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STARTER UNIT SPECIFICITY OF THE ASUKAMYCIN "UPPER" CHAIN POLYKETIDE SYNTHASE AND THE BRANCHED-CHAIN FATTY ACID SYNTHASE OF *STREPTOMYCES NODOSUS* SUBSP. *ASUKAENSIS*

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This paper is dedicated to Professor Satoshi Omura on his 70th birthday

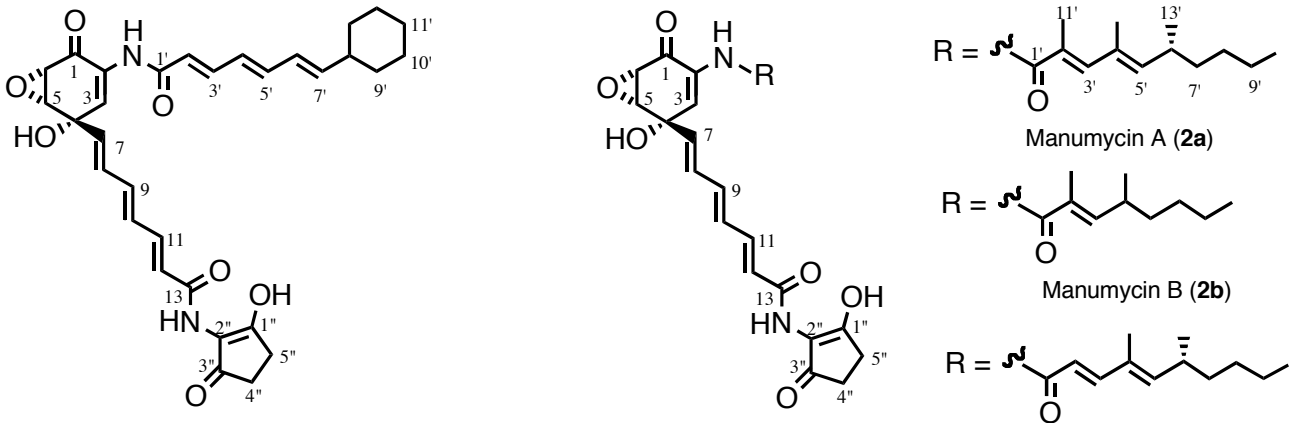
Abstract – Asukamycin, a member of the manumycin family of antibiotics, obtained from *Streptomyces nodosus* subsp. *asukaensis*, contains two amide-linked polyketide chains, one of which, the "upper" chain, is initiated by cyclohexanecarboxylic acid as the starter unit. Analysis of the congeners of asukamycin present in the fermentation and of the fatty acid spectrum of the mycelial lipids revealed that the "upper" chain polyketide synthase (PKS) and the branched-chain fatty acid synthase (FAS) of *S. nodosus* ssp. *asukaensis* share qualitatively, although not quantitatively, the same starter unit selectivity for branched chain and alicyclic carboxylic acids. Feeding of various alicyclic carboxylic acids of different ring size resulted in the formation of new asukamycin analogs as well as changes in the fatty acid spectrum. Feeding of various alicyclic carboxylic acids also shut off the endogenous production of cyclohexanecarboxylic acid.

INTRODUCTION

The antibiotic asukamycin (**1**) was isolated by Omura and co-workers from *Streptomyces nodosus* ssp. *asukaensis*.[1-3] It is a polyketide containing two polyene chains linked to a central mC_7N unit derived from 3-amino-4-hydroxybenzoic acid (3,4-AHBA).[4,5] The "lower" polyketide chain is initiated by 3,4-AHBA

and terminates with an amide linkage to 2-aminocyclopent-2-en-3-olone (C_5N unit), a product of intramolecular cyclization of 5-aminolevulinic acid. The "upper" chain is initiated by cyclohexanecarboxylic acid (**4**) (probably as its CoA thioester), making it a member of the small group of natural products containing a singly substituted cyclohexane ring,[6] and terminates in an amide linkage to the mC_7N unit. Cyclohexanecarboxylic acid in turn is derived from shikimic acid by a series of dehydrations and reductions.[7,8] Biosynthetic feeding experiments have shown that both polyketide chains and the C_5N unit are assembled and connected to each other before a terminal oxidative modification of the mC_7N unit to the final epoxyquinol structure.[5]

Figure 1



Asukamycin belongs to the broader manumycin family of antibiotics,[9] exemplified by manumycin A (**2a**) (Scheme 1),[10-12] members of which have been isolated from different Actinomycetes. Although this has not been reported for asukamycin, the manumycins are often produced as a group of co-metabolites, i.e., a single strain produces more than one manumycin type compound. These co-metabolites differ from each other primarily in the structure of the "upper" chain, e.g., manumycin A (**2a**), manumycin B (**2b**) and manumycin C (**2c**) are produced by *Streptomyces parvulus* Tü 64 (Figure 1),[13] manumycins E, F and G by *Streptomyces* sp. WB-83,[14] EI-1511-3 and EI-1511-5 by *Streptomyces* sp. EI-1511[15,16] and TMC-1A, 1B, 1C and 1D by *Streptomyces* sp. A-230.[17] This diversity of the manumycins is attributed to a relaxed substrate specificity of the "upper" chain PKS. Manumycin antibiotics display different biological activities, which may be related to the structural variations of the "upper" chain. Most prominently, manumycin A, with a methyl-branched "upper" chain, is an inhibitor of Ras protein farnesyltransferase and shows activity against various cancers.[18] Asukamycin, with an unbranched "upper" chain, does not possess this activity. Thus, the nature of the "upper" chain seems to be important for biological activity, and the biological activities may be modulated by modification of this structural element.

Formation of the "upper" chains of the known manumycins is initiated by the CoA thioesters of straight chain, branched chain or alicyclic carboxylic acids. Short straight chain and branched chain acids also serve as starter units for fatty acid synthesis in Actinomycetes.[19] Fatty acid synthases and polyketide synthases are two branches of the same superfamily of enzymes and mediate the biosynthesis of primary and secondary metabolites, respectively. They involve the same or similar mechanisms of chain initiation and extension, but differ in starter unit and chain elongation substrate specificity and particularly in the processing steps (ketoreduction, dehydration, enoate reduction) following each chain extension cycle.[20]

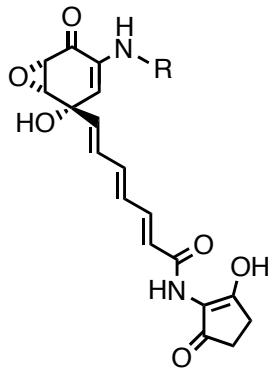
In this study we have compared the starter unit specificity of the asukamycin "upper" chain PKS and the *S. nodosus* subsp. *asukaensis* FAS by analyzing the spectrum of asukamycins and lipid fatty acids produced by the organism under normal fermentation conditions and upon feeding various natural and unnatural starter units.

