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REVIEW

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Microbial natural products: molecular blueprints for antitumor drugs

Lesley-Ann Giddings · David J. Newman

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Abstract Microbes from two of the three domains of life, the Prokarya, and Eukarya, continue to serve as rich sources of structurally complex chemical scaffolds that have proven to be essential for the development of anticancer therapeutics. This review describes only a handful of exemplary natural products and their derivatives as well as those that have served as elegant blueprints for the development of novel synthetic structures that are either currently in use or in clinical or preclinical trials together with some of their earlier analogs in some cases whose failure to proceed aided in the derivation of later compounds. In every case, a microbe has been either identified as the producer of secondary metabolites or speculated to be involved in the production via symbiotic associations. Finally, rapidly evolving next-generation sequencing technologies have led to the increasing availability of microbial genomes. Relevant examples of genome mining and genetic manipulation are discussed, demonstrating that we have only barely scratched the surface with regards to harnessing the potential of microbes as sources of new pharmaceutical leads/agents or biological probes.

Keywords Microbial natural product · Microbial genome · Cancer drugs · Clinical trial

The opinions expressed in this article are those of the authors and not necessarily those of the US government.

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Introduction

Although Man has used natural product extracts to treat diseases for thousands of years, the era of modern chemotherapy began in the 1940s with the use of nitrogen mustards, hormones, and folic acid antagonists. During World War II (1939–1945), the demand for penicillin, in addition to other new antibiotics, led to major pharmaceutical companies forming large research programs centered on natural products discovery. These programs aimed to identify new secondary metabolites with a variety of novel biological activities, including anticancer activities. During this time, academic laboratories and government agencies, such as the National Cancer Institute, also assisted pharmaceutical companies and spearheaded research programs that led to the discovery of many natural products, which are either currently used in the clinic or in active development.

The fields of microbiology, chemistry, and pharmacology have been instrumental in the search for novel agents that treat or ameliorate cancer. Throughout the last 70 years, natural products from the *Prokarya* predominately the actinomycetes, and *Eukarya* or their corresponding derivatives have played extremely important roles in drug discovery. Notably, microbial secondary metabolites, especially now from the marine environment, are the major source of drugs used for direct treatment as well as scaffolds upon which chemists can selectively modify to modulate activities against tumor cell growth. As a result of these efforts, there are currently 189 small molecule agents (excluding biologicals or vaccines, but including warheads on monoclonal antibodies) available worldwide that can be categorized as the following : N (29; 16 %), NB (1, 0.5 %), ND (59; 31 %), S (44; 23 %), S/NM (19; 10 %), S* (20; 11 %), and S*/NM (17; 9 %) [150–152]. The definitions are given as a footnote in Table 1.

Table 1 lists the 189 approved (by the FDA or equivalent) small molecule drugs, of which 89 (47 %) fall under the N, NB or ND classifications (see footnote in Table 1 for definitions) and to this number we have added Ara-C, which has also been reported from microbes, thus increasing the denominator to 90. Of these 90 natural product-related approved antitumor drugs, 30 (33 %) are either directly produced by microbes or are derivatives of microbial secondary metabolites. Of the remaining drugs on the list, some are based on pharmacophores derived from microbial natural products (e.g., vorinostat/mitoxantrone HCl), and nucleoside derivatives, which, with the exception of floxuridine (5-FU) and mercaptopurine, can be traced back to Bergmann's reports in the 1950s on sponge-derived arabinoside nucleosides [15–17], and thus the recognition that one can modify the sugar while still maintaining biological activity.

Since the 1990s, large pharmaceutical companies have deemphasized natural product discovery due to the advent of high-throughput screening programs based on molecular targets and combinatorial chemistry. These research programs were projected to speed up the drug development process and reduce costs. Ironically, instead of creating large libraries, combinatorial chemistry is now used to build small, focused collections that resemble the core scaffolds of natural products. In addition, even with this shift in focus, there is still a shortage of lead compounds entering clinical trials. Approximately 50 % of all small molecules that were approved by the FDA between 2000 and 2006 were not the new chemical entities derived from combinatorial chemistry but rather based upon natural products [150]. With increasingly available genomic sequences and the recent advances in metagenomic analyses, we now have more access to the sequences of biosynthetic gene clusters, especially those that are silent, which often may comprise a significant fraction of the microbial genome [181]. This review describes the brief history of nominally terrestrial microbial-sourced antitumor agents (secondary metabolites and derivatives based upon either their basic structure and/or pharmacophores), new agents based upon these scaffolds, and marine-sourced materials that are either presumptively by structural analogy or directly from microbes due to their chemical novelty and potency. Lastly, the influence of microbial genomic information on the discovery of new secondary metabolites as well as the potential sources of well-known plant-sourced anticancer agents and their implications will be discussed.

Terrestrial sources

During the 1950s to 1990s, pharmaceutical companies led an exhaustive search for new drugs produced by microbes

Table 1 All world-wide approved (1930s to 31DEC2012) small molecule antitumor drugs with identification of those either directly produced by microbes or are derivatives of microbial secondary metabolites

Generic name	Year introduced	Source	Microbe
Testosterone	Pre-1970	N	
Streptozocin	Pre-1977	N	Yes
Leucovorin	1950	N	Yes
Carzinophilin	1954	N	Yes
Sarkomycin	1954	N	Yes
Mitomycin C	1956	N	Yes
Chromomycin A3	1961	N	Yes
Mithramycin	1961	N	Yes
Vincristine	1963	N	
Actinomycin D	1964	N	Yes
Vinblastine	1965	N	
Bleomycin	1966	N	Yes
Doxorubicin	1966	N	Yes
Daunomycin	1967	N	Yes
Asparaginase	1969	N	Yes
Neocarzinostatin	1976	N	Yes
Aclarubicin	1981	N	Yes
Peplomycin	1981	N	Yes
Masoprocol	1992	N	
Pentostatin	1992	N	Yes
Paclitaxel	1993	N	
Angiotensin II	1994	N	
Arglabin	1999	N	
Paclitaxel nanoparticles[a]	2005	N	
Trabectedin	2007	N	Yes
Paclitaxel nanoparticles[a]	2007	N	
Romidepsin	2010	N	Yes
3-Angeloylingenol	2012	N	
Homoharringtonine	2012	N	
Solamargines	1989	NB	
Ethinyl estradiol	Pre-1970	ND	
Fluoxymesterone	Pre-1970	ND	
Hydroxyprogesterone	Pre-1970	ND	
Prednisone	Pre-1970	ND	
Fosfestrol	Pre-1977	ND	
Norethindrone acetate	Pre-1977	ND	
Prednisolone	Pre-1977	ND	
Methylprednisolone	1955	ND	
Dexamethasone	1958	ND	
Medroxyprogesterone acetate	1958	ND	
Triamcinolone	1958	ND	
Nandrolone phenylpropionate	1959	ND	
Dromostanolone	1961	ND	
Teniposide	1967	ND	
Testolactone	1969	ND	

Table 1 continued

Generic name	Year introduced	Source	Microbe
Megestrol acetate	1971	ND	
Calusterone	1973	ND	
Methyltestosterone	1974	ND	
Mitobronitol	1979	ND	
Vindesine	1979	ND	
Estramustine	1980	ND	
Etoposide	1980	ND	
Elliptinium acetate	1983	ND	
Epirubicin hydrochloride	1984	ND	Yes
Triptorelin	1986	ND	
Pirarubicin	1988	ND	Yes
Vinorelbine	1989	ND	
Idarubicin hydrochloride	1990	ND	Yes
Cladribine	1993	ND	
Cytarabine ocfosfate	1993	ND	Yes
Formestane	1993	ND	
Miltefosine	1993	ND	
Irinotecan hydrochloride	1994	ND	
Zinostatin stimalamer	1994	ND	
Docetaxel	1995	ND	
Etoposide phosphate	1996	ND	
Topotecan hydrochloride	1996	ND	
Alitretinoin	1999	ND	
Exemestane	1999	ND	
Valrubicin	1999	ND	Yes
Gemtuzumab ozogamicin	2000	ND	Yes
Amrubicin hydrochloride	2002	ND	Yes
Fulvestrant	2002	ND	
Belotecan hydrochloride	2004	ND	
Hexyl aminolevulinate	2004	ND	
Talaporfin sodium	2004	ND	
Vapreotide acetate	2004	ND	
Temsirolimus	2007	ND	Yes
Ixabepilone	2007	ND	Yes
Pralatrexate	2009	ND	
Mifamurtide	2010	ND	Yes
Vinflunine	2010	ND	
Cabazitaxel	2010	ND	
Eribulin	2010	ND	
Abiraterone acetate	2011	ND	
Brentuximab vedotin	2011	ND	
BF-200 ALA	2012	ND	
Carfilzomib	2012	ND	Yes
Pasireotide	2012	ND	
Diethylstilbestrol	Pre-1970	S	
Razoxane	Pre-1977	S	
Semustine (MCCNU)	Pre-1977	S	
Chlorotrianisene	Pre-1981	S	
Levamisole	Pre-1981	S	
Nimustine hydrochloride	Pre-1981	S	
Triethylenemelamine	Pre-1981	S	
Busulfan	1954	S	
Chlorambucil	1956	S	
Cyclophosphamide	1957	S	
Mechlorethamine	1958	S	
Thiotepa	1959	S	
Melphalan	1961	S	
Pipobroman	1966	S	
Hydroxyurea	1968	S	
Procarbazine	1969	S	
Mitotane	1970	S	
Dacarbazine	1975	S	
Ifosfamide	1976	S	
Lomustine (CCNU)	1976	S	
Carmustine (BCNU)	1977	S	
cis-Diamminedichloroplatinum	1979	S	
Hexamethylmelamine	1979	S	
Aminoglutethimide	1981	S	
Flutamide	1983	S	
Carboplatin	1986	S	
Amsacrine	1987	S	
Lonidamine	1987	S	
Nilutamide	1987	S	
Ranimustine	1987	S	
Fotemustine	1989	S	
Bisantrene hydrochloride	1990	S	
Porfimer sodium	1993	S	
Sobuzoxane	1994	S	
Nedaplatin	1995	S	
Oxaliplatin	1996	S	
Lobaplatin	1998	S	
Heptaplatin/SK-2053R	1999	S	
Arsenic trioxide	2000	S	
Zoledronic acid	2000	S	
Sorafenib	2005	S	
Plerixafor hydrochloride	2009	S	
Miriplatin hydrate	2010	S	
Vismodegib	2012	S	
Nafoxidine	Pre-1977	S/NM	
Tamoxifen	1973	S/NM	
Camostat mesylate	1985	S/NM	
Toremifene	1989	S/NM	
Anastrozole	1995	S/NM	
Bicalutamide	1995	S/NM	
Fadrozole hydrochloride	1995	S/NM	
Letrozole	1996	S/NM	

Table 1 continued

Generic name	Year introduced	Source	Microbe
Imatinib mesylate	2001	S/NM	
Gefitinib	2002	S/NM	
Temoporfin	2002	S/NM	
Bortezomib	2003	S/NM	
Erlotinib hydrochloride	2004	S/NM	
Sunitinib malate	2006	S/NM	
Dasatinib	2006	S/NM	
Lapatinib ditosylate	2007	S/NM	
Nilotinib hydrochloride	2007	S/NM	
Pazopanib	2009	S/NM	
Enzalutamide	2012	S/NM	
Azacytidine	Pre-1977	S*	
Mercaptopurine	1953	S*	
Methotrexate	1954	S*	
Fluorouracil	1962	S*	
Thioguanine	1966	S*	
Uracil mustard	1966	S*	
Cytosine arabinoside	1969	S*	Yes
Floxuridine	1971	S*	
Ftorafur	1972	S*	
Carmofur	1981	S*	
Enocitabine	1983	S*	
Mitoxantrone hydrochloride	1984	S*	
Doxifluridine	1987	S*	
Fludarabine phosphate	1991	S*	
Gemcitabine hydrochloride	1995	S*	
Capecitabine	1998	S*	
Azacytidine	2004	S*	
Clofarabine	2005	S*	
Nelarabine	2006	S*	
Decitabine	2006	S*	
Pixantrone maleate	2012	S*	
Raltitrexed	1996	S*/NM	
Temozolomide	1999	S*/NM	
Bexarotene	2000	S*/NM	
Abarelix	2004	S*/NM	
Pemetrexed disodium	2004	S*/NM	
Tamibarotene	2005	S*/NM	
Vorinostat	2006	S*/NM	
Degarelix	2009	S*/NM	
Vandetanib	2011	S*/NM	
Crizotinib	2011	S*/NM	
Vemurafenib	2011	S*/NM	
Ruxolitinib phosphate	2011	S*/NM	
Axitinib	2012	S*/NM	
Bosutinib	2012	S*/NM	
Cabozantinib *S*-malate	2012	S*/NM	
Regorafenib	2012	S*/NM	
Radotinib	2012	S*/NM	

N natural product, *NB* natural product botanical (a defined mixture of compounds), *ND* derived from a natural product; usually a semi-synthetic modification, *S* totally synthetic drug, *S** made by total synthesis; pharmacophore from a natural product, *NM* natural product mimic (sub-category) [150, 152]

[a] Quite different preparations

from a variety of terrestrial environments. Based on the biological activities of the novel metabolites identified from these microbes, the bacteria of the order *Actinomycetales* have undoubtedly produced some of the most utilized structures directly from nature and semisynthetic modifications. These soil bacteria have been the leading producers of medically useful drugs and have provided also basic templates for synthetic modifications. Other rich sources of secondary metabolites include fungi and myxobacteria. Here, we highlight some of the key secondary metabolites isolated from terrestrial microbes that have been approved as drugs or provided significant leads in the drug discovery process.

