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MINI-REVIEW

S. Weist · R. D. Süssmuth

Mutational biosynthesis—a tool for the generation of structural diversity in the biosynthesis of antibiotics

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Abstract Natural products represent an important source of drugs in a number of therapeutic fields, e.g. antiinfectives and cancer therapy. Natural products are considered as biologically validated lead structures, and evolution of compounds with novel or enhanced biological properties is expected from the generation of structural diversity in natural product libraries. However, natural products are often structurally complex, thus precluding reasonable synthetic access for further structure-activity relationship studies. As a consequence, natural product research involves semisynthetic or biotechnological approaches. Among the latter are mutasynthesis (also known as mutational biosynthesis) and precursor-directed biosynthesis, which are based on the cellular uptake and incorporation into complex antibiotics of relatively simple biosynthetic building blocks. This appealing idea, which has been applied almost exclusively to bacteria and fungi as producing organisms, elegantly circumvents labourious total chemical synthesis approaches and exploits the biosynthetic machinery of the microorganism. The recent revitalization of mutasynthesis is based on advancements in both chemical syntheses and molecular biology, which have provided a broader available substrate range combined with the generation of directed biosynthesis mutants. As an important tool in supporting combinatorial biosynthesis, mutasynthesis will further impact the future development of novel secondary metabolite structures.

S. Weist · R. D. Süssmuth (✉)
Biologische Chemie/Institut für Chemie,
Technische Universität Berlin,
Strasse des 17. Juni 135,
10623 Berlin, Germany
e-mail: suessmuth@chem.tu-berlin.de
Fax: +49-30-31424205

Present address:
S. Weist
Department of Chemistry,
Massachusetts Institute of Technology,
77 Massachusetts Ave,
Cambridge, MA, 02139, USA

Introduction

The industrial drug finding process is based on two principles: screening for new lead structures on the one hand, and lead optimisation on the other. Sources of lead structures are either synthetic compound libraries or natural compound libraries. Although continuously under debate, natural products remain an important source for the development of new lead structures. Subsequent lead optimisation can be conducted by reduction of structural complexity followed, or accompanied by, chemical syntheses. However, structural simplifications of natural products often result in a decrease or loss of biological activity.

Compared to chemically synthesised compounds, an intrinsic advantage of many natural products is their enormous structural complexity combined with biological activity. Semi-synthetic approaches, especially in the development of new anti-infective agents, often make use of natural product core structures, which are modified in structure-activity relationship studies. Because of the chemical instability and/or structural complexity of natural products, particular functional groups and molecular entities often remain unaffected by such semi-synthetic modifications. Alternatively, manipulation of biosynthesis of the natural product can lead to novel structural diversity.

Precursor-directed biosynthesis and mutasynthesis

In order to generate novel structural diversity via biosynthetic manipulation, early research approaches involved "precursor-directed biosynthesis" (PDB) and "mutational biosynthesis/mutasynthesis" (MBS). Unfortunately, a misleading use of the terms PDB and MBS exists in the literature, which often considers both methods as PDB. More exactly, "precursor-directed biosynthesis" is the supplementation of growth media of wild-type secondary metabolite producers, e.g. bacteria or fungi, with compounds that are used competitively as building blocks for biosynthetic assembly (Fig. 1). In contrast, "mutational biosynthesis/mutasynthesis" is based on the feeding of compounds

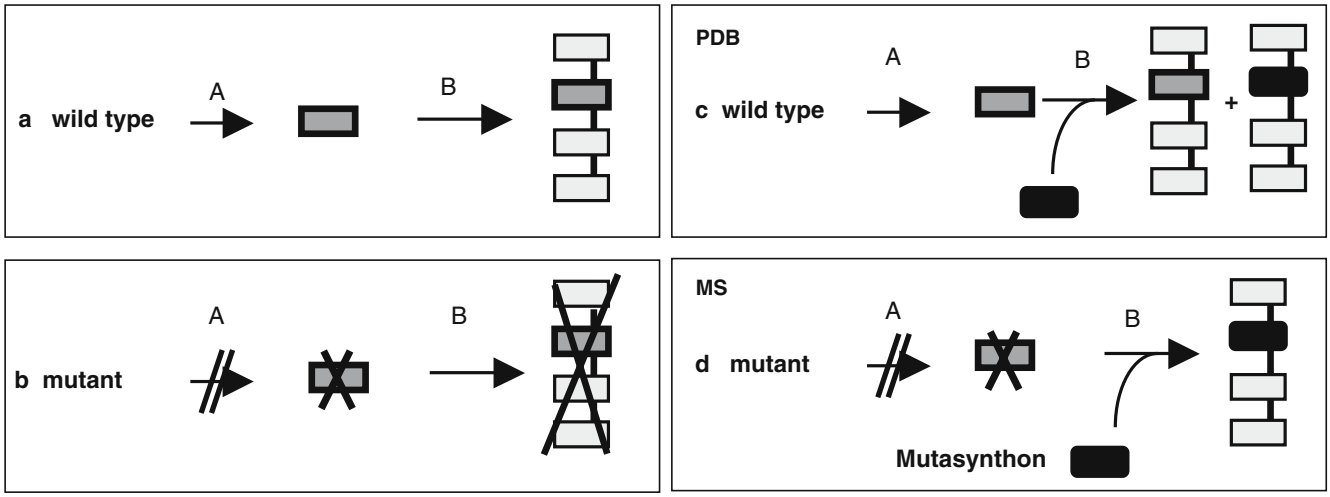

Fig. 1a–d Schematic illustration of mutational biosynthesis (MBS) and precursor-directed biosynthesis (PDB). **a** Biosynthetic pathway of wild-type metabolite. **b** Inactivated biosynthesis of intermediate *A*; the lack of *A* cannot be compensated, thus the final metabolite is not produced. **c** PDB: Integration of the supplemented building block competes with biosynthetic processing of the natural substrate, resulting in two metabolites. **d** MBS: Bypass of the biosynthetic block via supplementation of analogous mutasynthons: production of target metabolite with altered structure

("mutasynthons") to antibiotic biosynthesis mutants inactivated in an important step of antibiotic assembly. Due to structural similarities to the natural building blocks, the biosynthetic block can be bypassed in mutasynthesis experiments and an analog of the wild-type antibiotic is obtained (Fig. 1). MBS can address the processing of alternative substrates and intermediates that cannot be achieved in the same way by PDB. In both PDB and MBS, building blocks are commonly derived from biosynthetic building blocks, e.g. amino acids, carbohydrates or benzoic acids. The definition of mutasynthesis also includes the use of natural substrates, or isotopically labelled derivatives thereof, as mutasynthons. This makes mutasynthesis a useful tool also in biosynthesis investigations, which however is not the subject of this review.