RESULTS

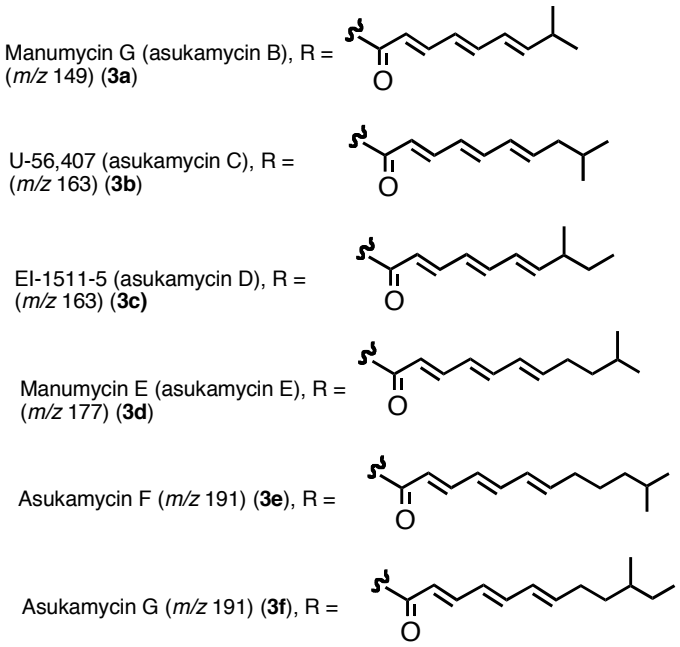
Asukamycin congeners from *Streptomyces nodosus* ssp. *asukaensis*

Since most producer organisms yield not just one manumycin antibiotic, but several related compounds, we analyzed extracts of *S. nodosus* ssp. *asukaensis* by chromatographic fractionation and NMR and mass spectrometry for the presence of asukamycin congeners. In addition to the main component, asukamycin (**1**), three more compounds (**3a** – **3c**), were isolated and characterized by NMR and MS, and another three (**3d** – **3f**), were detected and partially characterized by LC-MS/MS (Figure 2). All these

Figure 2



Asukamycin congeners isolated from or detected in *Streptomycesnodosus* ssp. *asukaensis (m/z refers to the molecular ion of the respective compound in the ESI-MS)*



compounds differ from each other only in the structure of the "upper" polyketide chain, reflecting solely the incorporation of different starter units. Four of the compounds, manumycin G (**3a**)[14] (from 2-methylpropanoate as starter unit), U-56,407 (**3b**)[21] (3-methylbutanoate starter), EI-1511-5 (**3c**)[15,16] (2-methylbutanoate starter) and manumycin E (**3d**)[14] (4-methylpentanoate starter) had been reported before as products of other Streptomycetes. Compounds **3b** and **3c** co-chromatographed; the ^{13}C NMR of the mixture closely matched that of EI-1511-5 but with an extra carbon signal at 23 ppm due to the two *geminal* methyl carbons of **3b**. ^{1}H-NMR decoupling experiments confirmed the presence the two separate alkyl groups of **3b** and **3c**, respectively, in a ratio of 1 : 3.7. Two of the minor constituents detected, asukamycin F (**3e**) and asukamycin G (**3f**), are new compounds; their structures were tentatively inferred from the *m/z* 191 of the mass spectral fragment of their "upper" chains and analogy to the structures of **3b** and **3c**. They might be formed from 5-methylhexanoate and 4-methylhexanoate, respectively, as starter units, but alternatives cannot be excluded.

	No addition	1 mM **4**	2 mM **4**	4 mM **4**	8 mM **4**
Manumycin G (**3a**)	10.1 %	5.6 %	2.8 %	2.6 %	2.3 %
U-56,407 (**3b**)	6.9 %	23.7 %	14.5 %	13.4 %	13.0 %
EI-1511-5 (**3c**)	25.4 % Σ 32.3%				
Manumycin E (**3d**)	11.8 %	4.0 %	2. 2 %	1.8 %	1.7 %
Asukamycin (**1**)	45.8 %	66.8 %	80.5 %	82.2 %	83.0 %

Table 1. Relative abundance of asukamycin congeners produced by *S. nodosus* ssp.*asukaensis* with and without addition of cyclohexanecarboxylic acid (**4**).

The relative abundance of the various asukamycin congeners in the *S. nodosus* ssp. *asukaensis* fermentation was determined by ESI-MS and is shown in the first column of Table 1. Although **1** is clearly the main metabolite at slightly less than 50 %, several of the newly detected compounds, such as **3c, 3a** and **3d**, are also quite prominent, each contributing more than 10 % to the mixture. The amounts of **3e** and **3f** were minimal (<1 %). The data suggest considerable promiscuity of the "upper" chain polyketide synthase. This raises the question to what extent the product distribution reflects precursor availability and whether it can be influenced by increasing precursor supply. To probe this, we added cyclohexanecarboxylic acid (**4**) to the fermentation in 1, 2, 4 and 8 mM concentration and reanalyzed the product distribution. As shown in Table 1, the fraction of **1** in the mixture increased substantially,

saturating at somewhat above 80 % at 2 – 4 mM cyclohexanecarboxylic acid, whereas the percentages of the other congeners decrease accordingly. Relatively pure **1** can thus be obtained by fermenting the organism in the presence of added **4**. Hence, the "upper" chain polyketide synthase indeed shows some plasticity in its substrate selection.

Fatty acid composition of *S. nodosus* ssp. *asukaensis* lipids

Branched chain fatty acids which must be derived from some of the same starter units as the newly detected asukamycins have been reported as prominent constituents of membrane lipids in various Streptomycetes.[22] It was therefore of interest to examine the fatty acid spectrum of the *S. nodosus* ssp. *asukaensis* lipids and to compare its relationship to the spectrum of asukamycins produced. To this end, the lipids were hydrolyzed with base, the fatty acid mixture methylated and then analyzed by GC-MS. The results, summarized in the first column of Table 2, indicate that qualitatively the branched chain fatty acid synthase and the "upper" chain asukamycin PKS of *S. nodosus* ssp. *asukaensis* share the same starter unit specificity. Not only are the same branched chain starter units used by both enzymes, but the lipids also contain ω-cyclohexylundecanoic acid, albeit only about 3.4 %. ω–Cyclohexyl fatty acids are found in some thermophilic, acidophilic bacteria, such as *Alicyclobacillus acidocaldarius*, where they constitute