Actinomycins, anthracyclines, and bleomycins

Waksman's discovery of the actinomycins in various species of soil *Streptomyces* [226] in 1940 led to many firsts in 1952, namely actinomycin C was the first crystalline antibiotic as well as the first antibiotic to demonstrate in vitro antitumor activity [76]. That same year, Schulte reported the first clinical studies with these agents [183]. In 1963, actinomycin D (Act D, **1**; Fig. 1) was approved for the treatment of highly malignant tumors. However, its use has been limited by its extreme cytotoxicity, thus doses of Act D are carefully calculated for each patient to primarily treat rhabdomyosarcoma and Wilms' tumor in children or for use in combination regimens.

Act D consists of a planar 2-aminophenoxazin-3-one chromophore and two large cyclic pentapeptide lactones. This agent is a DNA intercalator that competes for transcription factor DNA binding sequences, thereby inhibiting RNA and protein synthesis [70]. The antitumor activity of Act D has been reported to be attenuated by polyamines, such as spermine, but if a polyamine inhibitor such as methyl glyoxal-bis(guanylhydrazone) (MGBG) was added to the Act D treatment, then a synergistic effect was seen in a cell proliferation assay. Thus it is possible that such a combination can be exploited to enhance the anticancer

activity of the drug [228]. Several derivatives have been identified in various soil *Streptomyces* [19] and developed using precursor-directed biosynthesis and synthetic methods to modulate their cytotoxicity [134, 135]. In addition to terrestrial sources, Act D has also been isolated from a marine-derived strain (*Streptomyces* sp. MS449) from a sediment sample collected from the South China Sea [32].

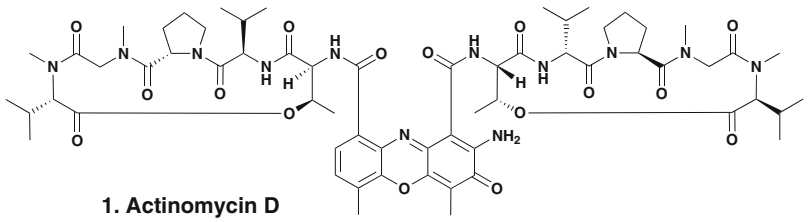
Anthracyclines

Following the discovery of the actinomycins, a wide variety of antibiotics, including the bleomycins, mitomycins, mithramycins, and anthracyclines, from terrestrial microbial sources were tested for antitumor activity and their core structures were subsequently developed for clinical use. Two of the most useful anthracyclines are daunorubicin (**2**; Fig. 1) and doxorubicin (adriamycin, **3**; Fig. 1) isolated from *Streptomyces peucetius* and various related strains. The FDA approved the use of daunorubicin and doxorubicin for cancer therapy in the 1960s. Daunorubicin is used in the treatment of acute lymphoblastic or myeloblastic lymphoma, whereas doxorubicin, which terminates in a primary alcohol instead of a methyl group, is used in the treatment of breast cancer, solid tumors in children, soft tissue sarcomas, and aggressive lymphomas. The major drawbacks to these compounds are their significant cardiotoxicity, which can lead to congestive heart failure, the development of resistance by tumor cells, and the fact that there is lifetime limit on the gross amounts used for treatment. To circumvent these problems, thousands of analogs of anthracyclines have been structurally modified or synthesized through semi- or total synthesis, but only a few compounds have reached the stage of clinical development and approval. These include the anthracycline analogs epirubicin (**4**; Fig. 1), pirarubicin (**5**; Fig. 1), idarubicin (**6**; Fig. 1), valrubicin (**7**; Fig. 1), amrubicin (**8**; Fig. 1), aclarubicin (**9**; Fig. 1), and a combination of anthracycline and anthracene dione structural classes, mitoxantrone hydrochloride (**10**; Fig. 1) and pixantrone dimaleate (**11**; Fig. 1). A comprehensive review of anthracyclines can be found in the two recent volumes edited by Krohn [115, 116].

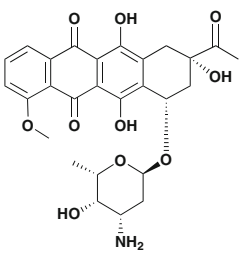
The following chemical modifications of the basic doxorubicin skeleton are currently being investigated in Phase I–II trials: sabarubicin hydrochloride (Menarini; ClinicalTrials.gov identifiers: NCT00027781, NCT00003982, and NCT00003028; **12**; Fig. 1), in which the major structural modification is the addition of a sugar moiety, completed Phase II clinical trials for the treatment of prostate cancer; annamycin, a liposomal variant of doxorubicin (Callisto Pharmaceuticals Inc; NCT00271063; **13**; Fig. 1) is in Phase I/II clinical trials for the treatment of leukemia; and berubicin hydrochloride (Reata Pharmaceuticals Inc; NCT00538343; **14**; Fig. 1), in which a benzyl ether is attached to the sugar moiety, completed Phase I clinical trials (NCT00538343) but is no longer in Phase II clinical trials for breast cancer patients with recurrent brain metastases due to low enrollment. In addition, several liposomal and PEGylated formulations are currently being explored. The FDA approved the use of the liposome-encapsulated doxorubicin Doxil® in combination regimens for the treatment of mainly recurrent ovarian cancer; however, the side effects are similar to those of free doxorubicin. As a result, other drug delivery systems, such as monoclonal antibody (NCT01101594) and peptide conjugates (NCT01698281), nanoparticles (NCT01655693), liposomes (NCT01170650) [30], and carbohydrates [202], several of which are currently in Phase I–III clinical trials, are now being explored for increased efficiency and direct uptake by cancer cells.

Bleomycins

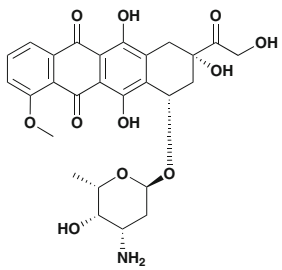
Another series of extremely important molecules from the *Actinomycetales* are the family of glycopeptide antibiotics known as the bleomycins, particularly bleomycin A_2 sulfate (**15**; Fig. 2). Bleomycins share a core structure but differ based on the presence of various positively charged functional groups and disaccharides. These molecules were originally isolated from *Streptomyces verticillus* by Umezawa's group at the Institute of Microbial Chemistry in Tokyo, and were developed as antitumor agents by Bristol Myers. Bleomycins are currently used in the treatment of squamous cell carcinomas, germ cell tumors, and select lymphomas. Their original mechanism of action was elucidated by Hecht and coworkers who demonstrated that a metal ion (Cu^{2+} or Fe^{2+}) is required to activate the sequence-specific oxidative cleavage of the DNA [83, 196] and RNA [84]. The early work in the synthesis, mechanism, and DNA–RNA interactions of bleomycins were described by Hecht in a 2005 review [85] with an update in 2012 [86]. In 2006 a potential RNA target for bleomycins was reported [205], and in 2008, strong DNA-binding motifs for metal-free bleomycins (since the clinical relevance of zinc and iron-bound bleomycin is unknown) were identified [5]. The therapeutic efficacy of bleomycin(s) is limited due to its extreme pulmonary toxicity. A new bleomycin antibiotic, NC-0604 (**16**; Fig. 2), was isolated from the fermentation broth of *Streptomyces verticillus* var. *pingyangensis* n. sp. and reported to exhibit stronger cytotoxicity in a variety of human tumor cell lines compared to bleomycin [31]. Notably, NC-0604 has lower pulmonary toxicity compared to other bleomycins and is currently in preclinical trials. Alternative drug delivery



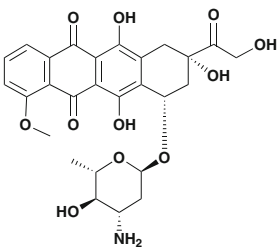
1. Actinomycin D



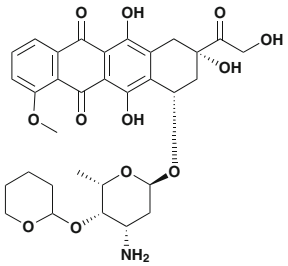
2. Daunorubicin



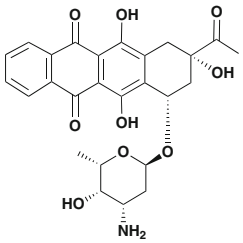
3. Doxorubicin



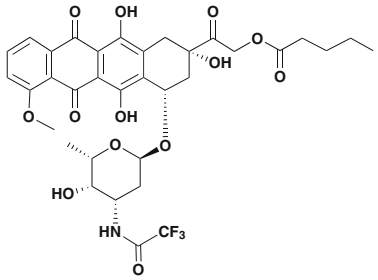
4. Epirubicin



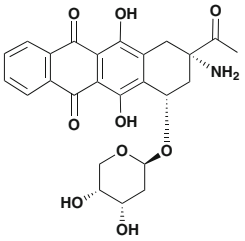
5. Pirarubicin



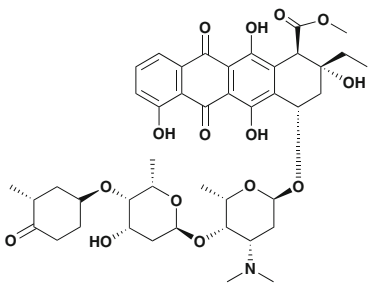
6. Idarubicin



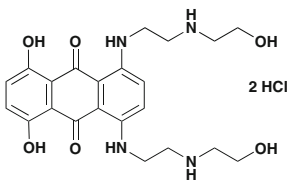
7. Valrubicin



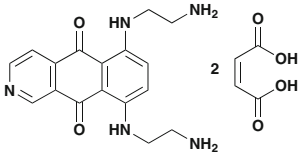
8. Amrubicin



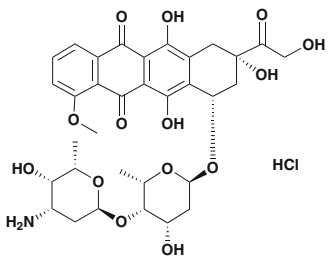
9. Aclarubicin



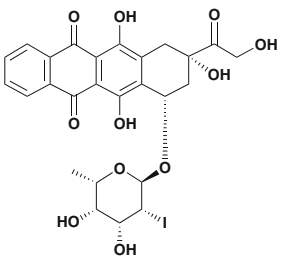
10. Mitoxanthrone hydrochloride



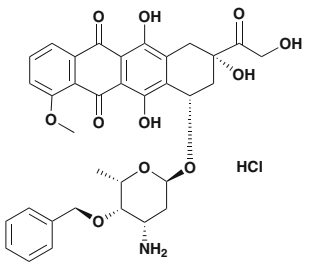
11. Pixantrone maleate



12. Sabarubicin hydrochloride



13. Annamycin

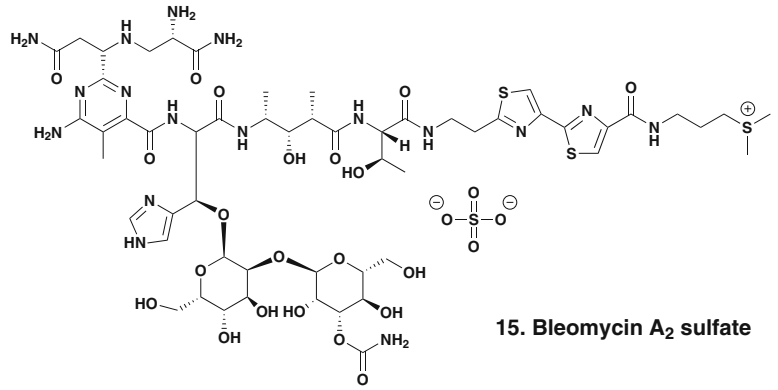


14. Berubicin hydrochloride

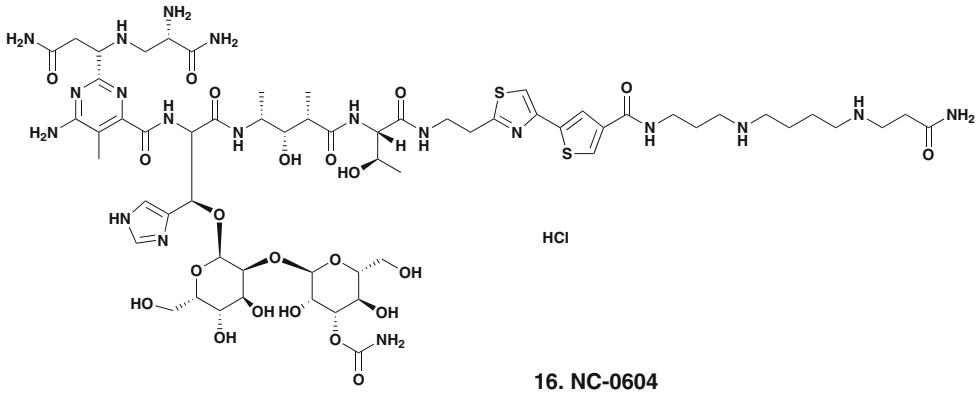
◀**Fig. 1** The chemical structures of actinomycin D and representative anthracyclines. (**1**) Actinomycin D, (**2**) daunorubicin, (**3**) doxorubicin, (**4**) epirubicin, (**5**) pirarubicin, (**6**) idarubicin, (**7**) valrubicin, (**8**) amrubicin, (**9**) aclarubicin, (**10**) mitoxantrone hydrochloride, (**11**) pixantrone maleate, (**12**) sabarubicin hydrochloride, (**13**) annamycin, and (**14**) berubicin hydrochloride

methods, such as photochemical internalization, are also currently being investigated in Phase II clinical trials to improve the incorporation of bleomycins into endosomal membranes for the treatment of recurrent squamous cell carcinoma (NCT01606566).

