Bluegenics: Bioactive Natural Products of Medicinal Relevance and Approaches to Their Diversification

Joseph S. Zarins-Tutt, Emily R. Abraham, Christopher S. Bailey and Rebecca J.M. Goss

Abstract Nature provides a valuable resource of medicinally relevant compounds, with many antimicrobial and antitumor agents entering clinical trials being derived from natural products. The generation of analogues of these bioactive natural products is important in order to gain a greater understanding of structure activity relationships; probing the mechanism of action, as well as to optimise the natural product's bioactivity and bioavailability. This chapter critically examines different approaches to generating natural products and their analogues, exploring the way in which synthetic and biosynthetic approaches may be blended together to enable expeditious access to new designer natural products.

1 History of Natural Products in Medicine

For millennia, natural products have been utilised by humans for medicinal purposes, with some of the earliest examples documented being that of the ancient Egyptians, who would use honey to aid wound healing and opium-soaked cloths during surgical procedures (Simon et al. 2009; Brownstein 1993). Today, we are far more aware of the chemistry and biology that surround their use. For example, the antimicrobial activity of honey has been shown to be the result of peroxide activity (Irish et al. 2011) and the natural product morphine **1** has been isolated and characterised from the opium poppy *Papaver somniferum*. To this day, medicinal grade honey is utilised as a surgical dressing, whilst morphine and codeine are used extensively to control pain. It was not until the first half of the twentieth century that natural products from microbes came to the forefront of modern medicine, with the seminal discovery by Sir Alexander Fleming that the mould *Penicillum notatum* was able to produce compounds with antibiotic properties (Fleming 1945). In 1945 the structure of the bioactive compound was determined by Dorothy Hodgkin revealing a central beta-lactam motif, the warhead of the penicillins **2** (Robinson 1949).

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Such early discoveries inspired the search for further medicinally useful natural products from both plants and microbes and resulted in the discovery of numerous compounds with a diverse array of chemical architectures and biological activities. Examples include antimicrobial compounds such as erythromycin **3**, daptomycin **4** and vancomycin **5**, the cholesterol lowering lovastatin **6**, the antiparisitic avermectin **8** and from plants the anticancer drug taxol **7** and the antimalarial artemisinin **9** (Fig. 1). The current and historical medicinal importance of natural products to society was acknowledged through the 2015 Nobel Prize in Physiology or Medicine being awarded for the discovery of avermectin **8** and artemisinin **9** (Egerton et al. 1979; Tu et al. 1981).

2 The Role of Natural Products in Nature

For many secondary metabolites it is unclear as to what function they have evolved for. For some compounds, it is clear that they have a role in defence. For instance, plants and bacteria may produce antimicrobial agents for protection against pathogens or as means of killing off their competition. Other molecules are used for signalling, for example in quorum sensing or as siderophores, chelating and uptaking metals, that are in limited abundance. However, the function for many natural products, if they indeed have one, remains unknown; whilst investment in the synthesis of natural products is energetically and resource costly for an organism, it provides an evolutionary advantage to have the ability to access diverse series of compounds and to rapidly modify the portfolio of compounds that are generated. It is also questionable as to whether the effects that we observe under an artificial laboratory environment are akin to those in nature. For example many compounds classified as antimicrobials in a laboratory environment may function purely as warning signals and not as the agents of death that their artificially high laboratory titres result in (Ratcliff and Denison 2011). Regardless of "evolution's original intentions", modern science has shown many of these molecules to function medicinally by binding specific protein targets and it has been suggested that 'during evolution, there has existed a natural product ligand that could bind to all protein folds' (Dixon et al. 2007). Despite being evolutionarily separated from many of the natural product producers, conserved protein folds may have therefore led to unintentional secondary function of natural products which we can exploit.

3 The Antibiotic Crisis and the Need for New Medicine

Antibiotics are chemical agents that are capable of inhibiting or perturbing bacterial growth. In order to exert a toxic effect, the chemical agent must prevent or interfere with cellular processes that are essential to the survival and proliferation of the bacterium. For this to be of medicinal use, there must be a high level of selectivity,

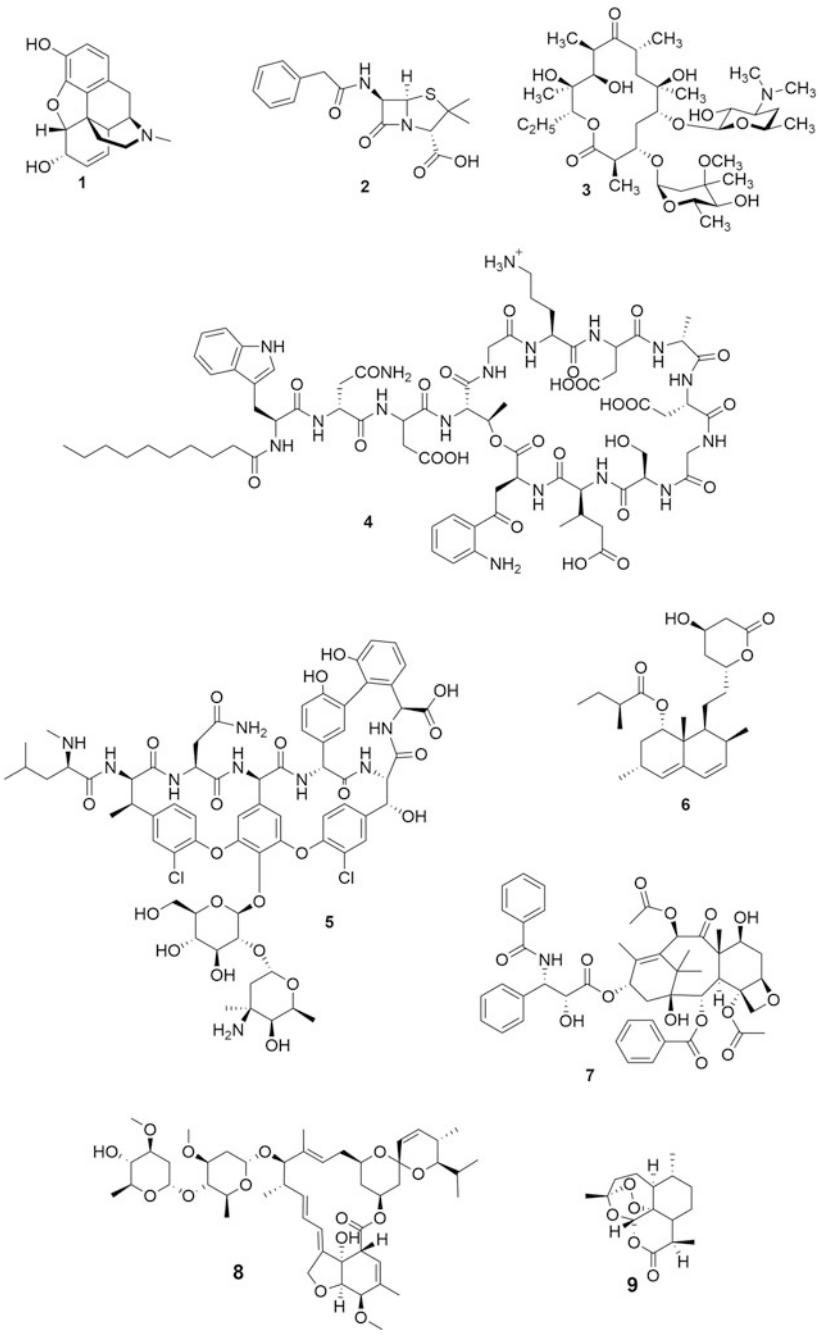


Fig. 1 Clinically relevant natural products: morphine **1**, penicillin G **2**, erythromycin **3**, daptomycin **4**, vancomycin **5**, lovastatin **6**, taxol **7**. Avermectin A1b **8** and artemisinin **9**

exploiting differences between the biochemistry of the bacterium and the host such that the bacterium is targeted but the animal host is not. There are six main targets: cell wall biosynthesis, protein synthesis, cell membrane integrity, RNA synthesis, folate synthesis and DNA synthesis and integrity; these are summarised in Fig. 2a. Antibiotic resistance is however a serious threat, resistance to antibiotics is observed alarmingly soon after a new antibiotic is introduced (Fig. 2b).

4 Antibiotic Resistance

Antibiotic resistance is by no means a new phenomenon, indeed, Fleming in his 1945 lecture (Fleming 1945) warned of the danger of resistance resulting from the inappropriate usage of antibiotics, by exposing bacteria to concentrations not sufficient to kill them to train them to be resistant. Antibiotic resistance has now been observed for all clinically used classes of antibiotics (Fig. 2b) and multidrug resistant (MDR) strains exist including strains that are resistant to all clinically available drugs—extreme drug resistance (EDR). The problem is exacerbated by the lack of new antibiotics with novel structures and new modes of action being introduced into the clinic. This means that there exist few alternatives for the treatment of resistant strains. The rise in antibiotic resistance has many causes, however, poor antibiotic stewardship has no doubt exacerbated the problem (Goossens et al. 2005).