Fatty acid	No addition	1 mM **4**	2 mM **4**	4 mM **4**	8 mM **4**
iso-C_{14}	3.8 %	4.4 %	4.2 %	4.3 %	4.0 %
normal-C_{14}	1.0 %	0.4 %	0.7 %	0.5 %	0.6 %
iso-C_{15}	6.7 %	7.5 %	6.9 %	6.8 %	6.4 %
anteiso-C_{15}	13.7 %	18.8 %	19.1 %	18.7 %	16.8 %
normal-C_{15}	1.5 %	<0.1 %	<0.1 %	<0.1 %	<0.1 %
iso-C_{16}	30.0 %	23.8 %	24.2 %	24.4 %	21.6 %
normal-C_{16}	11.5 %	10.6 %	9.6 %	10.6 %	9.6 %
iso-C_{17}	5.6 %	3.5 %	3.5 %	3.0 %	4.6 %
anteiso-C_{17}	19.2 %	13.9 %	14.2 %	12.2 %	13.9 %
normal-C_{17}	1.6 %	<0.1 %	<0.1 %	<0.1 %	<0.1 %
iso-C_{18}	1.3 %	0.1 %	0.2 %	0.1 %	0.4 %
normal-C_{18}	0.7 %	0.8 %	0.5 %	1.4 %	0.3 %
cyclohexyl-C_{15}	-	0.7 %	0.8 %	0.8 %	1.4 %
cyclohexyl-C_{17}	3.4 %	15.4 %	15.6 %	17.1 %	19.7 %
cyclohexyl-C_{19}	-	0.1 %	0.4 %	0.2 %	0.8 %
Total cyclohexyl	3.4 %	16.2 %	16.8 %	18.1 %	21.9 %

Table 2. Fatty acid composition of *Streptomyces nodosus* ssp. *asukaensis* with and without addition of cyclohexanecarboxylic acid (**4**)

70-90 % of the membrane lipids.[23] Just as the "upper" chain PKS, the fatty acid synthase also responds to an external supply of **4** with an increased output of cyclohexyl fatty acids and a corresponding reduction in normal and branched chain fatty acids. However, this system saturates at a much lower percentage, slightly over 20 %, of the cyclohexyl derivatives in the mixture. The fatty acid synthase and the asukamycin "upper" chain polyketide synthase of *S. nodosus* ssp. *asukaensis* share a qualitatively similar starter unit specificity, although there are clearly quantitative differences. The similarities and differences become particularly evident when one compares the utilization of different starter units in a normal fermentation, as shown in Table 3. First of all, while 16.3 % of the fatty acids are derived from short

Starter unit	Fatty acid	Fatty acid (%)	Antibiotic	Antibiotic(%)
Acetate	$n_{(2n)}$	13.2	none	-
Propionate	$n_{(2n+1)}$	3.1	none	-
2-Methylpropionate	$iso_{(2n)}$	35.1	Manumycin G (**3a**) Manumycin E (**3d**)	21.9
2-Methylbutanoate	$iso_{(2n+1)}$	12.3	U-56,407 (**3b**) Asukamycin F (**3e**)	6.9
3-Methylbutanoate	$anteiso_{(2n+1)}$	32.9	EI-1511-5 (**3c**) Asukamycin G (**3f**)	25.4
Cyclohexanecarboxylic acid (**4**)	$cyclohexyl_{(2n+1)}$	3.4	Asukamycin (**1**)	45.8

Table 3. Relative utilization of different starter units by the *S. nodosus* ssp. *asukaensis* fatty acid and "upper" chain polyketide synthases.

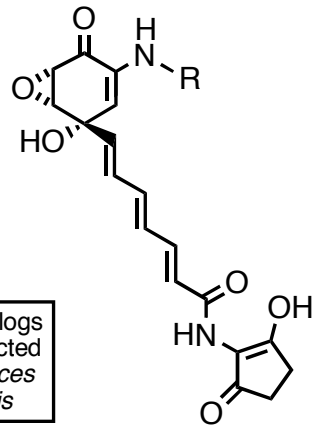
straight chain carboxylic acids, acetate and propionate, there are no corresponding asukamycin congeners in the antibiotic mixture. This is in marked contrast to the situation in the manumycin-producer, *S. parvulus* Tü 64, where the "upper" chain PKS is more relaxed in the choice of chain extension substrates, but uses only short, straight chain acids as starter units. There is some evidence that normal chain fatty acids in Streptomycetes can be produced by a different chain initiation process or enzyme than branched chain fatty acids.[24,25] If that is true, it appears that the *S. parvulus* "upper" chain PKS mimicks this straight-chain fatty acid synthesis process of Streptomycetes or the *E. coli* fatty acid synthase, whereas the asukamycin "upper" chain PKS more closely resembles branched chain fatty acid synthases. The two *S. nodosus* ssp. *asukaensis* enzymes show comparable preferences between different branched chain starter units. However, compared to the fatty acid synthase, the "upper" chain PKS has a

very pronounced preference for cyclohexanecarboxylic acid over branched alkyl chain starter units. Based on the data with and without feeding of **4**, this preference is between 4 and 13 fold.

Precursor-directed formation of unnatural asukamycins and fatty acids

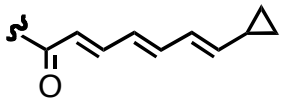
Given the starter unit promiscuity of the *S. nodosus* ssp. *asukaensis* branched chain fatty acid synthase and "upper" chain PKS and their substrate plasticity evident from the cyclohexanecarboxylic acid feeding experiments, it seemed likely that the enzymes might also accept unnatural substrates and produce unnatural analogs of asukamycin and fatty acids. This notion was explored in feeding experiments with a series of cycloalkanecarboxylic acids, ranging from cyclopropane- to cycloheptanecarboxylic acid. Each of the compounds was separately added to a fermentation at a 4 mM concentration and the products structurally analyzed and quantitated by mass spectrometry. New asukamycin analogs with the structures shown in Figure 3 were indeed formed. While cyclopropanecarboxylic acid, as shown in Table 4, was a poor substrate of the "upper" chain PKS, cyclobutane- and particularly cyclopentanecarboxylic acid where utilized very efficiently to give cyclobutyl-asukamycin (**5b**) and cyclopentyl-asukamycin (**5c**), respectively. Cycloheptanecarboxylic acid was again a less efficient substrate, but still gave the cycloheptyl-asukamycin at about 10 % of the product mixture. The "upper" chain PKS thus exhibits a preference for cycloalkanecarboxylic acids with ring sizes of 4 – 6 carbon atoms, with apparently cyclopentanecarboxylic acid the optimal substrate. Cyclobutane- and cyclopentanecarboxylic acid were such good substrates of the "upper" chain PKS that they very effectively outcompeted short branched chain carboxylic acids as starter units, dramatically suppressing

Figure 3

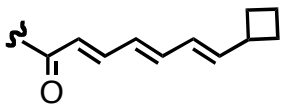


Unnatural asukamycin analogs obtained by precursor-directed biosynthesis in *Streptomyces nodosus* ssp. *asukaensis*