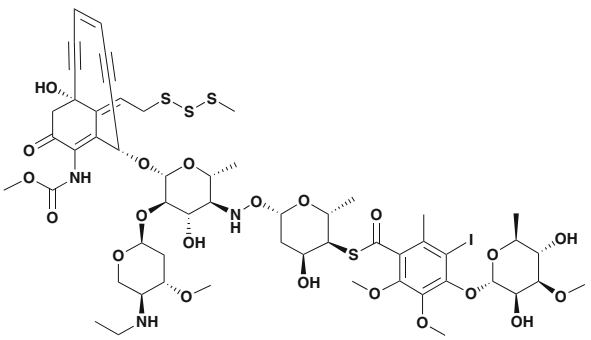
Fig. 2 The chemical structures of representative bleomycins and enediynes. (**15**) Bleomycin A_2 sulfate, (**16**) NC-0604, (**17**) calicheamicin-$\gamma 1'$, (**18**) sporalide A, (**19**) sporalide B, (**20**) cyanosporaside A, and (**21**) cyanosporaside B



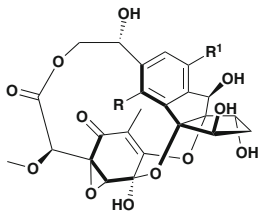
15. Bleomycin A_2 sulfate



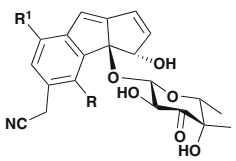
16. NC-0604



17. Calicheamicin-$\gamma 1^I$



18. Sporolide A; R = Cl, R^1 = H
19. Sporolide B; R = H, R^1 = Cl



20. Cyanosporaside A; R = Cl, R^1 = H
21. Cyanosporaside B; R = H, R^1 = Cl

Enediynes

The enediynes are a structurally unique class of antitumor antibiotics, containing one of the most important approved microbial compounds, calicheamicin $\gamma 1^I$ (**17**; Fig. 2). The ten-membered calicheamicins were first reported in 1987 from the bacterium *Micromonospora echinospora* spp. *calichensis*. Although calicheamicin $\gamma 1^I$ has sub-picomolar in vitro cytotoxic activity it was not developed for many years due to its extreme cytotoxicity. It and a close relative, dynemicin A, became the progenitors of a new chemical class of natural products, the enediynes. This class now comprises 13 enediynes, including the recognition of the much older neocarzinostatin chromophore [79] and the rearrangement products of the putative enediynes sporolides A and B (**18** and **19**, respectively; Fig. 2) and cyanosporasides A and B (**20** and **21**, respectively; Fig. 2). The enediyne core is composed of two acetylenic groups conjugated by a double bond within either a nine- or ten-membered ring. Enediynes have been thoroughly investigated as both warheads and from a biosynthetic perspective by the Wyeth discoverers [79] with an update in 2012 [80], who described calicheamicin $\gamma 1^I$ as a representative ten-membered enediyne core, and members of Shen's research group at the University of Wisconsin-Madison [218].

The biosynthesis of the enediyne core structure remains to be fully elucidated. Shen's group demonstrated the lack of biosynthetic divergence between the enediyne-specific iterative type I polyketide synthases involved in the biosynthesis of nine- and ten-membered enediynes, by demonstrating that the same major product (a heptaene metabolite) was made with all combinations of nine- and ten-membered polyketide synthases and cognate thioesterase domains, indicating that there were no pathway-specific interactions [95]. However, more recently, Belecki et al. [14] speculated that heptaene was a shunt product and showed that, in the absence of its cognate thioesterase domain, a heterologously expressed CALE8 polyketide synthase produces the expected octaketide polyene product with a beta-hydroxy moiety. The biosynthesis of the enediyne core will become clearer in the near future as the role of the unique beta-hydroxy group in the cyclization of enediynes is fully elucidated.

Upon activation, enediynes undergo an unprecedented rearrangement and interact with DNA, resulting in cleaved double-stranded DNA and subsequent cell death. In 2000, the FDA approved gemtuzumab ozogamicin, an anti-CD33 humanized antibody linked to a semi-synthetic calicheamicin derivative as the first antibody-warhead conjugate for use against chronic myeloid leukemia and possibly the most potent approved antitumor drug to date. This particular construct was subsequently withdrawn by Wyeth in 2010 from the US market. However, at the time of writing, the compound is still in use in Japan and there are at least six clinical trials in the USA and other countries currently recruiting patients for Phase II to Phase IV trials. Calicheamicins linked to other monoclonal antibodies are also being investigated. Phase I clinical trials of CMD-193, an anti-Lewis Y antigen linked to a calicheamicin toxin for the treatment of solid tumors were completed [88]. CMC-544 (inotuzumab ozogamicin), an anti-CD22 monoclonal antibody conjugated to *N*-acetyl-γ calicheamicin 1,2-dimethyl hydrazine dichloride is in Phase III trials in combination with rituximab (NCT01232556 and NCT01564784) coupled to other cytotoxic drugs. The conjugate was also in a recently completed Phase II trial (NCT00868608) for the treatment of indolent non-Hodgkin's lymphoma. The detailed review by Ricart [171] should be consulted for further information on the impacts of monoclonal antibodies attached to *N*-acetyl-γ calicheamicin 1,2-dimethyl hydrazine dichloride and read together with information in the 2012 update by Hamann et al. [80].

Rapamycins and epothilones

The rapamycins and epothilones are two exemplary families of compounds that have demonstrated how modern medicinal chemistry can be used to produce microbial antitumor agents and other pharmaceuticals that are currently either approved for use or in clinical trials.

Rapamycins

The 31-membered macrocyclic antibiotic rapamycin (**22a**) was originally reported in 1975 to be a potential antifungal agent produced by the fermentation of *Streptomyces hygroscopicus* isolated from soil samples in Rapa Nui (Easter Island) [11, 185, 220]. Rapamycin was unsuccessful as an antifungal agent due to its immunosuppressant effects. However, its potential as an antitumor agent against syngeneic murine tumors was later reported in 1984 by Sehgal and coworkers [58] at Ayerst Research Laboratories. At this time, the initial antitumor activity of rapamycin was not further developed, but the rapamycin base structure (**22**) has since led to the production of several molecules with a variety of different pharmacological activities. In the early 1990s, rapamycin's molecular target (target of rapamycin, TOR) in yeast [87] and its mammalian homolog (mTOR) [24] were discovered, leading to the development of a wide variety of anticancer agents.

Initially, modifications were made at the carbon atom at C^{43} on the rapamycin base structure (numeration as in Zech et al. [243] rather than the alternative numbering system of McAlpine et al. [136] based upon a comparison with FK506, which would be a C^{40} substitution), leading to a

total of four clinically approved drugs, sirolimus, everolimus, temsirolimus, and zotarolimus. In 1999, sirolimus (rapamycin) (**22a**; Fig. 3) was approved as an immunosuppressive agent and now the same molecule is in Phase I/II and II trials for the treatment of various cancers. Similarly, everolimus (**22b**; Fig. 3) was initially launched in 2004 as an immunosuppressive agent and subsequently in 2009, 2010, 2011, and 2012 approved for the treatment of kidney, brain, pancreatic, and breast cancers, respectively. In 2012, everolimus was released by Abbot to be used as a stent in the treatment of coronary and peripheral arterial diseases in the USA. This compound is also in Phase III trials for diffuse large B cell lymphoma (NCT00790036), liver (NCT01035229), and stomach (NCT00879333) cancers. Temsirolimus (CCI-779) (**22c**; Fig. 3) was approved as a treatment for renal carcinoma in the USA in 2007, later approved in Japan in 2010, and is currently in Phase II trials for the treatment of various carcinomas in the USA, mainly under the support of the National Cancer Institute. Zotarolimus (**22d**; Fig. 3) was launched in the USA in 2005 for the treatment of arterial restenosis and recently, the EU approved a stent containing novolimus, a metabolite of rapamycin that has a C^{7}-hydroxy group.

Currently, Merck & Co. and Ariad Pharmaceuticals have collaborated to develop another rapamycin derivative, ridaforolimus (AP-23573); **22e**; Fig. 3), which is in Phase III clinical trials for the treatment of soft tissue carcinoma (NCT00538239) and bone cancer (NCT00538239). Wyeth Pharmaceuticals developed a rather interesting derivative of rapamycin with a modified ring structure (**23**; Fig. 3), ILS-920. The modification of the triene portion of the molecule was designed to disrupt mTOR binding. However, ILS-920 appears to have a different target as it is a non-immunosuppressive neurotrophic rapamycin analog that has been reported to exhibit over a 900-fold higher binding affinity for FKBP52 over FKBP12 compared to that of rapamycin, promotes neuronal survival and outgrowth in vitro, and binds to the β1 subunit of L-type calcium channels (CACNB1) [179]. ILS-920 was under development for treating stroke [2] and a Phase I clinical trial for the treatment of acute ischemic stroke (NCT00827190) was completed. Interestingly, FKBP52 inhibition affects tubulin interactions in cells [28] and has been exploited to screen natural products that inhibit the formation of a complex between FKBP52 and androgen receptors, which play a role in the progression of prostate cancer [48]. Thus, ILS-920 may exhibit antitumor activity, although no reports of such activity have been published yet.

In addition, there are two prodrugs of rapamycin, Abraxis' ABI-009 (a nanoparticle encapsulated formulation of rapamycin) and Isotechnika's TAFA-93 (structure not yet published), both in Phase I clinical trials. The structures of these molecules either include the rapamycin core structure or only have modifications at the C^{43} hydroxyl group, avoiding both the FKBP-12 and TOR binding sites as modifications anywhere else are thought to negate the basic biological activity of these derivatives [111, 148].

Epothilones

The identification of the 16-membered macrolides epothilones A and B (**24** and **25**, respectively; Fig. 3) from *Sorangium cellulosum* So ce90 by Reichenbach and Hoefle in the mid to late 1980s [92, 93, 169], and their activity as tubulin stabilizers (a similar mechanism to that of paclitaxel) [20], led to a surge in the number of chemical, biochemical, and genomic modifications to further explore the utility of the epothilone base skeleton. These efforts led to the FDA approval of Bristol-Myers' 16-aza-epothilone B (**26**; Ixabepilone; Fig. 3), a semisynthetic epothilone, where the lactone bridge was replaced by an amide linkage, for the treatment of breast cancer in October 2007.

Epothilone A was determined to be less active in preclinical trials than epothilone B [112], which has subsequently advanced to further stages of clinical development. Interestingly the only difference between these two compounds is the presence of a methyl group at C^{12}. Epothilone B was in Phase III clinical trials for the treatment of ovarian cancer, but these trials were discontinued by Novartis Oncology in 2010 because the product did not demonstrate a significant overall survival advantage. Currently, epothilone B is in Phase II trials for the treatment of various cancers, including central nervous system metastases (NCT00450866), as well as prostate (NCT00411528) and brain (NCT00219297). In addition, a synthetic epothilone derivative, sagopilone (ZK-EPO, **27**; Fig. 3), a close chemical relative of epo B is in Phase II trials under Bayer-Schering for the treatment of melanomas (NCT00598507) and other cancers. For further details on the epothilones and their mechanisms of action, see the latest review by Ferrandina and colleagues [64] in addition to those by Danishefsky [45] and Altmann and colleagues [6], which describe opportunities for the synthesis of epothilones and other derivatives.