Mutasynthesis, a term coined by Rinehart (1977), is a means of obtaining new structural diversity that was originally proposed as an alternative method to PDB (Birch 1963). According to Rinehart, the mutasynthesis approach consists of five central steps: (1) generation of the biosynthetic block mutant; (2) generation of the mutasynthon; (3) integration of the mutasynthon; (4) isolation of the target compound (metabolite); (5) evaluation of biological activity.

An increasing number of examples of novel mutasynthesis have been published recently. Reasons for this renaissance are the availability of a broad substrate range from chemical libraries, and the advanced repertoire of synthetic chemists. Furthermore, characterization of biosynthetic enzymes combined with sequencing of biosynthetic gene clusters from secondary metabolites has led to a deeper understanding of antibiotic biosyntheses. Modern methods of molecular biology allow the generation of directed biosynthetic mutants, which had previously relied on the screening of vast numbers of randomly generated mutants.

In this article, we focus on recent advances in mutasynthesis, which has been reviewed earlier (Daum and Lemke 1979). An excellent review on PDB has been published more recently (Thiericke and Rohr 1993). There is a considerable number of examples of MBS from diverse antibiotic classes, comprising aminoglycosides, macrolides, peptide antibiotics, and derivatives with mixed biosynthesis pathways, e.g. nikkomycins and novobiocins. Recent examples are discussed in the following sections.

Aminoglycoside/aminocyclitol antibiotics

The mutasynthesis principle was first demonstrated at the example of aminoglycoside antibiotics (Shier et al. 1969). In the first step, biosynthetic mutants of the neomycin producer *Streptomyces fradiae* were generated by mutagenesis in the presence of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), which is known to randomly generate point mutations in chromosomal DNA. After screening for a suitable mutant, related aminocyclitols streptamine and 2-epistreptamine (both available by chemical synthesis) were incorporated to yield novel hybrimycins. In a similar experiment, *S. fradiae* mutants were supplemented with 30 streptamine mutasynthons, which resulted in three novel neomycin analogs (Fig. 2) (Rinehart 1977). Other mutasynthesis examples of aminoglycoside antibiotics include studies with butirosins (Takeda et al. 1978) and sagamicins (Kitamura et al. 1982).

Siderophores (pyochelin)

Siderophores are low molecular weight iron chelators produced in response to iron deprivation by microorganisms, in which they mediate high affinity iron uptake mechanisms.

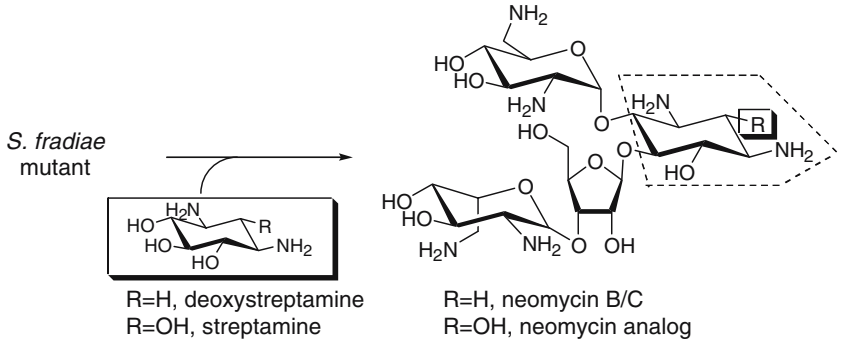

Fig. 2 Mutasynthesis of neomycin antibiotics (Rinehart 1977) with a *Streptomyces fradiae* mutant supplemented with streptamine mutasynthons

Pyochelin (Fig. 3), a phenolic siderophore, is produced by Gram-negative *Pseudomonas aeruginosa* and other *Pseudomonas* species. The group of Cox and Rinehart (Ankenbauer et al. 1991) employed MBS to obtain novel siderophores of the pyochelin-type by supplementation of a salicylic acid biosynthesis mutant of *P. aeruginosa* with salicylic acid analogs. From 13 derivatives fed, three salicylic acid analogs were accepted as substrates (Fig. 3). All three pyochelin derivatives showed iron transport properties, whereas only methylpyochelin showed increased transport activity compared to pyochelin. The acceptance of 3-fluorosalicylate in contrast to 3-chlorosalicylate by the mutant indicated the steric restraints conferred by the biosynthetic enzymes. Electronic factors were thought to be responsible for the rejection of e.g. the 2-hydroxy-nicotinic acid mutasynthon.

Nucleoside antibiotics (nikkomycin)

Nikkomycins (Fig. 4) are nucleoside-peptide antibiotics produced by *Streptomyces tendae* Tü901. They are potent inhibitors of chitin synthetase, and display antifungal, insecticidal, and acaricidal activity. Uracil and 4-formyl-4-imidazoline-2-one are variable bases of natural nikkomycin. A *pyr*-mutant of *S. tendae*, deficient in uracil metabolism, was fed with 23 *N*-heterocyclic compounds including pyrimidines, purines and imidazoles (Delzer et al. 1984). Three pyrimidines—thymine, 4-hydroxymethyluracil and 5-formyluracil—gave novel nikkomycins with antibiotic activity similar to nikkomycins *X* and *Z* produced by the wild-type.

A more recent approach generated a directed mutant in lysine-2-aminotransferase, which is crucial for the first step in the piperidine-2-carboxylate pathway necessary for peptide side-chain biosynthesis of nikkomycin. Resting cell cultures of this mutant were fed with benzoic acid derivatives (Bormann et al. 1999). Cultures supplemented with 3-hydroxypicolinic acid, 3-hydroxmethylpyridine, 6-hydroxynicotinic acid and 4-hydroxybenzoic acid gave antibiotically active metabolites, however in very low yields (<3 mg/l). Only with benzoic acid supplementation, nikkomycins B_x and B_z could be isolated (Fig. 4). From the very limited acceptance of 4-hydroxybenzoic acid it was deduced that introduction of the 4-hydroxy group occurs at a late biosynthetic step. However, no antibiotic testing of the mutasynthesis products was reported in this case.