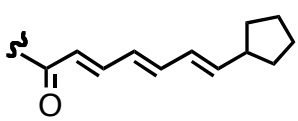
Cyclopropyl-asukamycin (**5a**), R =



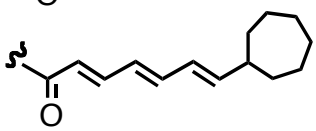
Cyclobutyl-asukamycin (**5b**), R =



Cyclopentyl-asukamycin (**5c**), R =



Cycloheptyl-asukamycin (**5d**), R =

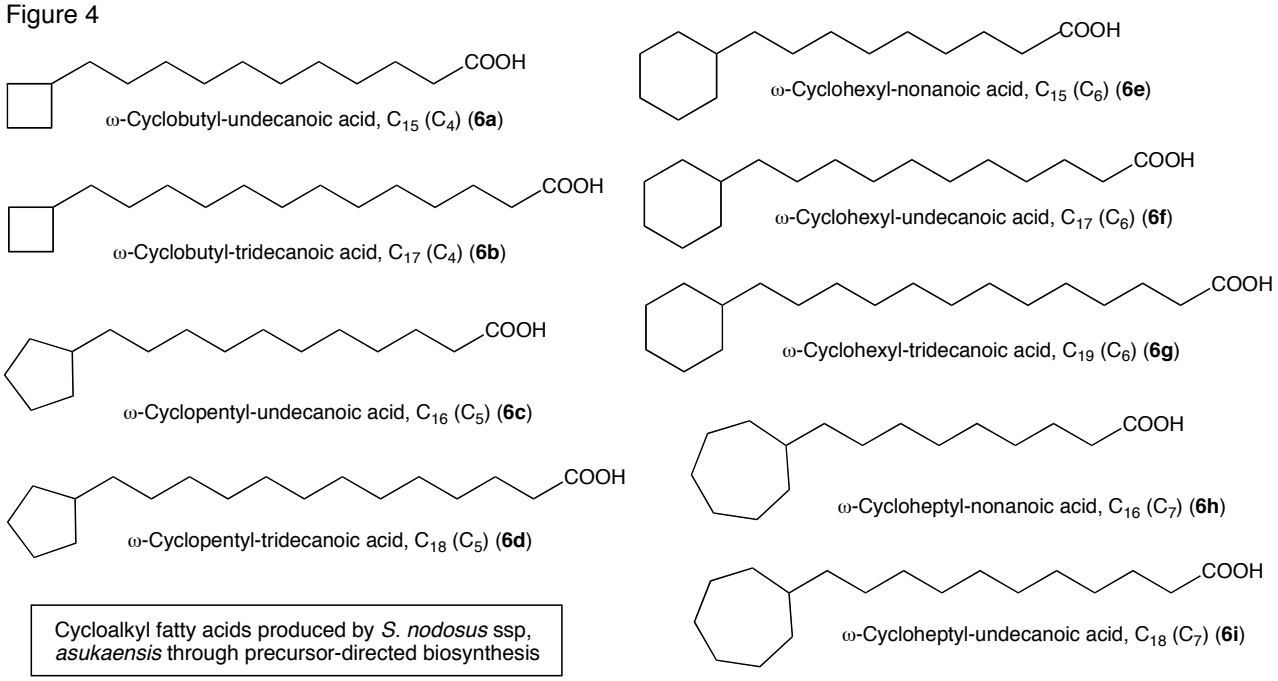


Product distribution	No addition	Cyclo-propane-COOH	Cyclo-butane-COOH	Cyclo-pentane-COOH	Cyclo-hexane-COOH (**4**)	Cyclo-heptane-COOH
Manumycin G (**3a**)	12.9 %	10.9 %	0.8 %	1.5 %	2.6 %	13.0 %
U-56,407 (**3b**) EI-1511-5 (**3c**)	36.9 %	36.2 %	2.4 %	0.7 %	13.4 %	57.8 %
Manumycin E (**3d**)	6.9 %	10.0 %	1.2 %	0.8 %	1.8 %	16.2 %
Cyclopropyl-asukamycin (**5a**)		4.2 %				
Cyclobutyl-asukamycin (**5b**)			68.6 %			
Cyclopentyl-asukamycin (**5c**)				92.6 %		
Asukamycin (**1**)	43.3 %	38.7 %	26.9 %	4.3 %	82.2 %	2.6 %
Cycloheptyl-asukamycin (**5d**)						10.4 %

Table 4. Relative abundance of asukamycin analogs produced by *S. nodosus* ssp. *asukaensis* in the presence and absence of 4 mM cycloalkanecarboxylic acids

the abundance of the asukamycin congeners (**3a** – **3d**) in the product mixture. The preference of the "upper" chain PKS for ring sizes of 4 – 6 carbon atoms was confirmed in a competition experiment, in which all five cycloalkanecarboxylic acids, including **4**, were added to the fermentation simultaneously at a 1 mM concentration each. The product mixture contained no cyclopropyl-asukamycin (**5a**), 18.6 % cyclobutyl-asukamycin (**5b**), 39.8 % cyclopentyl-asukamycin (**5c**), 35.4 % asukamycin (**1**) and 6.2 % cycloheptyl-asukamycin (**5d**), demonstrating again the preference of the enzyme for cyclopentanecarboxylic acid as the optimal substrate.

Figure 4



The lipid fatty acids from the feeding experiments with the individual cycloalkanecarboxylic acids were also analyzed and found to contain new cycloalkyl fatty acids (Figure 4). Their abundances in the total fatty acid mixtures, shown in Table 5, reveal that the *S. nodosus* ssp. *asukaensis* fatty acid synthase has essentially the same ring size preference among the cycloalkanecarboxylic acid chain starter units as the "upper" chain PKS. No significant amounts of cyclopropyl fatty acids were detectible after feeding

Cycloalkyl fatty acid	No addition	Cyclopropane-COOH	Cyclobutane-COOH	Cyclopentane-COOH	Cyclohexane-COOH (**4**)	Cycloheptane-COOH
Cyclo-C_{15}[a]	C_6[b] **6e** <0.1 %	C_6 **6e** 0.1 %	C_4 **6a** 0.9 % C_6 **6e** 0.4 %		C_6 **6e** 0.8 %	
Cyclo-C_{16}				C_5 **6c** 22.6 %		C_7 **6h** 1.4 %
Cyclo-C_{17}	C_6 **6f** 3.0 %	C_6 **6f** 2.0 %	C_4 **6b** 3.1 % C_6 **6f** 2.5 %	C_6 **6f** 0.5 %	C_6 **6f** 17.1%	C_6 **6f** 0.2 %
Cyclo-C_{18}				C_5 **6d** 18.4 %		C_7 **6i** 1.0 %
Cyclo-C_{19}					C_6 **6g** 0.2 %	

Table 5. Percentage of cycloalkyl fatty acids produced by *S. nodosus* ssp, *asukaensis* with and without addition of 4 mM cycloalkanecarboxylic acids. The remainder of each mixture consists of straight and branched chain fatty acids. [a] Subscript = total number of carbon atoms; [b] subscript = number of carbon atoms in ring.