Additional agents have been derived from work originating in Danishefsky's laboratory at Memorial Sloan-Kettering and further developed by Kosan Biosciences. Phase II trials with (*E*)-9,10-didehydroepothilone D or KOS-1584 (**28**; Fig. 3) for the treatment of non-small cell lung cancer were completed but no recent development of this compound has been reported [232]. Isoxazolefludelone or KOS-1803 (**29**; Fig. 3) is another derivative that has been jointly developed by Memorial Sloan Kettering and Kosan Biosciences [37]. This compound is currently in

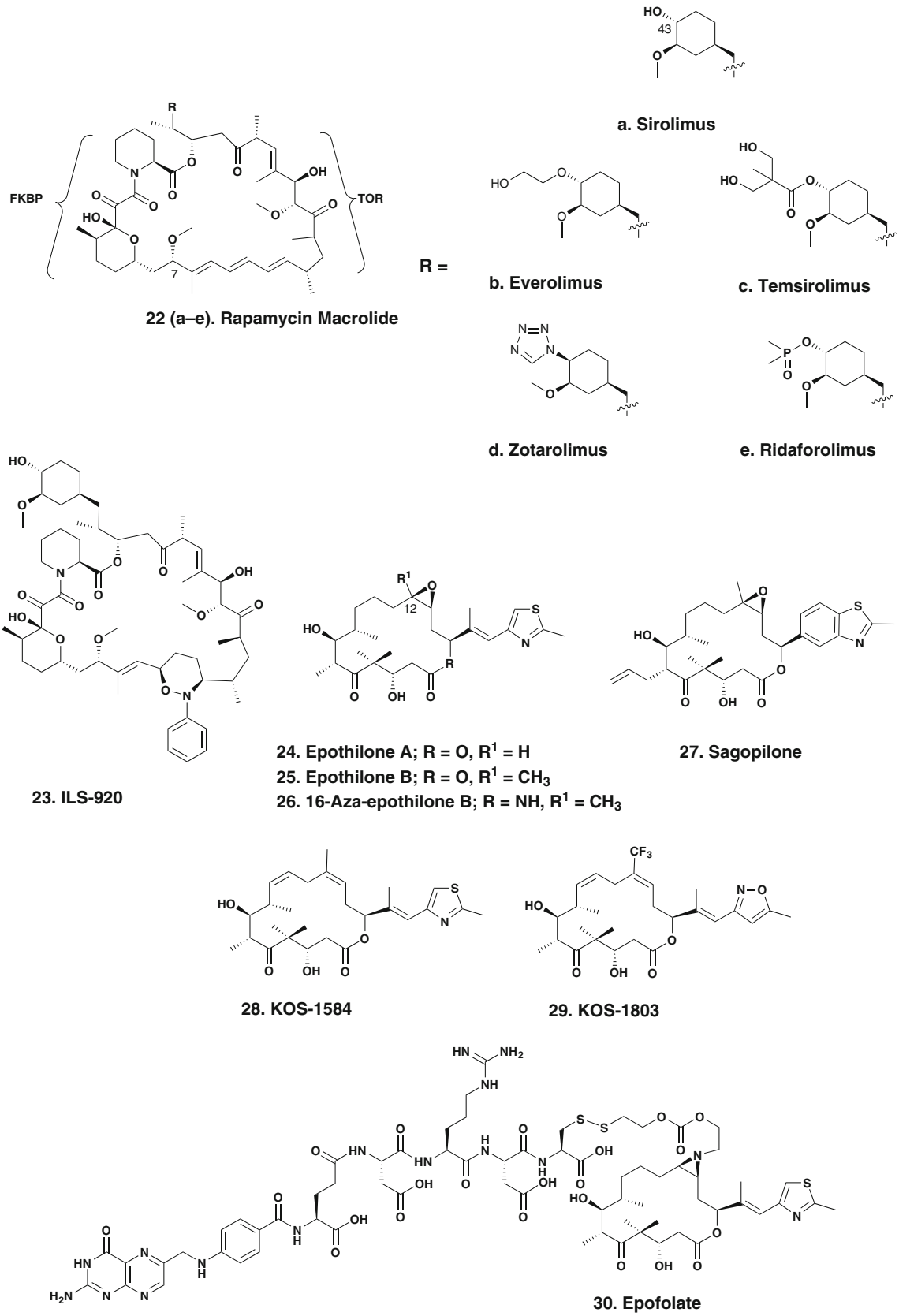


Fig. 3 The chemical structures of representative rapamycins and epothilones. (**22a**) Sirolimus, (**22b**) everolimus, (**22c**) temsirolimus, (**22d**) zotarolimus, (**22e**) ridaforolimus, (**23**) ILS-920, (**24**) epothilone A, (**25**) epothilone B, (**26**) 16-aza-epothilone B, (**27**) sagopilone, (**28**) KOS-1584, (**29**) KOS-1803, and (**30**) epofolate

Phase I clinical trials (NCT01379287) for the treatment of solid tumors. A discussion of the chemistry leading to KOS-1803 and other derivatives was published by Danishefsky's research group in 2008 [38].

A collaboration between Endocyte (folate targeting) and Bristol-Myers Squibb (epothilone derivative) led to the development of a folate receptor-targeted molecule, epofolate (BMS-753493; **30**; Fig. 3), in which folic acid was linked to an aza-modified epothilone [119], with the synthetic information covered in a 2010 publication from Endocyte (folate linker) and Bristol-Myers Squibb (azaepothilone) [222]. Epofolate was in early clinical trials at Bristol-Myers Squibb for the treatment of patients with advanced solid tumors. However, these trials terminated in 2011 but to date there have been no publications covering the results or reasons for cessation.

With the availability of the genetic sequence of the *S. cellulosum* myxobacterium, more combinatorial biosynthetic products with modifications to the epothilone base skeleton are being reported. For example, Menzella and coworkers [139] have reported the whole-cell biocatalysis of novel epothilone analogs using *Escherichia coli*. In addition, Tang and colleagues have reengineered epothilone polyketide synthases found in *S. cellulosum* and introduced them into another myxobacterium (*Myxococcus xanthus*) that is more amenable to genetic manipulation [204]. For additional details on epothilones produced via genetic engineering, see the review by Park and coworkers [161].

Geldanamycin derivatives and HSP90 inhibitors

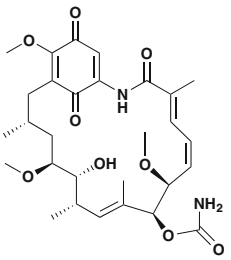
The production of the benzoquinone ansamycin antibiotic geldanamycin (**31**; Fig. 4) by S*treptomyces hygroscopicus* var *geldanus* was first reported by The Upjohn Company in 1970 [50]. Subsequent studies revealed that this compound had antitumor properties, which were initially thought to result from the inhibition of the tyrosine specific kinase (v-Src) involved in regulating growth and cell proliferation as well as several signal transduction pathways [216, 217]. In 1994 however, this compound was determined to bind to heat shock protein 90 (HSP90) by Whitesell and coworkers [233]. In 1997, Stebbins et al. reported that geldanamycin specifically binds to an ATP site on HSP90, altering its chaperone activity and indirectly leading to cell death [192]. The history of the various modifications of geldanamycin and the initial development of tanespimycin (17-AAG; Kosan Biosciences/Institute of Cancer Research UK/NCI; **32**) was described by Snader in 2005 [190], with further details of the biological activity reported by Kingston and Newman [109] in 2008, culminating in a 2012 review by Snader [191].

The geldanamycin derivative tanespimycin entered clinical trials in 1999 as the first example of a signal transduction modulator under the auspices of the NCI and was subsequently licensed to Kosan Biosciences for development. Phase III trials of tanespimycin for the treatment of relapsed and refractory multiple myeloma were completed and the compound is still in Phase II trials for the treatment of thyroid cancer. The 18,21-dihydro-derivative of tanespimycin, IPI-504 or retaspimycin (Infinity Pharmaceuticals; **33**; Fig. 4) [200], is in Phase II clinical trials for the intravenous treatment of relapsed or refractory stage IIIb or stage IV non-small cell lung cancer (NSCLC) and other solid tumors. Due to the superiority of retaspimycin, Infinity Pharmaceuticals is no longer developing the oral formulation of 17-aminogeldanamycin (**34**; Fig. 4; IPI-493), which was in Phase I trials for the treatment of advanced malignancies [121]. In the future, we expect reports of several other derivatives as the genes involved in geldanamycin biosynthesis are known. Novel analogs, such as thiazinogeldanamycin (**35**; Fig. 4) and 19-hydroxy-4,5-dihydrogeldanamycin (**36**; Fig. 4), have already been reported from engineered strains of *S. hygroscopicus* JCM4427 in addition to other known derivatives [34].

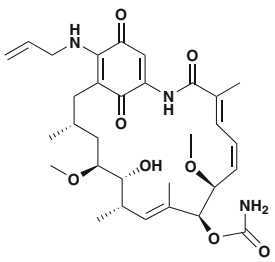
Staurosporine derivatives

The indolocarbazole alkaloid staurosporine (**37**; Fig. 4) was first identified as an antifungal agent by Omura et al. in 1977 [159] and later reported as a nanomolar protein kinase C (PKC) inhibitor by Tamaoki et al. [201] in 1986. These results led to several pharmaceutical companies searching for selective PKC inhibitors via synthesis and screening indolocarbazole compounds. For an in-depth review of the discovery, biosynthesis, and biological activity of staurosporine and derivatives, one should consult the 2009 review by Nakano and Ōmura [147].

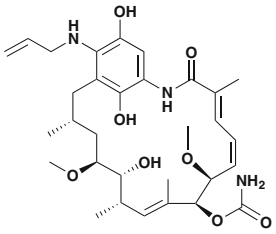
Currently, there are several variations of staurosporine in clinical trials. The first is the rebeccamycin (a naturally occurring halogenated staurosporine-like molecule) derivative becatecarin (**38**; Fig. 4), which has been in early clinical trials at the NCI for the treatment of acute lymphocytic leukemia [22], small cell lung cancer [184], and solid tumors [154]. Becatecarin was also in Phase III clinical trials by Exelixis and Helsinn for the treatment of bile duct tumors, but another antitumor agent exhibited superior activity and the trials were discontinued. No recent development has been reported for becatecarin. The second variation is N-benzoyl staurosporine or midostaurin (**39**; Fig. 4), which is a potent inhibitor of FMS-like tyrosine kinase-3 (FLT-3) and is in Phase III clinical development in combination with other antitumor agents for the



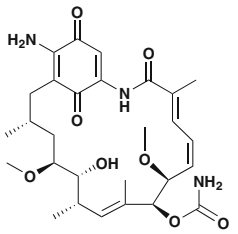
31. Geldanamycin



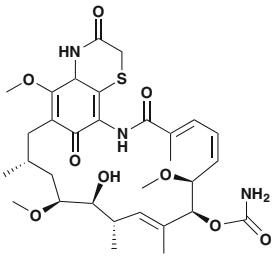
32. Tanespimycin



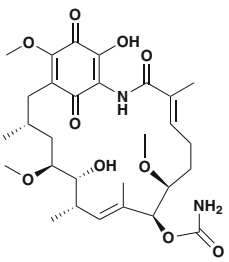
33. Retaspimycin



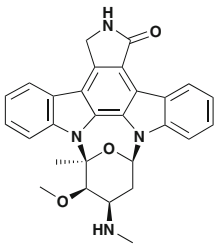
34. 17-Aminogeldanamycin



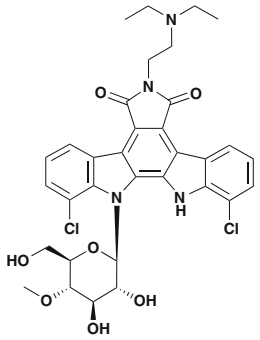
35. Thiazinogeldanamycin



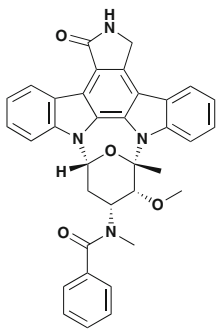
36. 19-Hydroxy-4,5-dihydrogeldanamycin



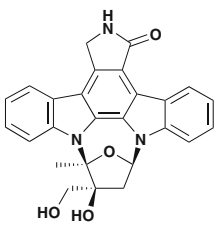
37. Staurosporine



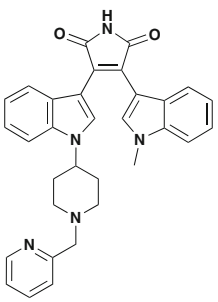
38. Becatecarin



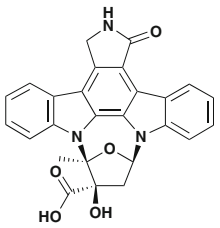
39. Midostaurin



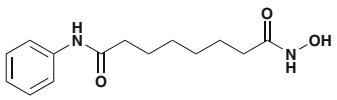
40. Lestaurinib



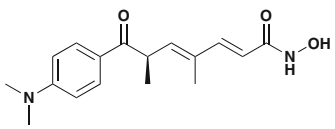
41. Enzastaurin



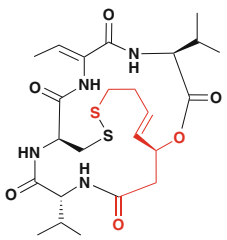
42. K-252A



43. Suberoylanilide hydroxamic acid (SAHA)



44. Trichostatin A



45. Romidepsin

Fig. 4 The chemical structures of geldanamycin and representative HSP90 inhibitors, staurosporine derivatives, HDAC inhibitors, and derivatives. (**31**) Geldanamycin, (**32**) tanespimycin, (**33**) retaspimycin, (**34**) 17-aminogeldanamycin, (**35**) thiazinogeldanamycin, (**36**) 19-hydroxy-4,5-dihydrogeldanamycin, (**37**) staurosporine, (**38**) becatecarin, (**39**) midostaurin, (**40**) lestaurtinib, (**41**) enzastaurin, (**42**) K-252A, (**43**) suberoylanilide hydroxamic acid (SAHA), (**44**) trichostatin A, and (**45**) romidepsin