Bacteria acquire resistance to antibiotics through a number of different pathways; these include proactive responses such as mutations in pre-existing genes to render antibiotic targets resistant (for example resistance to rifampicin through mutations in the RNA polymerase) (Srivastava et al. 2011; Ho et al. 2009; Floss and Yu 2005; Mariani and Maffioli 2009), horizontal gene transfer (HGT), where resistance genes are transferred between environmental species through transfection, conjugation or transformation, and physiological and behavioural changes such as biofilm formation in which the bacterial cells are protected by an extracellular polysaccharide matrix and metabolic dormancy enables persister cells to escape inhibition (Cantón 2009).

The majority of antibiotics in use are natural products, or derivatives of natural products, where the organisms generating them have an endemic resistance system built in so that their own survival is not compromised through the production of the antibiotic compound. As such, these resistance genes are already present in nature and can be transmitted between bacterial species. These genes include detoxifying enzymes, such as the beta lactamases which break down beta-lactam antibiotics through the hydrolysis of the beta-lactam ring, genes that encode the modification of the target so that the antibiotic can no longer bind effectively and genes responsible for the rapid export or efflux of the bioactive compound. Due to this widespread resistance, there is an urgent need for new antibiotics with novel structures that are not recognised by or susceptible to the most common and prevalent mechanisms of resistance and preferably compounds that inhibit clinically unexploited targets.

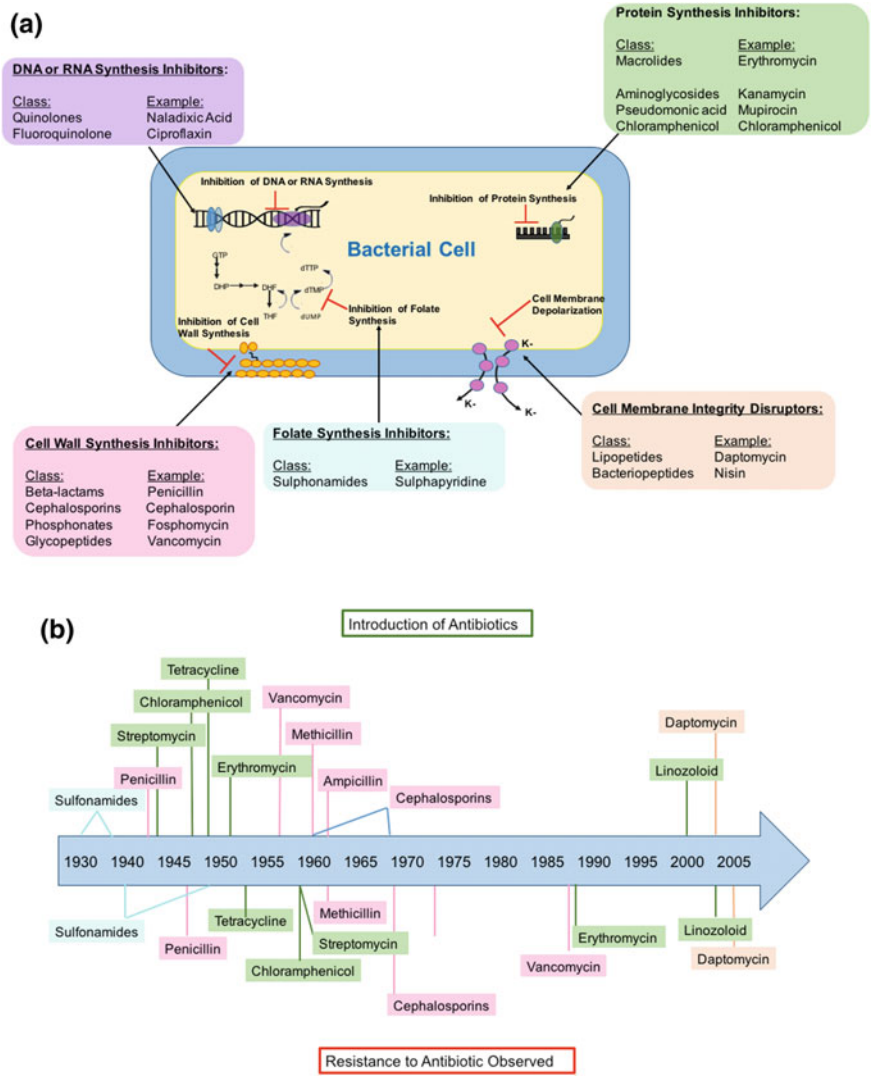


Fig. 2 Main antibiotic targets with clinical examples alongside a timeline highlighting when resistance to the clinical example was first observed. **a** The cartoon illustrates the main antibiotic targets and highlights classes of drugs which affect each bacterial target and clinical examples which belong to each class. Cartoon adapted from Clatworthy et al. (2007). **b** Timeline illustrating the introduction of antibiotics, (*top*) compared with when resistance to the antibiotic was first observed. The colours of the antibiotic correspond to the antibiotic's target shown in Fig. 2a. Figure adapted from Clatworthy et al. (2007)

5 Natural Products and the Clinic. Towards New Antibiotics in the Battle Against Resistance

In the past three decades over 70% of antibiotics entering clinical trials have been based on natural products, compounds generated by organisms, in particular microbes (Van Lanen and Shen 2006). Strikingly, investment into exploration of streptomyces, perhaps the most chemically characterised microbial genus to date, has resulted in the discovery of over two-thirds of all known antibiotics.

Whilst the direct use of a natural product only currently accounts for 6% of all currently clinically used medicines, a further 27 and 17% result from derivatisation of a natural product or the inclusion of a pharmacophore from a natural product respectively. This means a staggering 50% of medicines are based on natural products (Fig. 3), with further synthetic compounds designed to inhibit targets that have been revealed through the use of natural products (Newman and Cragg 2012).

Figure 4 displays an overview of the number of new antibiotics that have reached the clinic between 2000 and 2012 and highlights examples of synthetic and natural product derived molecules in phase III clinical trials, illustrating the importance of natural product research in this therapeutic arena. However, only a small proportion (0.5%) of antibiotic-producing microbes are currently cultivable in the lab, although innovative culturing methods have enabled success in antibiotic discovery with the recent identification of teixobatin from a previously uncultured microbe (Ling et al. 2015). The marine environment represents a rich source of biologically active compounds, with marine organisms accounting for approximately half of the earth's biodiversity. Though the marine microbiome has been

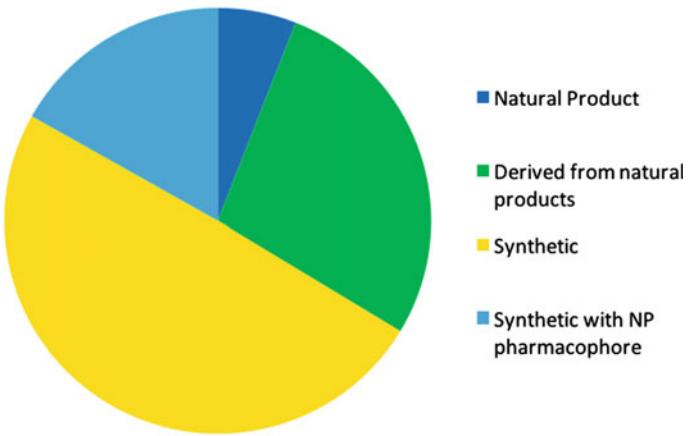


Fig. 3 Percentage and origin of all clinically used drugs. Adapted from Newman and Cragg (2012)

