Total synthesis approaches to natural product derivatives based on the combination of chemical synthesis and metabolic engineering

Andreas Kirschning,* Florian Taft and Tobias Knobloch

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Secondary metabolites are an extremely diverse and important group of natural products with industrial and biomedical implications. Advances in metabolic engineering of both native and heterologous secondary metabolite producing organisms have allowed the directed synthesis of desired novel products by exploiting their biosynthetic potentials. Metabolic engineering utilises knowledge of cellular metabolism to alter biosynthetic pathways. An important technique that combines chemical synthesis with metabolic engineering is mutasynthesis (mutational biosynthesis; MBS), which advanced from precursor-directed biosynthesis (PDB). Both techniques are based on the cellular uptake of modified biosynthetic intermediates and their incorporation into complex secondary metabolites. Mutasynthesis utilises genetically engineered organisms in conjunction with feeding of chemically modified intermediates. From a synthetic chemist's point of view the concept of mutasynthesis is highly attractive, as the method combines chemical expertise with Nature's synthetic machinery and thus can be exploited to rapidly create small libraries of secondary metabolites. However, in each case, the method has to be critically compared with semi- and total synthesis in terms of practicability and efficiency. Recent developments in metabolic engineering promise to further broaden the scope of outsourcing chemically demanding steps to biological systems.

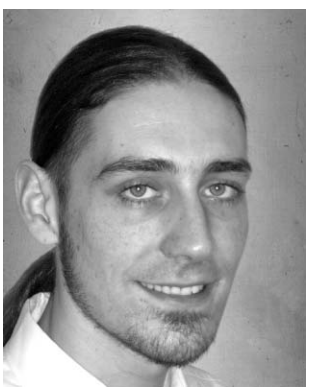
Institute of Organic Chemistry, Leibniz University Hannover, and Center of Biomolecular Drug Research (BMWZ), Schneiderberg 1b, 30167 Hannover, Germany. E-mail: andreas.kirschning@oci.uni-hannover.de; Fax: (+49)-(0)511-762 3011

1. Introduction

The fundamental basis of chemistry is still and will be the chemical synthesis for creating new molecules or materials with target

Andreas Kirschning

Andreas Kirschning was born in Hamburg (Germany) in 1960. He studied chemistry at the University of Hamburg and Southampton University (UK). In Hamburg, he joined the group of Prof. E. Schaumann and received his Ph.D. in 1989 working in the field of organosilicon chemistry. After a postdoctoral stay at the University of Washington (Seattle, USA) with Prof. H. G. Floss, supported by a Feodor-Lynen scholarship of the Alexander-von Humboldt foundation, he started his independent research at the Clausthal University of Technology in 1991, where he finished his habilitation in 1996. In 1997, he held a position as guest professor at the Humboldt-University in Berlin. He served as a visiting professor at the universities of Wisconsin in Madison (U.S.A.) (1989), Rennes (France) (2003) and Le Mans (France) (2007). In 2000 he moved to the Leibniz University of Hannover and became director of the institute of organic chemistry. He is one of the editors of RÖMPP online, Natural Product Reports and Beilstein Organic Chemistry. His research interests cover the total synthesis and mutasynthesis of natural products, glycochemistry, and synthetic technology (solid-phase assisted synthesis, microreactors).



Florian Taft

Florian Taft was born in Celle (Germany) in 1980. He studied chemistry at the Leibniz University of Hannover, obtaining his diploma in chemistry in 2006 working in the field of advanced polyketide synthesis. Currently he is in the second year of his Ph.D. in organic chemistry under the supervision of Prof. Andreas Kirschning. His research work is focused on mutasynthetic approaches towards natural product derivatives, especially of the highly potent antitumor agent ansamitocin.

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Tobias Knobloch

Tobias Knobloch was born in Hannover (Germany) in 1982. He studied biology and life science at the Leibniz University of Hannover, obtaining his B.Sc. in the latter field in 2004 and followed by his M.Sc. in 2006 while being supported by a scholarship of the Studienstiftung des deutschen Volkes and working in the field of cyclising enzymes in polyketide biosynthesis. He is currently in the first year of his Ph.D. in the group of Prof. Andreas Kirschning. His current research activities are focused on enzymatic macrolactamization in the biosynthesis of ansamycin polyketide antibiotics such as ansamitocin and mutasynthetic approaches towards natural product derivatives.

properties. Due to the continuous development of new reactions, new reagents and catalysts, new synthetic concepts such as domino transformations or multicomponent reactions, stereocontrolled organic synthesis has seen a dramatic sophistication over the last three decades. Nowadays it is justified to consider total synthesis approaches for preparing complex natural products or derivatives on an industrial scale. In fact, this has recently been demonstrated for the complex natural products with antitumor activity discodermolide **1**[1] and the epothilone derivative ZK-EPO **2** (Fig. 1).[2] The major advantage of total synthesis approaches is associated with the opportunity to modify a natural product in every possible manner including alterations of the carbon backbone. However, so far mainly semisynthesis of natural products, particularly chemical modifications of the natural product itself, has served for the preparation of small libraries of natural product derivatives useful for studying structure–activity relationships (SAR).[3,4] This approach, however, is commonly limited to selected transformations, so that in comparison to chemically driven medicinal chemistry and high throughput synthesis, natural products have lost some of their attraction in industrial drug research.

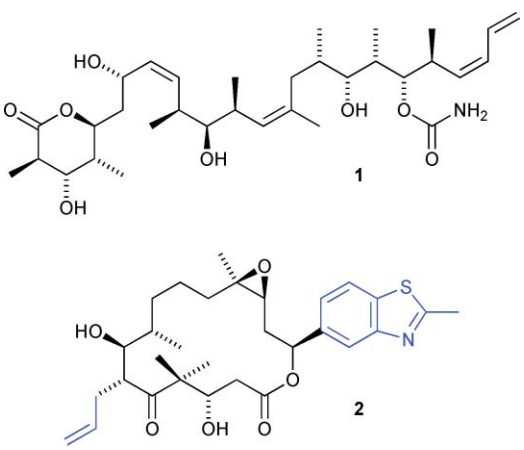

Fig. 1 Discodermolide **1** and Schering's epothilone derivative **2** (ZK-EPO, synthetic changes with respect to epothilone are marked in blue) prepared in an industrial scale.

Still, because of being optimised and preevaluated by Nature to serve as ligands for proteins and receptors, natural products represent a very important source of drugs in several therapeutic fields such as antiinfectives, immunosuppressants and in cancer therapy.[4,5] Therefore, there is a quest for improved techniques for generating natural product-derived libraries. In addition to total- and semisynthesis, new concepts based on interference with the biosynthesis of natural products have been added to the portfolio of methods for structural diversification (Scheme 1).[6] One of the oldest strategies to generate novel structural diversity of microbially produced secondary metabolites utilises the respective wild-type strain producers (Scheme 1; case I) for precursor-directed biosynthesis. PDB is performed by supplementing biosynthetic precursor-analogues to the fermentation broth (Scheme 1; case II). Due to the fact that wild-type producers are used, PDB is a straightforward concept for exploiting the biosynthetic machinery of secondary metabolite producing organisms without time-consuming genetic manipulations using methods of modern molecular biology. However, due to the internal competition of natural and unnatural precursors, yields of the desired analogues are often low and this problem is consequently accompanied by difficulties in separation of these compounds in the presence of the natural metabolites.

A very interesting strategy combines chemical synthesis with biosynthesis using genetically engineered microorganisms. Occasionally, this approach has been termed mutational biosynthesis (MBS) or in short mutasynthesis.[6c,7] According to Rinehart,[8] mutasynthesis involves the generation of biosynthetic block mutants, feeding of mutasynthons to these mutants and their integration into novel metabolites, followed by isolation of the unnatural products for a final evaluation of their biological activities (Scheme 1, case III). Recently, mutasynthesis has experienced a renaissance since the number of fully sequenced biosynthetic gene clusters coding for pharmaceutically potent natural products has substantially increased, setting the stage for easier creation of specific block mutants and therefore efficient access to modified drug candidates.

The application of advanced genetic engineering offers another principal strategy. While the approaches discussed previously make use of any pre-existent enzymatic machinery, the concept of combinatorial biosynthesis recruits different biosynthesis systems for the assembly of engineered pathways in a host system of choice (Scheme 1, case IV).[9]

The efficiency and selectivity of enzymatic catalysis forms the basis for the preparative generation of natural products, and when presented with non-natural precursors the enzymes are required to exhibit broad substrate tolerance for the successful utilisation of the aforementioned methods. While these prerequisites seem contradictory at first glance, the examples discussed in this article show that a practical compromise between selectivity and flexibility of enzymatic machineries exists and can be exploited.

