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Delineating the earliest steps of gilvocarcin biosynthesis: role of GilP and GilQ in starter unit specificity†

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Received 22nd April 2010, Accepted 23rd June 2010
First published as an Advance Article on the web 8th July 2010
DOI: 10.1039/c0ob00036a

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Published on 08 July 2010 on http://pubs.rsc.org | doi:10.1039/C0OB00036A

***In vivo* and *in vitro* investigations of GilP and GilQ, two acyltransferases encoded by the gilvocarcin gene cluster, show that GilQ confers unique starter unit specificity when catalyzing an early as well as rate limiting step of gilvocarcin biosynthesis.**

Introduction

Gilvocarcin V (GV, **1**) is a structurally unique anticancer antibiotic produced by several *Streptomyces sp.* including *Streptomyces griseoflavus* Gö3592.[1] Notable structural features of **1** include a polyketide-derived benzo[*d*]naphtho[1,2-*b*]pyran-6-one backbone which emerged from a benz[*a*]anthracene (angucyclinone) intermediate through an unusual oxidative rearrangement.[2,3] Furthermore, there is an unusual *C*-glycosidically linked D-fucofuranose moiety, and a C-8 vinyl side chain. The latter two functional groups are critical requirements for GV's biological activity. GV has been found to mediate a unique cross-linking of DNA to histone H3, a major inner core component of the histone complex responsible for DNA replication and packaging.[4–7] Specifically, the C8 vinyl side chain undergoes a photo[2+2]cyclo-addition with thymine residues of DNA,[8,9] while the D-fucofuranose moiety is likely to interact with histone H3.[10] Along with the principle product GV, two congeners, gilvocarcin E (GE, **2**) and gilvocarcin M (GM, **3**), are naturally produced by various *Streptomyces sp.*, both are considerably less active than GV.[1,11–13] All gilvocarcins (GV, GE and GM) are structurally identical apart from the C-8 side chain, which is determined by the incorporation of two distinct starter units, either acetate or propionate. Because of the decreased biological activity of the GE and GM congeners, *i.e.* compounds without the crucial vinyl side chain found in GV, these compounds have to be considered unwanted side products of GV biosynthesis. In order to reduce the production of the less favorable GE and GM congeners it is imperative to understand the early steps of gilvocarcin biosynthesis and the enzymes involved in starter unit specificity.

The isolation and characterization of the gilvocarcin (*gil*) gene cluster revealed gilvocarcins to be produced by a type II polyketide synthase (PKS) consisting of two ketosynthase subunits ($KS_{\alpha/\beta}$), an acyl carrier protein (ACP), two malonyl-CoA:acyl carrier protein transacylase (MCAT) homologues, and several post-PKS tailoring enzymes including oxygenases, methyltransferases, and a *C*-glycosyltransferase.[14] The inclusion of two MCAT homologues encoded by the *gil* gene cluster, GilP (47% amino acid (aa)-similarity/33% aa-sequence identity compared to FabD) and GilQ (30% aa-similarity/19% aa-identity compared to FabD), is unusual for a type II PKS. In contrast to fatty acid synthases (FAS) and type I PKS systems, typical type II PKSs do not contain a dedicated MCAT, and it was suggested that the minimal type II PKS ($KS_{\alpha/\beta}$ and ACP) recruits an outside fatty acid synthase (FAS) MCAT to transfer malonyl-CoA to the ACP.[15–20] However, it was also found for the actinorhodin PKS that self-malonylation of the ACP is possible and that MCAT is not required.[21–23] It remains an ongoing subject of debate whether and how the MCAT that was found outside of the *act* cluster interacts with the actinorhodin PKS ACP.[24–26] Whether loaded by MCAT or not, the malonyl-CoA building block is used for both initiation and elongation of well studied acetate-primed polyketides, such as actinorhodin and tetracenomycin, where the acetate primer is generated by decarboxylation of malonyl-ACP through KS_{β}, and is not derived from acetyl-CoA.[17,18,23,27] In stark contrast to this apparently generally accepted view, our recent studies with components of the gilvocarcin type II PKS show that acetyl-CoA is in fact a requirement of the *gil* pathway, as polyketide production was not observed in the absence of acetyl-CoA.[28]