treatment of acute myeloid leukemia (NCT00651261) [65]. Midostaurin is also in Phase I and II trials for the treatment of systemic mastocytosis (NCT00233454) [72] and adenocarcinoma (NCT01282502), respectively. Lestaurtinib (**40**; Fig. 4) is another derivative that has completed several Phase II trials at Cephalon for the treatment of myeloproliferative diseases. This agent is also in Phase I trials at the NCI for the treatment of recurrent or refractory high-risk neuroblastoma in pediatric patients (NCT00084422). Enzastaurin (**41**; Fig. 4), an open-ringed, derivatized version of the base staurosporine carbocycle, is a selective PKC-β inhibitor in Phase III clinical development at Eli Lilly & Co as oral treatment of diffuse large B-cell lymphoma (NCT00332202). This agent was compared to the alkylating drug lomustine (CCNU) in Phase III trials (NCT00295815) after a successful Phase II trial for patients with recurrent glioblastoma multiforme. However, the study was terminated due to the low median overall survival. Because enzastaurin was more tolerated than lomustine, enzastaurin may be more successful in other combination therapies [234]. Enzastaurin is also in Phase II trials both alone and in combination with other agents for the treatment of gliomas (NCT00475644), renal cancer (NCT00709995), and a variety of lymphomas (NCT00475644, NCT00542919, and NCT00451178). Finally, a PEGylated version of the known indocarbazole K252a (**42**; Fig. 4) has completed Phase II clinical trials for the treatment of the proliferative disease psoriasis vulgaris at Creablis SA (NCT00995969 and NCT01465282).

New derivatives with biological activity are still being synthesized via synthetic modifications to a variety of positions on the indocarbazole base structure [7, 8]. However, with the increasing number of known gene clusters and reduced specificity of their corresponding biosynthetic enzymes, combinatorial biosynthesis can be expected to provide a wider pool of structurally diverse indocarbazoles candidates with antitumor properties [180].

Histone deacetylase inhibitors

Histone deacetylases (HDACs) are typically found in multiprotein complexes that regulate transcription by altering the acetylation of histone proteins and other non-histone targets [96, 100, 238]. The expression of HDACs is altered in tumors, making them useful targets for preventing epigenetic abnormalities that lead to cancer. A total of 18 human HDACs [49, 54, 75, 104, 114, 160] have been identified and can be divided into four classes (I–IV) based on their homology to known yeast HDACs, subcellular localization, and enzymatic activities. Class I and II are Zn^{2+}-dependent amidohydrolases that share some degree of sequence homology and are homologous to yeast Rpd-3 and Hda1, respectively. Class IV is also Zn^{2+}-dependent, homologous to yeast HDAC HOS3, and has a known member, HDAC11, that shares weak homology with the catalytic core regions of Class I and II enzymes [160]. The Class III sirtuins are outliers as they require the cofactor NAD^{+}, have different cellular localizations depending upon the isoform(s), and are homologous to the Sir2 yeast protein [140].

HDAC inhibitors (HDACis) typically consist of a tripartite structure consisting of an aromatic enzyme binding group, a hydrophobic spacer group, and an inhibitor group [170, 239, 241]. Suberoylanilide hydroxamic acid (SAHA, **43**; Fig. 4) is a good example of an HDACi with this tripartite structure as it consists of a 6-carbon aliphatic chain (the spacer), the inhibitory end being the zinc-binding hydroxamic acid, and the aromatic enzyme binding group being the phenyl-amino ketone group. SAHA is a synthetic hybrid polar compound structurally related to the potent (nanomolar level) microbial HDACi trichostatin A (TSA, **44**; Fig. 4) [172, 173, 240], which was isolated from *S. hygroscopicus* in 1976 and determined to be an antifungal agent [212]. By combining the structure of TSA and data from hybrid polar compounds, SAHA was synthesized and tested as an HDACi. In 2006, SAHA was approved by the FDA to be used as an oral treatment for lymphoma under the generic name vorinostat. SAHA is currently in multiple Phase I–III trials for the treatment of a variety of cancers.

The only other approved HDACi is romidepsin (FK228), a fermentation product isolated in 1994 from the Gram-negative bacterium *Chromobacterium violaceum* isolated from a Japanese soil sample [215]. Romidepsin is a bicyclic depsipeptide with an unusual disulfide bond connection between a thiol and D-cysteine. In 1998, romidepsin was identified as a broad-spectrum HDACi that induces epigenetic changes in a variety of cancer cells [146]. Furumai et al. [67] demonstrated that romidepsin was a naturally occurring prodrug that inhibited Class I HDACs presumably by the reduced sulfhydryl group(s) interacting with the active site Zn^{2+}. In 2009 and 2011, romidepsin was approved by the FDA for the treatment of relapsed cutaneous cell lymphoma and relapsed/refractory peripheral T-cell lymphoma, respectively. Romidepsin has also completed early clinical trials for the treatment of several cancers, including solid tumors (NCT00019318), lung (NCT00086827), thyroid (NCT00098813), and prostate (NCT00106418) cancers, mainly under the auspices of the NCI. For an in-depth overview of the preclinical and clinical development of romidepsin, see the 2012 review by Harrison and coworkers [82].

With the sequence of the gene clusters involved in romidepsin biosynthesis now known, reengineering strategies are predicted to produce several analogs via fermentation [36, 167]. A patent (PCT/US2008/053473) was filed by Cheng on the sequences involved in romidepsin

biosynthesis to generate analogs [35]. In the meantime, a variety of synthetic methods have been employed [73, 122]. In 2008, Wen and colleagues used a macrolactamization protocol to form the amide bond in the larger ring last instead of macrolactonization to form the lactone last, bypassing the challenges in the macrolactonization of less reactive substrates to produce synthetic romidepsin analogs [229]. Additional modifications are also being made to the HDACi tripartite skeleton to develop more selective inhibitors [178, 193, 206], including those without a Zn^{2+}-chelating group in order to prevent undesirable interactions with other metalloenzymes [221]. For a summary of other reported HDACis produced by bacteria, see the 2012 review by Ho and coworkers [91].

In addition, Di Maro et al. [52] demonstrated the isosteric replacement of the synthetically challenging (3*S*,4*E*)-3-hydroxy-7-mercaptoheptenoic acid (shown in red in **45**; Fig. 4) in order to make romidepsin analogs from the easily assembled starting materials, L-aspartic acid and a cysteamine. To accomplish this, the *trans* double bond was replaced by an isosteric amide group and the macrolide lactone was replaced by an amide linkage. However, the resulting isosteres were not as potent as romidepsin. In 2009, Bowers and coworkers [23] also reported the synthesis of an amide isostere of romidepsin but the compound exhibited 50-fold lower inhibition against HDACis. Based on computational analysis, these results were speculated to arise from small differences in the position of the free thiol group when the molecule was docked in the enzyme active site. Thus, understanding the subtle differences between the structures of HDACi analogs may confer changes in their potency and provide direct routes to inhibitors with enhanced activity.

Marine sources

The ocean continues to be one of the largest unexplored sources of specialized metabolites due to its inaccessibility as more than 70 % of the Earth is covered by water. The rich diversity found in the world's oceans has provided a plethora of structurally diverse specialized metabolites with a variety of carbon skeletons and degrees of halogenation. Although numerous compounds have been reported with cytotoxic activities, very few have been definitively proven to originate from invertebrates, but there is sufficient evidence invoking microbial production of a significant number of these bioactive metabolites [124]. A significant number of compounds have been isolated from blue-green algae (cyanobacteria), other bacteria [145], and fungi that were isolated from sediments (both shallow and abyssal) or from invertebrates, where there is no question of the actual producer as fermentations produced the compounds of interest. Over the past 30–40 years, a number of these cytotoxic compounds have been isolated and tested for biological activity by the NCI. Here, we describe a select number of marine natural products that entered, are still in clinical trials, or have been approved by the FDA (or corresponding agencies in other countries) and who either have or may well have, a microbe involved in their production.

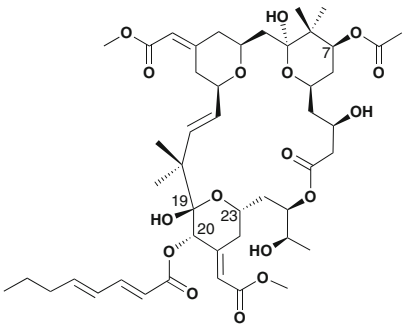
Bryostatins, dolastatins, and analogs

Bryostatin and analogs

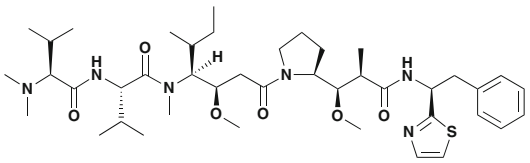
Bryostatin 1 (**46**; Fig. 5) and dolastatin 10 (**47**; Fig. 5)) are two prime examples of marine-derived compounds that required heroic efforts to isolate enough material for initial clinical trials from the invertebrate *Bugula neritina* and nudibranch *Dolabella auricularia*, respectively. Details of the early to relatively late history of the bryostatins can be found in reviews by Newman [149] and Trindade-Silva [208], and by Flahive and Srirangam [66] for the dolastatins.

Bryostatins are a family of 20 macrocyclic lactones that originate from the marine invertebrate *Bugula neritina*. All metabolites in this family generally share a 20-member macrolactone core and three remotely functionalized polyhydropyran rings. Bryostatins differ from one another by substitution at C^7 and C^{20} and placement of the γ-lactone at either C^{19} or to C^{23} in the polyhydropyran ring. These metabolites are thought to be produced by the uncultured microbe *Candidatus Endobugula sertula*, but definitive proof was lacking at the time of the discovery of this microbe. The most promising piece of evidence at that time was the reduction of the amount of bryostatin 1 in *B. neritina* colonies treated with antibiotic-treated larvae [46, 126]. However, later work by Haywood and coworkers led to the isolation and cloning of the gene cluster involved in the biosynthesis of the base ring structure of bryostatins but the large, trans-AT PKSs have deterred their heterologous expression at this time [127].

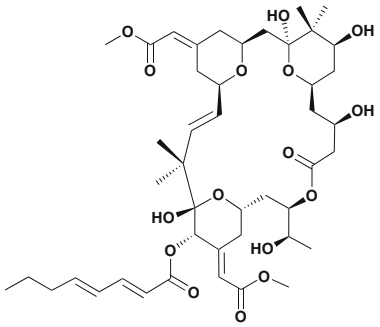
The most studied member of the bryostatin family is bryostatin 1. Phase I and II trials against hematological cancers have been completed with this agent either alone or in conjunction with a cytotoxin. As a result of these trials, bryostatin 1 appeared to be more promising when administered in combination with other antitumor agents, such as paclitaxel and vincristine [13, 143]. Currently, there are three Phase II trials with bryostatin plus a cytotoxin or signal transduction inhibitor listed in the NCT clinical trials database but all have an "unknown" status. Thus, these trials have probably ceased, though there is one Phase I trial (NCT00112476) with temsirolimus and bryostatin listed as recruiting as of the end of 2012.