In this report we provide an overview on recent examples of mutational biosynthesis including innovative examples of PDB. Furthermore, we shall cover examples that require advanced metabolic engineering which are expected to lead the reader into the most recent developments in this field of natural product research. The main perspective chosen in this article is the one of a synthetic chemist. Thus, we will occasionally point out the synthetic value of the examples presented with respect

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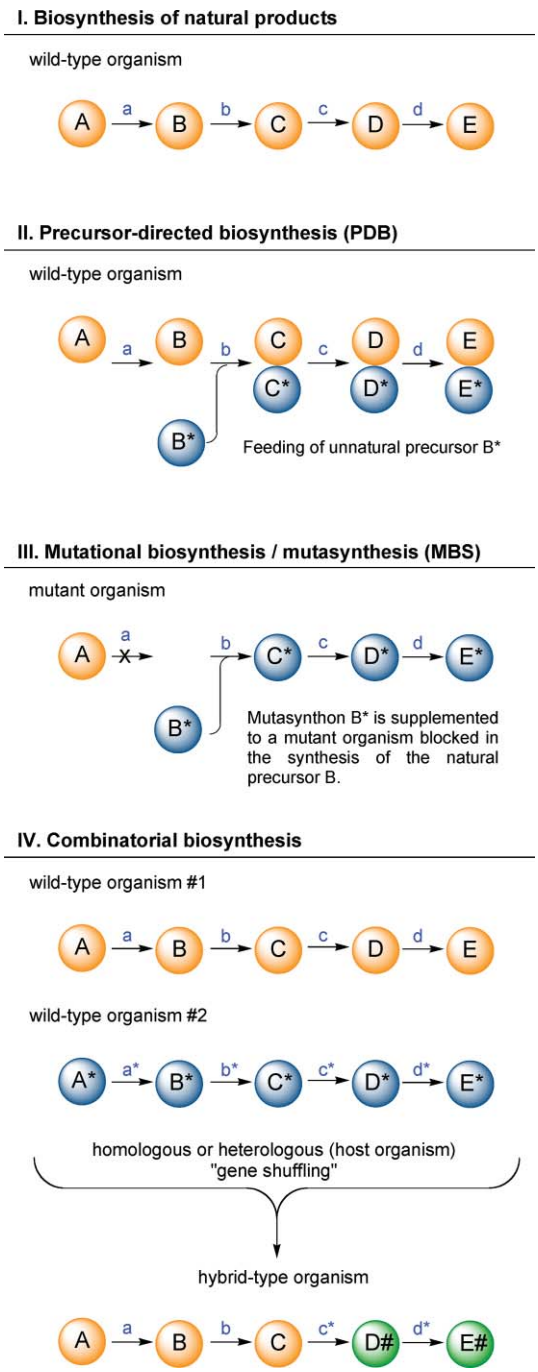


Scheme 1 Biosynthetic concepts for natural product synthesis and derivatives (a–d = enzymes; A = starter building block; B–D = biosynthetic intermediates; E = natural product or derivative).

to practicability, structural novelty and diversity. Considering that elucidation of the required gene sequences and genetic manipulation can be a time consuming task, not every mutational synthesis approach or metabolic engineering concept will make sense, when the same synthetic goal can straightforwardly be achieved by semi- or total synthesis.

This overview is intended to highlight selected examples and is far from comprehensive. For an excellent and more comprehensive review on mutasynthesis the reader is kindly referred to ref. 7.

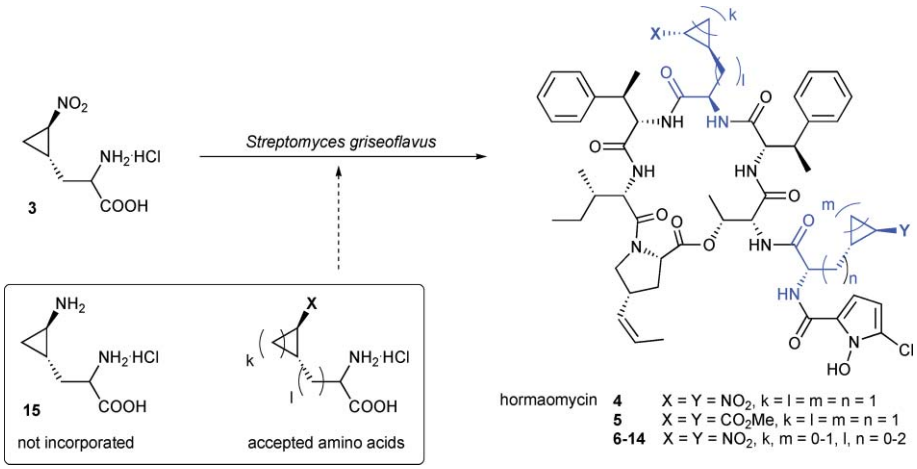
2. Precursor directed biosynthesis (PDB)

An efficient application of PDB for the generation of novel secondary metabolites requires basic knowledge concerning the cultivation of the producer organism and an elementary understanding of the target natural product's biosynthetic assembly process. Once a suitable type of precursor has been chosen for supplementation experiments, several uncertainty factors remain, those being (a) whether these analogues will be assimilated by the organism, (b) will they exhibit non-predictable toxic side-effects, (c) will they be accepted by the biosynthetic machinery in the presence of the natural building block and finally (d) will it be possible to separate novel analogues from the natural variants with a reasonable amount of work. The following examples will demonstrate that PDB is a worthwhile approach, even though the aforementioned complications may occur.

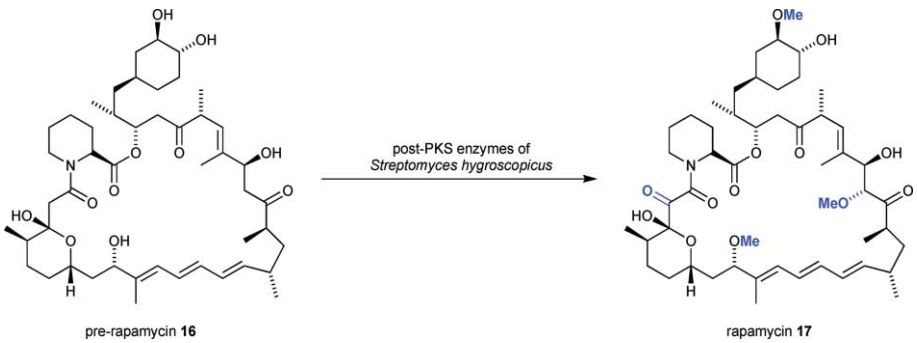
A comprehensive example of PDB was recently applied by Zeeck, de Meijere and coworkers who generated new analogues of hormaomycin **4**, a peptide lactone from *Streptomyces griseoflavus* with a broad spectrum of biological activities, including antibacterial as well as antimalaria activities (Scheme 2). Preliminary feeding experiments[10] with standard deuterium-labelled building blocks pointed to 2-nitrocyclopropylalanine **3** as a suitable precursor for the cyclopropane units. Interestingly, 2-aminocyclopropylalanine **15** is neither an intermediate nor an acceptable substrate for the multienzyme complex. Subsequent feeding experiments[11] with a variety of building blocks resulted in the incorporation of unnatural amino acids, thus leading to novel hormaomycins **5–14**. However, the authors noted difficulties in separation of the analogues from the competitively produced hormaomycin **4**, which is a principal problem for PDB. Nonetheless, structure–activity relationship (SAR) studies were conducted and revealed the unexpected antibacterial activity for one derivative against the opportunistic fungal pathogen *Candida albicans* in an order of magnitude equivalent to the antimycotic agent nystatine.

The immunosuppressant rapamycin **17** isolated from *Streptomyces hygroscopicus* is initially assembled by a polyketide synthase (PKS) to yield pre-rapamycin **16** and further transformed to **17** by post-PKS modifications (Scheme 3). With the intention of employing a PDB approach, 4,5-dihydroxycyclohex-1-enecarboxylic acid **18** (Scheme 4) was regarded as the most likely starter unit for polyketide chain assembly.[12] In a preliminary survey, a series of 21 carboxylic acids was fed to *S. hygroscopicus*, resulting in several new rapamycin analogues **19–21** which exerted comparable or weaker immunosuppressant activity. Due to the broad acceptance of different starter acids the rapamycin loading module seems to possess some degree of flexibility in recognizing carboxylic acids. These results suggest that transfer of the rapamycin loading module to other modular PKS systems could allow the introduction of new building blocks selectable by this system into other metabolites, representing a facet of the combinatorial biosynthesis approach (Scheme 1; case IV).

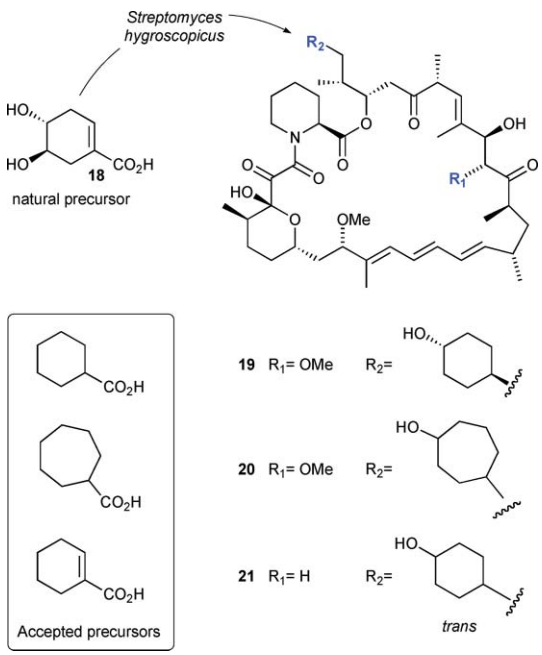
A recent example for the extraordinary potential of PDB was disclosed by Graziani *et al.*[13] Instead of employing deficiency mutants (*vide supra*) the authors demonstrated that enzyme inhibitors can also be exploited to shut down the biosynthesis of rapamycin **17** at an early stage (Scheme 5). Thus, by feeding (±)-nipecotic acid **27** to cultures of *Streptomyces hygroscopicus* the rapamycin biosynthesis was shut down. The inhibitor **27**



Scheme 2 Precursor-directed biosynthesis of novel hormaomycins **5–14**.