Unlike actinorhodin and tetracenomycin C, gilvocarcins utilize two distinct starter units. Early labeling studies revealed these starter units to be derived from acetate and propionate which condense with 9 malonate extender units to produce C_{20}- and C_{21}-decaketides, respectively.[3,29] Subsequent intramolecular aldol condensations and several complex post-PKS modifications produce **2** and **3**, respectively.[2,10,14,30–32] At a yet unknown step during the biosynthesis of **2**, oxidation of the ethyl side chain by GilOIII produces the vinyl functional group found in **1**.[10]

The use of propionyl-CoA in a type II PKS-system is relatively uncommon and has been observed in only a handful of natural products belonging to the class of multi-cyclic, aromatic polyketides. Most notably, the daunorubicin (**6**) and doxorubicin (**7**) pathways, which share early PKS enzymes, have been studied extensively in determining starter unit specificity.[33–39] The daunorubicin gene cluster (*dps*), when compared to other type II PKS systems, has several unique features including the presence of a β-ketoacyl:ACP synthase III (KAS III) homologue (DpsC) usually found as a component of a type II FAS, and a single MCAT homologue (DpsD), more recently proposed to be an acetyl-ACP thioesterase.[34,39–42] When the function of DpsC and DpsD were investigated as determinants of starter unit specificity it was found that DpsC primarily conferred specificity for propionyl-CoA incorporation in *in vivo* and cell-free synthetic systems.[35,37] Interestingly the *gil* cluster lacks this unique KAS III homologue found in the daunorubicin as well as in several other pathways for which unique starter units are required, *i.e.* frenolicin

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† Electronic supplementary information (ESI) available: Full details of *in vivo* and *in vitro* experimentation. See DOI: 10.1039/c0ob00036a

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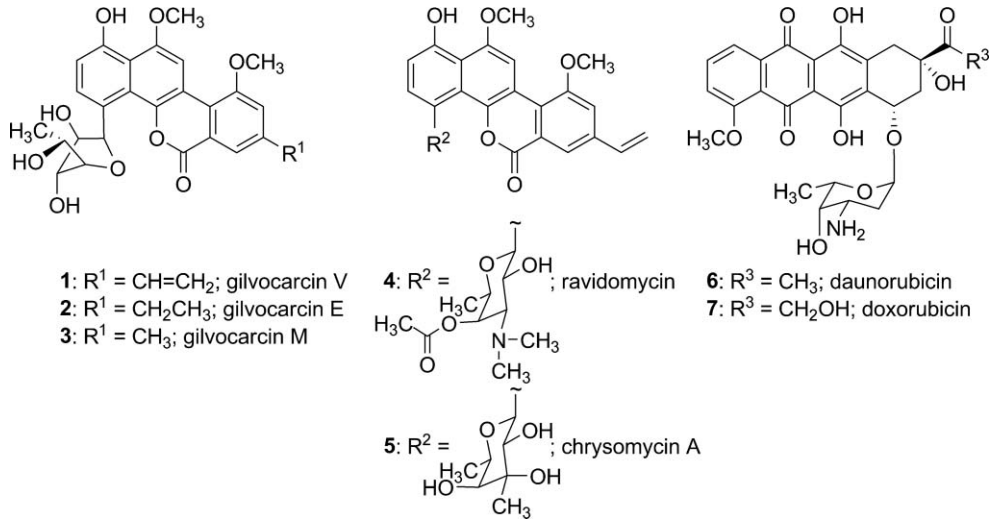
Table 1 Production distribution of gilvocarcins



HPLC trace	Strain	(**3**) % GM[a]	(**1**) % GV[a]	(**2**) % GE[a]
(A)	*S. lividans* TK24 (cosG9B3)	34.6 ± 1.8	65.4 ± 1.8	0
(B)	*S. lividans* TK24 (cosG9B3-P$^-$)	36.5 ± 1.6	52.3 ± 2.2	11.2 ± 1.8
(C)	*S. lividans* TK24 (cosG9B3-Q$^-$)	77.2 ± 0.9	22.8 ± 1.2	0
(D)	*S. lividans* TK24 (cosG9B3-pGilQGilOIII)	16.5 ± 0.7	83.5 ± 0.7	0
(E)	*S. lividans* TK24 (cosG9B3-pGilQ)	11.6 ± 0.7	83.7 ± 0.6	4.7 ± 0.01

[a] Percentage of gilvocarcins calculated by the relative values of area under the curve taken from HPLC-MS chromatogram traces (n = 3).

