 17-O-demethyl-geldanamycin, a cytotoxic ansamycin polyketide, by
*Streptomyces hygroscopicus* DEM20745

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Abstract
The actinomycete DEM20745, collected from non-rhizosphere soil adjacent to *Paraserianthes falactaria* trees (Cangkringan, Indonesia), is an efficient producer of the anticancer ansamycin polyketide 17-O-demethyl-geldanamycin (17-O-DMG), a biosynthetic precursor of the Hsp90 inhibitor geldanamycin (GDM). In DEM20745, 17-O-DMG is the major ansamycin product observed reaching a maximum titre of 17 mg/L in the fermentation broth. 17-O-DMG has potential to be a key starting material for the semi-synthesis of GDM analogues for use in anticancer therapy. Thus this preferential biosynthesis of 17-O-DMG facilitates easy access to this important molecule and provides further insight the biosynthesis of the geldanamycins.

Keywords
17-O-demethyl-geldanamycin/actinomycete/polyketide/ansamycin/cytotoxic
1. Introduction

The ansamycin polyketide geldanamycin (GDM) (Deboer et al. 1970, Rinehart et al. 1970) was first isolated from the soil actinomycete Streptomyces gelanamycinicus (reclassified from Streptomyces hygroscopicus var. geldanus var. nova (UC-5208)) (Goodfellow et al. 2007). GDM and its analogues were subsequently shown to inhibit heat-shock-protein 90 (Hsp90), a key anticancer target, thorough competitive binding in the ATP pocket (Franke et al. 2013). This prompted a search for an effective anticancer drug based on the molecular structure of GDM, resulting in the preparation and biological evaluation of many semi-synthetic GDMs, typically through modification at the C-17 position (Franke et al. 2013 and Kitson & Moody 2013). Examples include tanespimycin (17-AAG) and alvespimycin (17-DMAG), both of which have shown efficacy in the clinical treatment of cancer, but have limited applicability due to poor bioavailability and off-target hepatotoxicity, linked to the quinone ring acting as a Michael acceptor (Cysyk et al. 2006) (Figure 1).

Research has therefore been directed towards improved GDM analogues, through (a) semi-synthetic derivation of the parent (Kitson et al. 2013), (b) synthetic biology approaches involving the manipulation of the biosynthetic pathway (Patel et al. 2004 and Buchanan et al. 2005), (c) the search for related natural products (Hu et al. 2004, Zhang et al. 2010, Liu et al. 2011, Li et al. 2013 and Ni et al. 2014) and (d) mixed semi-synthetic/synthetic biology approaches (Lee et al. 2008), with a particular focus on non-quinone containing systems and (Wu et al. 2011 and Hermene et al. 2015). Despite these efforts an anticancer agent based on the GDM pharmacophore has yet to reach the clinic. Herein we present our investigation into the production of 17-O-demethyl-geldanamycin (17-O-DMG) by the actinomycete DEM20745. 17-O-DMG is of interest due to its potential use as starting material for the development of new semi-synthetic GDMs for clinical evaluation. For example the 17-arylgedanamycins, prepared via a triflation/Suzuki coupling approach starting from synthetic 17-O-DMG, have been shown to be potent inhibitors of Hsp90 (Le Brazidec et al. 2004). However further synthetic work has been restricted by the limited availability of 17-O-DMG, it being previously observed as only a minor product in native GDM producers (Barzilay et al. 2004 and Tadtong et al. 2007) and more recently via the fermentation of a $\Delta gdmMT$ strain of S. autolyticus (Yin et al. 2011) or accessed through synthetic modification of GDM itself (Rinehart et al. 1977 and Le Brazidec et al. 2004).

2. Results and Discussion

DEM20745 was identified as a potential source of bioactive secondary metabolites as part of a bioassay guided screening program of the Demuris actinomycete collection. The organism was isolated from non-rhizosphere soil adjacent to the tropical legume Paraserianthes falactaria in Cangkringan near Yogyakarta, Java, Indonesia (Sembiring et al. 2000) and was
shown to produce a greyish aerial spore mass (which later turned black) with a greyish yellow reverse colour (Figure S1) but did not form melanin pigments on peptone extract iron yeast agar. In the present study, strain DEM20745 was recovered in the *S. violaceusniger* 16S rRNA gene clade together with the type strains of *S. demannii*, *S. endus*, *S. hygroscopicus subsp. hygroscopicus* and *S. sporocinereus*, a taxon supported by the results from the maximum-likelihood and maximum parsimony analysis and by a 98% bootstrap value (Figure S2, S3 and S4). DEM20745 grew well on yeast extract-malt extract agar forming an abundant aerial spore mass with moist liquefied (hygroscopic) areas and spiral chains of rugose ornamented spores, properties typical of members of the *S. violaceusniger* 16S rRNA gene clade (Figures S5, S6 and S7). Thus DEM20745 was amended to be a member of the earlier described species, *Streptomyces hygroscopicus* (Goodfellow *et al.* 2012).

Fermentation of *S. hygroscopicus* DEM20745 in liquid culture (ISP2 medium) resulted in production of a water soluble purple compound that displayed a pronounced colorimetric pH response. Initial cytotoxicity testing towards the human liver carcinoma cell line HepG2 gave an IC$_{50}$ of approximately 0.3 µg/mL (Figure S5), prompting us to carry out further investigations. Large scale fermentation of DEM20745 allowed isolation, via multiple chromatography steps, of 27 mg of a purple crystalline compound (4).