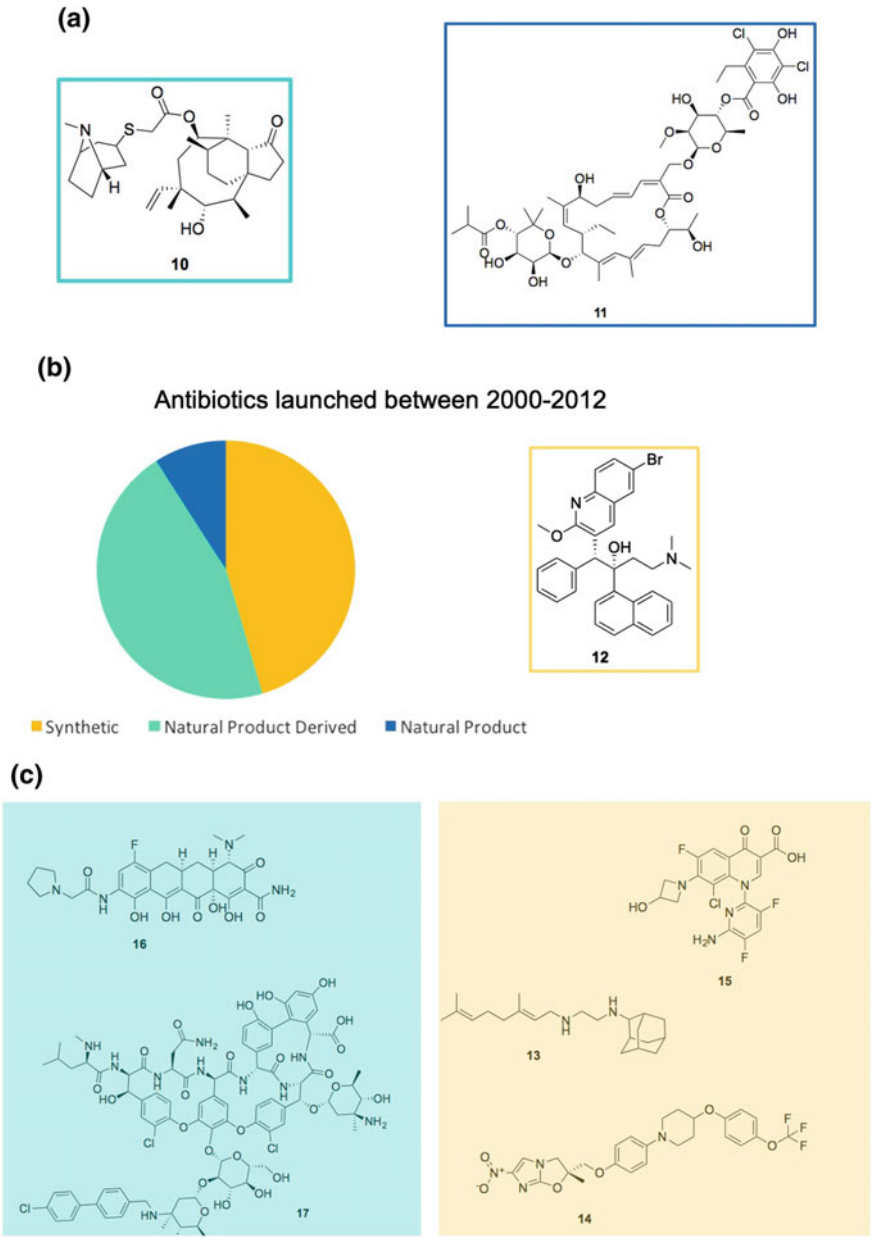


Fig. 4 An overview of the number of new antibiotics that have reached the clinic between 2000 and 2012 as well as an example of both synthetic and natural product derived molelcules in phase III clinical trials, displaying the continued importance of natural products in this therapeutic arena. Included within these 22 new drugs, are three novel structural classes spanning both synthetic and natural product origins. **a** examples of new antibiotic classes launched for human use since 2000 including natural product derived retapamulin **10**, the natural product fidaxomicin **11**, and the synthetic bedaquiline **12**. **b** chart displaying the number of new antibiotics launched in the clinic between 2000 and 2012. **c** Example of synthetic compounds in phase III clinical trials SQ109 **13**, delamanid **14** and delafloxacin **15** and of natural product derived compounds in phase III trials eravacycline **16** and oritavancin **17**. Data obtained from Butler et al. (2013)

little explored for compound discovery, evidence indicates searches will be fruitful. In July 2014 a total of 7 marine-derived compounds had already received EU/FDA approval, with a further 25 in phase i–iii clinical trials. Studies thus far, have focussed predominantly on collection and examination of marine invertebrates, (such as sponges, and tunicates) rather than microbes, leaving this a largely unexplored resource. Marine microbes therefore represent an important discovery opportunity; in many of these systems the mutualistic microbes are suspected, and in some cases shown, to be the originators of the isolated bioactive compounds.

6 A Growing Focus on Marine Natural Products

There is a growing focus on natural products from the marine environment. New drug leads, with rich and novel chemical architectures, have been identified and include the novel antibacterial polyene marinomycin A **18**, the proteasome inhibitor salinosporamide A **19**, (Gulder and Moore 2010) and the cyclic peptide patellamide A **20** (Schmidt et al. 2005). A number of novel marine scaffolds, such as analogues of plinabulin **21**, plitidepsin **22** and bryostatin **23** (Mayer et al. 2010) have been progressed into clinical trials as anticancer agents (Fig. 5).

The oceans cover two-thirds of the earth's surface and provide a plethora of distinct environmental niches giving rise to huge diversity of species. In order to survive in these unique environments, many of the organisms have developed unique adaptations and relationships, which are reflected in the chemical diversity found in the oceans. Many marine tunicates have symbiotic relationships with a number of bacteria that are capable of producing highly toxic compounds (Donia et al. 2011). Furthermore, many marine-derived compounds are halogenated, due in part to the natural concentrations of bromine and chlorine (Gerwick and Moore 2012). With such chemical diversity, many of the clinically approved marine natural products can be deemed 'first in class' (Gerwick and Moore 2012). Currently, a total of seven marine or marine-derived natural products have been approved clinically, two of which are unmodified natural products (Gerwick and Moore 2012). These seven drugs span four therapeutic areas; anticancer; Yondelis® **24**, Cytosar-U® **25** and Halaven® **26**, antiviral, pain; Prialt® **27** and hypertriglyceridemia (Fig. 6) (Mayer et al. 2010; Gerwick and Moore 2012).

As described above, natural products have evolved for utility within their producing organisms, therefore it is essential to access analogues of natural products not only to determine the target of interaction and the way in which the natural

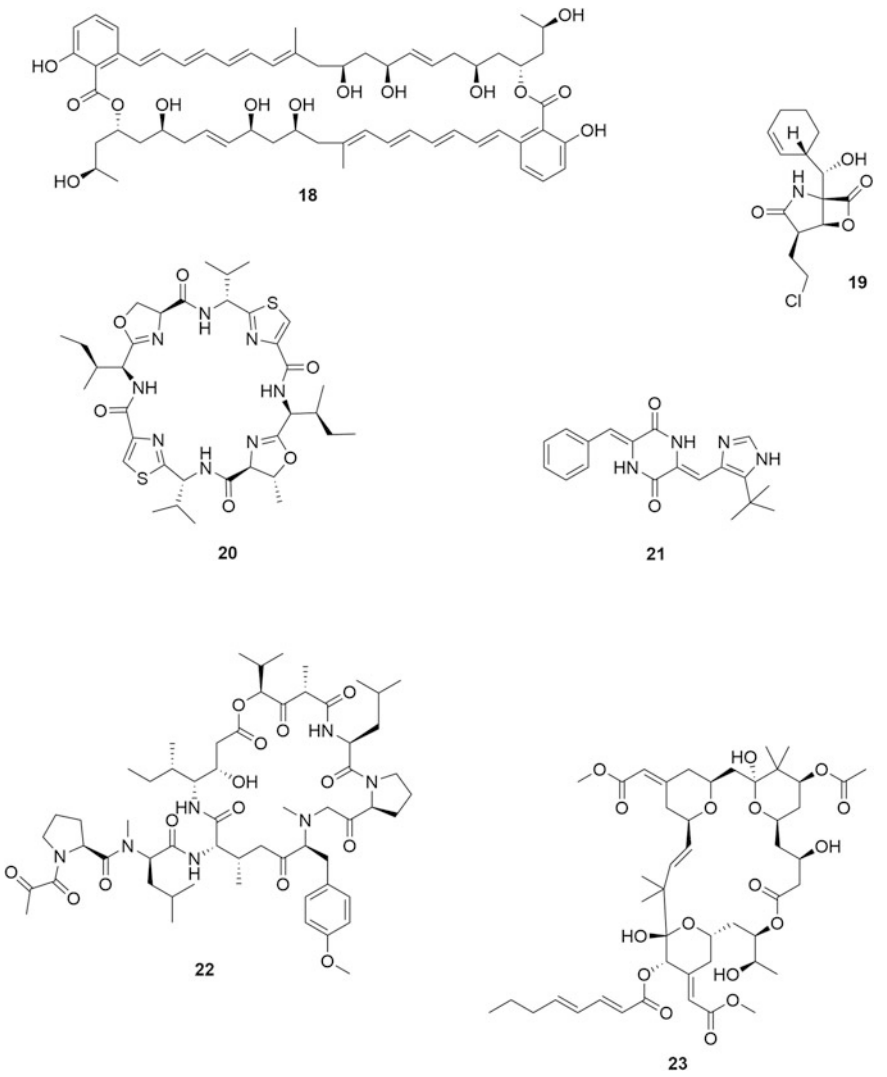


Fig. 5 Marine natural products. Marinomycin **18**, salinosporamide A **19**, patellamide A **20**, plinabulin **21**, plitidepsin **22**, bryostatin **23**

product interrelates with it at the molecular level, but also to generate materials with improved activities and oral bioavailability. ***The remainder of this chapter looks particularly at approaches that may be utilised to enable access to series of analogues of natural products, with a particular focus on natural products with antibiotic activities***.