Scheme 3 Rapamycin **17** and pre-rapamycin **16** (post-PKS modifications are labelled in rapamycin).



Scheme 4 Precursor-directed biosynthesis of novel rapamycin derivatives **19–21**.

specifically interferes with the biosynthesis of L-pipecolate **22**, a building block that is naturally incorporated just prior to final ring closure. In the presence of thiaanalogues of L-pipecolate, **25** and **26**, incorporation and hence formation of new thiarapamycins **23** and **24** was achieved. Remarkably, processing included all post-PKS transformations while no competition with the natural building blocks occurred. As a result, the yield of the thiaderivative **23** was comparable to rapamycin production, while for derivative **24** a tenfold lower yield was encountered. Despite the fact that the activity assay indicated drastic weaker target binding affinities, this approach revealed deeper insights into SARs.

Other remarkable studies exemplifying the potential of PDB include the generation of soraphen-derivatives employing its natural producer *Sorangium cellulosum*[14] and formation of novel rhamnopyranosides exploiting *Streptomyces griseoviridis*.[15,16]

3. Mutational synthesis

3.1. When is mutational biosynthesis beneficial? A synthetic chemist's perspective

When the administration of unnatural precursors to wild-type organisms leads to complex product mixtures that are difficult to separate, or does not give rise to any novel products at all, this may be due to the internal competition with natural precursors. These problems can be overcome by application of a mutant organism blocked in key biosynthesis steps leading to the targeted natural precursor of choice. The concept of complementing these deficiencies by feeding exogenous precursors ('mutasynthons') to the mutant is termed mutational biosynthesis or mutasynthesis (MBS). Even though random mutagenesis and selection can in

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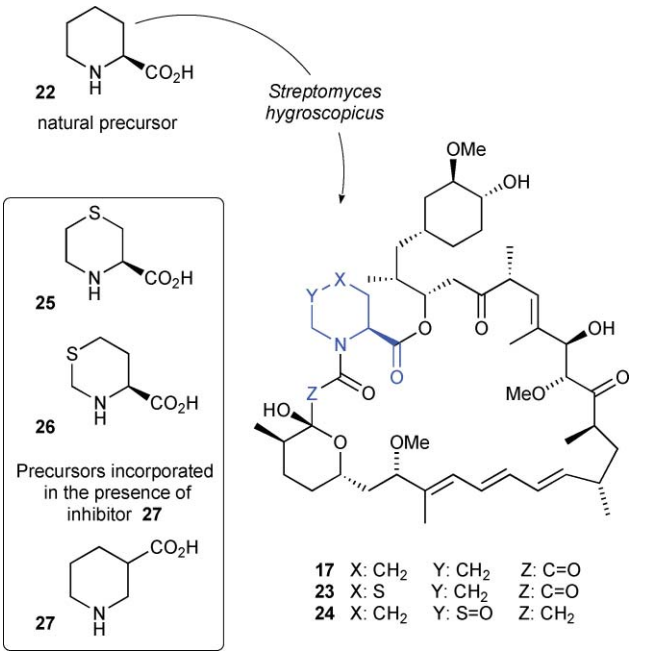


Scheme 5 Efficient generation of rapamycin thiaderivatives *via* precursor-directed biosynthesis using inhibitor **27**.

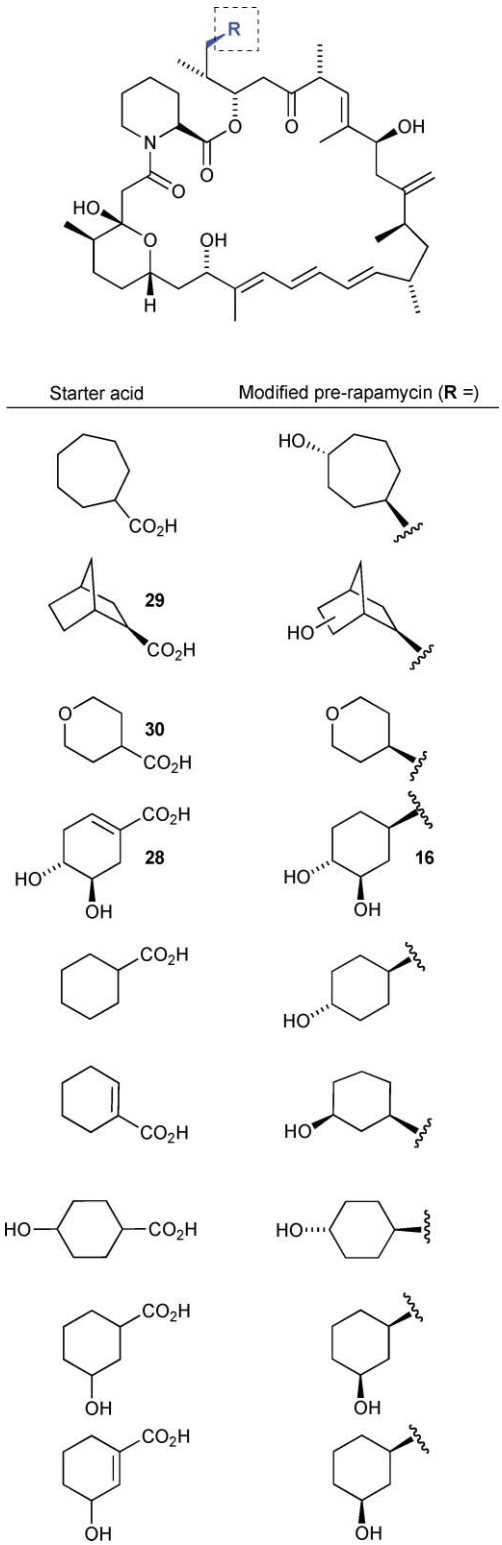
principal provide for the required mutant strains, information concerning the genetic and functional background of the targeted natural product's biosynthesis greatly simplifies the process and may be considered a prerequisite. MBS cannot be employed when the natural precursor requiring elimination is essential for the growth of the organism, so that the entry point has to be reconsidered and more advanced precursors have to be used. When a wild-type organism is not amenable to genetic manipulation, sophisticated methods of metabolic engineering can solve this problem (*vide supra*).

From a synthetic chemist's point of view the concept of mutasynthesis is highly attractive, as the method combines chemical expertise with Nature's synthetic machinery. The best of both worlds are merged, combining the chemist's flexibility in creating any structural assembly and Nature's efficiency in carrying out multistep linear sequences with excellent preciseness and stereocontrol. As a result, this synthesis–biosynthesis hybrid technique rapidly generates small libraries derived from natural products, typically with predictable structural alterations. It only makes sense to spend much effort on sequencing and genetic manipulations if the secondary metabolite has excellent biological activity in a pharmaceutically relevant field or for cell biology. The secondary metabolite should be structurally complex, making mutational synthesis highly competitive over semi- and total synthesis approaches.

In the following, selected recent examples on mutational synthesis are presented and evaluated as far as being useful from a synthetic point of view.

3.2. Sensible examples of mutational biosynthesis

The elimination of internal competition being the main advantage of MBS over PDB, it does not surprise that MBS can lead to improved yields of novel products also available *via* PDB. This was recently demonstrated by Leadlay *et al.* using a mutant strain of the rapamycin producer *S. hygroscopicus*, disrupted in the biosynthesis of the natural starter acid (4*R*,5*R*)-4,5-dihydroxycyclohex-1-ene carboxylic acid **28** as well as in all post-PKS modification steps, facilitating access to pre-rapamycin derivatives (Scheme 6).[17] Besides a significant enhancement of yield (up to 100 mg L^{-1} of



Scheme 6 Mutational biosynthesis of novel rapamycin derivatives.

pre-rapamycin analogue compared to 5 mg L^{-1} of the corresponding completely processed rapamycin analogue by PDB), the application of MBS allowed the incorporation of unusual precursors, such as norbonane carboxylic acid **29** and tetrahydro-2*H*-pyran-4-carboxylic acid **30**, which had in previous PDB experiments with the wild-type strain failed to result in novel products.[18]

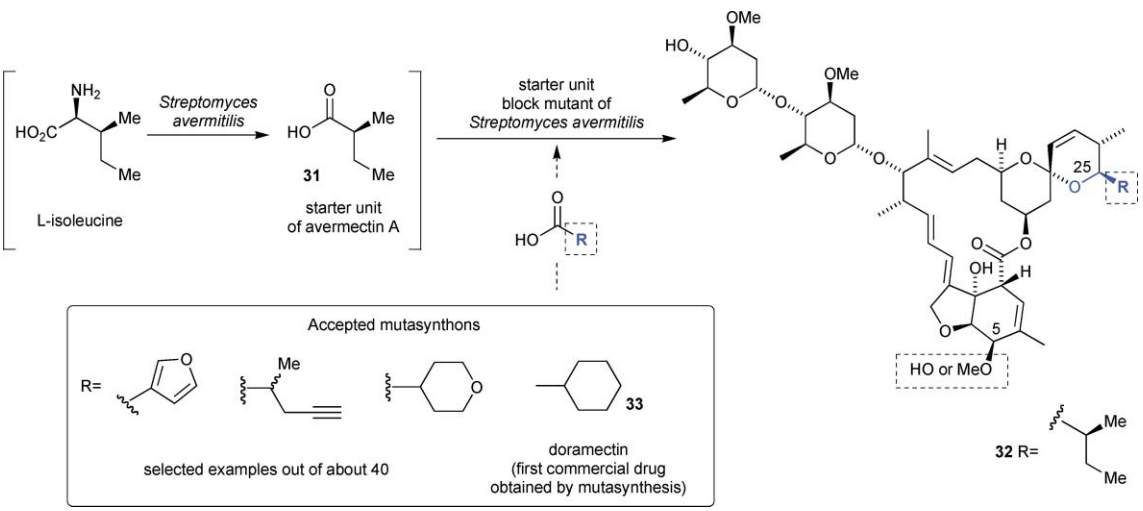
Additional feeding experiments with this mutant strain revealed that incorporation of precursor acids required either a hydrogen bond acceptor, preferably at the 4-position, while alternatively this position had to be kept vacant, retaining the possibility for enzymatic oxidation there.[19] This became evident when fluorine-substituted cyclohexanoic acid derivatives were not accepted by the PKS, probably due to the substantially different electronic properties, thereby preventing enzymatic oxidation.