Novobiocin and other aminocoumarin derivatives

The family of aminocoumarin antibiotics exhibits inhibitory activity towards DNA gyrase. Of the three aminocoumarins produced by *Streptomyces* species—novobiocin, clorobiocin (Fig. 5) and coumermycin—novobiocin is the best-known and most important. However, aminocoumarins possess considerable toxicity towards eukaryotic cells, so that less toxic derivatives of this class of antibiotics are being sought for pharmaceutical applications. Since the biosynthetic gene clusters of all three compounds are known, and the directed generation of gene inactivation mutants had been established, mutasynthesis experiments could be performed. This was achieved by employing a mutant deficient in the biosynthesis of the 3-dimethylallyl-4-hydroxybenzoyl moiety (DMAHB, ring A), which is coupled via an amide synthetase to the aminocoumarine ring (ring B, Galm et al. 2004a). In a biochemical study, the amide transferases of all three coumarin antibiotics (CloL, NovL and CouL) were heterologously expressed in *Escherichia coli* and screened for promiscuity in substrate conversion. Among these three enzymes the amide synthetase CloL was found to be most promising and therefore subsequent mutasynthesis experiments were preferentially performed with the prenyltransferase-deficient *cloQ*-mutant unable to synthe-

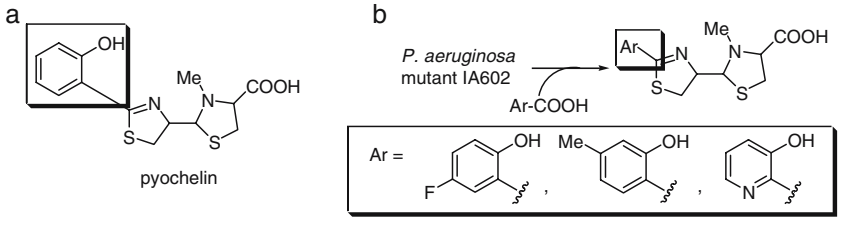

Fig. 3a,b Mutasynthesis of siderophore analogs. **a** Structural formula of pyochelin. **b** MBS of 5-fluoropyochelin, 4-methylpyochelin and 6-azapyochelin (Ankenbauer et al. 1991)

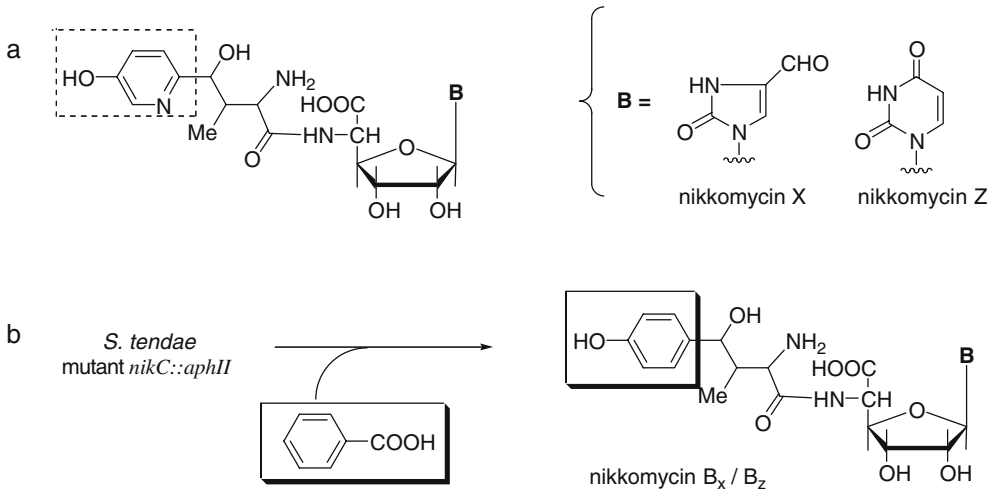

Fig. 4a,b Mutasynthesis of nikkomycins B_x and B_z (Bormann et al. 1999). **a** Nikkomycins *X* and *Z* with the substituents uracil and 4-formyl-4-imidazoline-2-one. **b** Supplementation of the *nikC* gene inactivation mutant with benzoic acid to yield novel nikkomycins B_x and B_z

size the DMAHB precursor. From 13 benzoic acids fed at a relatively low concentration of 20 mg/l, nine aminocoumarines (1.7 mg/derivative) with variations in the A-ring were isolated and characterized (Fig. 5). However, subsequent antibiotic testing of the 28 so-called novclobiocins ranged from inactive derivatives to derivatives half as active as clorobiocin (2-fold activity compared to novobiocin, Galm et al. 2004b).

Polyketide antibiotics—analogs of avermectin, erythromycin, rapamycin and enterocin

Polyketide antibiotics represent one of the major classes of antibiotics. Biosynthetic gene clusters of a considerable number of important PKS-type antibiotics have been sequenced, and their biosyntheses investigated. Thus, a variety of mutasynthesis examples exists especially for this compound class, which have even been combined with combinatorial biosynthesis approaches. Here, we present selected examples that illustrate advancements in this field.

