

# Exploring Marine Rare Actinomycetes: Untapped Resources of Bioactive Compounds in Clinical Development

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**Abstract.** Marine Actinomycetes represent a rich and valuable source of distinct and promising substances. The genus *Streptomyces* in particular, has been extensively studied due to its ability to produce bioactive compounds and its abundance of biosynthetic gene clusters (BGCs). However, the exclusive focus on *Streptomyces* has resulted in the rediscovery of known compounds. On the other hand, marine rare Actinomycetes (MRA), comprising Actinomycetes species beyond *Streptomyces*, also harbor a significant number of BGCs. In this article, we summarize the chemical composition, biological activity, and biosynthetic pathways of compounds sourced from MRA that have been tested in clinical trials for their potential in infection, pain relief, and anticancer treatments. Our particular emphasis lies on compounds derived from MRA associated with marine invertebrates, an area that has been comparatively underexplored when compared to MRA isolated from marine sediment and water. Some notable compounds include rifamycin SV, staurosporine, and tetrodotoxin, which are produced by actinomycetes from the genera *Salinospora*, *Micromonospora*, and *Nocardioopsis*. The findings of this overview shed light on the potential of MRA associated with marine invertebrates to yield intriguing compounds that could be developed into drugs. Exploring the natural products from these bacteria holds the promise of discovering novel compounds with remarkable bioactivities.

## 1 Introduction

The Earth's surface is predominantly covered by oceans, constituting approximately 70% of its total expanse. Oceans serve as a pivotal resource in meeting various human requirements, encompassing both sustenance and pharmaceutical applications. The aquatic realm has bestowed upon us a plethora of bioactive compounds, particularly those with profound implications in oncology, cholesterol regulation, and analgesic efficacy [1].

A spectrum of anticancer agents, notably cytarabine, trabectedin, eribulin mesylate, and brentuximab vedotin, finds their origins in the aquatic milieu [2–5]. Further, pharmaceutical substances renowned for their cholesterol-reducing attributes, such as omega-3 fatty acids,

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alongside potent analgesics exemplified by conotoxin, trace their lineage to marine invertebrate sources [6,7]. Advances in diving technology have facilitated deeper oceanic exploration, contributing to an exponential surge in screening campaigns aimed at isolating potentially therapeutic compounds from both macro- and microorganisms inhabiting the marine ecosystem[8].

Culturable bacteria in marine environment are source of potential natural products for drug discovery and development, despite the number of culturable bacteria approximately only 1%. It has been subjected to rigorous investigation in the context of natural product discovery [9]. It is noteworthy that the quest for novel bacterial species and compounds from culturable bacteria continues unabated, evidenced by the perpetual growth in the number of natural products isolated from marine microbial sources [10]. The isolation of culturable bacteria presents researchers with an invaluable opportunity to scrutinize entire genome sequences, exercise precision in manipulating growth conditions through the One Strain Many Compounds (OSMAC) approach [11,12], experiment with co-culturing methodologies [13], and harness heterologous expression techniques [14]. These multifaceted endeavours are geared toward the discovery of hitherto unknown compounds endowed with potent bioactive properties, especially for Actinomycetes. Nevertheless, a persistent conundrum in the screening campaigns is the rediscovery of previously known compounds[15].