These insights into the requirements and limitations of the biosynthetic machinery responsible for rapamycin production pave the way for a more efficient generation of additional derivatives.

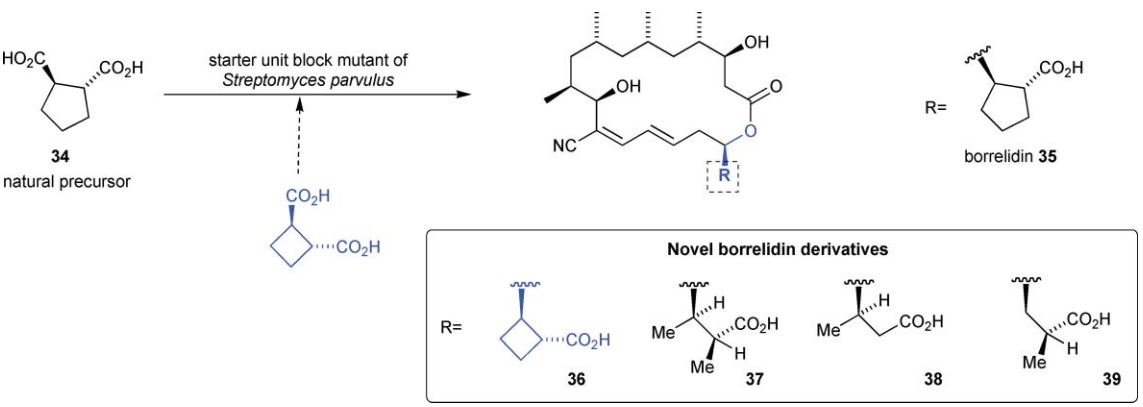
A remarkable example for the generation of structural derivatives not easily accessible *via* semi- or total synthesis is given by an early mutasynthetic lead optimisation approach reported in 1991 (Scheme 7).[20] The avermectins are a group of antiparasitic macrolides produced by *Streptomyces avermitilis*. Mutational biosynthesis served to create new avermectin derivatives modified at C-25. This was achieved by feeding different carboxylic acids or their biosynthetic precursors to a block mutant lacking the ability to form *S*-2-methylbutyric acids **31** from the 2-oxo acid/amino acid precursor. It was found that the substrate tolerance of the avermectin loading domain was rather broad, allowing the successful administration of about 40 different carboxylic acids. From these studies, doramectin **33** was generated, the first commercial drug obtained by mutasynthesis.

Based on these findings and particularly the possibility to generate doramectin by mutational biosynthesis, Reynolds and coworkers introduced the cyclohexylcarboxyl-CoA biosynthetic gene cluster present in the ansatrienine producer *Streptomyces collinus* into the block mutant of *Streptomyces avermitilis*. This engineered strain was able to produce doramectin without supplementation of cyclohexylcarboxylic acid, a strategic approach that represents a creative facet of combinatorial biosynthesis (Scheme 1; case IV).[21]

Another impressive application of MBS for lead optimisation was disclosed by Wilkinson *et al.* and dealt with the polyketide derived angiogenesis inhibitor borrelidin **35** (Scheme 8).[22] Using a non-producing strain disrupted in the biosynthesis of the starter unit (1*R*,2*R*)-cyclopentane-1,2-dicarboxylic acid **34**, supplementation with various analogues resulted in a set of novel borrelidins **36–39** differing in the C-17 side chain. Assays



Scheme 7 Mutational biosynthesis of avermectins **32** and access to doramectins **33** (5-OMe: A series; 5-OH: B series).



Scheme 8 Mutational biosynthesis of borrelidins **35–39**.

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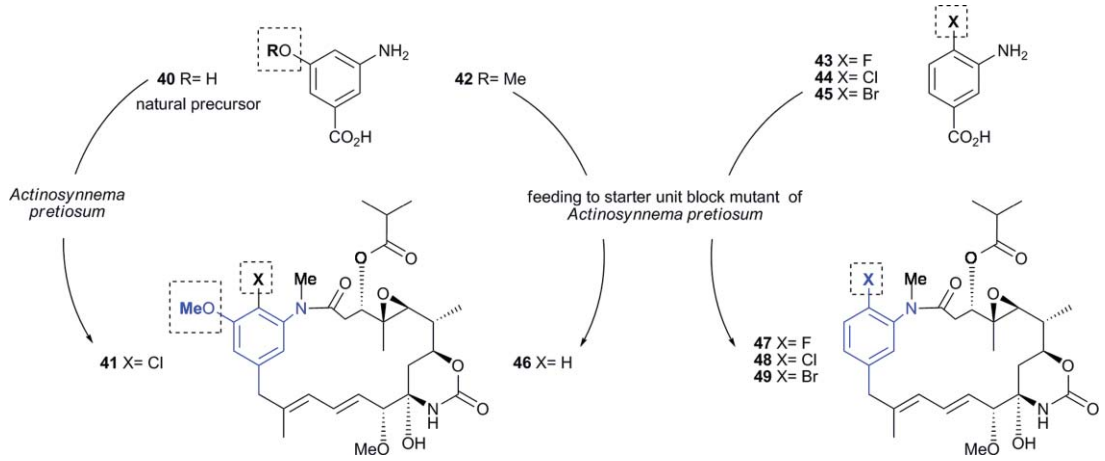

Scheme 9 Successful mutasyntheses of ansamitocin P-3 **41** derivatives.

examining their antiproliferative activity indicated a significantly improved selectivity of the C17-cyclobutyl analogue **36** for *in vitro* angiogenesis inhibition over cytotoxicity. These results underline that MBS can be successfully applied for lead optimisation, whereas total- or semisynthetic approaches towards such compounds would be extremely laborious.

Another unusual starter unit, 3-amino-5-hydroxybenzoic acid **40** (AHBA), is required for the biosynthesis of ansamycin antibiotics, a distinctive class of polyketide-based metabolites (Scheme 9). Ansamitocins are one of the few most cytotoxic compounds known, evident in the inhibition of different leukemia cell lines as well as human solid tumors at very low concentrations (10^{-3} to 10^{-7} μg ml^{-1}). They are accessible *via* their bacterial producer *Actinosynnema pretiosum* and differ in the nature of the acyl side-chain, with ansamitocin P-3 (AP-3) **41** being an important example.[23] The group of ansamitocins has recently re-attracted attention due to their extraordinary potency and are currently being evaluated in phase I studies for their use as warheads in target-directed antibody conjugates.[24] Meanwhile, work towards generation of novel ansamitocin analogues employing a mutant blocked in the biosynthesis of the unique starter unit AHBA has been conducted.[25] By supplementing cultures of the *A. pretiosum* mutant with benzoic acid derivatives **42–45**, novel AP-3 derivatives **46–49** could be generated in amounts suitable for structural identification and activity analysis (Scheme 9).[26] The analogues exhibited strong antiproliferative activity against several tumor cell lines (IC_{50} values in pg mL^{-1}-range). It should be noted that total synthesis has not provided new AP-3 derivatives while semisynthesis has mainly addressed ester side chain modifications and dehalogenation.[23]

Vancomycin **50** is a glycopeptide antibiotic in clinical use. Due to vancomycin-resistant bacteria strains there is an intensive search for novel vancomycin-type derivatives, but semisynthetic approaches have primarily addressed the peripheral glycon unit whereas the structure of the tricyclic aglycon has been left unaltered except for some amide side chain modifications (Fig. 2).[27] From a pharmaceutical point of view, Süssmuth, Wohlleben and coworkers reported one of the most remarkable applications of mutasynthesis by utilizing different block mutants of the actinomycete *Amycolatopsis balhimycina*, the producer of the vancomycin-type glycopeptide antibiotic balhimycin **51**.[28] Elimination of internal β-hydroxytyrosine biosynthesis allowed the introduction of structural variations at the 3-chloro-β-hydroxytyrosine moieties by means of mutational biosynthesis. In fact this approach led to the first fluorinated vancomycin-type glycopeptide antibiotic (Scheme 10).[28b] Additionally, they utilised a deletion mutant blocked in the biosynthesis of 3,5-dihydroxyphenylglycine (Scheme 11)[29] and obtained a second set of new vancomycin-type derivatives modified in the AB macrocycle.