Avermectins (Fig. 6) are important antiparasitic macrolides produced by *Streptomyces avermitilis*. As early as 1988, MBS was employed for the generation and production of novel avermectins (Hafner et al. 1988) by supplementing cultures of an avermectin null-mutant inactivated in branched chain 2-oxo acid dehydrogenase. This mutant type was used to generate 36 novel avermectin derivatives from 800 mutasynthons (Dutton et al. 1991). Remarkably, the avermectin biosynthesis machinery showed an unusually broad substrate tolerance at an early stage of PKS biosynthesis, not only for compounds related to the biosynthetic precursor (*S*)-2-methylbutyric acid and isobutyric acid, but also towards unsaturated rings and heterocyclic compounds

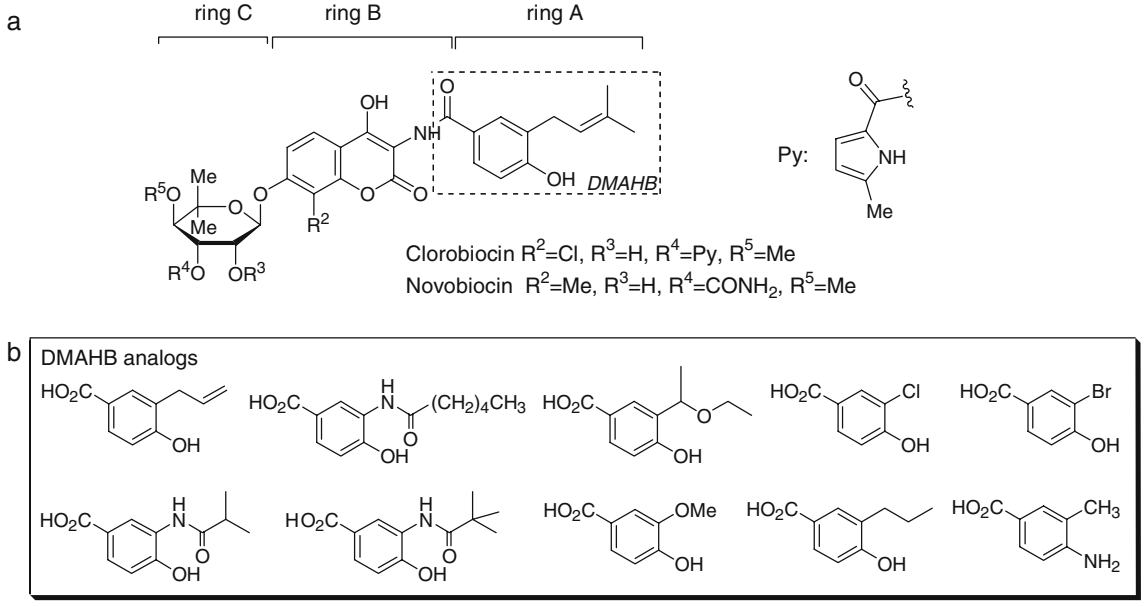

Fig. 5 **a** Structural formulae of clorobiocin and novobiocin. **b** 3-Substituted 4-hydroxybenzoic acid mutasynthons successfully employed for the supplementation of a novobiocin *cloQ*-inactivation mutant (Galm et al. 2004a,b)

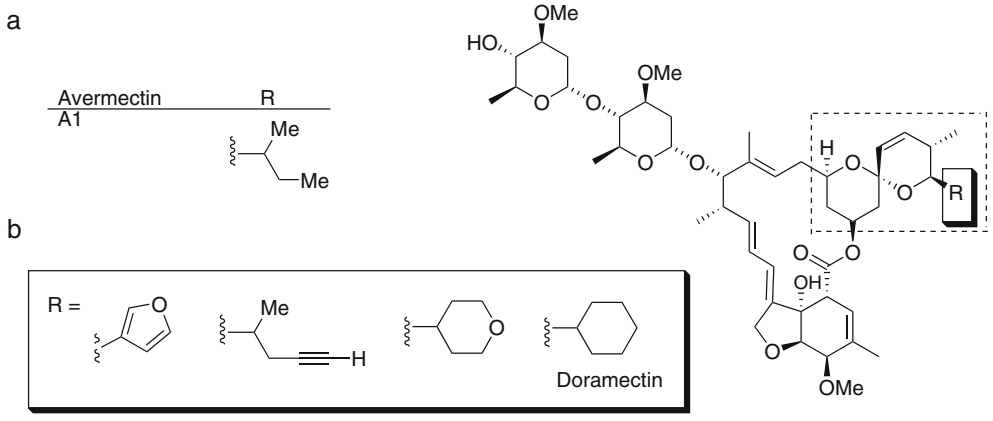

Fig. 6 **a** Structural formula of avermectin A1. **b** Mutasynthesis examples with avermectins; 17 different subclasses and a total of 36 novel compounds were obtained (Dutton et al. 1991)

(Fig. 6). Although no quantitative data were given by Dutton et al. (1991), all avermectins showed high killing rates against *Caenorhabditis elegans* and blowfly (*Lucilia cuprina*) larvae in bioassays for anti-parasitic activity.

These contributions formed the basis of mutasynthetic production of the commercial antiparasitic agent doramectin from a *S. avermitilis* mutant in the presence of the mutasynthon cyclohexanecarboxylic acid (CHC) (Hafner et al. 1988; McArthur 1998). To our knowledge, doramectin is the only drug on the market produced by MBS to date. However, critical points for industrial production of drugs by mutasynthesis are (1) inefficient transport into the cell, (2) toxicity of the mutasynthon to the producing strain, and (3) the relatively high costs of mutasynthon synthesis. Thus, in a recent approach, biosynthetic genes for CHC from *Streptomyces collinus* were heterologously expressed in the *S. avermitilis* mutant, which paved the way for mutasynthon-independent industrial production of doramectin by means of a biotechnologically engineered strain (Cropp et al. 2000).

The importance of erythromycin and its analogs as antibiotics in clinical use has resulted in extensive biosynthesis studies and attempts to engineer the erythromycin producer strain *Saccharopolyspora erythrea*. Early studies on the glycosylation of fluorinated erythromycins were related more to bioconversion than to MBS (Toscano et al. 1983). The biosynthesis of 6-deoxyerythronolide (6-dEB), a biosynthetic precursor of erythromycin, is performed by the polyketide synthases DEBS1 (loading domain+module 1/2), DEBS2 (module 3/4) and DEBS3 (module5/6+Te-domain). Biochemical studies revealed an extended substrate tolerance of DEBS1 for primer units such as acetyl and butyryl coenzyme A (CoA, Pieper et al. 1995a) or *N*-acetylcysteamine (SNAc) thioesters (Pieper et al. 1995b) of their corresponding diketides. These results initiated the in vivo testing of SNAc thioesters by MBS to generate novel erythromycin analogs (Jacobsen et al. 1997). For this purpose, a ketosynthase mutant (DEBS KS1°) in module 1 was generated, bearing a plasmid (pJRJ2) carrying deoxyerythronolide B synthase genes (DEBS 1–3). The plasmid was transformed into the heterologous host *Streptomyces coelicolor* CH999. Biosynthetic activity of this mutant, incapable of synthesising 6-dEB derivatives, was restored upon feeding of synthetic SNAc-thioesters (Fig. 7). The acceptance of an aromatic substituent indicated considerable substrate tolerance toward bulky residues of all subsequent DEBS polyketide synthases (modules 2–6). By means of bioconversion, the isolated aglyca from the *S. coelicolor* mutant were further processed to give C6-oxidised and glycosylated erythromycin D derivatives when submitted to the erythromycin producer *Saccharopolyspora erythrea* (Fig. 7). Subsequent investigations of that system involved modifications at the 12-position (Jacobsen et al. 1998) or 13-position (Kinoshita et al. 2003) of the 6-dEB backbone. The latter contribution also highlights more detailed investigations of the stereochemistry, and of triketide