(butyrate), R1128 (short-chain fatty acids), and aclacinomycin (propionate).[43–46] This indicates that the minimal gilvocarcin PKS likely incorporates propionyl-CoA utilizing a protein with unique function not seen in similar pathways. It is in this context, through *in vivo* and *in vitro* characterization, we report here the MCAT homologue GilQ being the key determinant for propionate incorporation during gilvocarcin biosynthesis.

Results and discussion

Traditional *in vivo* methods for investigating gilvocarcin biosynthesis are severely hindered due to the inability to introduce genetic information into wild type *S. griseoflavus* Gö3592. This was remedied by the isolation of cosG9B3, a pOJ446-derived cosmid harboring the entire genetic information for gilvocarcin production.[14] Through PCR-targeting REDIRECT technology[47] and heterologous expression, several unique gilvocarcin biosynthetic enzymes have been assigned and characterized, respectively.[2,10,28,30–32] To determine the exact biosynthetic role of GilP and GilQ, two cosG9B3 mutants were constructed harboring an in-frame-deletion of GilP (cosG9B3-P$^-$) and GilQ (cosG9B3-Q$^-$), respectively (see electronic supplementary information (ESI)†). Conjugal transfer of these cosmids into *Streptomyces lividans* TK24 produced three strains: *S. lividans* TK24(cosG9B3-P$^-$), *S. lividans* TK24(cosG9B3-Q$^-$) and as a control *S. lividans* TK24(cosG9B3). Fermentation and HPLC-MS analysis of each strain were conducted in triplicate (see ESI†), and the resulting distribution profiles of gilvocarcins produced are summarized in Table 1. The distribution profile of *S. lividans* TK24(cosG9B3-P$^-$) is comparable to that of the control strain. In both strains, GV accounts for roughly 55–65% of all gilvocarcins produced. The remaining 35% and 0–10% make up GM and GE, respectively (Fig. 1). This observation clearly shows that the absence of GilP from the gilvocarcin biosynthetic machinery does not affect the overall production of gilvocarcins. This was not unexpected, as it had been shown that many type II minimal PKS systems can recruit an outside FAS MCAT.[17,18] Furthermore, this suggests GilP functions as a typical MCAT,

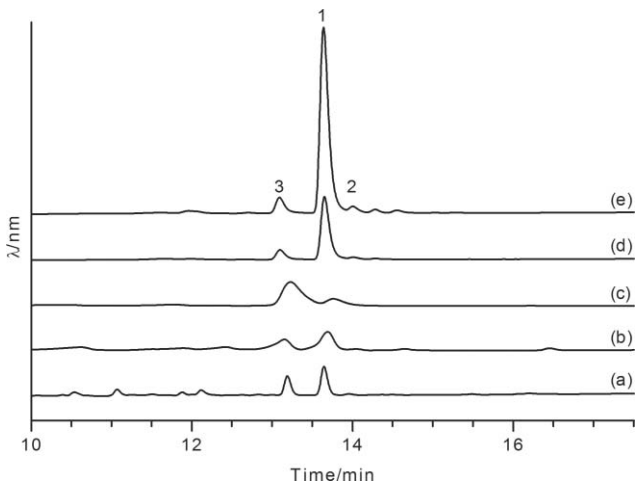


Fig. 1 HPLC traces of *S. lividans* TK24 containing cosG9B3 (a); cosG9B3-P$^-$ (b), cosG9B3-Q$^-$ (c), cosG9B3-pGilQGilOIII (d), and cosG9B3-pGilQ (e). **1** = gilvocarcin V; **2** = gilvocarcin E; **3** = gilvocarcin M.

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and does not play an important role in the selection of propionate during GV biosynthesis.