The structure of 4 was elucidated through a combination of UV/Vis, $^1$H, $^{13}$C and 2-D NMR and HRMS analysis. The UV/Visible spectrum in acidic aqueous MeCN (Figure S9) showed a $\lambda_{\text{max}}$(abs) at 312 nm whilst the molecular formula of 4 was determined to be C$_{30}$H$_{36}$N$_2$O$_9$ on the basis of HRMS (m/z 569.2474 [M+Na]$^+$ and 1115.5048 [2M+Na]$^{2+}$) (Figure S10). Analysis of the $^{13}$C NMR spectrum showed a total of 26 distinct carbon resonances, including four carbonyls (two amides and two quinone carbons), eight sp$^2$ carbons and thirteen sp$^3$ carbons. Further analysis of the $^1$H NMR showed five vinylic protons, two methoxy groups, four methyl groups and two geminally coupled methylenes, whilst COSY/HMBC analysis revealed the presence of two large coupling systems (CCH$_2$CH(CH$_3$)CH$_2$CH(OCH$_3$)CH(OH)CH(CH$_3$)CH and C(CH$_3$)CH(OCONH$_2$)CH(OCH$_3$)CH=CHCH=C(CH$_3$)) allowing us to identify compound 4 as the ansamycin polyketide 17-O-demethyl-geldanamycin (17-O-DMG) (Figure S11, Table S1, partial NMR data for 17-O-DMG has been previously reported Barzilay *et al.* 2004 and Tadtong *et al.* 2007). The molecular structure of 4 was subsequently confirmed by X-ray crystallography, crystals being grown from MeOH solution by slow evaporation (Figure 2 and Table S2).

With the structure of 17-O-DMG (4) unambiguously assigned we re-examined the fermentation of DEM20745 to assess the production titre of 4, and to look for the presence of
other related ansamycin polyketides. Thus a 20 L stirred tank bioreactor was inoculated with an exponentially growing culture of DEM20745 in ISP2 media at 30 °C. 450 mL samples were taken daily and analysed by RP-HPLC for the presence of 17-O-DMG (4) and related molecules (Figure S8). Interestingly, alongside the previously observed 17-O-DMG (4), we detected the production of GDM (1) in the fermentation broth of DEM20745, the production of both compounds commencing after 40 hours and reaching a maximum after approximately 90 hours, after which the supernatant concentration of both decreased. 17-O-DMG (4) was the major biosynthetic product, being detected at a peak concentration of ~17 mg/L, whilst GDM (1) was only produced at a maximum of ~3 mg/L. It has been reported that GDM (1) can undergo hydrolysis at high pH to form 17-O-DMG (4) (Le Brazidec et al. 2004). To confirm that 17-O-DMG (4) was not being formed through this route under the fermentation conditions, commercial GDM (1) was stored at pH’s 4, 7 or 9 in aqueous solution for 32 hours, at which point no conversion of GDM (1) into 17-O-DMG (4) could be detected, thus confirming the preferential biosynthesis of 17-O-DMG (4).

The biosynthesis of the polyketide core of GDM (1) has been previously established through the cloning and sequencing of the GDM biosynthetic cluster from three related strains of *S. hygroscopicus* (Rascher et al. 2003, Rascher et al. 2005, He et al. 2008, Hong et al. 2004 and Shin et al. 2008). However the order of the post-PKS tailoring steps are less well understood, with Hong and Kirschning (Hong et al. 2004, Shin et al. 2008 and Eichner et al. 2012) suggesting that C-17 hydroxylation, 17-O-methylation and C-21 oxidation occur first, followed by O-carbamoylation and finally C-4/5 desaturation, in *S. hygroscopicus* JCM4427. Whilst Shens investigation of the gene encoding for the “missing” 17-O methyltransferase (*gdmMT*), resulting in its discovery 17-kb upstream from the boundary of the GDM cluster (Yin et al. 2011), suggesting that in “*S. autolyticus*” CGMCC 0516 17-O-methylation was in fact the final post-PKS tailoring step in the biosynthesis of GDM. Subsequent upstream searching of the genome of *S. hygroscopicus* strains JCM4427 and NRRL 3602, by 15-kb and 20-kb respectively, suggesting some similarity to CGMCC 0516 (Yu et al. 2002, Tahlan et al. 2004, Ostash et al. 2007 and He et al. 2010). DEM20745 is taxonomically remote from known GDM producers, thus our observation of the preferential production of 17-O-DMG (4) is suggests that in this strain either the expression of *gdmMT* is uncoupled from the production of the polyketide core or that GdmMT is less active compared to other DMG producers. Thus further investigation into the biosynthetic gene cluster of 17-O-DMG/GDM and post-PKS tailoring steps in DEM20745 will be the focus of future work.

3. Conclusions

In conclusion, we have reported the first example of a high titre, preferential biosynthesis of 17-O-DMG (4) from a wild type organism. Due to the interest in novel GDM analogues in the
development of anticancer agents this work provides the basis for a future production strain for this potentially valuable compound along with providing further insight into the biosynthesis of the GDMs.

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**References**


Figure 1: Structure of 1 Geldanamycin (GDM), X = OMe
2 Tanespimycin (17-AAG), X = NHCH₂CH=CH₂
3 Alvespimycin (17-DMAG), X = NHCH₂CH₂NMe₂

Figure 2: ORTEP diagram and chemical structure of 4 17-O-DMG.