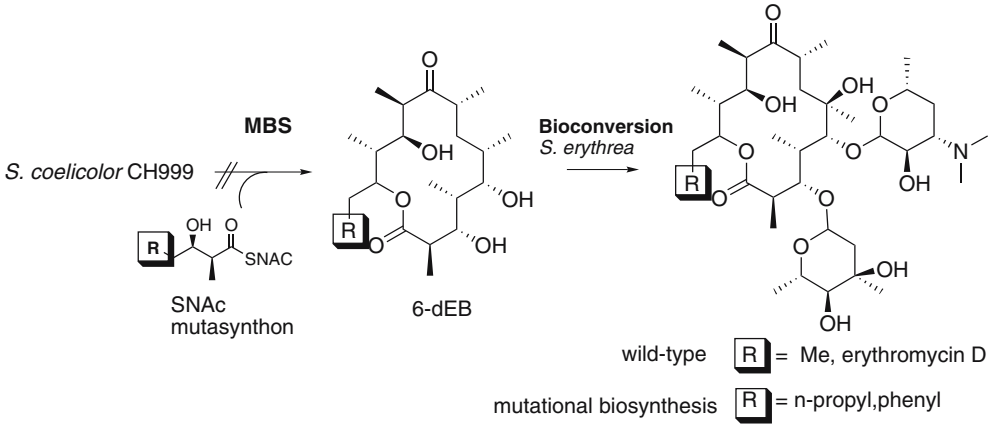

Fig. 7 Examples of MBS of 6-dEB, erythromycin D (R=Me) and analogs (Jacobsen et al. 1997)

lactone formation with *Streptomyces coelicolor* CH999/pCK16 harboring DEBS1+Te(KS1°), as well as kinetics with overexpressed DEBS+Te. The formation of unusual 16-membered lactones by MBS was explored with three additional triketide thioesters (Kinoshita et al. 2001). Other important in vitro studies deal with the biochemical analysis of DEBS1+Te-systems in order to investigate the selectivity of erythromycin PKS (Weissman et al. 1998; Cane et al. 2002).

The stability of SNAc precursors during mutational biosynthesis as well as the timepoint of feeding are important factors influencing the success and yields of MBS studies (Leaf et al. 2000). This topic was investigated with the above-mentioned *Streptomyces coelicolor* CH999/pJRJ2, which was fed with three different triketide-SNAc derivatives as mutasynthons. It was found that 6-dEB production increased with mutasynthon concentration (1–4 mM). The feeding of mutasynthons at an early stage of the culture (<2 days) resulted in higher 6-dEB yields. Optimal feeding concentrations depended on the diketide used. This was explained by a combination of factors such as solubility, cellular uptake, product stability, and DEBS specificity. The investigation of the fate of diketide thioester mutasynthon showed that 11% were incorporated into the 6-dEB analog, 34% remained in the medium and >50% were degraded. Interestingly, the feeding of two enantiomers did not decrease or inhibit 6-dEB production. The analogous *Saccharopolyspora erythrea* KS1° mutant was also evaluated for direct production of glycosylated erythromycins by MBS (Frykman et al. 2001). However, quantitative data on mutasynthon incorporation and production rates were lower compared to the heterologous host *Streptomyces coelicolor*. Furthermore, higher titres of 13-substituted erythromycins were obtained by MBS with *Saccharopolyspora erythrea* (40 mg/l versus 0.7 mg/l) compared to PDB with the wild-type strain.

The broad substrate tolerance of the avermectin starter unit elucidated by MBS led to experiments aimed at producing an engineered avermectin-erythromycin hybrid polyketide synthetase (Marsden et al. 1998). The PKS-loading module *avr*-IM of avermectin producer *Streptomyces avermitilis* was fused with the *eryAI* (DEBS1) of the erythromycin biosynthesis gene cluster and expressed in *Saccharopolyspora erythreae*. This engineered strain produced novel erythromycin analogs with variations in the starter unit; however, this occurred in an MBS-independent manner from substrates produced by the strain itself.

Because of its immunosuppressant properties and structural similarities to FK506, rapamycin is an antibiotic of growing interest. In a contribution on MBS for the generation of novel rapamycins, a *rapL* mutant inactivated in lysine cyclodesaminase (RapL) was used (Khaw et al. 1998). RapL is responsible for the synthesis of the pipecolic acid building block incorporated prior to the final ring closure of the macrolide. Upon feeding of hydroxyproline (15 l fermentor), three prolyl-rapamycins were isolated in low yields (3–15 mg). Interestingly, with regard to steric requirements, the five-ring proline was accepted in place of the six-ring structure of the natural pipecolic acid building block. In another contribution, inhibitors of RapL were fed (Graziani et al. 2003). Concomitantly with these inhibitors, thiazane carboxylic acids were supplemented to the culture medium, and two novel sulfur-containing rapamycin analogs were obtained, which were however less active than rapamycin. The authors designated these experiments as PDB. Indeed, with the addition of an enzyme inhibitor to the wild-type, only a "transient mutation" is generated, which reverts in the absence of inhibitor.