Similar work on mutasynthesis of calcium dependent lipopeptides was reported, however, without full structural assignment of the new products or presentation of any biological data.[30] Enterocin **52** and wailupemycin **53** are secondary metabolites from the marine bacterium *Streptomyces maritimus* and exert bacteriostatic properties.[31] These polyketide-based natural products originate from a common biosynthetic intermediate assembled from a benzoyl-CoA starter unit. Moore and coworkers[32a] administered a series of aryl acids (monosubstituted benzoates and heteroaromatic carboxylates) and their corresponding SNAC esters (*vide infra*), as well as cyclohex-1-enecarboxylate to a mutant blocked in the initial step of the internal degradative starter unit generation starting with L-phenylalanine (Scheme 12). The structural complexity of **53** hampers easy access to aryl analogues by semi- or total synthesis.[32b] In contrast to mutasynthesis experiments involving the usually rather tolerant modular type I PKS, the iterative type II PKS systems appeared to be more discriminating toward mutasynthons, given that only few successful mutasyntheses could be conducted. Nevertheless, chemical access to these wailupemycin derivatives by synthetic routes is expected to be very laborious.

Nature utilises the highly unusual polyketide starter unit *p*-nitrobenzoate in the biosynthesis of aureothin, a polyketide–shikimate hybrid metabolite from the soil bacterium *Streptomyces thioluteus*. Following elimination of starter unit biosynthesis, cultures of the respective mutant strain were supplemented with a range of *p*-substituted benzoates.[33] Since the first crucial step leading to incorporation of modified analogues represents starter unit activation and loading of the substrate onto the PKS apparatus, feeding was carried out using either free acids or *N*-acetyl cysteamine (NAC) thioesters. The NAC adducts serve as activated acyl-CoA mimics[34] that may enter the bacterial cells

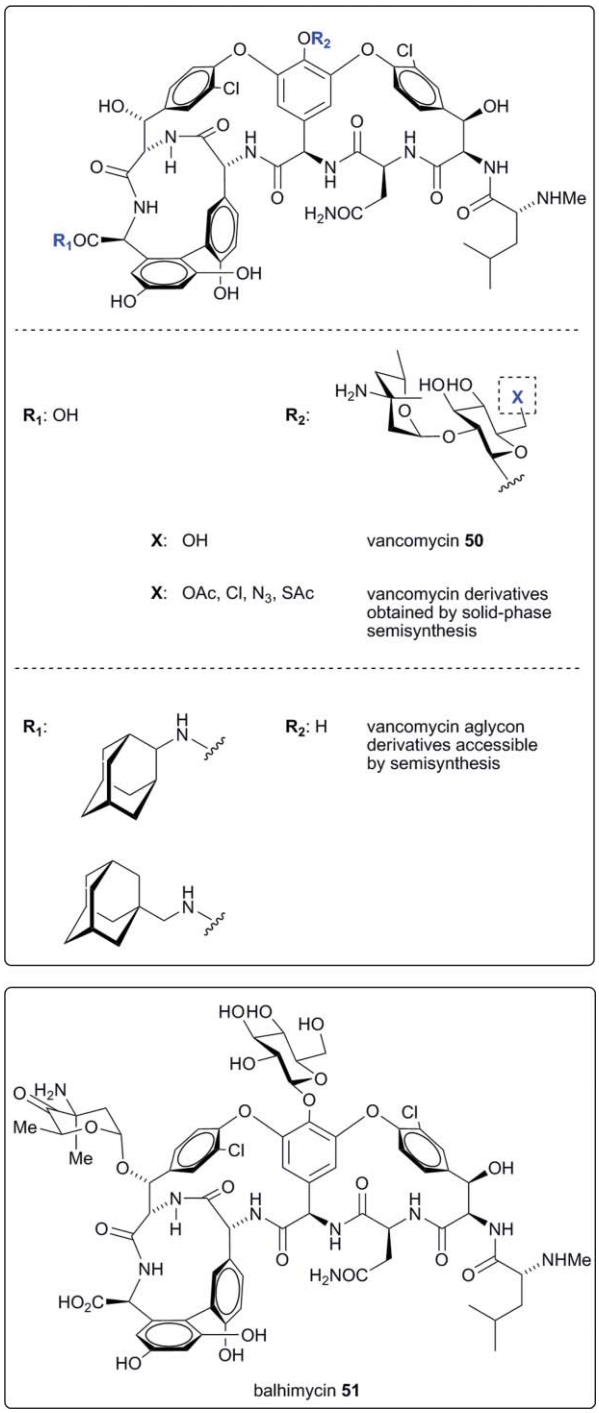

Fig. 2 Selected examples of semisynthetically derived analogues of vancomycin **50** and the structure of balhimycin **51**.

more easily than the free acids and, depending on the requirements of the loading mechanism involved, may bypass a potential CoA-activation bottleneck. However, only *p*-cyanobenzoate was accepted and completely processed to the novel aureothin derivative aureonitrile. Surprisingly, aureonitrile exhibits significantly enhanced antiproliferative activities.[33]

While a great number of additional studies employing mutasynthesis have appeared in the literature, these experiments are not discussed here in detail due to the following reasons. From a synthetic point of view some of these secondary metabolites lack complexity so that total synthesis strategies can be considered instead of accessing derivatives by feeding mutasynthons to block mutants. From our point of view the siderophore pyochelin[35] and the chitin synthetase inhibitor nikkomycin[36] are typical examples. While other examples of mutasynthesis allowed modification of fairly complex natural products, the modifications introduced were of a type that should in principle have been accessible *via* semisynthetic means. The gyrase inhibitor clorobiocin[37] is such an example where MBS was employed to create altered amide side chains. However, amide formation is a straightforward chemical transformation with the advantage of larger substrate flexibility compared to approaches based on biotransformations. Admittedly, many of these examples were published in the eighties and nineties, when mutasynthesis was still in its infancy and the structural diversifications aimed at by those studies primarily served to prove the principle concept.

3.3. How complex can a mutasynthon be?

The previous chapter dealt with structurally fairly simple mutasynthons that could be supplemented to cultures of mutant producers blocked in the biosynthesis of the respective natural small building blocks. But is mutasynthesis limited to small building blocks and how complex can such a mutasynthon be? The generation of mutasynthons based on lead structures of advanced biosynthetic intermediates *via* sophisticated chemical synthesis further increases the flexibility of mutasynthesis since structural modifications normally dictated by downstream biosynthetic steps can be implemented. Apart from making the introduction of modifications at unusual positions possible, the use of advanced biosynthetic analogues promises a larger probability of mutasynthon acceptance due to the smaller number of potentially specificity-restricted biosynthetic steps required for final processing. When administering more complex structures, their assimilation by the producer organism, the cell membrane and transport systems integrated therein have to be regarded as substantial obstacles. So far, the application of mutasynthesis for advanced mutasynthons appears to be no popular strategy as only a very limited number of examples is described in the literature. This can be attributed to the usually substantial synthetic efforts required prior to feeding.

Studies integrating sophisticated methods of molecular biology and chemical synthesis were carried out with the aim of elucidating the acceptance of advanced intermediates by the 6-deoxyerythronolide B synthase (DEBS) of *Saccharopolyspora erythraea*, the producer of the broad spectrum antibiotic erythromycin B **54**. The DEBS system represents the most extensively characterised modular polyketide synthase and for a more detailed description of the insights gained into its utilization of advanced intermediates, the reader is directed to the work of Ward *et al.*[38] Studies were carried out with mutants of the natural producers, as well as with modified DEBS systems reconstituted in different heterologous hosts. The benefits of using the latter systems will be discussed in the next chapter of this review, with the following paragraph concentrating solely on the DEBS system itself. Due to the selectivity of the DEBS loading module for standard biosynthetic building blocks such as propionyl-CoA, the elimination of internal precursor competition is not possible by blocking the respective

