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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 Hermane *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-arylgeldanamycins, 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 Δ*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 λ_{max}(abs) at 312 nm whilst the molecular formula of **4** was determined to be $C_{30}H_{36}N_2O_9$ on the basis of HRMS (m/z 569.2474 $[M+Na]^+$ and 1115.5048 $[2M+Na]^+$) (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 ($CCH_2CH(CH_3)CH_2CH(OCH_3)CH(OH)CH(CH_3)CH$ 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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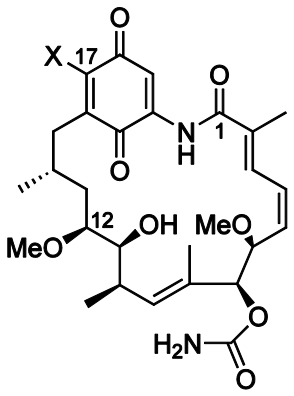
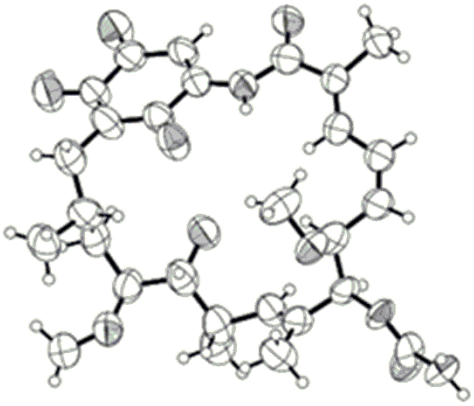


1 Geldanamycin (GDM), X = OMe
2 Tanespimycin (17-AAG), X = $NHCH_2CH{=}CH_2$
3 Alvespimycin (17-DMAG), X = $NHCH_2CH_2NMe_2$

Figure 1: Structure of **1** GDM, **2** 17-AAG and **3** 17-DMAG.



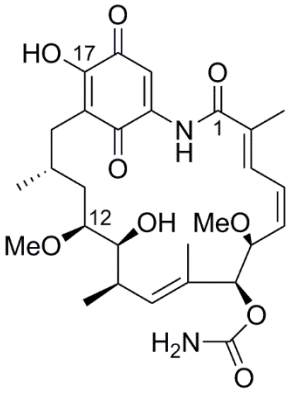


Figure 2: ORTEP diagram and chemical structure of **4** 17-O-DMG.