cyclopropanecarboxylic acid, but cyclobutanecarboxylic acid and cycloheptanecarboxylic acid produced 4 % and 2.4 %, respectively, of cyclobutyl- and cycloheptyl fatty acids, and cyclopentanecarboxylic acid was a more efficient substrate than **4**. The formation of cycloalkyl fatty acids has also been observed upon feeding of cycloalkanecarboxylic acids to a branched-chain a-keto acid dehydrogenase mutant of the avermectin producer, *S. avermitilis*.[26] Cycloheptyl fatty acids have been isolated from the thermophile, *Alicyclobacillus cycloheptanicus*, where they represent the main components of the membrane lipids.[27] The cycloheptane ring moiety is formed by a ring expansion of phenylacetic acid.[28] Cyclobutyl fatty acids have apparently not yet been encountered in nature, but a series of cyclopentenyl fatty acids are present in the seeds of several genera of *Flacourtiaceae*, where they arise from the non-proteinogenic amino acid, cyclopentenylglycine.[29] The high efficiency of cyclopentanecarboxylic acid as a starter unit, leading to a remarkable 41 % of cyclopentyl fatty acids in the mixture, demonstrates that the fatty acid synthase of *S. nodosus* ssp. *asukaensis* shows the same ring size preference among cycloalkanecarboxylic acids as starter units as the "upper" chain PKS.

Inhibition of cyclohexanecarboxylic acid formation.

Further examination of the data in Table 4 shows that the feeding of cycloalkanecarboxylic acids with 4, 5 or 7-membered rings not only led to the formation of the corresponding asukamycin analogs, but also to a drastic decrease in the amount of asukamycin (**1**) produced. For example, in the presence of cyclopentanecarboxylic acid as external substrate, the amount of **1** formed is decreased 10 fold. While one might ascribe this to the favorable competition of the unnatural starter units with cyclohexanecarboxylic (**4**) for binding to the "upper" chain PKS, the cycloheptanecarboxylic acid experiment shows that this phenomenon is still observed when the unnatural precursor is a relatively modest substrate. Here, only 10.4 % of cycloheptyl fatty acids are produced, yet **1** formation is reduced almost 17 fold compared to the unsupplemented fermentation. As a consequence the amounts of compounds **3b**, **3c** and **3d** are substantially increased. This leads to an alternative conclusion, namely that the external cycloalkanecarboxylic acids not only compete with **4** for use as substrate by the "upper" chain PKS, but that they also suppress the endogenous formation of **4**. Presumably they must inhibit one or more of the steps in the conversion of shikimic acid into **4**. An earlier feeding experiment with [7-^{13}C]-**4** (99 % ^{13}C), which gave **1** with the same 99 % isotopic enrichment,[30] shows that this is even true for **4**, i.e., external **4** completely shuts off the endogenous production of **4**. The data in Table 5 from the analysis of the fatty acids are also consistent with a reduced availability of **4** in the presence of external cycloalkanecarboxylic acids, although the effect is somewhat less pronounced. Cyclopentanecarboxylic acid, a good fatty acid synthase substrate, and cycloheptanecarboxylic acid, a poorer substrate, reduce the amount of cyclohexyl fatty acid 6 and 15 fold, respectively.

DISCUSSION AND CONCLUSIONS

Fatty acid biosynthesis in Streptomycetes, as in other bacteria,[31] is mediated by a type II dissociated fatty acid synthase (FAS).[32] Starter unit specificity rests with a ketosynthase III (KSIII, FabH), which catalyzes the first condensation step between the starter acyl-CoA and malonyl-acyl carrier protein (malonyl-ACP).[25,33] The resulting 3-ketoacyl-ACP then serves as the substrate for the other enzymes of the FAS complex which catalyze the further condensation, reduction and dehydration steps. The substrate specificity of FabH, in addition to the precursor supply,[34] is the major determinant of the structures, straight chain or branched chain, of the fatty acids produced. The *E. coli* FabH, for example, selects for short straight chain acyl-CoAs, acetyl-CoA and propionyl-CoA,[35] whereas the FabH from a Streptomycete prefers branched chain acyl-CoAs, isobutyryl-, 2-methylbutyryl- and 3-methylbutyryl-CoA, as starter units, although it can also, apparently less efficiently, prime the synthesis of straight chain fatty acids from acetyl- and propionyl-CoA.[25] There is also some evidence for a possible minor, FabH-independent path for the synthesis of straight chain fatty acids in Streptomycetes.[24,25]

Very little is known about the nature of the polyketide synthase (PKS) catalyzing the assembly of the two polyketide chains of **1**. Based on feeding experiments which ruled out most of the plausible assembly intermediates, we at one time thought that the entire molecular framework might be assembled on a single type I modular PKS.[5] However, the recent cloning and preliminary analysis of the asukamycin (*asu)* biosynthetic gene cluster showed the absence of any type I PKS genes, but revealed the presence of type II fatty acid synthase genes, including two homologues of *fabH*.[36] Thus, the two polyene chains of **1** are apparently synthesized by fatty acid synthase-like enzymes rather than by a more typical PKS. One of the two *fabH* homologues, *orf 21*, is most closely related (54 % at aa level) to a *fabH* from *S. coelicolor* (NP626634), whereas the other, *orf 20*, is less closely related (35 %) to a *fabH* from *Clostridium tetani* (NP780842). Also present are a ketoreductase and a dehydratase gene, but no enoate reductase homologue, consistent with the processing of the polyketide chains only through the dehydration step.[36]

As the antibiotic product spectrum of the *S. nodosus* ssp. *asukaensis* fermentation indicates, the starter unit for the "lower" polyene chain is rather invariant. Therefore, the FabH responsible for this initiation step seems to have a fairly stringent substrate specificity. In contrast, the "upper" chain can be initiated by a range of branched chain and alicyclic acyl-CoAs, implying a FabH of rather broad substrate range. The results reported here reveal a remarkable similarity between the starter unit pattern of the asukamycins and the fatty acids produced by *S. nodosus* ssp. *asukaensis*, albeit with a several fold bias of asukamycin biosynthesis towards alicyclic acyl-CoAs as starter units relative to fatty acid biosynthesis. Similarly, both processes show comparable plasticity in their response to externally added starter unit substrates, both natural and unnatural ones. These observations imply that the FabH initiating "upper" chain formation must be very similar to FabHs involved in branched chain fatty acid synthesis in Streptomycetes. It is therefore plausible to suggest that Orf 21 has this function, although there is at the moment no direct experimental evidence for or against this notion.