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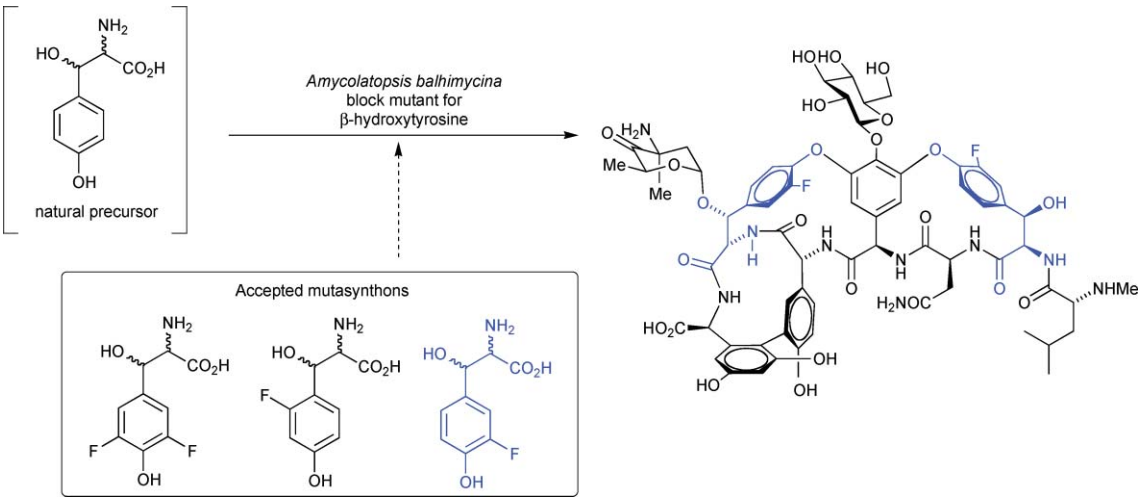
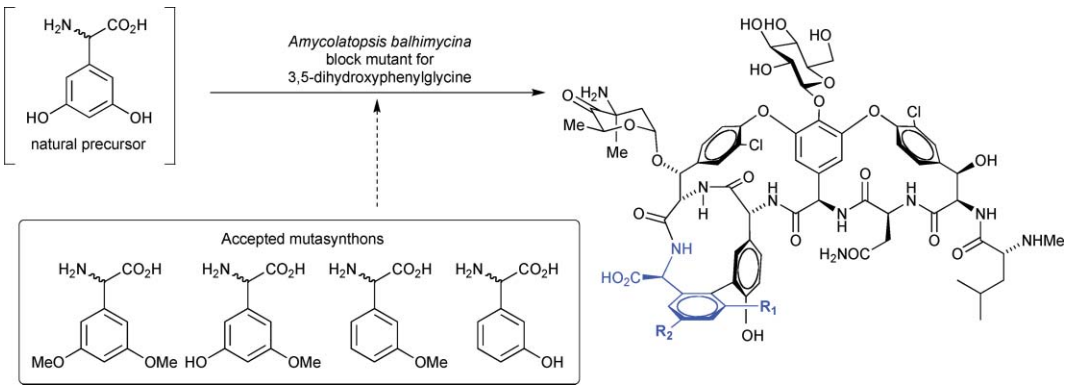
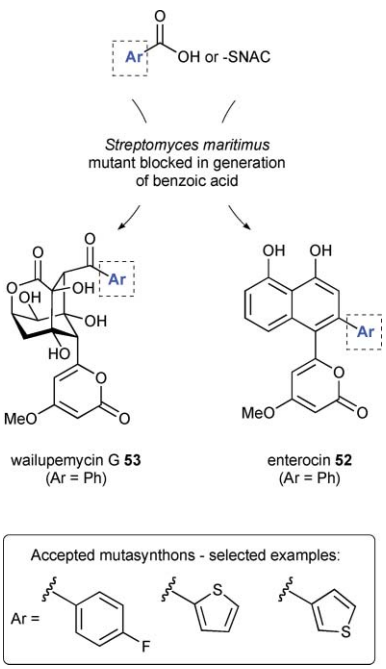


Scheme 10 Mutational biosynthesis of novel vancomycin-type derivatives (part 1).



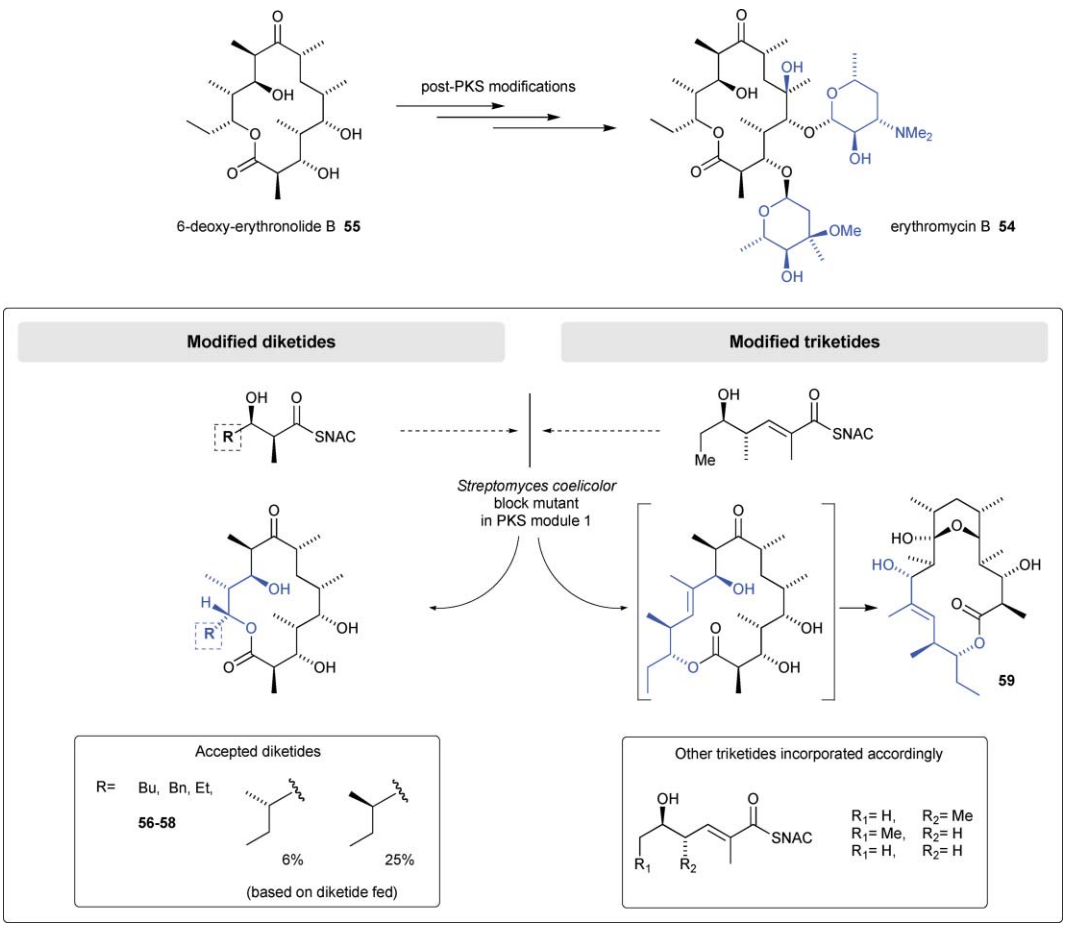
Scheme 11 Mutational biosynthesis of novel vancomycin-type derivatives (part 2).



Scheme 12 Mutational biosynthesis of enterocin **52** and wailupemycin **53** derivatives.

starter unit biosynthesis. To make the DEBS PKS suitable for a mutasynthesis approach, a different strategy was employed. A point mutation was introduced into the active site of the first PKS ketosynthase (KS1) domain, thereby blocking diketide formation based on the available internal starter units.[42] These engineered KS1^0 DEBS systems were shown to convert the natural diketide as well as modified diketides and triketides into analogues of 6-deoxy-erythronolide B **55**.[39,40] These advanced precursors could be further modified into novel erythromycins **56–58** by application of a *S. erythrea* mutant unable to synthesise the core polyketide, but equipped with the full set of post-PKS tailoring enzymes. Remarkably, when SNAC-esters of 2,3-unsaturated triketide derivatives were administered, polyketide elongation and macrolactonisation yielded 16-membered lactones **59** which spontaneously formed the corresponding lactols (Scheme 13).[41] The triketide analogues were apparently accepted as surrogates for the absent diketide precursors and treated accordingly by the biosynthetic machinery, finally resulting in an increased ring size. In a more recent study it was demonstrated that removal of the DEBS loading domain and first module rather than a catalytic inactivation of the latter resulted in an increased utilisation of supplemented diketide precursors by the engineered PKS system.[38]

Apart from the generation of novel compounds, MBS has also served as a tool in biosynthetic investigations, such as

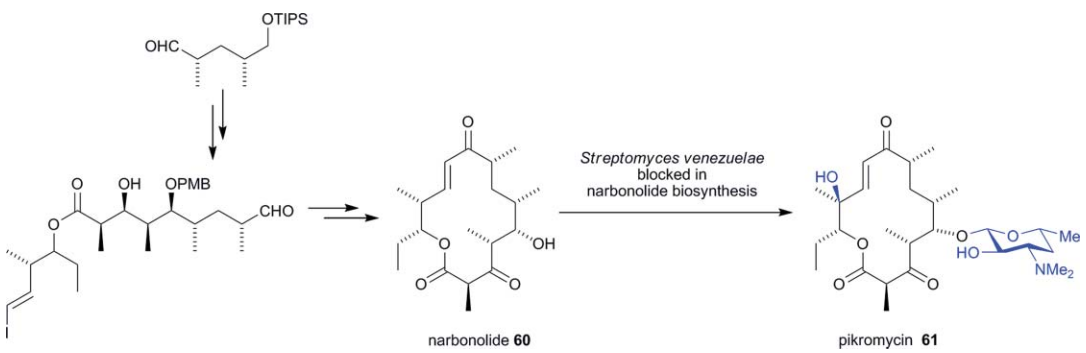


Scheme 13 Successful incorporations of advanced mutasynthons into 6-deoxy-erythronolide B derivatives.

the stereochemical assignment of biosynthetic intermediates by feeding different stereoisomeric ketides and monitoring their acceptance.[42,43] In this context, evidence has been accumulated that advanced ketides are often not efficiently loaded on the relevant polyketide synthase modules, so that addressing the postketide enzymes holds greater promise. In fact, as asymmetric synthesis has considerably matured over the past two decades, particularly when preparing polyketide-type backbones, several examples have recently appeared in the literature.