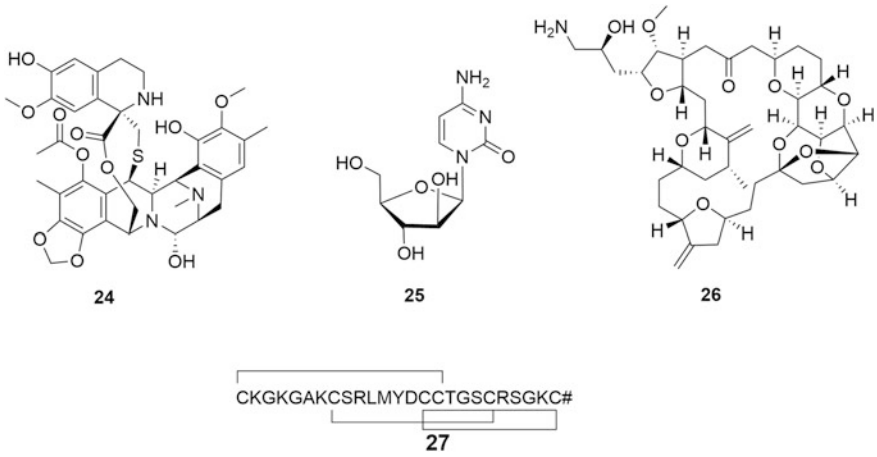


Fig. 6 Example of marine natural products in clinical use. trabectedin (Yondelis®) **24**, cytarabine (Cytosar-U®) **25**, eribulin (Halaven®) **26**, ziconotide (Prialt®) **27**

7 Generating Natural Product Analogues

Natural products are made by biomolecules and therefore naturally predisposed to interact with biomolecules, thus as discussed above, they make an excellent starting point for drug discovery. The ability to access series of natural product analogues is crucial in order to gain understanding as to the structure activity relationship of a natural product, investigate its precise interactions with its target at the molecular level and develop compounds with improved bioactivity as well as other desirable qualities such as good solubility, low toxicity, appropriate stability, and oral bioavailability. Due to the structural and chemical complexity of natural products they have in been perceived to be "unmedchemable", and challenging to make analogues of. This viewpoint comes from focussing on the more traditional approaches to analogue generation, including semisynthesis and total synthesis (Fig. 7), however opportunities are being developed that utilise synthetic biology and synthetic chemistry combining the best of both worlds in order to more readily generate designer analogues of natural products.

Today, approaches to natural product analogue generation include semisynthesis (in which a natural product is directly chemically modified), total synthesis (chemically generating the compound de novo from simple readily abundant starting materials) and a variety of ways in which biosynthesis is harnessed.

Fig. 7 Image demonstrating drugs which are available for use, which have been derived from natural products. For example, simvastatin 6b is a semi synthetic derivate of the natural statin lovastatin 6, and is used to treat patients suffering from high cholesterol levels

7.1 Total Synthesis

Total synthesis and semisynthesis are the most traditional methods of accessing natural product analogues. Total synthesis is particularly useful in cases where the natural product is small and chemically simplistic, where the natural product is available in extremely limited quantities or not at all, or where the producing organism is not readily manipulated. Total synthesis also is regularly proven essential in the determination of the absolute structure of recently isolated natural products. With total synthesis, the approach is simply to synthesise the entire compound from inexpensive and readily available starting materials. Derivatives can be prepared by modifying either the reactants or the reactions used in each step of the synthesis. Whilst this approach still affords the greatest potential for accessing diverse analogues, the syntheses are often long, complex, inefficient, and give low yields. Furthermore, for particularly complex natural products, the initial synthetic route may be difficult to establish.

It is rare to be able to access more than a few milligrams of any one bioactive compound from certain marine organisms. Tunicates and sponges are far from simple to culture in laboratories making sustainable access to bioactive compounds isolated from such organisms challenging. For such systems total synthesis of the

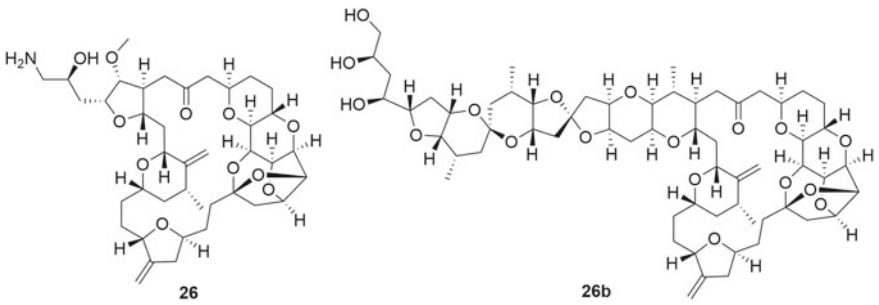
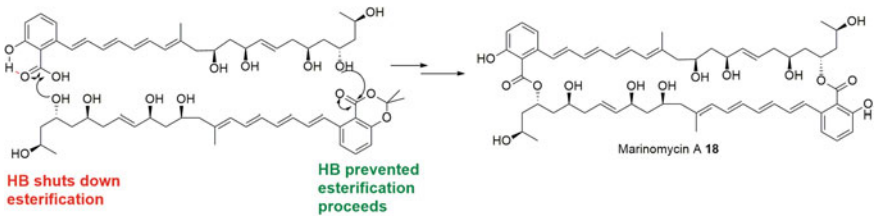


Fig. 8 Eribulin mesylate **26**, a derivative of halichondrin B **26b**, has stereogenic centres and is the most synthetically challenging drug to be generated by the pharmaceutical industry to date

parent compound and any analogues is often the only option. Eribulin mesylate **26** (Fig. 8), marketed by Esai Ltd. as Halaven is a polyketide macrocycle that has potent anticancer activity and is used in the treatment of breast cancer. Eribulin mesylate **26** is an analogue of the natural product halichondrin B **26b**, produced by the *Halichondria* genus of sponge. Total synthesis however, in 1998, revealed the active portion of the molecule, this allowed for simpler structural analogues be developed. This ultimately resulted in the delivery of eribulin **26** to the clinic (Yu et al. 2011). This structurally elaborate natural product analogue is accessed through an impressive total synthesis. Halaven **26** is considerably more structurally complex than other marketed pharmaceutical that has been accessed through total synthesis and represents a step change demonstrating the enhanced skill and determination of the pharmaceutical industry in order to access this important anticancer agent in scale. It is noteworthy that investment in the industrial total synthesis of a drug that attracts significant revenue, such as anticancer agents, due to the need of the patient to take it for long periods is viable. However, the revenue from the sales of antibiotics, which are typically administered as short, one to two week courses are currently considerably less. Complex syntheses of antibiotics are not currently a viable option for industry.

Marinomycin **18**, a polyene of polyketide origin, was isolated from the marine actinomycete *Marinospora* CNQ-140 by Fenical (Kwon et al. 2006) and showed excellent antibiotic activity (0.1–0.6 uM) against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faceium* (VREF) as well as promising and selective activity against a variety of cell lines associated with skin cancer. A key drawback of this potentially promising, new structural class of antibiotic is its high propensity to photoisomerise (Kwon et al. 2006). Nevertheless, the compound produced in low titre by the microbe (0.5–2 mg/L) (Kwon et al. 2006) has attracted significant attention as a target for total synthesis with the compound initially being synthesized by Nicolaou et al. (2007). Evans and his team were elegantly able to assemble the challenging dimeric compound demonstrating the selective deactivation and activation of a salicylate



Scheme 1 Marinomycin **18** and its curious salicylate switching mechanism

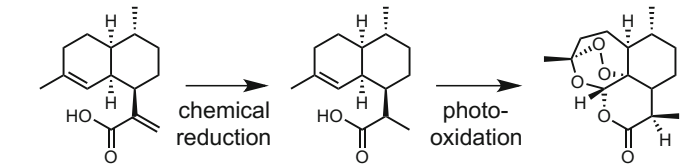
moiety, through the formation of a short strong hydrogen bond, to esterification—a switching mechanism that perhaps operates naturally in controlling the macrolatonisation of the two dimers (Scheme 1) (Evans et al. 2012). Semisynthetic studies around marinomycin **18** to generate the diacetonide and hexa-acetate derivatives both resulted in slightly reduced antibiotic activity (Kwon et al. 2006).

7.2 Semisynthesis

Semisynthesis is the direct chemical derivitisation of an extracted and isolated natural product. The nature of chemical derivitisation possible and the regiochemistry of the diversification is dictated by the chemical reactivity of the natural product and in particular the presence of reactive and chemically orthogonal functional groups. It also can be utilised to convert an abundant natural product into a desired compound that is naturally in short supply. Trabectedin **24**, for example was originally isolated from the marine tunicate *Ecteinascidia turbinate* at a yield of 0.0001% (Cuevas and Francesch 2009). As such it required a semisynthetic routes from the structurally similar cyanosfracin B (Cuevas and Francesch 2009).