Kalaitzis et al. (2003) reported the generation by MBS of wailupemycin and enterocin analogs from *Streptomyces maritimus* (Fig. 8), which is the first example of the use of MBS for type-II PKS. From previous experiments with *Streptomyces hygroscopicus*, the producer of the structurally related vulgamycin, it was known that fluorinated benzoic acids were accepted as biosynthetic precursor analogs in PDB (Kawashima et al. 1985). The application of PDB to *S. maritimus* however, showed that other benzoic acid analogs were not accepted as substrates. In order to enforce the acceptance of non-natural substrates, *encP*—responsible for cinnamate biosynthesis as a precursor for the starter unit of type-II PKS—was inactivated by an in-frame deletion. Upon supplementation with five mutasynthons, the corresponding wailupemycin and enterocin analogs were obtained and characterized by HPLC-MS. Parallel studies on substrate specificity of overexpressed

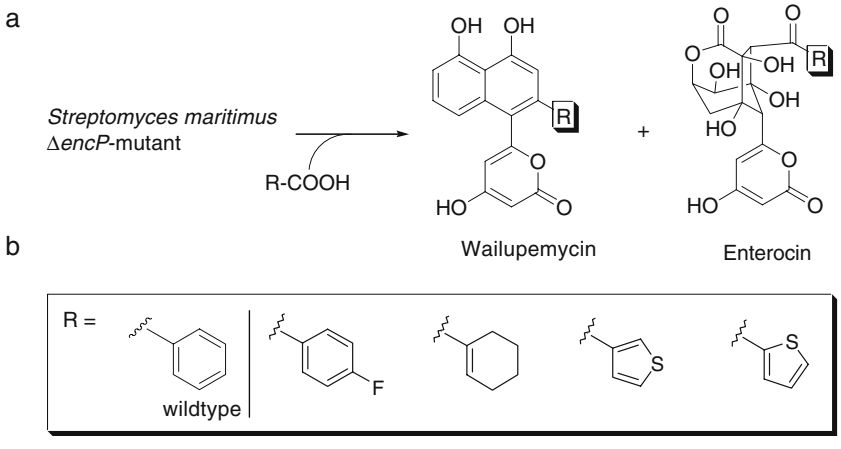

Fig. 8 **a** Mutasynthesis of wailupemycin and enterocin analogs (Kalaitzis et al. 2003). **b** Residues of mutasynthons incorporated into wailupemycin and enterocin structures

CoA ligase EncN were in agreement with the results from MBS.

Peptide antibiotics

Compared to polyketide antibiotics, MBS with peptide antibiotics is still widely unexplored. Recent examples of successful MBS experiments with peptide antibiotics have been independently performed for calcium-dependent antibiotic (CDA; Hojati et al. 2002) and vancomycin-type glycopeptide antibiotics (Weist et al. 2002, 2004). In both examples, aromatic amino acids were the preferred substrates.

Calcium-dependent antibiotic

In the CDA producer *Streptomyces coelicolor*, a gene inactivation mutant was generated in the biosynthesis operon of the non-proteinogenic amino acid 4-hydroxyphenylglycine (Hpg). This mutant, which was unable to produce CDA, was supplemented with mandelic acids, glyoxylic acids and phenylglycines bearing H-, F-, Cl- and OMe substituents in the 4-position of the aromatic side chain (Fig. 9) (Hojati et al. 2002). Interestingly, the fluorine substituent could replace the hydroxy group with 4-fluorophenylglycine, 4-fluorophenylpyruvate, and 4-fluoromandelic acid as substrates to yield CDA2fb. Phenylglycine was also accepted as a substrate and led to the generation of the CDA analog CDA2d lacking the hydroxy group in the four-position. Bulky chlorine or methoxy substituents did not yield CDA analogs.

Vancomycin-type glycopeptide balhimycin synthesised by non-ribosomal peptide synthetases

Investigation into the biosynthesis of the vancomycin-type glycopeptide antibiotic balhimycin (*Amycolatopsis balhimycina*) (Süssmuth and Wohlleben 2004) gave access to a number of biosynthesis mutants that were used for mutasynthesis experiments with aromatic amino acids. Understanding glycopeptide biosynthesis was of particular importance in obtaining information on the nature of the amino acid building blocks. Essential building blocks of all glycopeptide antibiotics are the non-proteogenic aromatic amino acids β-hydroxytyrosine (Hty), 3,5-dihydroxyphenylglycine (Dpg), and 4-hydroxyphenylglycine (Hpg).

The first series of mutasynthesis experiments was performed with an in-frame deletion mutant of the *bhp* perhydrolase gene (Puk et al. 2002) inactivated in Hty biosynthesis. Glycopeptide production of this null-mutant was restored upon feeding of Hty. The supplementation of chemically synthesised racemic 3-fluoro-β-hydroxytyrosine restored antibiotic activity of the culture filtrate (Fig. 10a) (Weist et al. 2002). Fluorobalhimycin was isolated from large-scale fermentation and characterised by means of FTICR-MS and 2D-NMR. Other mutasynthetically generated fluorobalhimycines were obtained by feeding 2-fluoro- or 3,5-difluoro-β-hydroxytyrosine, respectively (Fig. 10b). All novel fluorbalhimycines showed antibiotic activity against the indicator strain *Bacillus subtilis*. D/L-Tyrosine, or other phenylserines lacking the 4-OH group, were not accepted as substrates. Unfortunately, the productivity of the mutant was too low to obtain sufficient amounts of glycopeptides for MIC-tests.

In a second set of experiments MBS was performed with Dpg-analogs (Weist et al. 2004). Dpg is the C-terminal amino acid of type I–IV glycopeptide antibiotics. The *dpgA*-deletion mutant is inactivated in the first step of Dpg biosynthesis from four acetate units (Pfeifer et al. 2001). A set of racemic Dpg analogs was synthesised and tested in MBS assays. Antibiotic activity was restored after feeding the *dpgA* mutant. Besides the natural substrate Dpg, four variations of mono- and di-substituted hydroxy- and methoxy-substituents were accepted as substrates and assembled into the glycopeptide (Fig. 11). Glycopeptides were analysed by HPLC-ESI-MS and high-resolving ESI-FTICR-MS. Supplementation experiments generated more than 20 metabolites with different glycosylation patterns. Interestingly, the feeding of 3-monosubstituted phenylglycines resulted in derivatives that were partially lacking the AB-ring in the aromatic side chains of ^{7}Dpg and ^{5}Hpg (Weist et al. 2004). It was concluded that replacement of the hydroxy group with the bulkier methoxy-substituents may suffice for acceptance as a substrate, and even for AB-ring formation.