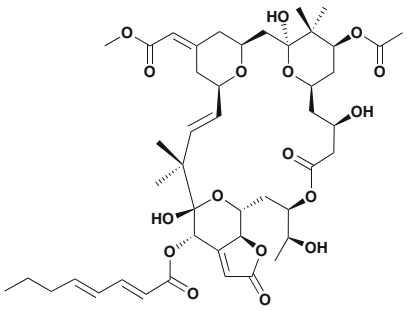
46. Bryostatin 1



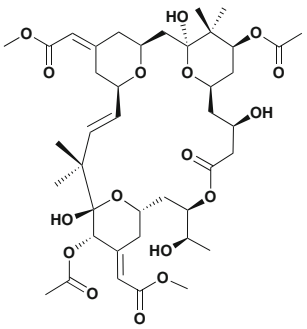
47. Dolastatin 10



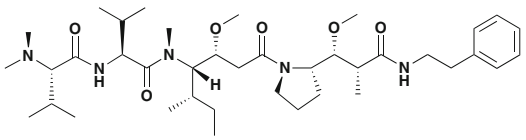
48. Bryostatin 2



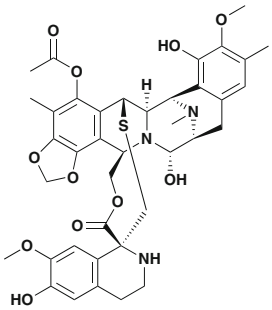
49. Bryostatin 3



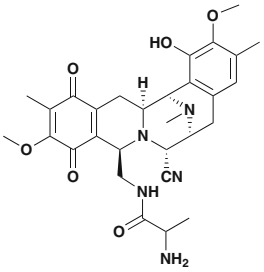
50. Bryostatin 7



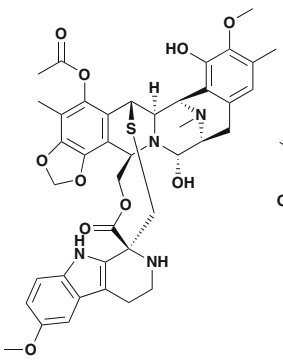
51. Auristatin PE



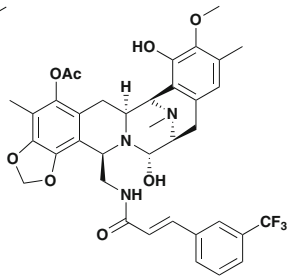
52. Trabectedin



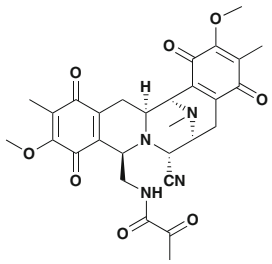
53. Cyanosafracin B



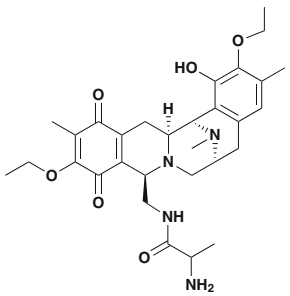
54. Lurbinectedin



55. Zalypsis



56. Saframycin A



57. Safracin A

Fig. 5 The chemical structures of trabectedin as well as representative bryostatins, dolastatins, and derivatives. (**46**) Bryostatin 1, (**47**) dolastatin 10, (**48**) bryostatin 2, (**49**) bryostatin 3, (**50**) bryostatin 7, (**51**) auristatin PE, (**52**) trabectedin, (**53**) cyanosafracin B, (**54**) lurbinectedin, (**55**) zalypsis, (**56**) saframycin A, and (**57**) safracin A

Several bryostatins and analogs have been synthesized using methods such as function-oriented synthesis as employed by Wender and other workers to develop simplified analogs with comparable or improved activities [51, 209, 230, 231], but the economical production of bryostatins via synthesis requires further investigation. Bryostatins 2 (**48**; Fig. 5) [61], 3 (**49**; Fig. 5) [157], and 7 (**50**; Fig. 5) [105, 132] have been made via total synthesis utilizing more than 70 steps. To address this problem, Trost and coworkers [210] have developed a 39-step, stereoselective synthesis of the bryostatin polyhydropyran ring C and the Keck group recently published an impressive, asymmetric 60-step synthesis of bryostatin 1 [107].

Bryostatins have a high binding affinity for protein kinase C (PKC) isozymes [113], which have been used to identify specific targets and develop other analogs [106]. Furthermore, PKC activity plays a major role in learning and memory [1], and from animal studies, bryostatin 1 may have potential to treat cognitive diseases [198, 199, 227]. Bryostatin 1 was approved for a Phase II trial (NCT00606164) for the treatment of Alzheimer's disease at the Blanchette Rockefeller Neurosciences Institute, but there have been no updates on the progress of this study.

Dolastatins and analogs

The linear pentapeptide dolastatin-10 was initially isolated from the sea hare *Dolabella auricularia*, and then isolated from a cyanophyte of the genus *Symploca* many years later [130]. Thus, in contrast to the bryostatins, the actual producing organism could be isolated and identified. Dolastatin 10 was later found to inhibit the assembly of microtubules [56] and Phase II clinical trials for the treatment of several solid tumors, including pancreatic (NCT00003677) and kidney (NCT00003914) were completed. However, these trials revealed that dolastatin 10 exhibited minimal responses in cancer patients and no recent development of this compound has been reported [108, 165, 224]. Phase II clinical trials for the treatment of non-small cell lung cancer (NCT00061854) and metastatic soft tissue sarcoma (NCT00064220) of auristatin PE (**51**; Fig. 5), a synthetic derivative of dolastatin 10, have been completed, but no recent developments has been reported for this agent. However, an anti-CD30 antibody-conjugated monomethyl auristatin E (brentuximab vedotin or SGN-35, Seattle Genetics) was approved by the FDA in 2011 for the treatment of various lymphomas. The details of the development of brentuximab vedotin are discussed in the 2012 review by Younes and colleagues [242].

Currently, several other antibody-conjugates using a variety of stable linker systems attached to monomethylauristatin E (licensed from Seattle Genetics) are either in or approaching clinical trials. Phase II clinical trials for the treatment of breast cancer (NCT01156753 and NCT00704158) and resectable stage III or stage IV melanoma have been completed for CR011-vcMMAE (Curagen Corp), where monomethyl aurostatin E is linked to a CR011 antibody against the melanoma antigen glycoprotein NMB, which is also expressed in some metastatic breast cancers. The results of NCT01156753 were promising as efficacy was shown in the following cohorts: patients with triple-negative breast cancer, triple-negative breast cancer and significant glycoprotein NMB expression, and only high levels of glycoprotein NMB [27]. Triple negative breast cancers do not express any of the three receptors (estrogen, progesterone or her-) commonly found in most breast carcinomas and are therefore difficult to treat with current receptor-targeted drugs. Thus these findings are of definite import for the design of treatment protocols.

A Phase II trial for the treatment of prostate cancer (NCT1695044) using PSMA-ADC (Progenics), where monomethyl auristatin E is tethered to a prostate-specific membrane antigen, is in progress. A Phase I trial of DCDS-4501A (an anti-CD79b monoclonal antibody linked to monomethyl auristatin E via a protease cleavable linker; Genentech) in conjunction with rituximab is underway for the treatment of non-Hodgkin's lymphoma and chronic lymphocytic leukemia (NCT01290549). Using the same linker and warhead, but to a different monoclonal antibody, a humanized IgG1 anti-CD-22, Genentech are sponsoring a Phase II trial of this agent (DCDT-2980S) against follicular and diffuse large B-cell lymphomas, in conjunction with other monoclonal antibodies as part of a complex treatment regimen (NCT01691898). Phase I clinical trials for the treatment of a variety of cancers using other antibody-auristatin conjugates utilizing different linkers, such as MLN-0264 (Millenium Pharmaceuticals), DSTP-3086S (Genentech), AGS-22M6E (Astellas Pharmas Inc/Seattle Genetics/Agensys Inc), and AGS-5 ADC (Seattle Genetics/Agensys Inc), are also underway or actively recruiting. Thus, the promising concept of linking auristatin and derivatives to monoclonal antibodies targeted at specific epitopes may lead to an increase in the number of agents and derivatives in cancer therapy in due course.

Trabectedin

The tetrahydroisoquinoline alkaloid trabectedin (ET-743, PharmaMar; **52**; Fig. 5) is the first compound "directly from the sea" to be approved for the treatment of cancer. In 1969, ethanol extracts of the Caribbean tunicate *Ecteinascidia turbinata* were first reported to have antiproliferative properties by Sigel and colleagues [188]. It took at least another 17 years for the first report of the isolation and

structural characterization of ecteinascidin derivatives to be published [94], followed by two papers in 1990 [175, 235]. The compound was later licensed to the Spanish pharmaceutical company PharmaMar where its isolation was optimized from large-scale collections from marine environments and also from in-sea and on-land aquaculture of the source tunicate. Several years of aquaculture/isolation provided enough material for the initial and early clinical trials [41]. Simultaneously, synthetic efforts were also made to obtain more trabectedin. The first total synthesis was accomplished by Corey's research group, which was inspired by the probable biosynthetic route to trabectedin [40]. Several other syntheses of trabectedin were published but ultimately did not meet the requirements for the commercial production of trabectedin. However, PharmaMar developed a short, semisynthetic synthesis to produce gram quantities of trabectedin [43] starting with the antibiotic cyanosafracin B (**53**; Fig. 5) [99], a fermentation product of a marine-derived *Pseudomonas fluorescens* [42].

Trabectedin has since been developed by PharmaMar and approved by the EMEA in September 2007 for the treatment of sarcoma and in 2009 for the treatment of ovarian cancer. For more information about the discovery and mechanism of action of trabectedin, see these recent reviews [44, 162]. In 2009, PharmaMar also launched this drug in the Philippines for the treatment of ovarian cancer, then in 2011 trabectedin was approved in Japan for the treatment of malignant soft tissue tumors accompanied with chromosomal translocation. In the US, an application was filed for the approval of the use of trabectedin in combination with docetaxel for the treatment of recurrent ovarian cancer. However, the FDA recommended that an additional Phase III trial be conducted, which led to the voluntary withdrawal of the New Drug Application by Johnson & Johnson.

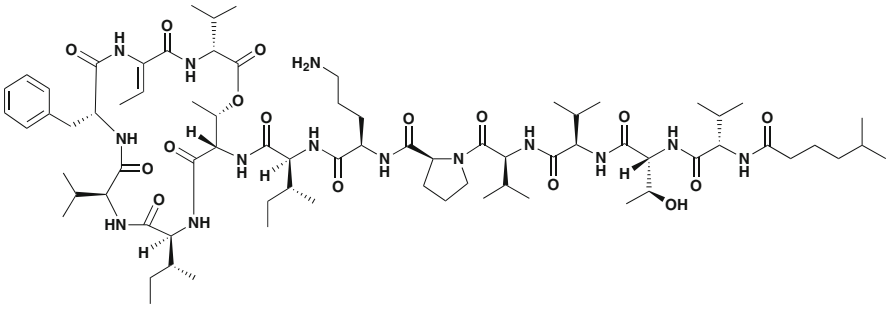
Trabectedin is still in many Phase III and earlier clinical trials for the treatment of various cancers. PharmaMar has also developed the trabectedin analog lurbinectedin (PM01183, **54**; Fig. 5), which has a tetrahydro-β-carboline moiety instead of the tetrahydroisoquinoline present in ring C. Phase I and II clinical trials with lurbinectedin are currently underway for the treatment of advanced tumors (NCT01405391) and metastatic breast cancer (NCT01525589), respectively. A Phase II trial against Ewing's sarcoma (NCT01222767) using the closely related compound zalypsis (PM00104, **55**; Fig. 5), bearing structural similarities to ET-743 and the jorumycins with a trifluoromethyl-phenyl substituent on the Southern edge, just completed. This compound was also in two European Phase II trials EudraCT Number 2010-020994-18 for the treatment of cervical carcinoma (completed but no published details as yet) and EudraCT Number 2009-016054-40 against refractory melanoma.

Trabectedin is structurally related to the saframycin and safracin classes of known antibiotics (see **56** and **57** in Fig. 5 for examples of these structural classes, respectively), which share similar tetrahydroisoquinoline frameworks. Thus, trabectedin and other compounds were thought to have microbial components involved in their production. Recently, Rath and coworkers [168] have demonstrated that a microbial consortium derived from *Ecteinascidia turbinata* via metagenomic sequencing was the probable producer of trabectedin. Using the known gene clusters of the saframycin [123] and safracin [219] metabolites as markers, the contig encoding the NRPS biosynthetic enzymes involved in trabectedin production was identified as well as the producing organism γ-proteobacterium *Candidatus Endoecteinascidia frumentensis* (AY054370). These results confirm previous reports speculating that bacterial candidates from Caribbean and Mediterranean *E. turbinata* [144, 164] are involved in trabectedin production as *Candidatus Endoecteinascidia frumentensis* was found in *E. turbinata* in both geographic locations. With these new findings, we expect to see more reports on the genetic engineering of the trabectedin biosynthetic genes to produce several new analogs with promising biological activity.

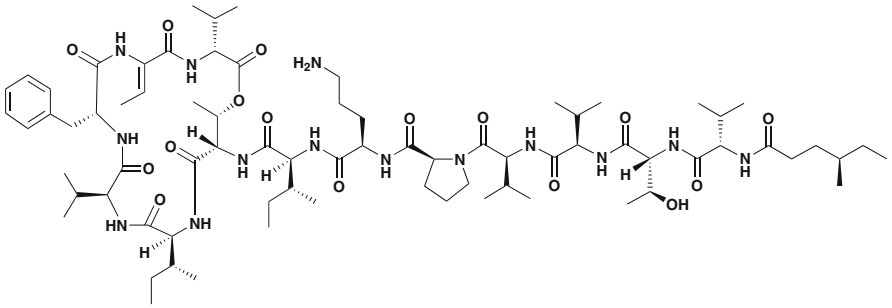
Kahalalide F

The cyclic depsipeptide kahalalide F (**58**; Fig. 6) is one of the most active antitumor metabolites of the kahalalide family that was first isolated from the herbivorous Sacoglossan mollusk, *Elysia rufescens,* which grazes on the green macroalga, *Bryopsis* sp. [78]. Following its isolation and identification, kahalalide F was also reported to be found in less concentrated amounts in the alga compared to the mollusk based on wet weight [77]. These observations suggest that this compound is likely to be a specialized metabolite produced by a symbiont. Total synthesis has mainly been used to access kahalalide F and derivatives using solid phase peptide techniques [101, 103, 128]. However, Hill and coworkers filed an international PCT application, in which they describe the isolation of kahalalide F and other analogs from *Vibrio mediterranei/shilonii* isolated from *Bryopsis* and *E. rufescens* [89]. Therefore, large-scale fermentation may be able to generate renewable supplies of the depsipeptide. For more details on the isolation, structural elucidation and biological activity of kahalalide F and analogs, see the 2011 review by Gao and Hamann [68].