The inactivation of GilQ, however, severely altered the production of gilvocarcins, as seen in *S. lividans* TK24(cosG9B3-Q^{-}). GV production drops to ~20% of total gilvocarcins produced, while GM accounts for the remaining ~80% (Fig. 1). The dramatic decrease of GV production shows GilQ primarily influences the use of propionate over acetate. Interestingly, the complete abolishment of GV was not observed, demonstrating that GilP or a host MCAT may have the ability, albeit at a much reduced rate, to load and transfer propionate to the minimal gilvocarcin PKS.

With *in vivo* results supporting the role of GilQ as the determinant of propionate selection during GV biosynthesis, an additional strain containing cosG9B3 and pGilQ (a plasmid conferring GilQ over-expression) was produced to increase the propensity for utilizing a propionate-starter unit (see ESI†). The expected increase of C_{21}-decaketides produced by such a strain may saturate GilOIII, thereby leading to an incomplete formation of the desired vinyl-side chain and an increased production of GE (**2**) as opposed to an ideal increase of just the desired GV (**1**) congener. To account for this, a second strain containing cosG9B3 with pGilQGilOIII (a plasmid ensuring both GilQ and GilOIII over-expression) was generated to completely flux all intermediates towards GV (see ESI†). The resulting strains; *S. lividans* TK24(cosG9B3-pGilQ) and *S. lividans* TK24(cosG9B3-pGilQGilOIII) were fermented and analyzed by HPLC-MS as discussed earlier, Table 1 (see also ESI for experimental details†). Both constructs increased GV production to ~85% of total gilvocarcins and reduced GM to ~15% compared to ~65% GV and ~35% GM in the control. While *S. lividans* TK24(cosG9B3-pGilQ) still produced detectable amounts of GE (**2**) , the inclusion of GilOIII in *S. lividans* TK24(cosG9B3-pGilQGilOIII) completely converted all intermediates to **1**, and no **2**-production was observed. Thus, by providing additional GilQ in the heterologous system, the minimal gilvocarcin PKS was able to increase the usage of propionate starter units for the production of poly-β-ketide thioesters, which consequently led to greater GV yields. This finding further validates the unique role of GilQ in starter unit selection for GV biosynthesis.

Surprisingly, along with the increased GV : GM ratios in strains ensuring GilQ overexpression, a dramatic overall increase of gilvocarcin production was observed, as shown in Table 1. Total gilvocarcins produced by wild type *S. griseoflavus* and *S. lividans* TK24(cosG9B3) are 21 ± 4 mg L^{-1}. *S. lividans* TK24(cosG9B3-pGilQGilOIII) and *S. lividans* TK24(cosG9B3-pGilQ) increased total gilvocarcin yields to 201 ± 13 mg L^{-1} and 450 ± 79 mg L^{-1}, respectively, correlating to a 10- to 20-fold increase in total gilvocarcin production compared to the wild type and control strains (see ESI†). This finding provides indirect evidence that GilQ not only catalyzes the initial step of gilvocarcin biosynthesis and plays an important role in starter unit specificity, but also catalyzes a rate limiting step.