A further example of the application of semisynthesis, relating to the supply of the desired natural product, is the generation of the antimalarial drug artemisinin **9** from artemisinic acid **28** (Ro et al. 2006). Artemisinin **9** is generated by the plant *Aretemisia annua* (sweet wormwood) which takes eight months to reach maturity. Though much of the artemisinin **9** used clinically is extracted directly from plants alternative and sustainable approaches to access the compound are sought. The total synthesis for this compound has been established, but using this approach it is not possible to produce sufficient quantities for the clinic (Kennedy 2008). Instead, artemisinic acid **28** is extracted from an extensively engineered strain of *Saccharomyces cerevisiae*, and modified in two steps to artemisinin **9** (Scheme 2) (Kennedy 2008). Using this method, artemisinin **9** can be produced in sufficient quantities to meet the demand for this drug, and cheaply enough to be accessible to the developing world (Kennedy 2008).

There are a number of considerations when using this approach. First, there must be sufficient amounts of the natural product to use as a reactant. Second, there are often many similar functional groups in natural products and so selectively reacting



artemisinic acid **28** artemisinin **29**

Scheme 2 Artemisinic acid **28**, available from the fermentative culture of *S. cerevisiae*, then chemically modified to artemisinin **9** (Kennedy 2008)

only certain ones requires either highly specific reactions or the extensive use of protecting groups. Third, the possible modifications are limited by the molecule itself; the stability of the molecule towards certain reagents and the presence or absence of certain groups may prevent or hinder certain modifications.

Extension and modification of the pharmaceutical scaffold is a common strategy employed to combat drug resistance. By changing the drug scaffold, it may no longer be recognised as a substrate by the enzyme involved in drug resistance. This is also for true for resistance mechanisms which modify the drug target as opposed to the drug itself. This is because extension of drug scaffolds can change physical as well as chemical properties such as lipophylicity, electronegativity and sterics, all of which control binding and recognition.

Penicillin **2**, whose serendipitous discovery is attributed to Fleming, a β-lactam antibiotic produced by the fungus *Penicillium notatum*, acts by inhibiting bacterial cell wall biosynthesis. Specifically, it prevents the natural cell wall rigidification that is enabled by the cross-linking of the constituent peptidoglycan chains. Peptidoglycan is a key component in bacterial cell walls, providing much of the strength and rigidity. It is composed of a β-1,4-linked glycan backbone of alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) units. Pendant pentapeptide chains are attached to the 3-position of each muramic acid with the terminal residues being D-Ala-D-Ala; neighbouring this motif are either L-lysine or *meso*-diaminopimelic acid (DAP) residues. Cross-linking of these residues by amide bond formation serves to rigidify the polymer. The introduction of penicillin **2** into the clinic in 1943 was soon followed by the rapid emergence of wide spread resistance due to the spread of β-lactamase activity. To overcome this resistance, semisynthetic analogues were developed.

The macrolides are a series of antibiotics that include erythromycin **3**, from the actinomycete *Saccharapolyspora erythraea* and inhibit protein synthesis. Drug resistance mechanisms against erythromycin include *ermB*, a *N*-6-methyltransferase that modifies the adenine base of adenosine 2058 in 23S rRNA thereby enabling translation and protein synthesis to occur in the presence of erythromycin **3** and *mefA*, an efflux pump (Roberts et al. 1999). These resistance mechanisms are found to be inducible rather than constituitive.

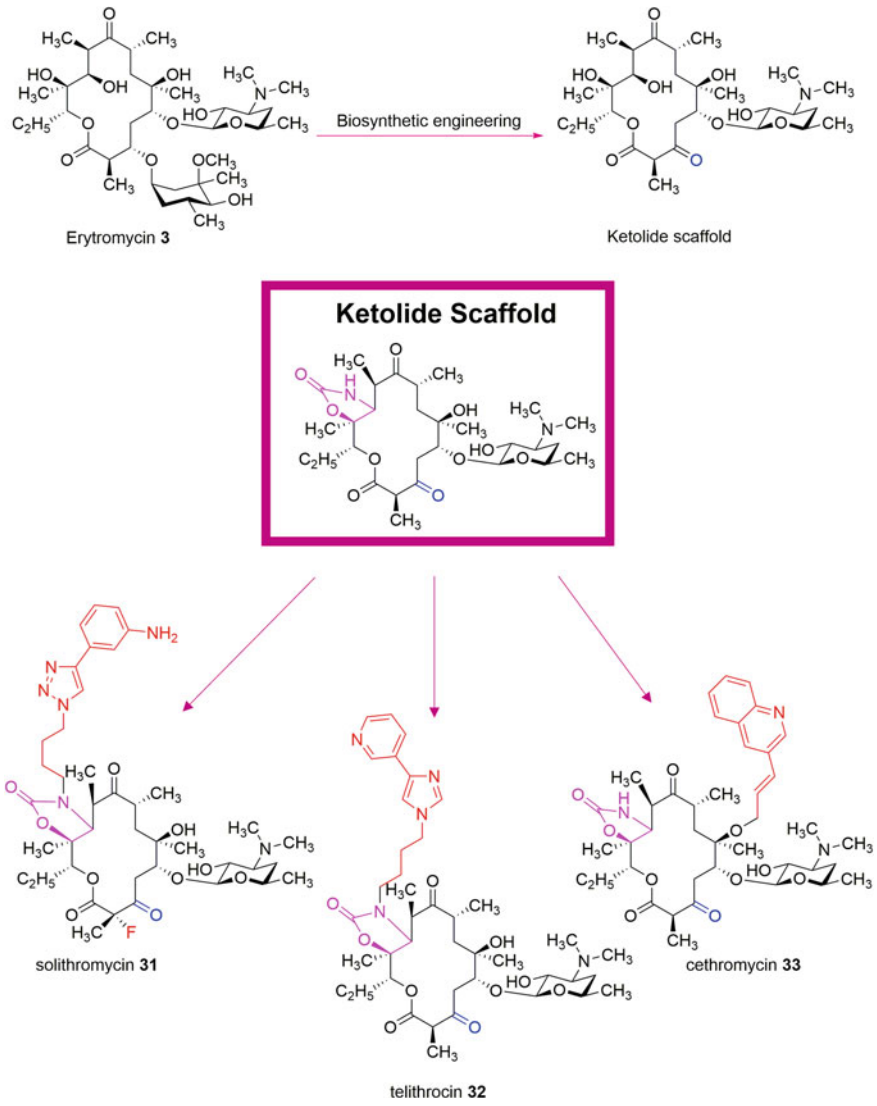
The ketolides are an important example of clinically successful extended scaffolds accessed by semisynthesis (as well as biosynthetic engineering). Whilst the

polyketide core of erythromycin **3** is elaborated by post PKS glycosylation with both a D-desosamine and L-cladinose sugar, in the ketolides the L-cladinose sugar moity is absent and replaced by a ketone (as found naturally in the antibiotic pikromycin **29**). A semisynthetic preparation of the ketolide scaffold is achieved through selective cleavage of the L-cladinose followed by oxidation (Zhanel et al. 2002). Access to 5-*O*-desosaminyl erythronolide A **30** through biosynthetic engineering provides a precursor that may simply be oxidized to the ketolide (Zhanel et al. 2002). The ketolide analogues lacking the L-cladinose do not induce resistance (Zhanel et al. 2002). Whilst the lack of the L-cladinose results in poorer binding to the ribosome, this can be more than compensated for by the semisynthetic addition of an 11,12 carbamate extension. Further semisynthetic extension of the scaffold results in the drugs solithromycin **31**, telithrcin **32** and cethromycin **33** (Scheme 3). The ketolides remain biologically active in strains expressing efflux pumps, perhaps due to their higher binding affinity to their target, and an ability to bind to methylated ribosomes (Capobianco et al. 2000).

Vancomycin **5**, generated by the bacterium *Amycolatopsis orientalis*, is often referred to as the antibiotic of last resort. It has been used in the treatment of Gram-positive bacterial infections including methicillin-resistant *S. aureus* for 6 decades, however in recent times its potency has been observed to have reduced and vancomycin-resistant *Enterocci* and *S. aureus* strains have emerged (Courvalin 2006). The generation synthetic and semisynthetic analogues of vancomycin **5** are sought to combat resistant strains (Nakama et al. 2010; Xie et al. 2011). Vancomycin **5** prevents cross-linking by forming 5 hydrogen bonds to D-Ala-D-Ala to overcome this resistant strains have evolved in which replacement of D-Ala-D-Ala with D-Ala-D-Lac reduces hydrogen bonding to vancomycin **5** (Pinchman and Boger 2013). Analogues capable of killing vancomycin-resistant strains are being sought. Recent work in this area includes the Boger lab's work on vancomycin aglycone converting the chloride to a boronic acid giving rise to a variety of function groups via cross-coupling modification (Fig. 9) (Pinchman and Boger 2013).