Whether the same FabH initiating "upper" chain formation also serves to prime fatty acid biosynthesis in *S. nodosus* ssp. *asukaensis* or whether, more likely, the genome contains another, closely related FabH dedicated to fatty acid biosynthesis, can not be decided based on available evidence. In the first case, the quantitative differences in the starter unit utilization for fatty acid and asukamycin formation would have to be due to differences in the substrate preferences of downstream enzyme. It has been shown in *S. glaucescens* that the FabH priming fatty acid biosynthesis exhibits high specificity for FAS ACPs and does not react with the ACP from the tetracenomycin type II aromatic PKS, allowing the two systems to function independently.[37] On the other hand, in recent work,[38] deletion of the KSIII initiating alkylprodiginine biosynthesis, RedP, in *S. coelicolor* resulted in reduced overall alkylprodiginine

production, but formation of new prodiginines from branched chain starter units. Expression of the FabH from the *S. glaucescens* FAS in this mutant increased overall alkylprodiginine titers, but gave the same mixture of normal and new compounds. Clearly, in this more FAS-like system, the FAS FabH can substitute for the dedicated KSIII of the secondary metabolic pathway. Thus the limited precedence available does not really suggest a distinction between the two possibilities outlined above.

The notable starter unit flexibility of the asukamycin upper chain PKS allows the precursor-directed biosynthesis of **1**-analogs with modified upper chains, as shown in a number of demonstration cases with different cycloalkanecarboxylic acids. The scope of this approach in terms of the structural range of unnatural substrates that is accepted remains to be explored. A similar approach has been used for the mutational biosynthesis of a large number of avermectin analogs,[39] including the commercially important doramectin.[40] Since, surprisingly, the endogenous formation of cyclohexanecarboxylic acid is inhibited by the unnatural homologs utilized, this approach can be practiced, in this case, without the need to block the endogenous synthesis of the natural substrate by mutation. However, the fact that fatty acid synthesis in this organism also responds to the external unnatural chain initiation substrates could conceivably be a limitation, if unnatural fatty acids are formed that disrupt membrane function or are otherwise toxic to the organism. That did not seem to be a problem, however, in the demonstration cases reported so far.

EXPERIMENTAL

Materials

Streptomyces nodosus ssp. *asukaensis* (ATCC 29757) was obtained from the American Type Culture Collection. Fermentation ingredients were purchased from Difco and Sigma, chemicals from Aldrich and Sigma.

Instrumentation and chromatography

^{1}H- and ^{13}C-NMR spectra were obtained on Bruker AF 300 and AM 500 spectrometers. Gas chromatography-mass spectrometry (GC-MS) was carried out on a Hewlett-Packard 5790A series gas chromatograph coupled to a Kratos Profile HV-3 mass spectrometer. Electrospray ionization mass spectra (ESI-MS) and tandem mass spectra (ESI-MS/MS) were recorded on a Bruker Esquire ion trap mass spectrometer. Liquid chromatography-mass spectrometry (LC-MS) was carried out with 2 Shimadzu LC-10AD pumps, a SPD-10AV UV-Vis variable detector and a Micromass Quattro II Tandem Quadrupole mass spectrometer. Fermentations were carried out in a New Brunswick G25 controlled environment incubator shaker or in an Adolf Kühner ISF-4-V rotary shaker cabinet. Analytical thin

layer chromatography (TLC) was performed on 60F-254 precoated silica gel plates from EM Science. Flash column chromatography was performed on 230-400 mesh silica gel from Aldrich or on reverse-phase C_{18} silica gel from Whatman. High pressure liquid chromatography (HPLC) was conducted with a Beckman model 116 isocratic pump and Beckman model 166 absorbance detector, monitoring absorbance at 260 and 310 nm, using C_{18} reverse-phase analytical or semi-preparative columns.

Fermentations and feeding experiments

S. nodosus ssp. *asukaensis* was grown on yeast-malt extract agar plates, incubated at 28 ºC for 4 days and then stored in a refrigerator. A loop of *S. nodosus* ssp. *asukaensis* was transferred into 100 mL of culture medium in a 500 mL baffled Erlenmeyer flask and grown on a rotary shaker at 300 rpm for 2 days at 28 ºC. Ten mL of this seed culture was used to inoculate each 100 mL of culture medium in 500 mL baffled Erlenmeyer flasks. The culture medium contained glucose 20 g, Bacto Peptone 5 g, K_2HPO_4 0.25 g, $MgSO_4$ 0.25 g, $(NH_4)_6Mo_7O_{24}x4H_2O$ 5 mg, $FeSO_4x7H_2O$ 50 mg, $CuSO_4x5H_2O$ 5 mg, $ZnSO_4x7H_2O$ 5 mg, $MnCl_2x4H_2O$ 10 mg, deionized water to 1000 mL, pH 7.0. Media were sterilized for 20 min at 121 ºC in an autoclave.

The precursor compounds were added in the concentrations indicated at the time when the production culture was inoculated: Cyclopropanecarboxylic acid, cyclobutanecarboxylic acid, cyclopentanecarboxylic acid and cycloheptanecarboxylic acid, 4 mM; cyclohexanecarboxylic acid 1 mM, 2 mM, 4 mM and 8 mM. The cultures were then incubated at 28 ºC with shaking at 300 rpm.

Isolation, purification and identification of asukamycins

S. nodosus ssp. *asukaensis* production cultures were harvested 72 h after inoculation. The fermentation broth was centrifuged at 9000 rpm for 25 min. The supernatant was saturated with NaCl and extracted three times with ethyl acetate. The combined extracts were dried and the solvent was evaporated *in vacuo*. The crude products were purified on a silica gel column eluting with methylene chloride/methanol (100:3). The products were further purified using semi-preparative C_{18} reverse-phase HPLC eluting with acetonitrile / water. Three HPLC fractions (1, 2 and 4) containing asukamycin-type compounds were obtained and the following compounds were identified in them:

HPLC fraction 1: Manumycin G (asukamycin B) (**3a**): R_f 0.42 (MeOH/$CHCl_3$ 10:1). T_{ret} 6.4 min (acetonitrile / water 70:30, flow rate 4 mL/min). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 189.9, 166.9, 165.6, 147.0, 143.5, 142.8, 141.5, 140.5, 139.4, 132.3, 131.4, 129.2, 129.2, 128.2, 128.2, 124.1, 123.1, 115.5, 71.9, 57.8, 53.4, 32.1, 22.3, 22.3. ESI-MS/MS $[M+H]^+$ 507, daughter ions 489, 149. $[M-H]^-$ 505, daughter ions 487, 469, 374, 357, 339, 322, 216, 178.