In parallel to the above described *in vivo* study, we took an *in vitro* approach to further characterize GilP and GilQ. Four proteins (GilP, GilQ, RavC, and Svp) were expressed in *Escherichia coli*, and purified by immobilized metal affinity chromatography (IMAC) (see ESI†). RavC is the ACP homologue of GilC, from the closely-related ravidomycin (**4**) biosynthetic pathway,[48] and was used because the natural ACP of the *gil* cluster, GilC, failed to over-express in soluble form upon expression in *E. coli*, despite numerous attempts and various conditions. Several homologues of GilC have been recently characterized from the closely related ravidomycin and chrysomycin biosynthetic pathways; RavC and RavC1 (aa-similarity/aa-identity 62%/42% and 57%/38%) as well as ChryC and ChryC1 (69%/48% and 52%/40%).[48] Note that in contrast to the *gil* gene cluster, both the recently cloned ravidomycin (**4**) and chrysomycin (**5**) gene clusters contain two distinct ACP-genes. Two ACP-encoding genes were also previously found in gene clusters of other type II PKS-pathways, in which non-acetate starter units were utilized, *e.g.*, the frenolicin and R1128 pathways.[49,50] Specifically, the secondary ACP, ZhuG, has been shown to be indispensable for the activity of the priming module of the R1128 pathway.[44,51] This led to the hypothesis that RavC and RavC1 as well as ChryC and ChryC1 may also play distinct roles in their biosynthetic pathways. It is likely that the secondary ACPs of the ravidomycin and chrysomycin pathways are functionally identical, as alluded by the recent observation that either RavC or RavC1 alone can function with the remaining minimal *gil* PKS proteins (GilA and GilB) to produce early shunt products of gilvocarcin biosynthesis, such as RM20b/c or SEK 43.[28,52,53] Additionally, initial *in vitro* acyl transfer assays using radio-labeled acyl-CoAs did not show any preference for RavC or RavC1 by either acyl-GilP or acyl-GilQ (data not shown). Based on these observations and the fact that it could be expressed in soluble form in *E. coli*, RavC was chosen as a GilC replacement for further *in vitro* testing.

As expected, the expression of RavC predominantly produced functionally inactive *apo*-RavC in *E. coli*. The promiscuous phosphopantetheinyl transferase, Svp, was then used to convert purified *apo*-RavC to the active *holo* form (see ESI†).[54] To evaluate the extent of self loading of RavC (ACP), we compared the initial rates of ^{14}C-malonyl-CoA-ACP formation from self loading of *holo*-RavC alone to that of GilP (MCAT)-assisted acylation. The GilP assisted formation of ^{14}C-malonyl-CoA-RavC showed a 120-fold increase in initial rate over self loading of *holo*-RavC alone (for experimental details, see ESI†). Thus, under the specific conditions tested, self loading appeared to be a negligible component of malonylation. An acyl transfer assay was utilized in which purified GilP and GilQ were incubated separately with *holo*-RavC and individual ^{14}C-acyl-CoA molecules (^{14}C-acetyl-CoA, ^{14}C-malonyl-CoA, ^{14}C-propionyl-CoA, and ^{14}C-methylmalonyl-CoA) (see ESI†). The ability of GilP and GilQ to load and transfer specific ^{14}C-acyl-CoAs to *holo*-RavC (^{14}C-acyl-CoA-RavC) were monitored by scintillation counting, and represented as specific activity in Table 2 (for experimental details, see also ESI†). The specific activity of GilP (malonyl-CoA > methylmalonyl-CoA > propionyl-CoA > acetyl-CoA) shows a clear preference for malonyl-CoA, and is comparable to other *Streptomyces* MCAT proteins.[19] Interestingly, GilP shows similar activity with methylmalonyl-CoA, a possible artifact of MCAT binding to the carboxylic acid moiety of acyl-CoA molecules.[25] Additionally, the results show that GilP is able to transfer propionyl-CoA, but only at a fifth of the rate of malonyl-CoA transfer. This substantiates the *in vivo* results where, in absence of GilQ, gilvocarcins with a propionate starter unit were still produced, but only at ~23% of the total gilvocarcin production. The specific activity of GilQ (propionyl-CoA > malonyl-CoA > acetyl-CoA = methylmalonyl-CoA) is in agreement with the *in vivo* results, which

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Table 2 Specific activity of GilP and GilQ

Acyltransferase	^{14}C-labeled substrates	Specific activity/unit mg^{-1}[a]	Relative activity
GilP	^{14}C-acetyl CoA	16×10^3	1
GilP	^{14}C-malonyl CoA	463×10^3	29
GilP	^{14}C-propionyl CoA	86×10^3	5
GilP	^{14}C-methylmalonyl CoA	301×10^3	19
GilQ	^{14}C-acetyl CoA	16×10^3	1
GilQ	^{14}C-malonyl CoA	27×10^3	2
GilQ	^{14}C-propionyl CoA	51×10^3	3
GilQ	^{14}C-methylmalonyl CoA	15×10^3	1