Fecik and co-workers carried out a *de novo* synthesis of the macrocyclic polyketide narbonolide **60** and fed it to a mutant strain of *Streptomyces venezuelae* incapable of synthesising the pikromycin core polyketide **60** due to elimination of the first PKS protein bearing the loading domain and elongation modules 1 and 2 (Scheme 14).[44] The substrate was further processed by the post-PKS tailoring enzymes (oxidation at C-12 and glycosylation at O-5) so that a formal total synthesis of pikromycin **61** was achieved.

The enantioselective total synthesis of proansamitocin **62**, a key biosynthetic intermediate of the antitumor agent ansamitocin P-3 **41**, was disclosed by Kirschning *et al.* (Scheme 15).[45] Feeding of proansamitocin to a mutant of *Actinosynnema pretiosum* with eliminated starter unit biosynthesis yielded ansamitocin P-3 **41** as well as dechloroansamitocin P-3 **46**. In tests with different cultured



Scheme 14 Mutasynthetic total synthesis of pikromycin **61** using narbonolide **60** as mutasynthon.

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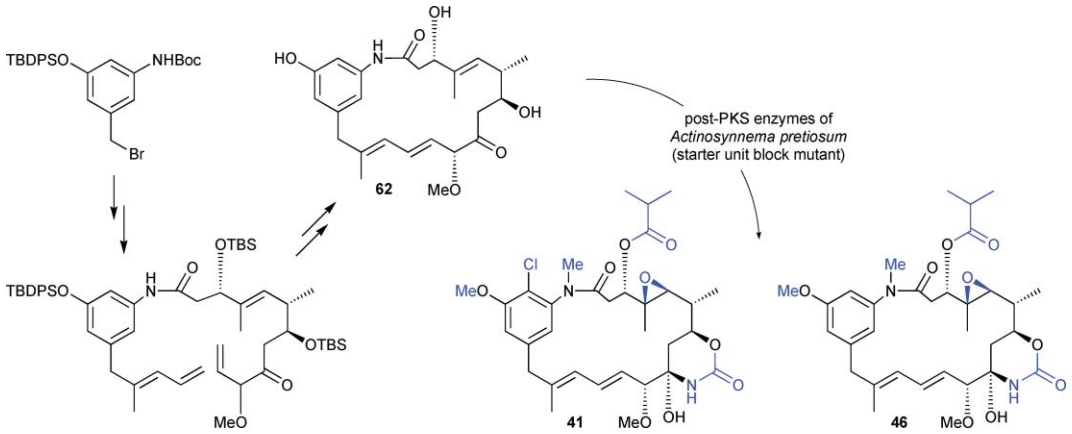


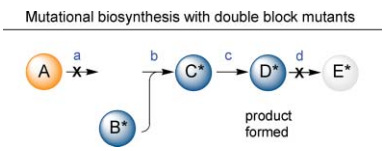
Scheme 15 Mutasynthetic total synthesis of ansamitocin P-3 **41** using proansamitocin **62** as mutasynthon.

human tumor cell lines, **46** showed strong antiproliferative activity with IC_{50} values down to 10 pg mL^{-1}.

The last two examples clearly reveal that very advanced biosynthetic intermediates are able to cross the cell membrane by means of unknown uptake mechanisms for further processing to the final product by post-PKS enzymes. These studies pave the way to process modified advanced intermediates, thus targeting other parts of secondary metabolites for structural diversification.

4. Advanced metabolic engineering

While the conventional mutasynthetic approach allows the introduction of modifications at specific points in the biosynthesis of natural compounds, its flexibility can be broadened by combining the primary genetic blockages with secondary inactivating mutations, for instance addressing the enzymes responsible for peripheral decoration of a core compound (Scheme 16).

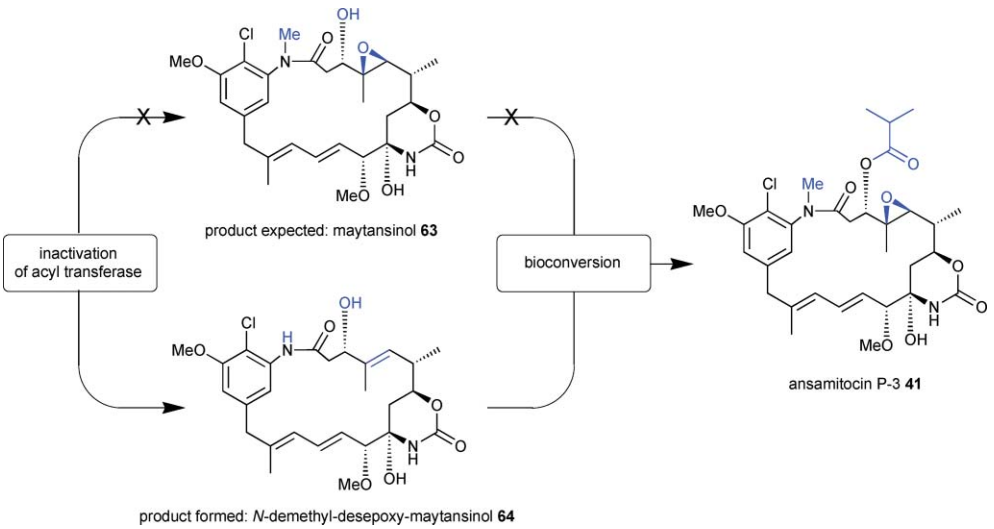


Scheme 16 Additional mutations add further variety and flexibility to the mutational biosynthesis concept.

An example of this strategy represents the mutasynthetic production of pre-rapamycin analogues employing a *S. hygroscopicus* strain both deficient in the production of the starter acid, as well as the post-PKS modifications of the macrolactone (see also Scheme 3).[46] In principle, the selective expression of late-stage enzymes can be employed for the engineered production of intermediates with desired functionalities suitable for further chemical modification and detailed SAR studies. However, intermediates partially lacking peripheral decorations are often accumulated in lower yields than their fully modified counterparts. Furthermore, omittance of a specific modification in the biosynthetic assembly logic might interfere with the substrate specificities of downstream core-decorating enzymes, thus not leading to the accumulation of the desired compound, but to partially modified intermediates. For instance, the inactivation of the acyltransferase in the ansamitocin pathway of *Actinosynnema pretiosum* did not lead to the expected maytansinol **63**, but to its *N*-demethyl-desepoxy analogue **64** (Scheme 17).[47]

The corresponding methyltransferase and epoxidase are situated downstream of the acyltransferase in the biosynthetic pathway grid and do not accept precursors bearing the free hydroxyl group. Likewise, the acyltransferase has been shown to utilise multiple pathway intermediates, but refused to exert its activity when presented with semisynthetically prepared maytansinol bearing *N*-methyl and epoxide functionalities. It is therefore not suitable as a catalyst for chemoenzymatic attachment of linker side-chains facilitating the formation of tumor-directed immunoconjugates such as DM1 (Immunogen).[48]

Though a given organism's biosynthetic potential offers the means and selectivity to access complex molecules, it also imposes restrictions on any attempts to generate novel compounds *via* PDB and mutasynthesis. One set of limitations originates from the highly complex nature of checks and balances making up an organism's inner mechanisms, while another is given by the regulation of pathway-associated enzymes, their substrate flexibility and given set of transformations. The fields of process engineering and metabolic engineering provide means to address these problems. For a more elaborate introduction into the latter field, the reader is directed to the review written by Burkart.[49] Optimisation of fermentation procedures and strain improvement routinely employed to provide for industrial strains can improve yields and growth characteristics. The relocation of a set of enzymes or entire pathways to heterologous hosts is another strategy to deal with these problems, while in addition, the well-known systems selected for this approach offer the advantages of sophisticated procedures for genetic manipulation and cultivation. The aspects of using heterologous hosts not only for the production of proteins but to access complex secondary metabolites have been reviewed with a special focus on polyketides and nonribosomal peptides by Pfeifer and Khosla[50] and recently by Watanabe and Oikawa.[51] While this may provide for high-level production, especially if closely related strains already optimised for production of related compounds are used,[52] the benefits of different host systems have to be balanced against their disadvantages. The reported biosynthesis of myxobacterial epothilone in *E. coli* certainly allows for the facile genetic manipulation of the pathway, but the reported yields for epothilone C and D have been disappointing with titers of less than 1 μg l^{-1} of fermentation culture[53] as

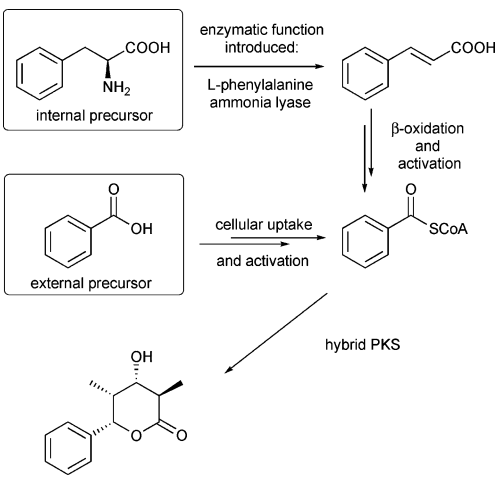


Scheme 17 Logic of acyl chain incorporation in ansamitocin biosynthesis.

compared to more complex *Streptomyces*-based systems generating up to a hundred times as much product.[54] In addition, the step-by-step reassembly of biosynthetic pathways in a foreign environment devoid of interfering background transformations can give rise to refined insights into the functions of gene products.[55]

Apart from changes in the absolute presence or absence of substrates and catalysts, biological systems are highly susceptible to changes in the relative levels of interacting components. The over-expression of a pathway-inherent glycosyltransferase during landomycin biosynthesis was described to lead to the novel landomycin J bearing a tetrasaccharide side-chain, as well as the previously unobserved monoglycosylated landomycin I (Fig. 3).[56] The latter product was speculated to have arisen by depletion of the shared substrate pool for the glycosyltransferase attaching the second sugar moiety by the over-expressed enzyme, which catalyzes the fourth glycosylation step.