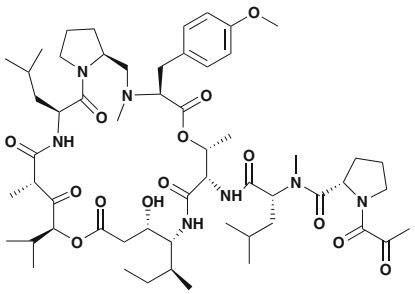
In the 1990s, kahalalide F (PM-92102) was licensed to PharmaMar by the University of Hawaii and entered preclinical development. Kahalalide F entered Phase I clinical trials in Europe in December 2000 for the treatment of



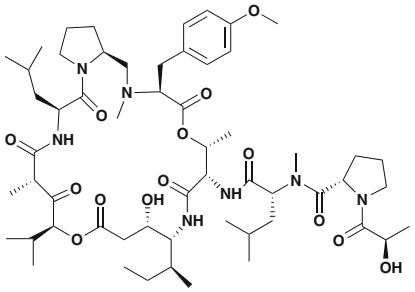
58. Kahalalide F



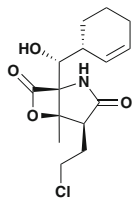
59. Isokahalalide F



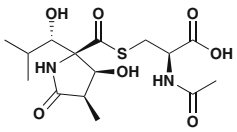
60. Aplidine



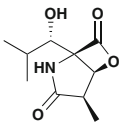
61. Didemnin B



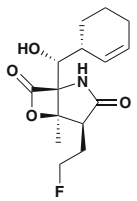
62. Salinosporamide A



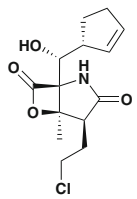
63. Lactacystin



64. Omuralide



65. Fluorosalinosporamide A



66. Salinosporamide X7

Fig. 6 The chemical structures of representative kahalalides, didemnins, salinosporamides, and derivatives. (**58**) Kahalalide F, (**60**) isokahalalide F, (**61**) aplidine, (**62**) didemnin B, (**63**) salinosporamide A, (**64**) lactacystin, (**65**) omuralide, (**66**) fluorosalinosporamide A, and (**67**) salinosporamide X7

androgen-independent prostate cancer and is currently in Phase II clinical trials against a variety of cancers including, hepatocellular carcinoma, non-small cell lung cancer, androgen-independent prostate cancer, and malignant melanoma. However, aside from a Phase II trial in Spain (EudraCT Number 2004-001253-29) for the treatment of non-small cell lung cancer that has been ongoing since 2004, there have been no recent developments reported for this compound for the treatment of cancer. Most antitumor agents induce programmed cell death or apoptosis, but kahalalide F appears to have a unique mechanism of action of primarily inducing oncosis [186, 197], which is the process of passive cell death accompanied by swelling. Several other kahalalide F targets have been identified to

play a role in inducing cytotoxic activity, such as lysosomes [69, 197], plasma membrane [197], and proteins involved in the Erb3 and phosphatidylinositol 3-kinase-Akt/PKB signal pathways [102, 166].

A closely related isomer of kahalalide F, isokahalalide F (PM-02734, elisidepsin, **59**; Fig. 6) has also been reported to be cytotoxic and was produced by solid-phase synthesis and developed at PharmaMar [42]. A completed Phase I clinical trial (NCT00884845) with this compound in combination with erlotinib for the treatment of solid tumors demonstrated that the combination was not efficacious [71].

Aplidine

The isolation of the cytotoxic depsipeptide aplidine (dehydrodidemnin B, **60**; Fig. 6; PharmaMar) from the ascidian *Aplidium albicans* was first reported by Rinehart and colleagues in 1991 [174]. This compound is structurally similar to didemnin B (NSC-325319, **61**; Fig. 6), which was isolated from the Caribbean tunicate *Trididemnum solidum* and reported to have antitumor and antiviral properties [176]. A detailed history of the isolation, biological activity, and clinical development of the didemnin family as well as aplidine can be found in the 2012 review by Lee and coworkers [120].

The only difference between the structures of aplidine and didemnin B is the presence of a lactyl hydroxyl group in the terminal side chain of didemnin B instead of its corresponding ketone form in aplidine. Interestingly, this small structural difference has resulted in aplidine exhibiting stronger antitumor effects and lower cardiotoxicity compared to didemnin B [125], which was the first marine natural product to enter clinical trials. Didemnin B was not further developed beyond Phase II clinical trials due to the lack of response, acute cardiotoxicity, and neurotoxicity [117]. Aplidine has become PharmaMar's second most advanced compound behind trabectedin as it has completed Phase II trials for the treatment of aggressive non-Hodgkin lymphoma (NCT00884286) and Phase III trials in combination with dexamethasone (NCT01102426) for the treatment of multiple myeloma [42].

Chemical synthesis has been used to produce aplidine [177] for clinical studies however bacterial fermentation may eventually be used to produce this compound, as other tunicate-derived didemnins have recently been demonstrated to be produced by free-living and potentially symbiotic bacteria [213]. Xu and coworkers [237] sequenced the genome of the marine α-proteobacteria *Tistrella mobilis*, revealing the didemnin gene cluster, and using imaging mass spectrometry, the real time conversion of didemnin X and Y precursors to didemnin B was observed. With the identification of the didemnin B gene cluster, it may be feasible to use genetic engineering to create renewable supplies of aplidine via microbial fermentation. Furthermore, metagenomic analyses of *Aplidium albicans* using *Tistrella mobilis* gene clusters as markers, may lead to the identification of the bacterial genes involved in aplidine biosynthesis in the tunicate as it has not yet been proven that the same free-living microbe produces both aplidine and its reduced congener, didemnin B.

Salinosporamide A

The cytotoxic proteasome inhibitor salinosporamide A (NPI-0052, **62**; Fig. 6; Nereus Pharmaceuticals) is one of the most interesting natural products as it has been isolated from cultivatable deep-sea, free-living microbes [62]. Its producing strain, *Salinispora tropica*, was isolated from deep-sea sediment collected in the Bahamas. *Salinispora tropica* is a marine streptomycete that can be propagated via saline fermentation and has truly showcased the capabilities of marine bacterial fermentation of natural products. Notably, bacterial fermentation afforded enough material (450 mg/l) for the clinical development of this compound [211], representing the first time in which saline fermentation was successfully performed on any scale with a marine-sourced microbe.

Salinosporamide A is structurally similar to the terrestrial bacterial product lactacystin (**63**; Fig. 6) and the lactacystin-biorearrangement derivative, omuralide (**64**; Fig. 6), the prototypical inhibitor of the 20S proteasome [53, 63]. The cytotoxicity of salinosporamide A is derived from its irreversible binding to the 20S proteasome through an ester linkage between its β-lactone carbonyl carbon and the hydroxyl group of an N-terminal threonine residue in the proteasome. Subsequent hydrolysis of the β-lactam of salinosporamide A leads to an intramolecular nucleophilic addition to the chloroethyl group, resulting in the elimination of chlorine to form a cyclic ether, which prevents the cleavage of the proteasome-inhibitor ester bond [74]. In addition, the unusual cyclohexene ring in salinosporamide A enhances its hydrophobic interactions with the proteasome S1 specificity pocket, further preventing the hydrolysis of the proteasome-inhibitor ester bond. Salinosporamide A also inhibits NF-κB activation, which is activated by proteasome activity [3].

Phase I clinical trials sponsored by Nereus Pharmaceuticals with Salinosporamide A commenced only 3 years after the report of its discovery, for the treatment of advanced malignancies (NCT00629473) and multiple myeloma (NCT00461045). A Phase I trial for the treatment of solid tumors and lymphoma has been completed (NCT00396864). Because studies have shown that

combinations of HDAC and proteasome inhibitors have significant therapeutic potential [142, 163], Phase I clinical trials are also being performed in combination with vorinostat for the treatment of non-small cell lung cancer, pancreatic cancer, and melanoma (NCT00667082). Thus far, the results of this combination have been positive as it exhibited highly synergistic antitumor activity and was deemed to be as safe as taking salinosporamide A and vorinostat separately [141].

Several synthetic methods have been used to generate many salinosporamide A derivatives [33, 153, 182]. However, the most interesting analog has been derived via the genetic engineering of the salinosporamide A gene cluster. Notably, the production of the fluorinated metabolite fluorosalinosporamide A (**65**; Fig. 6) and salinosporamide X7 (**66**; Fig. 6) by the Moore group demonstrated the utility of genetic engineering and chemical synthesis to produce novel marine-derived analogs [59]. Fluorosalinosporamide A was determined to be a slow reversible inhibitor of the 20S proteasome, whereas salinosporamide X7 was nearly three times more cytotoxic than salinosporamide A.

Carfilzomib

In 1992, Hanada et al. reported [81] the isolation and antitumor properties of the tetrapeptide epoxomicin (**67**; Fig. 7), a metabolite of an unidentified actinomycete strain No. Q996-17 with an epoxy-β-aminoketone moiety similar to that of eponemycin (**68**; Fig. 7). Epoxomicin entered preclinical trials with Bristol-Myers Squibb Research Institute in Tokyo but was dropped due to its unknown mechanism of action. In 1999, the Crews' group at Yale University published both the synthesis of epoxomicin [189] as they were not able to obtain its producing strain and the results of a labeling study [138], in which they were able to demonstrate that this compound inhibited the 20S proteasome. Notably, they did not observe any cross-inhibition with other proteases.

An N-terminal threonine residue in the proteasome was determined to covalently attach to epoxomicin by forming a morpholino ring adduct with the carbonyl of the epoxy ketone moiety. Other proteases typically do not have active sites at the N-terminus to form an N-morpholino ring. Thus, epoxomicin is very specific for the 20S proteasome. With this mechanism of inhibition in mind, the Crews' group in conjunction with Proteolix synthesized many epoxomicin derivatives. The final drug candidate, carfilzomib (**69**; Fig. 7), was the initial compound designed by Crews (YU-101, **70**; Fig. 7) [57] with a morpholino end group attached to improve its solubility, oral availability, and ADME properties [47]. This compound was approved by the FDA in July of 2012 for the treatment of multiple myeloma, with an analysis of the clinical trials leading to FDA approval published by McCormack [137]. As of July 2013, there are 53 ongoing clinical trials covering the treatment of hematological cancers at Phases I–III.

The story of carfilzomib demonstrates the power of modern synthetic chemistry coupled with key biochemical pull-down/binding experiments to identify a previously unknown target of a microbial product. In addition, synthesis enabled subsequent modification of the base molecule to produce a viable drug candidate. Effectively 90 % of the epoxomicin backbone, including the "warhead end", is a part of the structure of the synthetic drug, representing a prime example of a modified natural product skeleton leading to a drug entity. Importantly, carfilzomib is the second approved protease inhibitor after bortezomib (**71**; Fig. 7) but the first to not cause painful side effects, such as peripheral neuropathy [9].

Halichondrin B

In 1986, Uemura and coworkers [90] reported the isolation of the structurally complex natural product halichondrin B (**72**; Fig. 7) from 600 kg of marine sponge *Halichondria okadai*. The structural class of this molecule implied that it may well be produced by a protist, though the involvement of other microbes is possible. This compound exhibited potent cytotoxic activity against B-16 melanoma cell lines ($IC_{50} = 0.09$ nM) and in 1991, Bai and coworkers [10] demonstrated that halichondrin B functioned as a tubulin destabilizing agent.

At that time, total synthesis of halichondrin B seemed impossible. However in 1992, Kishi and coworkers [4] reported that they had synthesized halichondrin B and later worked with chemists and biologists at Eisai to develop over 200 derivatives of the natural product and evaluate their in vitro and in vivo activities. During this time, scientists at the NCI were also independently working with New Zealand scientists to isolate enough halichondrin B from the deep water New Zealand sponge *Lissodendoryx* sp. for preclinical studies. In 1998, scientists at both the NCI and Eisai collaborated to evaluate the two best synthetic analogs from Eisai and pure halichondrin B from the deep-water New Zealand collections. One of the synthetic compounds, a truncated halichondrin B analog, now known as eribulin (**73**; Eisai; structural similarities to **72** are shown in red in Fig. 7), showed significantly more potent activity. Specifically, eribulin exhibited an order of magnitude higher potency than halichondrin B against DLD-1 human colon cancer cells [110] and similar profiles against the NCI60 tumor cell lines. Based on its antitumor activity, eribulin was evaluated in clinical trials as a mesilate salt.