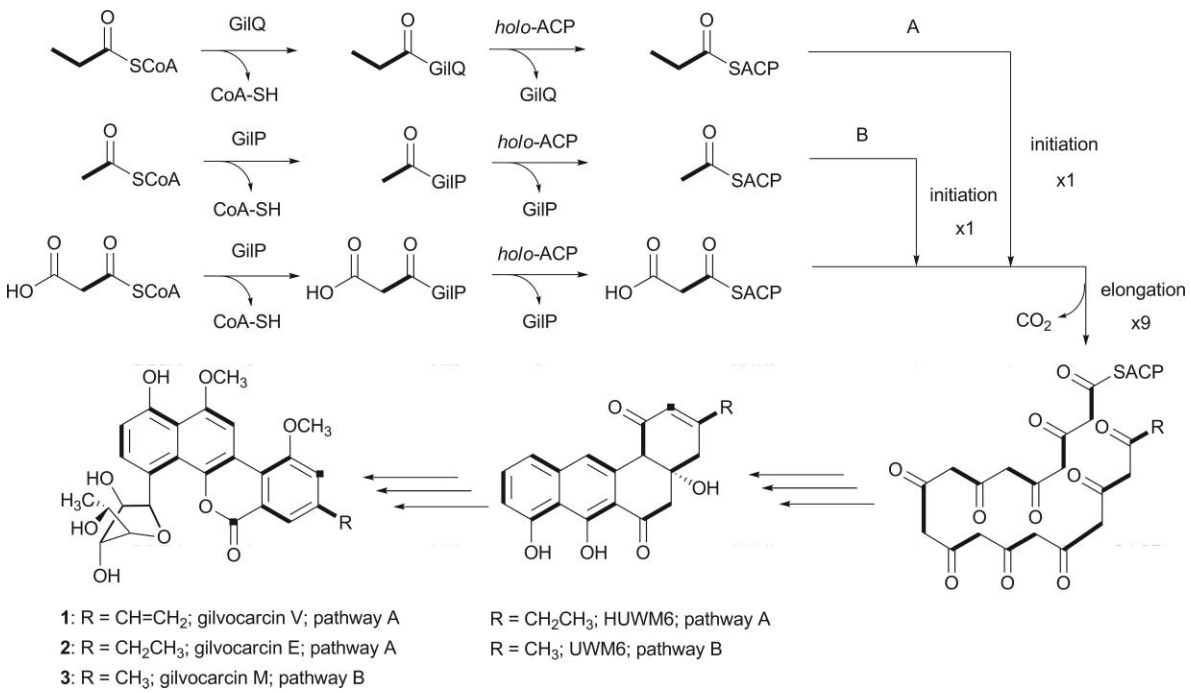
[a] One unit is defined as the amount of His_6-GilP or His_6-GilQ required to catalyze the synthesis of 1 pmol of acetyl-, malonyl-, propionyl- or methylmalonyl-RavC per sec at pH 7.5 and 30 °C with 8 μM RavC and 8 μM ^{14}C-labeled substrates.

suggests that the primary role of GilQ is transferring propionyl-CoA. Surprisingly, GilQ shows only a 2-fold higher preference for forming propionyl-RavC compared to malonyl-RavC, and is roughly 40% less active than GilP at producing propionyl-RavC. Although GilP shows slightly more activity than GilQ in loading propionyl-CoA under our *in vitro* assay conditions, the *in vivo* environment may be much different, given the fact that the *in vivo* concentrations of GilP and GilQ are unknown. When considering the relative activity of each acyltransferase it is clear that even though GilQ is less active than GilP in forming propionyl-RavC, GilP will almost exclusively sequester malonyl-CoA (5.4 : 1 preference of malonyl-CoA : propionyl-CoA), while GilQ will load and transfer propionyl-CoA (1.8 : 1 preference of propionyl-CoA : malonyl-CoA).