Metabolic flux can be redirected by the introduction of new key biosynthesis steps drawing from existing precursor pools and leading to the internal production of unnatural substrates. The introduction of a foreign gene encoding L-phenylalanine ammonia lyase into a *Streptomyces erythrea* strain circumvented the need for substrate supplementation. It provided an engineered hybrid PKS with activated benzoic acid starter units (Scheme 18).[57]



Scheme 18 Engineering of a new pathway for internal precursor supply.

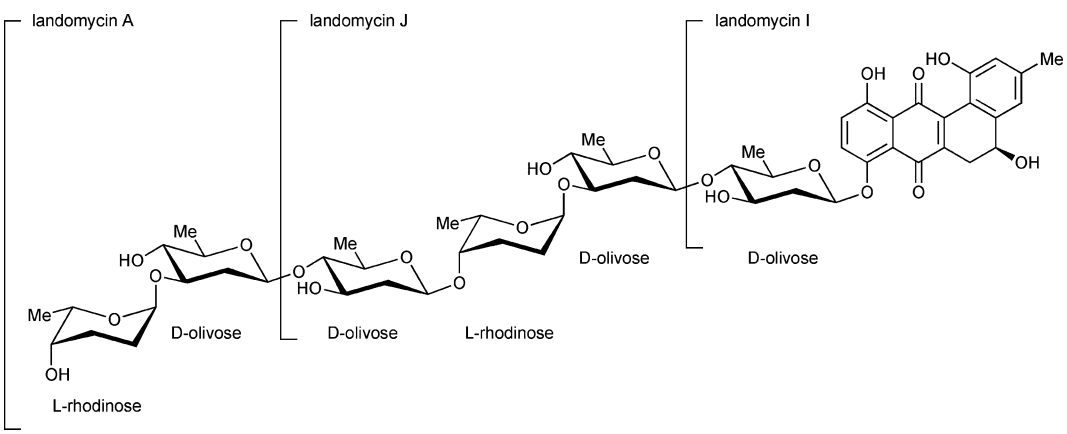


Fig. 3 Glycon-derivatives of landomycin.

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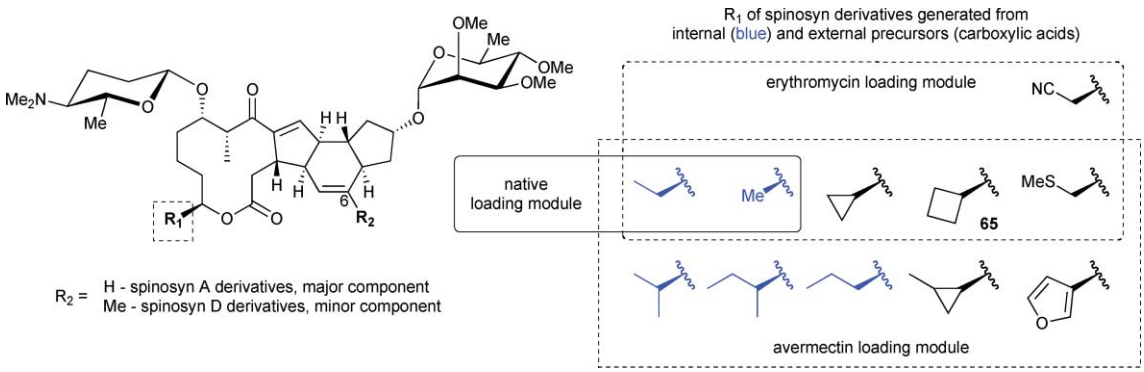

Scheme 19 Generation of novel spinosyns *via* loading module variation.

Depending on the host system used, a supplemented precursor might exhibit toxic effects in concentrations required to produce sensible amounts of metabolites. In systems requiring PKS-independent precursor activation (*e.g.* CoA-thioester formation), this may be due to the inefficient or absent processing mechanisms in the host system. The addition of exogenous substrates may even elicit detoxification mechanisms in versatile degraders such as streptomycetes, mainly resulting in decomposition but not integration of administered precursors. When benzoic acid was externally supplemented to the fermentation of *S. erythrea*, it was necessary to equip the organism with a benzoate:CoA ligase to provide the hybrid PKS's loading module with activated starter units.[57] This enabled the organism to channel the substrate into a detoxifying pathway leading to the production of the desired polyketide. When a different foreign benzoate:CoA ligase gene was used, polyketide production was tripled and no inhibition of strain growth could be observed, thus identifying starter unit activation as a major choke point in the system on hand.

In more general terms, the substrate specificity of loading modules usually represents the first barrier limiting the success for PDB and MBS approaches. This bottleneck can be widened by swapping the respective modules with their counterparts from other gene clusters known to display a selectivity spectrum fitting the studies' demands. Loading modules exhibiting selectivity for ubiquitous starter units such as CoA-thioesters of propionate and acetate result in biosynthetic arrangements only suitable for PDB,[58] while those utilizing unusual building blocks such as AHBA can give rise to systems for the application of mutasynthetic strategies.[59] Swapping loading modules is a popular engineering strategy and whilst often efficient in the qualitative generation of novel compounds, its preparative applicability is usually comparable to PDB and mutasynthesis strategies. Spinosyns represent a class of commercially important insecticides produced by *Saccharopolyspora spinosa*. The native loading module of the spinosyn PKS is of a usually rather restrictive type and recruits methylmalonyl-CoA and to a lesser extent malonyl-CoA *via* decarboxylation. In order to introduce different starter units, the loading module was replaced with different versions originating from the erythromycin and avermectin PKS.[58] These loading modules show flexibility towards a range of different coenzyme A-activated carboxylic acids and can be employed in engineered PDB systems. A variety of carboxylic acids was fed to engineered producer strains, relying in good faith on their internal activation prior to processing, and novel spinosyns originating from the respective loading module's selection of internal (*e.g.* propionyl-CoA) and external precursors could be generated (Scheme 19). Even though the engineered systems yielded only about 2–8% of the native spinosyn PKS (6–25 mg L^{-1} instead of 300 mg L^{-1}), some of the novel products could be isolated and characterised. Semisynthetic modification of the novel cyclobutyl-variant **65** of spinosyn A *via* hydrogenation led to its 5,6-dihydro-analogue displaying increased activity against several insect pests in comparison to the natural spinosyn A.

Similar to the advantages of mutasynthesis in comparison to PDB, the incorporation of advanced precursors can be improved by blocking biosynthetic steps upstream of a targeted entry point. This has been demonstrated for the model type I PKS producing the erythromycin aglycone 6-deoxyerythronolide B (Scheme 13, *vide supra*).

Finally, the previously mentioned concept of swapping loading modules between pathways can in principle be expanded to all enzymatic entities forming a pathway, resulting in the concept of combinatorial biosynthesis. The inherent potentials and problems have been reviewed by Floss[60] and recent developments outlined by Baltz[61] emphasizing the complementarity of the fields of molecular engineering and medicinal chemistry. The success of combinatorial biosynthesis is currently best ensured when closely related catalytic entities are swapped,[62] while their rearrangement to rationally design biosynthetic pathways still suffers from the inadequate understanding of their functional mechanisms and interaction.

5. Conclusions

Besides the well-established approaches of semi- and total synthesis, mutational biosynthesis will emerge as a third major method available for the generation of natural product libraries. This strategy combines advanced methods of chemical synthesis with those of molecular biology and microbiology required for modification of biosynthetic cascades and handling of their hosts. In fact, mutational biosynthesis often complements semisynthesis[63] as structural diversification can be achieved in positions that often cannot be addressed by semisynthesis. With respect to total synthetic approaches towards derivatives of complex natural products, mutasynthesis can be regarded as a short cut total synthesis strategy since linear synthetic sequences are included into the synthesis conducted by the microbial producer.

The applicability of enzyme catalysts for a transformation of choice is limited by their inherent substrate flexibility and by the

reactions performed. The optimisation of substrate specificities, which should include evolutionary techniques and the predictable construction of artificial biosynthesis cascades by sophisticated metabolic engineering, will certainly expand the scope of mutasynthesis in the near future. But it has to be pointed out that the intricacy and the principles of regulation of the biosynthetic machineries are not fully understood yet and many mutasynthetic approaches are far from being practical in terms of productivity. In fact, several publications lack data on the structural and biological characterisation of new mutaproducts. Structural proof is still often based on LC-MS data and insufficient amounts of material were generated to carry out SAR's. It will definitely prove beneficial for this field of natural product synthesis if more attention would be paid to this aspect in the future.

Acknowledgements

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63 Many of the secondary metabolites described in this report are of pharmaceutical importance so that derivatives obtained by semisynthesis can be searched and found in the patent literature.