HPLC fraction 2: U-56,407 (asukamycin C) (**3b**) and EI-1511-5 (asukamycin D) (**3c**): R_f 0.42 (MeOH/$CHCl_3$ 10:1). T_{ret} 8.2 min (acetonitrile / water 70:30, flow rate 4 mL/min). ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C 189.5, 165.9, 164.9, 144.9, 142.0, 141.2, 140.4, 139.5, 139.2, 131.3, 130.0, 129.0, 128.5, 128.1, 123.6, 123.3, 122.5, 114.7, 70.7, 57.3, 52.4, 38.9, 28.9, 23.0, 19.9, 11.7. ES-MS/MS $[M+H]^+$ 521, daughter ions 503, 163. $[M-H]^-$ 519, daughter ions 501, 483, 388, 357, 339, 322, 216, 178.
HPLC fraction 4: Asukamycin (**1**): R_f 0.42 (MeOH/$CHCl_3$ 10:1). T_{ret} 12.1 min (acetonitrile / water 70:30, flow rate 4 mL/min). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 190.0, 166.7, 165.6, 145.9, 143.2, 142.8, 141.7, 140.3, 139.3, 132.4, 131.4, 129.2, 128.6, 128.4, 124.1, 123.6, 115.3, 71.9, 57.9, 53.4, 41.8, 33.3, 26.7, 26.5. ES-MS/MS $[M+H]^+$ 547, daughter ions 529, 189. $[M-H]^-$ 545, daughter ions 527, 509, 414, 357, 339, 322, 216, 178.
Minor components detected by LC-MS/MS were tentatively assigned as: Manumycin E (asukamycin E) (**3d**): ES-MS/MS $[M+H]^+$ 535, daughter ions 517, 177. $[M-H]^-$ 533, daughter ions 515, 497, 402, 357, 339, 322, 216, 178. Asukamycin F (**3e**) and Asukamycin G (**3f**): ES-MS/MS $[M+H]^+$ 549, daughter ions 531, 191.

The unnatural asukamycins, which resulted from the feeding of alicyclic carboxylic acids, were: Cyclopropyl-asukamycin (**5a**): ES-MS/MS $[M-H]^-$ 503, daughter ions 485, 467, 372, 357, 339, 322, 216, 178. Cyclobutyl-asukamycin (**5b**): ES-MS/MS $[M-H]^-$ 517, daughter ions 499, 481, 386, 357, 339, 322, 216, 178. Cyclopentyl-asukamycin (**5c**): ES-MS/MS $[M-H]^-$ 531, daughter ions 513, 495, 400, 357, 339, 322, 216, 178. Cycloheptyl-asukamycin (**5d**): ES-MS/MS $[M-H]^-$ 559, daughter ions 541, 523, 428, 357, 339, 322, 216, 178.

The relative amounts of the various asukamycins in fermentations were estimated by direct ESI-MS analysis of the fermentation extracts from the intensities (ion currents) of the respective molecular ion peaks.

Fatty acids

After centrifugation of the fermentation broth, the mycelia were hydrolyzed with 2.5% KOH at 80 ºC overnight. The solution was filtered and the filtrate was extracted with 10 mL of n-hexane. The aqueous layer was acidified with 6 M HCl and re-extracted with 2 x 10 mL of n-hexane. The combined acidic hexane extracts were dried (Na_2SO_4) and the solvent was evaporated. The resulting fatty acids were methylated with diazomethane. The fatty acid methyl esters were analyzed by GC-MS. The initial column temperature was 60 ºC, the injector and detector temperature were both 250 ºC. After 2 min, the

column temperature was raised at 10 ºC/min to a final temperature of 290 ºC. Helium was used as the carrier gas.

The signals of the methyl esters of straight-chain, *iso*- and *anteiso*-branched chain fatty acids in the GC chromatograms were identified by comparison with authentic samples purchased from Sigma. The relative amounts of the fatty acid methyl esters were estimated from the relative intensities of their respective signals in the GC traces. The alicyclic fatty acid methyl esters obtained after feeding of alicyclic carboxylic acids were as follows: Methyl ω-cyclobutylundecanoate (C_{15}) (**6a**): T_{ret} 13 min 49 sec. GC-MS *m/z* (relative intensity) 254 (M^+, 5), 223 (23), 194 (33), 152 (42), 110 (50), 96 (64), 87 (94), 74 (100), 55 (97). Methyl ω-cyclobutyltridecanoate (C_{17}) (**6b**): T_{ret} 15 min 56 sec. GC-MS *m/z* (relative intensity) 282 (M^+, 8), 250 (46), 222 (63), 180 (46), 110 (67), 97 (83), 87 (89), 74 (93), 55 (98), 43 (100). Methyl ω-cyclopentylundecanoate (C_{16}) (**6c**): T_{ret} 15 min 5 sec. GC-MS *m/z* (relative intensity) 268 (M^+, 71), 225 (56), 199 (68), 143 (75), 87 (93), 74 (96), 55 (92), 43 (100). Methyl ω-cyclopentyltridecanoate (C_{18}) (**6d**): T_{ret} 17 min 6 sec. GC-MS *m/z* (relative intensity) 296 (M^+, 83), 253 (46), 199 (28), 143 (72), 87 (94), 74 (100), 55 (85). Methyl ω-cyclohexylnonanoate (C_{15}) (**6e**): T_{ret} 14 min 3 sec. GC-MS *m/z* (relative intensity) 254 (M^+, 18), 223 (3), 211 (6), 143 (18), 87 (76), 74 (100), 55 (68). Methyl ω-cyclohexylundecanoate (C_{17}) (**6f**): T_{ret} 16 min 13 sec. GC-MS *m/z* (relative intensity) 282 (M^+, 78), 251 (17), 239 (33), 199 (56), 143 (72), 87 (92), 74 (94), 55 (100). Methyl ω-cyclohexyltridecanoate (C_{19}) (**6g**): T_{ret} 18 min 7 sec. GC-MS *m/z* (relative intensity) 310 (M^+, 40), 279 (4), 267 (7), 143 (22), 87 (78), 74 (100), 55 (83). Methyl ω-cycloheptylnonanoate (C_{16}) (**6h**): T_{ret} 15 min 32 sec. GC-MS *m/z* (relative intensity) 268 (M^+, 23), 225 (8), 172 (16), 143 (37), 87 (75), 74 (100), 55 (74). Methyl ω-cycloheptylundecanoate (C_{18}) (**6i**): T_{ret} 17 min 33 sec. GC-MS *m/z* (relative intensity) 296 (M^+, 18), 265 (3), 199 (45), 143 (38), 87 (86), 74 (84), 55 (100).

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REFERENCES

1. S. Omura, C. Kitao, H. Tanaka, R. Oiwa, Y. Takahashi, A. Nakagawa, and M. Shimada, *J. Antibiot.*, 1976, **29**, 876.

2. K. Kakinuma, N. Ikegawa, A. Nakagawa, and S. Omura, *J. Am. Chem. Soc.*, 1979, **101**, 3402.

3. H. Cho, I. Sattler, J. M. Beale, A. Zeeck, and H. G. Floss, *J. Org. Chem.*, 1993, **58**, 7925.

4. R. Thiericke, A. Zeeck, A. Nakagawa, S. Omura, R. E. Herrold, S. T. S. Wu, J. M. Beale, and H. G. Floss, *J. Am. Chem. Soc.*, 1990, **112**, 3979.

5. Y. Hu and H. G. Floss, *J. Am. Chem. Soc.*, 2004, **126**, 3837.

6. See: B. S. Moore and C. Hertweck, *Nat. Prod. Rep.*, 2002, **19**, 70.

7. B. S. Moore, H. Cho, R. Casati, E. Kennedy, K. A. Reynolds, U. Mocek, J. M. Beale, and H. G. Floss, *J. Am. Chem. Soc.*, 1993, **115**, 5254.