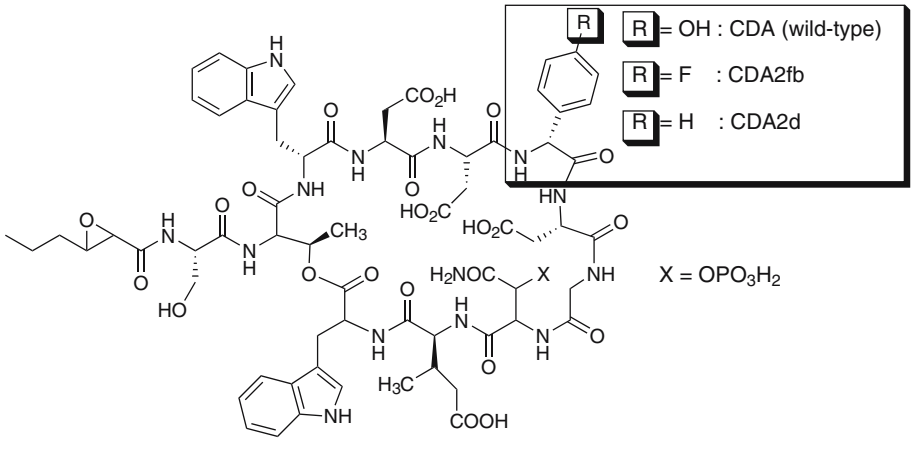

Fig. 9 Mutasynthesis of calcium-dependent antibiotic (CDA) analogs with phenylglycines (Hojati et al. 2002)

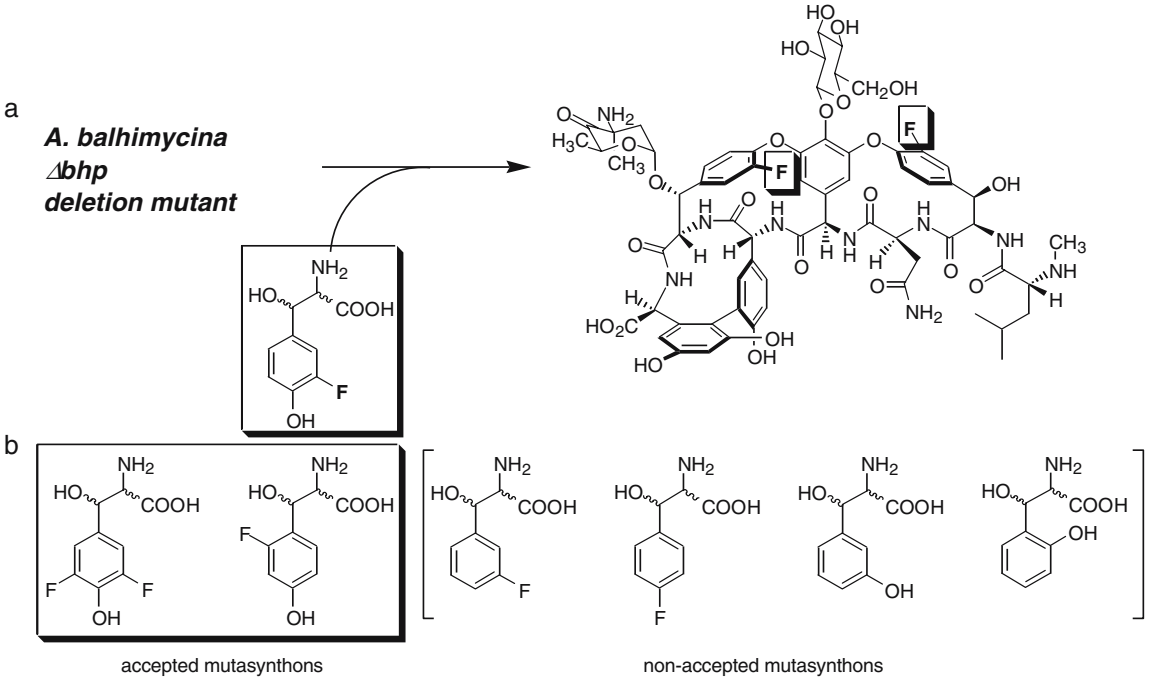

Fig. 10 Mutasynthesis of fluorinated glycopeptide antibiotics with an *Amycolatopsis balhimycina* mutant deficient in β-hydroxytyrosine (Hty) biosynthesis (Weist et al. 2002). **a)** 3-fluoro-β-hydroxytyrosine **b)** other fluorinated Hty analogs accepted as substrates in mutasynthesis are *boxed*. Phenylserine analogs in *brackets* were not accepted as mutasynthons

However, in AB-ring formation two positions for mono-OH/OMe-substituted derivatives obviously exist. Although not finally proven, it has to be assumed that the correct placement of the OH/OMe-group of mono-substituted phenylglycines activates biaryl-formation of the AB-ring by P450-dependent oxygenase OxyC, at the same time guaranteeing the atropisomery necessary for antibiotic activity. The subsequent feeding of mandelic acids and phenylacetic acids (Pfeifer et al. 2001), which are postulated precursors of Dpg biosynthesis, showed a strong restriction of substrate tolerance towards the natural 3,5-dihydroxy substrates.