Initial bioinformatic analyses revealed the expected conserved active site sequences xGHSxGE for the acyltransferases (GilP and GilQ) and LGxDSLxxVE for the ACP (RavC). To validate these expected active site residues *in vitro*, three mutant proteins ($GilPS_{90}A$, $GilQS_{111}A$, and $RavCS_{39}A$) were produced, in which the active site serine residues were replaced with alanine. Phosphorimaging showed both $GilPS_{90}A$ and $GilQS_{111}A$ were unable to load ^{14}C-malonyl-CoA and ^{14}C-propionyl-CoA, respectively (see ESI†). In addition, the removal of the active site serine in $RavCS_{39}A$ also inhibited loading of ^{14}C-malonyl-CoA (see ESI†).

Taken together, these results allow us to delineate the earliest steps of gilvocarcin biosynthesis. GilP and GilQ clearly function as acyltransferases, and work with the minimal gilvocarcin PKS to produce C_{20}- and C_{21}-decaketides, with acetate and propionate primed starter units, respectively. The principle function of GilQ is to load and transfer propionate to *holo*-ACP for the first condensation step of GE and GV biosynthesis (Scheme 1, path A). The incorporation of acetate as seen in GM biosynthesis is likely facilitated by GilP (Scheme 1, path B); however, unlike traditional acetate initiation which specifically involves the decarboxylation of malonyl-CoA by KS_β to produce acetyl-CoA, we propose the direct loading and transfer of acetyl-CoA to *holo*-ACP by GilP. This hypothesis is supported by the recent observation that the *gil* PKS absolutely requires both acetyl-CoA and malonyl-CoA to catalyze polyketide formation.[28] In addition, the highly conserved glutamine (or glutamate) residue found in type II KS_β to be essential for the decarboxylation process[27] is replaced by a shorter aspartate residue in the *gil* KS_β GilB, and might be too short to initiate decarboxylation of ACP-bound malonate.

The use of an acetate starter unit in GM biosynthesis is likely facilitated by GilP (Scheme 1, path B), as this is a common functionality of traditional MCATs.[17,18] Path B is also in agreement with recent results which show GilP and the minimal gilvocarcin PKS were able to catalyze the production of acetate primed GM intermediates only when both malonyl-CoA and acetyl-CoA were



Scheme 1 Role of GilQ and GilP in the biosynthesis of gilvocarcins.

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present in the reaction mixture.[28] Interestingly, GilP and GilQ show the same activity towards producing acetyl-ACP, therefore the possibility of GilQ contributing to path B cannot be completely dismissed. As path B leads to minor products in gilvocarcin biosynthesis, the primary role of GilP is to recruit and transfer malonyl-CoA for the chain-elongation process by the minimal *gil* PKS (Scheme 1).

The natural concomitant production of acetate and propionate primed decaketides by the *gil* PKS suggests GilP and GilQ as well the $KS_{\alpha/\beta}$ may possess intrinsic substrate flexibility. This is further exemplified by the ability of *gilQ*-deficient strains to continue to produce propionate primed decaketides. This is in stark contrast to many other non-acetate primed type II PKSs, which do not naturally produce acetate initiated congeners, such as daunorubicin and doxorubicin,[55,56] and has been attributed to acetyl-ACP thioesterase activity and strong starter unit specificity.[37,42] Additionally, the KAS III homologues encoded in these clusters have been indicated as an indispensible requirement for unique starter unit incorporation.[35,37,38] Interestingly, the *gil* cluster does not harbor any of these characteristics, and instead relies on MCAT-type activity to accomplish non-acetate starter unit incorporation.

To the best of our knowledge, GilQ is the first and only characterized MCAT-like acyltransferase shown to function in starter unit specificity in a type II PKS pathway. In this context, GilQ as well as RavQ and ChryQ from the closely related ravidomycin and chrysomycin pathways may comprise a unique category of type II PKS acyltransferases, which control the selection and utilization of the non-acetate starter unit propionyl-CoA.

Acknowledgements

This work was supported by the US National Institutes of Health (grant CA 102102 to JR). We would like to thank the Mass Spectrometry Core Facility and the Department of Biochemistry at the University of Kentucky for instrumentation usage as well as Professor Ben Shen (University of Wisconsin-Madison) for providing pBS18.

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Downloaded by Institute of Organic Chemistry of the SB RAS on 16 August 2010
Published on 08 July 2010 on http://pubs.rsc.org | doi:10.1039/C0OB00036A

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