8. B. S. Moore, K. Poralla, and H. G. Floss, *J. Am. Chem. Soc.*, 1993, **115**, 5267.

9. J. Sattler, R. Thiericke, and A. Zeeck, *Nat. Prod. Rep.*, 1998, **15**, 221.

10. A. Zeeck, K. Schröder, K. Frobel, R. Grote, and R. Thiericke, *J. Antibiot.*, 1987, **40**, 1530.

11. R. Thiericke, M. Stellwaag, A. Zeeck, and G. Snatzke, *J. Antibiot.*, 1987, **40**, 1549.

12. L. Alcarz, G. Macdonald, J. P. Ragot, N. Lewis, and R. J. K. Taylor, *J. Org. Chem.*, 1998, **63**, 3526.

13. I. Sattler, C. Gröne, and A. Zeeck, *J. Org. Chem.*, 1993, **58**, 6583.

14. Y. Z. Shu, S. Huang, R. R. Wang, K. S. Lam, S. E. Klohr, K. J. Volk, D. M. Pirnik, J. S. Wells, P. B. Fernandes, and P. S. Patel, *J. Antibiot.*, 1994, **47**, 324.

15. T. Tanaka, E. Tsukuda, K. Ochiai, H. Kondo, S. Teshiba, and Y. Matsuda, *J. Antibiot.*, 1996, **49**, 1073.

16. T. Tanaka, E. Tsukuda, Y. Uosaki, and Y. Matsuda, *J. Antibiot.*, 1996, **49**, 1085.

17. J. Kohno, M. Nishio, K. Kawano, N. Nakanishi, S. Suzuki, T. Uchida, and S. Komatsubara, *J. Antibiot.*, 1996, **49**, 1212.

18. M. Hara, K. Akasaka, S. Akinaga, M. Okabe, H. Nakano, R. Gomez, D. Wood, M. Uh, and F. Tamanoi, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 2281; M. Hara and M. Han, *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 3333; T. Ito, S. Kawata, S. Tamura, T. Igura, T. Nagase, J. Miyagawa, E. Yamazaki, H. Ishiguro, and Y. Matsuzawa, *Jpn. J. Cancer Res.*, 1996, **87**, 113; O. Kainuma, T. Asano, M. Hasegawa, T. Kenmochi, T. Nakagohri, Y. Tokoro, and K. Isono, *Pancreas*, 1997, **15**, 379; T. Nagase, S. Kawata, S. Tamura, Y. Matsuda, Y. Inui, E. Yamasaki, H. Ishiguro, T. Ito, and Y. Matsuzawa, *Int. J. Cancer*, 1996, **65**, 620; T. Nagase, S. Kawata, S. Tamura, Y. Matsuda, Y. Inui, E. Yamasaki, H. Ishiguro, T. Ito, Y. Miyagawa, H. Mitsui, K. Yamamoto, and Y. Matsuzawa, *British J. Cancer*, 1997, **76**, 1001.

19. T. Rezanka, J. Reichelova, and J. Kopecky, *FEMS Microbiol. Lett.*, 1991, **68**, 33; K. K. Wallace, B. Zhao, H. A. I. McArthur, and K. A. Reynolds, *FEMS Microbiol. Lett.*, 1995, **131**, 227.

20. D. O'Hagan : "The Polyketide Metabolites", Ellis-Horwood, New York, 1991.

21. T. F. Brodasky, D. W. Stroman, A. Dietz, and S. Miszak, *J. Antibiot.*, 1983, **36**, 950.

22. G. S. Saddler, A. G. O'Donnel, M. Goodfellow, and D. E. Minnikin, *J. Gen. Microbiol*., 1987, **13**, 1137; T. Kaneda, *Microbiol. Rev*., 1991, **55**, 288.

23. G. Darland and T. D. Brock, *J. Gen. Microbiol*., 1971, **67**, 9.

24. K. K. Wallace, S. Lobo, L. Han, H. A. McArthur, and K. A. Reynolds, *J. Bacteriol*., 1997, **179**, 3884.

25. L. Han, S. Lobo, and K. A. Reynolds, *J. Bacteriol*., 1998, **180**, 4481.

26. T. A. Cropp, A. A. Smogowicz, E. W. Hafner, C. D. Denoya, H. A. I. McArthur, and K. A. Reynolds, *Can. J. Microbiol*., 2000, **46**, 506.

27. G. Deinhard, J. Saar, W. Krischke, and K. Poralla, *Syst. Appl. Microbiol. 10*, 1987, 68; H. Allgaier, K. Poralla, and M. Jung, *Liebigs Ann. Chem*., 1985, 378.

28. B. S. Moore, K. Walker, I. Tornus, S. Handa, K. Poralla, and H. G. Floss, *J. Org. Chem*., 1997, **62**, 2173.

29. U. Cramer and F. Spener, *Eur. J. Biochem*., 1977, **74**, 495.

30. Y. Hu, and H. G. Floss, *J. Antibiot*., 2001, **54**, 340.

31. K. Magnusson, S. Jackowski, C. O. Rock, and J. E. Cronan, *Microbiol. Rev*., 1993, **57**, 522.

32. W. P. Revill and P. F. Leadlay, *J. Bacteriol*., 1991, **173**, 4379; W. P. Revill, M. J. Bibb, and D. A. Hopwood, *J. Bacteriol.,* 1995, **177**, 3946; R. G. Summers, A. Ali, B. Shen, W. A. Wessel, and C. R. Hutchinson, *Biochemistry*, 1995, **34**, 9389.

33. K. H. Choi, R. J. Heath, and C. O. Rock, *J. Bacteriol*., 2000, **182**, 365.

34. T. A. Cropp, A. A. Smogowicz, E. W. Hafner, C. D. Denoya, H. A. I. McArthur, and K. A. Reynolds, *Can. J. Microbiol*., 2000, **46**, 365.

35. J. T. Tsay, W. Oh, T. J. Larsen, S. Jackowski, and C. O. Rock, *J. Biol. Chem*., 1992, **267**, 6807.

36. M. Petricek, T. – W. Yu, H. G. Floss, and co-workers, unpublished results; see: H. G. Floss, *J. Nat. Prod*., 2006, **69**, 158.

37. G. Florova, G. Kazanina, and K. A. Reynolds, *Biochemistry*, 2002, **41**, 10462.

38. S. J. Mo, B. S. Kim, and K. A. Reynolds, *Chem. Biol*., 2005, **12**, 191.

39. C. J. Dutton, S. P. Gibson, A. C. Goudie, K. S. Holdom, M. S. Pacey, J. C. Ruddock, J. D. Bu'lock, and M. K. Richards, *J. Antibiot*., 1991, **44**, 357.

40. H. A. I. McArthur, 'Developments in industrial microbiology – BMP '97', ed. by C. R. Hutchinson and J. McAlpine, Society of Industrial Microbiology, 1998, pp. 43-48.