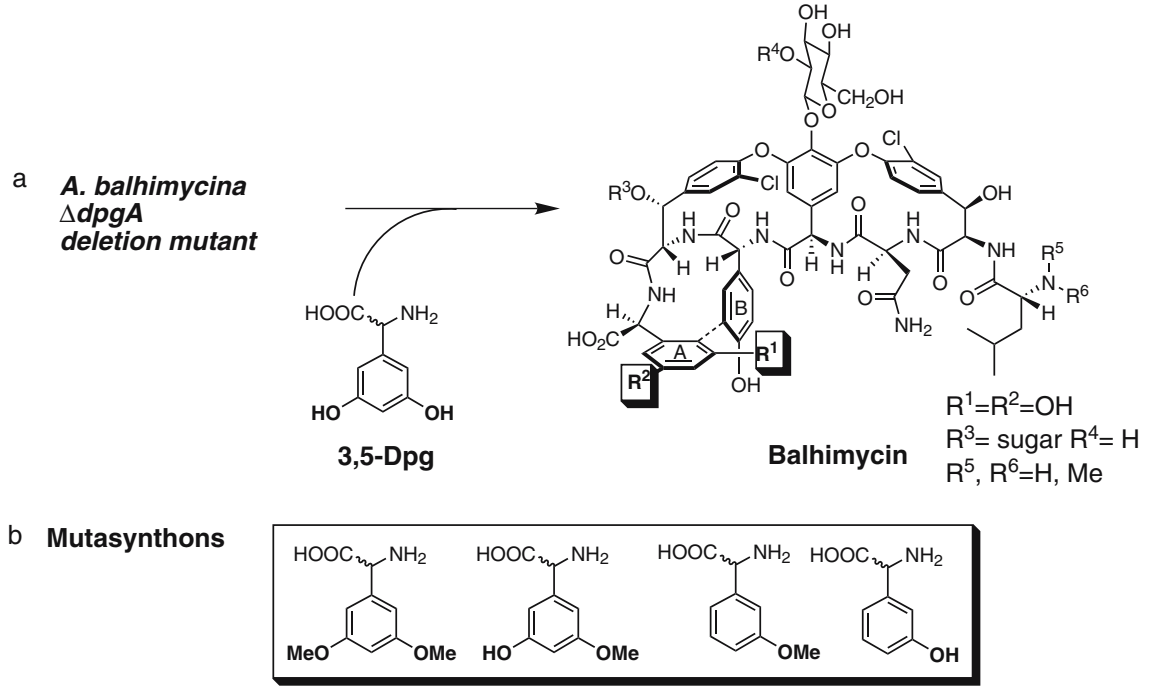

Fig. 11 **a)** Mutasynthesis of vancomycin-type glycopeptide antibiotics. **a)** Variation of 3,5-dihydroxyphenylglycine (Dpg) moiety (Weist et al. 2004). **b)** The generated metabolites were partially lacking the AB-ring-biaryl bond (*dashed line*)

The recent mutasynthesis experiments with CDA and balhimycin clearly demonstrate the existence of strong steric and electronic constraints for the acceptance of altered substrates, particularly for the non-ribosomal peptide synthetases (NRPS). The exchange of hydrogen for fluorine in the aromatic side chains of amino acids has been shown to be successful in several examples. This success is likely based on the similar van-der-Waals radii of hydrogen (120 pm) and fluorine (135 pm), and thus their similar steric requirements. As shown for CDA, bulkier chlorine (181 pm) and OMe substituents attached to mutasynthons are not accepted as substrates by NRPS. Surprisingly, in contrast to glycopeptide antibiotics, the absence of a phenolic group in a mutasynthon of CDA is tolerated. This can be explained by the essential role of OH groups in the formation of biarylether rings, which are mandatory for the antibiotic activity of glycopeptide antibiotics.

Conclusions

Until the advent of molecular biology techniques to generate directed mutants, MBS relied on the screening of chemically or UV-light mutagenised antibiotic producer mutants. Recent developments in the sequencing of antibiotic biosynthetic gene clusters, the cloning of genes, transformation techniques, and thus the generation of gene inactivation mutants, have greatly facilitated the application of MBS. These advances are complemented by the biotechnological engineering of enzymes and producing strains. However, other developments have also contributed to an increasing number of examples utilising MBS. Mutasynthons as building blocks are nowadays often commercially available, or can be synthesised more easily due to advancements in organic synthesis methods. More sensitive analytical methods, especially HPLC-MS, provide proof of successful mutasynthesis experiments, even if the yields are too low for isolation.

MBS has been performed for various structurally and biosynthetically diverse antibiotics. These comprise aminoglycosides as the first known examples, as well as polyketide antibiotics (PKS I and II) and peptide antibiotics (NRPS). Moreover, MBS has been performed for siderophores and antibiotics that cannot easily be assigned to one single biosynthetic pathway. Among macrolide antibiotics, avermectin and erythromycin are the most thoroughly investigated examples. These latter examples both show that starter units especially have the potential to generate novel derivatives by MBS. The field is still lagging somewhat behind with regard to peptide antibiotics synthesised by NRPS. The alteration of aromatic amino acids proved successful; however, substrate tolerance seems generally to be more restrictive than for macrolide antibiotics. However, still too little is known in order to predict the steric and electronic requirements for successful mutasynthesis experiments.

Obviously, a number of drawbacks still restrict the broader applicability of classical mutasynthesis. Despite advancements in gene inactivation of the producing organisms, it is still quite labourious to generate directed mutants blocked in a defined biosynthetic step. Unsurprisingly, the biosynthetic machinery often displays a high substrate specificity, manifested as acceptance of a limited substrate variety from the point of view of the researcher. Thus, the outcome in terms of structural diversity and metabolite yield is often disappointingly low. It therefore seems advisable to use mutants of high performance strains, or highly optimised antibiotic production conditions, in order to obtain reasonable amounts of mutasynthesis products (Leaf et al. 2000). A strong argument against MBS, and also PDB, is that the bioactivity of the mutasynthetically generated compounds is often below that of the wild-type metabolites. However, the important example of doramectin (Hafner et al. 1988; McArthur 1998) shows that MBS can be successfully used for industrial production of a drug.

In conclusion, MBS and PDB remain very appealing concepts, allowing easy access to a variety of complex natural products. With the progressive increase in understanding of antibiotic biosyntheses, especially on the level of complete biosynthesis gene clusters and of protein structure, extended evaluation of MBS and PDB will arise. A final evaluation of MBS cannot yet be undertaken. At present, MBS and PDB are about to merge with the dual approaches of engineering of novel biosynthesis enzymes, and combinatorial biosynthesis. This has already been shown with several contributions on the engineering of erythromycin biosynthesis. A further impressive example in this context is doramectin synthesis (Cropp et al. 2000), which has been transferred from MBS to production by an engineered strain possessing all required biosynthetic genes for mutasynthon-independent biosynthesis. The use of this strain in an industrial process appears possible in the near future.

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