In 2010, eribulin mesilate was approved and launched in the US for the treatment of patients with metastatic breast

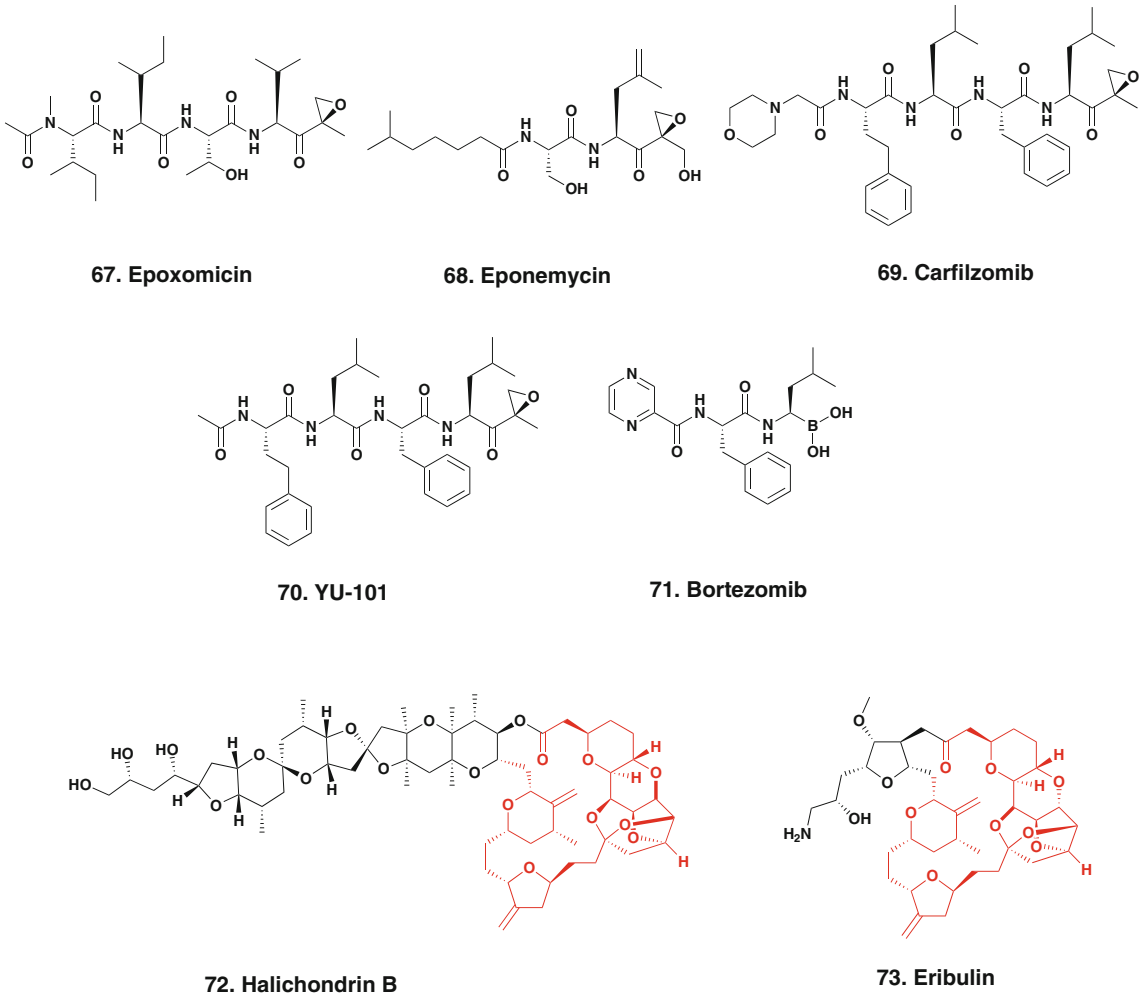


Fig. 7 The chemical structures of epoxomicin and halichondrin B and representative derivatives. (**67**) Epoxomicin, (**68**) eponemycin, (**69**) carfilzomib, (**70**) YU-101, (**71**) bortezomib, (**72**) halichondrin B, and (**73**) eribulin

cancer who have received and failed at least two chemotherapeutic regimens. A year later, this compound was approved in the EU and Japan for the treatment of breast cancer and later launched in Japan. Eribulin mesilate is currently in many clinical trials against various carcinomas either as a single agent (NCT01676818) or in combination with other compounds (NCT01534455, NCT01554371). To date, the actual producer is not yet identified but the fact that halichondrins have been isolated from many different classes of the *Porifera* from many geographical sites and depths, points to a microbial and or protist source.

Genetic blueprints for the production of microbial metabolites

For over 50 years, large and small pharmaceutical companies fermented millions of soil isolates in order to find new microbial agents and over time realized that they were isolating the same metabolites. This chemical redundancy is reflected by the significant decrease in the number of new secondary metabolites from terrestrial microbes that have been reported since the late 1960s. For a multiplicity of reasons, most companies decided to jettison their fermentation-based discovery programs from roughly the middle of the 1980s to early 2000s. However, with the development of new sequencing technologies, we now have access to microbial genomes and can use a variety of tools to fully elucidate and activate biosynthetic pathways.

Rapidly evolving next-generation sequence (NGS, post-Sanger sequencing) technologies have led to the quick and inexpensive high-throughput sequencing of multiple microbial genomes in parallel, providing biologists and chemists with high volumes of sequence data for genes involved in natural product biosynthesis. Sanger sequencing can provide read lengths of approximately 1,000 bp/read at a cost of at least $500/Mbp, but now some NGS technologies can rapidly produce at least 13–36 bps/read

for well under $3.00/Mbp [187]. As a result of the plummeting costs of sequencing, several large-scale sequencing initiatives have been initiated, such as the Genomic Encyclopedia of Bacteria and Archea [236] and the Human Microbiome Project [18]. As a result, the number of complete whole microbial genome sequences has skyrocketed, providing more opportunities for genome mining to discover novel new natural products.

We can now begin to fully exploit combinatorial biosynthesis by either utilizing unnatural starting substrates in order to alter the final product or mixing and matching gene clusters with the aim of producing novel structures. A prime example of the latter is the aforementioned engineering of fluorosalinosporamide from *S. tropica*. This fluorinated analog was generated by replacing the fluorination gene flA from *Streptomyces cattleya* with the salL chlorinase gene in *S. tropica* and growing the *salL*$^{-}$ *flA*$^{+}$ mutant strain in the presence of inorganic fluoride [60]. Fluorinated natural products are rare, yet 15 % of all marketed drugs contain fluorine, demonstrating how useful genetic engineering can be in incorporating unique and essential functionalities in molecules.

Furthermore, many genomic studies have revealed that most of the biosynthetic gene clusters in microorganisms are cryptic or silent. For example, sequencing *Streptomyces* genomes has revealed that each strain has the potential to produce at least 20 or more secondary metabolites, when only a fraction of these are produced by conventional fermentation methods [158]. The genetic capability of fungi has also been underestimated as the model soil fungus *Aspergillus nidulans* has 28 putative PKS and 24 NRPS gene clusters, demonstrating the potential to produce at least 52 secondary metabolites [223] and in a 2012 review, the Keller group at Wisconsin extended the analyses to eight other species of *Aspergillus* identifying between 33 and 79 putative clusters excluding any terpene synthases, on the eight chromosomes of this genus [181], thus the potential is immense.

We now have access to cryptic gene clusters and can focus on ways to activate them. One method reported by Tanaka et al. [203], was the addition of rare metals to the fermentation broth of *Streptomyces sloyaensis* thus activating the expression of nine genes that were previously silent or poorly expressed in *Streptomyces coelicolor* A3(2). In 2007, Udwary et al. [214] reported the sequence of *S. tropica*, identifying 17 potential biosynthetic gene clusters, including the salinosporamide locus, leading to studies by Fenical and coworkers eliciting the products of the products of some of these clusters via specific fermentation conditions, and now that the positions of the potential producing clusters have been identified, a combination of cloning, expression, and fermentation is being used to "unlock" these metabolites. Cocultivation with other microbes has been a successful method for "awakening" cryptic gene clusters. Recently, Nützmann and coworkers [155] reported the cocultivation of the soil-dwelling *Streptomyces rapamycinicus* (formerly *S. hygroscopicus*) with the fungus *A. nidulans*, activating a silent fungal polyketide synthase gene cluster involved in producing orsellinic and lecanoric acids as well as the cathepsin inhibitors F-9775A and F-9775B. The 2013 review by Ochi and Hosaka [156] should be consulted for an in-depth analysis of the current methods used to activate cryptic gene clusters.

The limited diversity of currently "culturable" microbes also contributed to the decline in the discovery of new natural products. Now, metagenomic sequencing has become very powerful in identifying gene clusters from environmental samples of mixed, unculturable organisms, providing further insight on the interactions between organisms within a community [225]. This will be extremely useful for identifying the biosynthetic gene clusters of marine natural products, for which one major challenge is working with unculturable organisms. Recently, Donia and coworkers [55] sequenced the complex microbiome underlying the symbiosis between the tunicate *Lissoclinum patella* and the uncultivated cyanobacterium *Prochloron didemni*, which demonstrated the secondary metabolite symbiosis between these organisms and also helped to identify other symbiotic bacteria for future studies. Metagenomic sequencing has also been used to direct culturing conditions such as the microbiota of the medicinal leech *Hirudo verbena,* which includes *Aeromonas veronii* and a *Rikenella*-like bacterium. High expression levels of mucin and glycan utilization genes were found in the *Rikenella*-like bacterium, and growing the microbe in media containing mucin instead of glucose led to the growth of pure cultures of this microbe [21]. Lastly, with the amount of data generated by NGS technologies, we expect an increase in the number of new databases thus facilitating the search for genes involved in the production of specific metabolites [26, 39, 97].

Plant metabolites and endophytes

Within the past two decades, there has been a significant increase in the number of reports of endophytes (microbes that live inside the living tissues of plants without having deleterious effects) producing valuable therapeutic plant secondary metabolites. For every one of the approximately 350,000 plant species on earth, each plant serves as a host to one or more endophytes [194]. This is a trend that was identified in the marine area where natural products that were initially thought to be invertebrate-derived were later found to be produced by symbiotic or commensal

microbes. Scientific data from the People's Republic of China has revealed a significant number of reports demonstrating that endophytic fungi produce the following four major classes of "plant-derived" natural products: taxanes, podophyllotoxins, camptothecins, and vinca alkaloids. Summaries of the known endophytic fungi that produce plant secondary metabolites were published in 2012 [29, 118].

In addition to endophytic fungi, there have also been reports of endophytic actinobacteria, albeit limited that enhance [207] and/or produce new secondary metabolites [195, 244]. In 2007, Lu and Shen [129] reported a new cytotoxic ansamycin, napthomycin K, produced by the endophytic *Streptomyces* sp. *CS* isolated from the medicinal plant *Maytenus hookeri*. More recently, Igarashi and coworkers [98] have identified the new anthraquinone lupinacidin C from the endophytic actinomycete, *Micromonospora lupini*, coexisting in the root nodules of the legume *Lupinus angustifolius*, which exhibits anti-invasive activity against murine colon cancer cells. These examples highlight the exciting new possibilities of endophytic microbes serving as an inexhaustible reservoir of new secondary metabolites with novel bioactivities.

Only a handful of mostly higher plants and their corresponding endophytes have been investigated, leaving the vast majority of plants to be studied. Not only can endophyte–plant interactions induce the production of new compounds, but endophyte–endophyte interactions within plants also have the potential to produce new secondary metabolites as plants are unlikely to be colonized by just a single microbe. Biosynthetic genes can be up- or down-regulated in endophytes as a result of interacting with other microorganisms within their environment [12, 155]. Signaling molecules analogous to bacterial quorum sensors and other elicitors are thought to be involved in activating cryptic biosynthetic gene clusters.

Recently, two papers were published in Nature that described using 454 sequencing to identify the bacterial microbiota colonizing the root rhizosphere, soil, and endophytic compartments (within the roots) of *Arabidopsis thaliana* [25, 131]. Both studies identified similar phyla of bacteria inhabiting the endophytic compartments of *A. thaliana* and demonstrated that they are significantly dissimilar compared to those found in plant-free soil and the root rhizosphere. Notably, the microbiota of the endophytic compartment is influenced by soil type, and some variation was observed among plants of different genotypes and developmental stages. All of these observations suggest that there is a large possibility of finding more unique endophytic microbes and symbiotic interactions that have the capability of producing new secondary metabolites. As more reports on the metagenomic sequencing of plant microbiomes are published, these will facilitate the dissection of endophyte-endophyte and endophyte–plant interactions.

Conclusions

Organisms from two of the three domains of life are not only producers of secondary metabolites with significant activity as both drugs and leads, but in the last few years, the investigation of the genomic sequences of such microbes has shown that we have overlooked most biosynthetic gene clusters by using axenic monoculture conditions. With the development of NGS technologies and the increasing availability of genetic blueprints, we can now make informed decisions (e.g., in silico predictions) [133] about how to characterize and fine tune the expression of genes, molecular interactions, cross-species gene expression, signal transduction, as well as the activation and silencing of various genes involved in the production of bioactive secondary metabolites. Most importantly, we are now developing the tools needed to uncover unexplored microbial genes and, correlating with the increasing numbers of genomic sequences available we also expect to see an increase in the number of new microbial-derived pharmaceuticals.

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