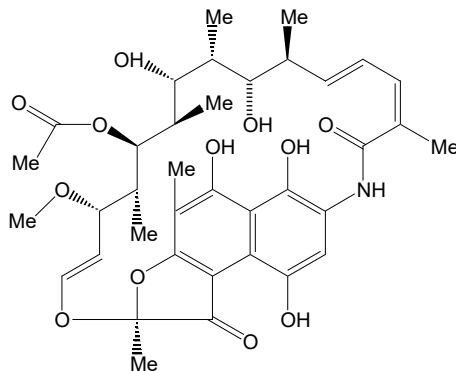
To address this issue, the focus of screening campaigns has shifted towards marine rare actinomycetes sourced from marine invertebrates. Several compelling reasons underpin this strategic shift: 1.) Actinomycetes, a prominent bacterial order, harbor a plethora of biosynthetic gene clusters (BGCs) capable of encoding a gamut of chemical entities. However, previous screening initiatives have been disproportionately focused towards *Streptomyces*, resulting in the rediscovery of known compounds [15]; 2.) Marine invertebrates have emerged as prodigious reservoirs of bioactive compounds, yet the actual biosynthetic origins of certain compounds, such as bryostatin, are attributed to symbiotic bacteria residing within these host organisms [16]; 3.) Marine rare actinomycetes, by virtue of their relative underexploration, constitute an alluring frontier. Despite the elucidation of BGCs within actinomycetes, the full spectrum of potential compounds remains largely uncharted, thus warranting dedicated scrutiny [17]; 4.) Prior endeavours targeting marine invertebrate-associated bacteria have yielded several instances of rare actinomycetes characterized by 16S rRNA sequences exhibiting less than 98% similarity which represent putative novel species [18].

In this article, we provide a succinct review encompassing the historical antecedents surrounding the discovery of compounds sourced from marine rare actinomycetes. Particular emphasis is directed towards rifamycin SV, staurosporine, and tetrodotoxin, compounds that have successfully traversed clinical trials. These agents hold promise in addressing an array of medical exigencies, encompassing traveler's diarrhea, leukemia, and pain management, respectively.

## 2 Rifamycin SV

Rifamycin SV (Figure 1) belongs to the rifamycin family of antibiotics, which originate from the bacterium *Amycolatopsis rifamycinica*, formerly known as *Amylocaptosis mediterranei* or *Streptomyces mediterranei* [19]. Rifamycin antibiotics, including rifampicin (rifampin), rifabutin, and rifapentine, are employed in the treatment of infections caused by various *Mycobacterium* species, such as *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). Due to their potent bactericidal properties against these bacteria, rifamycins are a crucial component of standard treatment protocols for TB and other mycobacterial infections [20].

Rifamycin SV, a semi-synthetic derivative of rifamycin B, boasts a complex polycyclic structure characterized by an ansa bridge, which is pivotal to its mode of action [20]. First introduced in 1963 for clinical use, rifamycin SV has historically been employed to treat lepromatous leprosy, a mycobacterial infection [21,22]. More recently, it has been investigated in clinical trials for its potential in treating traveler's diarrhea [23].



**Fig. 1.** Rifamycin SV.

Traveller's diarrhea is a prevalent and discomforting condition affecting millions of travellers annually, particularly in regions with subpar sanitation and hygiene standards. It results from the consumption of contaminated food or water and manifests as rapid-onset watery diarrhea, abdominal cramps, and other gastrointestinal symptoms [24]. In response to this issue, various antimicrobial agents have been developed, including Rifamycin SV, which has gained recognition for its effectiveness [25].

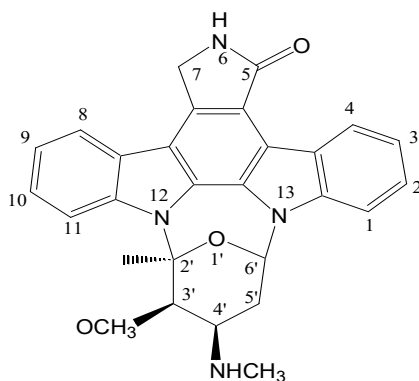
Rifamycin SV's primary mechanism of action centers on inhibiting bacterial RNA synthesis. Specifically, it targets the bacterial RNA polymerase enzyme, responsible for transcribing DNA into RNA. The antibiotic selectively binds to the  $\beta$ -subunit of the RNA polymerase enzyme, forming a stable complex that impedes RNA chain elongation. Upon binding to RNA polymerase, rifamycin SV acts as a physical barrier to the growing RNA chain, preventing the incorporation of new nucleotides and halting transcription. Consequently, the bacterium becomes incapable of synthesizing essential proteins vital for its survival and replication [26]. Notably, rifamycin SV's mechanism of action is not restricted to a particular group of bacteria, as it exhibits broad-spectrum activity against various Gram-positive and some Gram-negative bacteria. This versatility renders it effective against a wide array of potential pathogens responsible for traveler's diarrhea, including strains of *Escherichia coli*, *Salmonella*, and *Shigella* [23].