Complementary to this research, Miller has recently been taking semisynthesis in an exciting new direction utilising peptides, as catalysts, to control the site selective halogenation of the vancomycin **5** and the related glycopeptide antibiotic teicoplanin (Pathak and Miller 2012, 2013). Basing the design of the peptide agent on the vancomycin-binding site, regioselective halogenation has been achieved (Pathak and Miller 2012). Using screened peptide libraries, new semisynthetic routes utilising regiocontrolled halogenation of benzamides (Barrett and Miller 2013) and epoxidation of various isoprenols have been explored (Lichtor and Miller 2012). Recently, the groups have developed this approach to enable selective late stage epoxidation of series of compounds (Abascal et al. 2014).

Semisynthesis may also be used to modify potency and reduce toxicity, for example dolastatin, a peptide from the mollusc *Dolabella auricularia*, though likely to be of cyanobacterial origin, proved too potent to be used as a single agent. As such a synthetic analogue was developed called monomethyl auristatin E and is linked to a monoclonal antibody to be only the second antibody drug conjugate in clinical use (Schrama et al. 2006).



Scheme 3 Scaffold Extension. Semisynthetic ketolides can be accessed from the through the removal of the cladinose sugar of erythromycin **3** and oxidation to the ketode (*blue*). A cyclic carbonate is also added synthetically (*magenta*). Further synthetic elaboration of the scaffold (*red*) produces series of active compounds

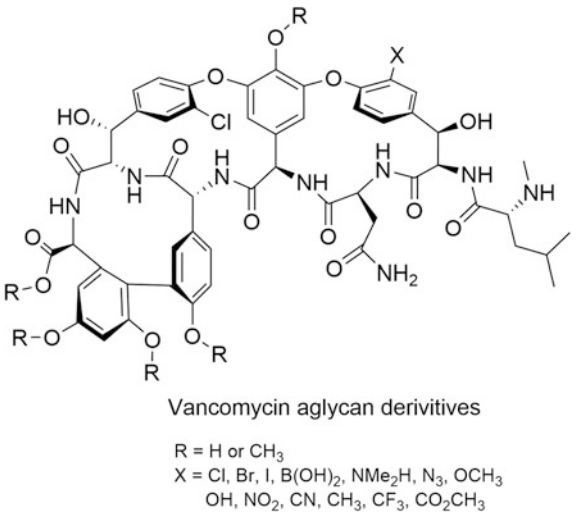


Fig. 9 Derivatives of the vancomycin aglycon E-ring aryl chloride to determine its effect on D-Ala-D-Ala binding and antimicrobial activity (Kwon et al. 2006)

7.3 Precursor-Directed Biosynthesis

Precursor-directed biosynthesis provides perhaps the least technologically demanding approach for harnessing a biosynthetic pathway to the natural product of interest, and takes advantage of the inherently flexible nature of many biosynthetic enzymes. If the biogenic assembly of the natural product is understood, analogues of the precursors may be designed, synthesised and administered to the producing organism. In other words, alternative biosynthetic building blocks may be supplied for the organism to utilise in the construction of a new to nature natural product. For multifunctional enzymes such as polyketide synthases and nonribosomal peptide synthetases the loading module at the beginning of the assembly process, which uploads and processes the first biosynthetic precursor, named the "starter unit" tends to be particularly flexible and able to tolerate a range of substrates. Scoping studies to explore the key components that are required within the precursor and investigate the extent of flexibility of the pathway are often useful. One example of a natural product with a strikingly flexible pathway is the uridyl peptide antibiotic pacidamycins **34** which is able to incorporate methylated and halogenated amino acids at high level into both the N and C termini (Grüschow et al. 2009; Ragab et al. 2010). Through scoping studies the flexibility for tolerance of bulky groups present at the 2 or 7 position of the indole ring is particularly apparent (Fig. 10).

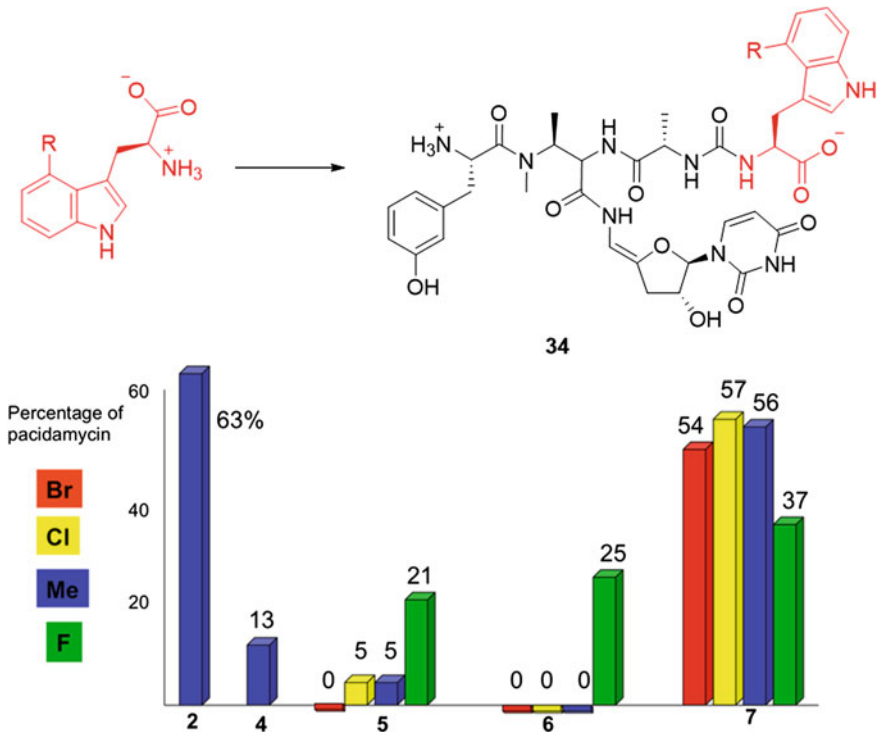


Fig. 10 Precursor-directed biosynthesis to yield analogues of pacidamycins **34**. Tolerance for tryptophans substituted at the 2 and 7 positions of the indole ring is particularly apparent (Grüschow et al. 2009)

7.4 Mutasynthesis

Mutasynthesis is a variation on precursor biosynthesis in which the organism is first engineered to either:

(i) Have extended substrate flexibility, as for example in the generation of the fluorinated erythromycin analogue (Goss and Hong 2005).
Or
(ii) Not be able to generate the natural starting material and so only be able to produce the compound of interest or its analogues if its media is supplemented with a suitable biosynthetic precursor.

Using the former approach, utilised to explore the generation of modified analogues of erythromycin **3** by *Saccharopolyspora erythraea*, a series of fluorinated precursor analogues were administered to the wild type strain and a strain engineered to incorporate the more flexible loading module from avermectin biosynthesis. Whilst the pathway in the wild type organism was unable to process extended fatty

Fig. 11 Exploration of utilisation of alternative biosynthetic precursors/building blocks by both wild type and engineered microorganisms

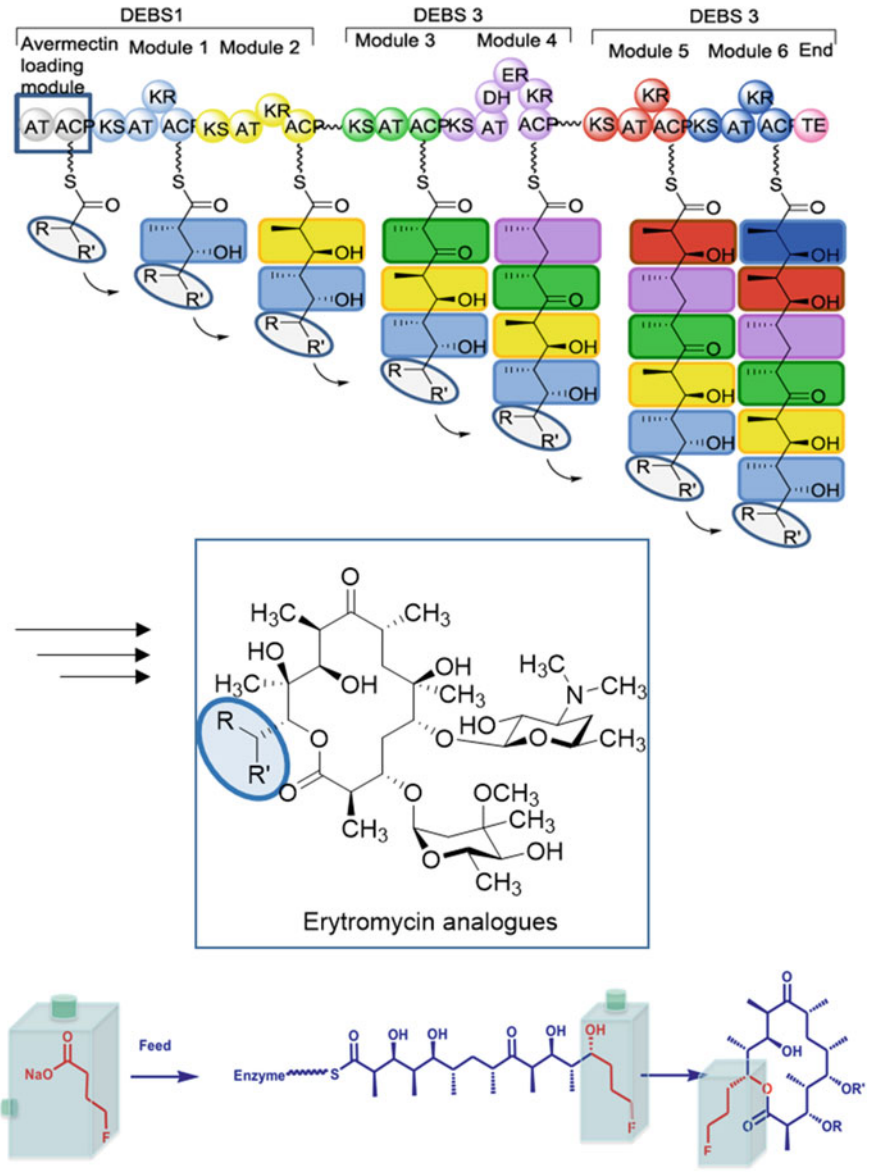
acids, the engineered strain enabled their incorporation into fully functionalised erythromycin analogues (Fig. 11) (Goss and Hong 2005; Marden et al. 1998).

The second approach has been applied to many systems including the generation of epothilone analogues and access to analogues of rapamycin **35** (Grüschow et al. 2009; Gregory et al. 2005; Goss et al. 2006) (Fig. 11 and Schemes 4 and 5).

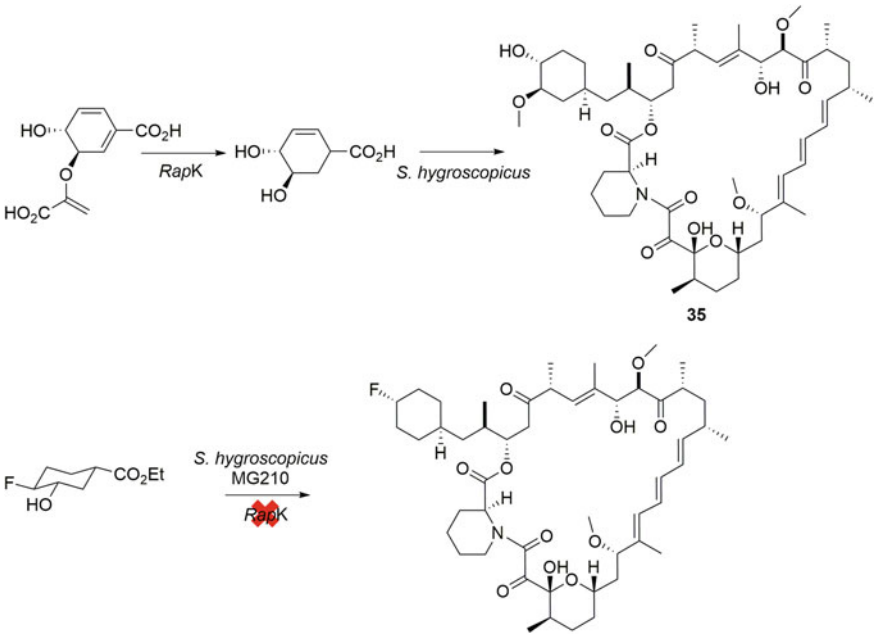
The fact that the organism is rendered incapable of suitable biosynthetic precursor analogue is particularly attractive. In precursor-directed biosynthesis the analogue is produced alongside the parent molecule and purification of the novel and desired compound that is generated can prove challenging. In this second approach to mutasynthesis, conveniently only the analogue and not the parent molecule may be fermentatively accessed, and purification of the desired new to nature compound is simplified. In the case of rapamycin **35** the generation of the dihydroxylated cyclohexenoic acid starter unit is catalysed by RapK which mediates the loss of pyruvate from chorismate. Mutants deficient in RapK can be utilised to enable selective access to series of analogues with modified starter units (Gregory et al. 2005). Initial scoping experiments reveal that only precursor analogues in which there is a hydrogen bond acceptor within the cyclohaxane ring are tolerated (Goss et al. 2006). This information enabled the design of fluorohydrins which could be synthesised, fed and incorporated into the natural product (Ragab et al. 2010).

7.5 Combinatorial Biosynthesis

Combinatorial biosynthesis describes the ambition to be able to utilise enzymatic components from various different biosynthetic pathways in a combinatorial fashion, mixing and matching genetic elements encoding enzymes and creating designer biosynthetic pathways at will. Before the dream of combinatorial biosynthesis in its fullest sense can be realised, there is a long way to go in terms of understanding multifunctional enzyme structures, how these enzymes interact and passage intermediates, and how their substrate specificity of individual domains as well as entire systems may be reprogrammed. So far combinatorial biosynthesis has been used to achieve ring contraction (Thomas et al. 2002; Wu et al. 2000, 2002) or



Scheme 4 **a** Mutasynthetic access to analogues of erythromycin **3**. The flexibility of the multifunctional enzyme is extended through the incorporation of the loading module from avermectin biosynthesis. *AT* acyltransferase, *ACP* acyl carrier protein, *KS* ketosynthase, *KR* ketoreductase, *DH* dehydratase, *ER* enoyl rductase, *TE* thioesterase. **b** This system may be used to access fluorinated analogues of erythromycin **3** (Goss and Hong 2005)



Scheme 5 Utilising mutasynthesis to generate analogues of rapamycin **35**

ring expansion (Kao et al. 1997), modification of the stereochemistry of moieties within polyketide synthases (Caffrey 2005) to alter glycosylation patterns (Shepherd et al. 2011; Pérez et al. 2008) and to generate new to nature halogenated metabolites (Heide et al. 2008; O'Connor 2012; Roy et al. 2010). The Goss group have been developing such studies during the Bluegenics programme. Recent example in this area being the impressive engineering of the fluorinase into the DEBS1-TE system (Walker et al. 2013).

One of the earliest examples of the combining of gene clusters or the introduction of a gene with a desired function into another cluster in order to transform a natural product in a desired way was the engineering of a bacterial strain by Omura et al. (Omura et al. 1986). This engineered strain produced the novel polyketide antibiotic mederrhodin **36**, a hybrid antibiotic produced by introducing the actinorhodin gene cluster heterologously into the medermycin producer (Omura et al. 1986). As part of this work, a plasmid containing part of or the entire actinorhodin gene cluster were introduced into the medermycin producing *Streptomyces* sp. *strain AM7161* and ex-conjugants were screened for the production of novel secondary metabolites. Two new secondary metabolites were isolated, mederrhodin A **36a** and mederrhodin B **36b** (Fig. 12). Mederrhodin A **36a** showed similar gram negative antibacterial activity to medermycin **37** but mederrhodin B 36b showed no activity against all Gram-positive and Gram-negative bacteria tested.

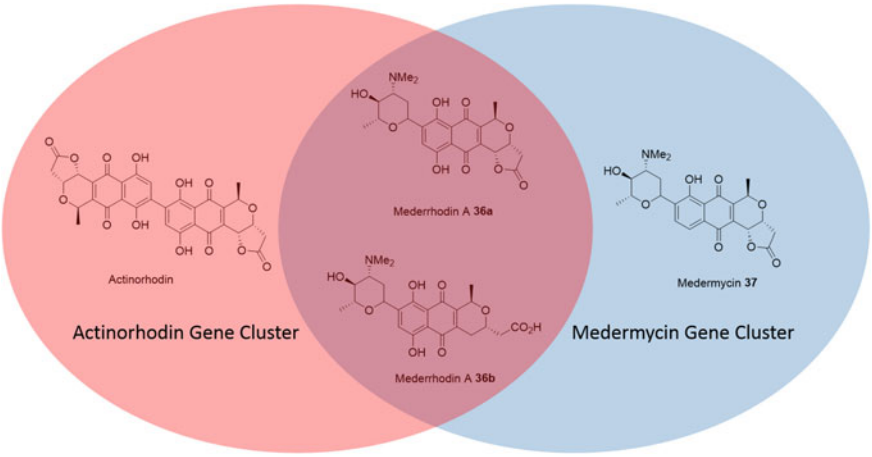


Fig. 12 Introduction of the actinorhodin gene cluster into the medermycin producer in a combinatorial biosynthesis approach yields the new antibiotics mederrhodin A **36a** and mederrhodin B **36b** (Omura et al. 1986)

Daptomycin **4** is a cyclolipopeptide used clinically for the treatment of skin infections caused by Gram-positive pathogens, bacteremia and endocarditis. Baltz and co-workers have generated analogues of daptomycin **4** by the exchange of single or multiple modules of the DptBC subunit of the NRPS and inactivation of a tailoring enzyme glutamic acid 3-methyltransferase from the producing organism *Streptomyces roseosporus*. Using this approach Baltz and co-workers were able to generate analogues with similar or better activities (Nguyen et al. 2006).

7.6 Genochemetics: *gene expression enabling synthetic diversification*

Genochemetics is a novel approach to the diversification of natural products. Described as 'gene expression enabling synthetic diversification (Roy et al. 2010), genochemetics uses molecular biological pathway engineering to add in a chemically orthogonal handle into a natural product that may be selectively modified using synthetic organic chemistry.

Genochemetics can be considered blending together complementary tools from combinatorial biosynthesis and semisynthesis. In genochemetics, an enzyme from an unrelated biosynthetic pathway that is capable of introducing a reactive, chemically orthogonal, and selectively functionalisable handle into a natural product of interest is genetically introduced into the producing organism. The system is designed such that the gene encoding the enzyme is expressed to enable the enzyme to be produced such that it acts in concert with the native biosynthetic machinery to

generate a modified, un-natural product—sometimes alongside the unmodified natural product. The presence of the reactive and chemically orthogonal handle enables subsequent chemical derivatisation to be performed selectively on the unnatural product, ideally as a component of the crude extracts and without need for prior purification or the use of protecting group chemistry.

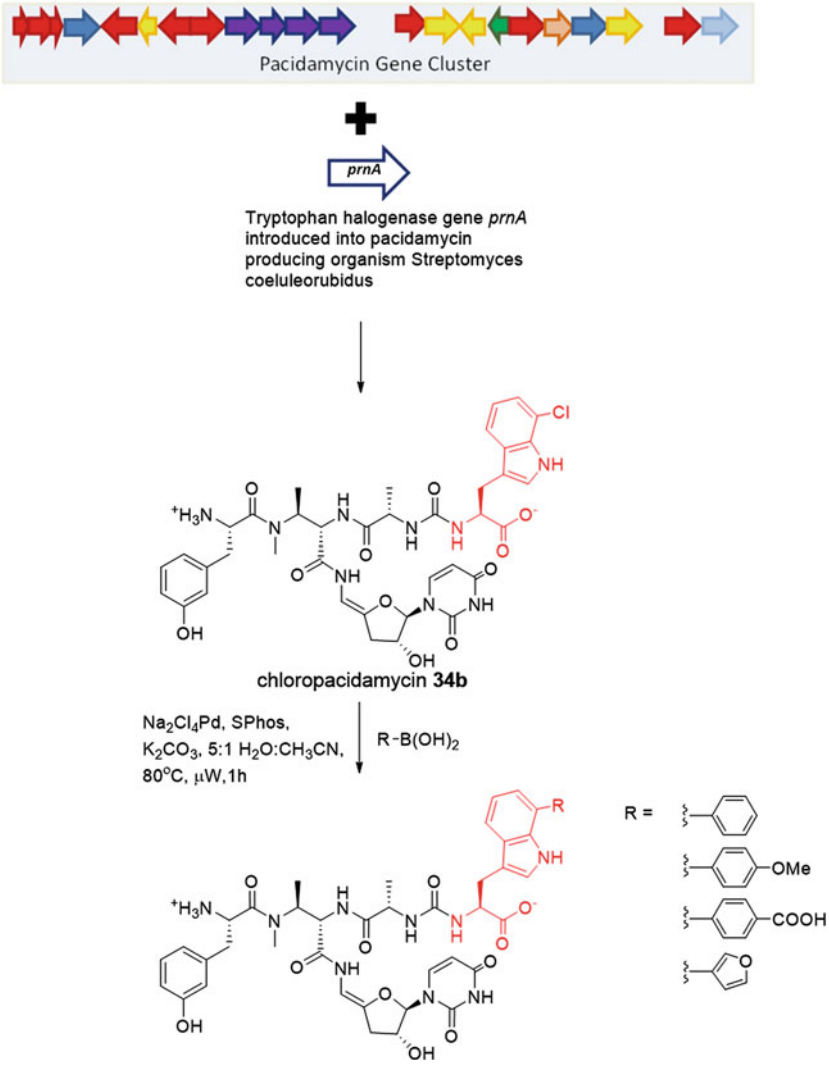
To facilitate the genochemetic approach to natural product diversification, handles must be enzymatically installable and should enable facile and selective chemistry (such as cross-coupling or click chemistries). Series of genetically installable handles are required and new mild chemistries that enable the selective modification of the new functionalised natural products. During the course of the Bluegenics programme, the Goss group have been particularly looking at new halogenases that might be developed and utilised within the genochemetic portfolio, investigating the stability of these natural and engineered compounds and developing new chemistries for the selective modification of halogenated natural and unnatural products.

The first example of genochemetic access to natural product analogues was demonstrated by the Goss group in 2010 (Scheme 6) using the uridyl peptide antibiotic pacidamycin **34** as the test bed (Roy et al. 2010). The aim was to biosynthetically introduce a carbon–halide bond, which could subsequently be modified using cross-coupling chemistries. The halotryptophan motif is found in several natural products, such as rebeccamycin **38** and pyrrolnitrin **39** (Fig. 13), and tryptophan halogenases have been widely studied (Smith et al. 2013). PrnA is responsible for the chlorination of tryptophan at the 7-position, which is the first step in pyrrolnitrin biosynthesis (Dong et al. 2005). The *prnA* gene was incorporated into the genome of the Pacidamycin producer *Streptomyces coeruleorubidus* under the control of the constitutive promoter *ermE**. This allowed production of the halogenase alongside the pacidamycin biosynthetic machinery, and resulted in in vivo generation of chlorinated pacidamycin **34b** (Scheme 6) (Roy et al. 2010).

The group developed this example of combinatorial biosynthesis into a full genochemitic system by exploiting the selective reactivity of the newly introduced halogen through aqueous, palladium-catalysed Suzuki-Miyaura cross-coupling chemistry under microwave irradiation, utilising a sulfonated form of the SPhos ligand developed by Buchwald et al. (Barder et al. 2005).

The conversion could be performed on the crude extracts of fermentation cultures without need for purification beforehand. Using a series of 5 boronic acids, pacidamycin analogues were produced and identified by LCMS/MS fragmentation patterns, in yields ranging from 33% to in excess of 95%. Two of these analogues, the phenyl and the *p*-methoxyphenyl substituted derivatives, were isolated in 52% and 67% respectively (Fig. 13) (Roy et al. 2010).

Recent work from the O'Connor group has presented the second example of genochemetic natural product analogue generation, this time in *Catharanthus roseus* as a development of their previous studies of the indole alkaloid metabolites of this plant (vide supra). The group employed cross-coupling chemistry similar to that previously used by the Goss group in order to functionalise halogenated metabolites generated both by precursor-directed biosynthesis and by combinatorial



Scheme 6 Integration of the tryptophan halogenase gene prnA into the pacidamycin **34** producer *Streptomyces coeluleorubidus* gave access to halogenated pacidamycin analogues which could be further functionalized as part of a genochematic approach using Suzuki-Miyaura cross-coupling chemistries, in aqueous solvents and under mild conditions (Roy et al. 2010)

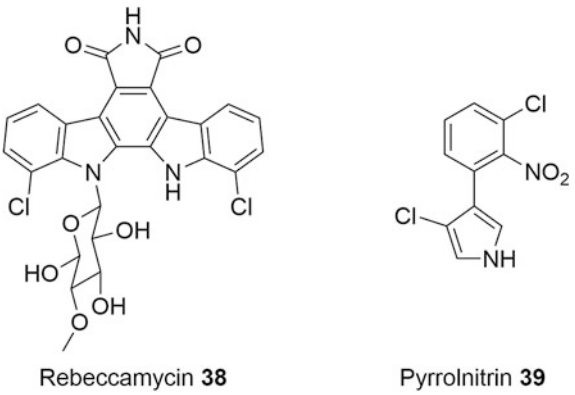


Fig. 13 Antibiotics such as rebeccamycin **38** and pyrrolnitrin **39** contain halogens derived from the flavin-dependent tryptophan halogenases RebH and PrnA. These enzymes can be introduced into other biosynthetic systems to generate halotryptophans in vivo

biosynthesis, with the latter giving the first eukaryotic genochemetic system (Roy et al. 2010; Runguphan and O'Connor 2013).

8 Concluding Remarks and Future Perspective

The marine environment holds much potential as a largely underexplored source of new compounds for human medicine. There is an urgent need to discover and develop new antibiotics with new scaffolds and modes of action, by carefully trawling the oceans new compounds to combat this crisis may be found. To fully exploit this potential new tools for the discovery of bioactive compounds are required as well as new approaches to finding, culturing and manipulating marine organisms and their biosynthetic pathways. By dovetailing biosynthetic and synthetic approaches analogues with designer properties may be accessed.

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