Rifamycin SV MMX, a specialized formulation of the antibiotic, is engineered to enhance its efficacy and reduce potential side effects by modifying its release and absorption characteristics in the gastrointestinal tract [27]. The "MMX" designation signifies the utilization of the "Multi-Matrix" technology in this formulation [28]. Rifamycin SV MMX has been specifically developed for medical conditions like traveler's diarrhea and certain inflammatory bowel diseases, such as Crohn's disease. By optimizing the release and absorption patterns of rifamycin SV, this formulation aims to offer an effective and well-tolerated treatment option for these conditions [23].

In 2006, rifamycin SV was discovered from the *Salinispora arenicola* strain M403, which was associated with the sponge *Pseudoceratina clavate* [29]. The bacterium was cultured on the medium containing starch – yeast extract – peptone (SYP) in artificial sea water at 28°C. This marked the first recorded instance of rifamycins originating from marine bacteria, expanding their potential sources beyond the genus *Amycolatopsis*. High-performance liquid

chromatography-tandem mass spectrometry (HPLC-MS-MS) analysis of the bacterium's extract revealed the production of both rifamycin B and SV [29]. From an ecological perspective, these compounds were tested against *Mycobacterium* strains found in the same sponge, indicating their role in competition with other members of the sponge's microbial community [30]. From pharmaceutical point of view, it is interesting to find the source that can produce rifamycin SV directly rather than semi-synthesize it from rifamycin B.

The biosynthesis of rifamycin B commences with the creation of a crucial building block called 3-amino-5-hydroxybenzoic acid (AHBA), which eventually forms the naphthalene aromatic part of rifamycin B (Figure 2). This AHBA compound is synthesized through a pathway known as the aminoshikimate pathway. This pathway closely resembles the shikimate pathway but utilizes a different starting material called 3,4-dideoxy-4-amino-D-arabino-heptulosonic acid 7-phosphate (aminoDAHP) instead of 3-deoxyD-arabino-heptulosonic acid 7-phosphate (DAHP). Once AHBA is synthesized, the assembly of the polyketide chain takes place. This is achieved through a hybrid enzyme complex known as a non-ribosomal peptide synthase/polyketide synthase (NRPS/PKS). This complex consists of several proteins, including *RifA*, *RifB*, *RifC*, *RifD*, and *RifE*. The modules in this complex have different functions, including ketosynthase (KS), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), and acyl carrier protein (ACP) domains. The starting point for this process is AHBA, and it requires eight units of methylmalonyl-coenzyme A (methylmalonyl-CoA) and two units of malonyl-coenzyme A (malonyl-CoA) to extend the polyketide to an undecaketide [31].



**Fig. 2.** Staurosporine.

The addition of the first three extended units is carried out by *RifA*, with the loading module on *RifA* being an NRPS module. All the other modules involved are of type I PKS. During this process, the precursor remains attached to the NRPS/PKS. After the undecaketide is fully synthesized, it is released from the complex with the assistance of *RifF*. *RifF* also catalyzes an intramolecular amide bond formation, a step proposed to generate a compound called proansamycin X [31].

The next steps involve further chemical transformations. Dehydrogenation and hydroxylation of proansamycin X result in the formation of an intermediate known as rifamycin W. An enzyme called *RifT* plays a crucial role in this stage of rifamycin B biosynthesis. While the exact function of *RifT* is not fully elucidated, bioinformatics suggest that it is an NADH-dependent dehydrogenase and is involved in converting proansamycin X into rifamycin W [31].

Subsequent to the formation of rifamycin W, the enzymes *Rif5*, *Rif20*, and *Rif14* come into play to convert it into rifamycin SV. *Rif5*, functioning as a monooxygenase, plays a key role in this conversion process, and its deletion results in the accumulation of rifamycin W.

*Rif20* acetylates a specific hydroxyl group at position C25 on an intermediate molecule downstream of rifamycin W, particularly in the context of ketal formation. *Rif14*, on the other hand, methylates another hydroxyl group at position C27 to yield rifamycin SV. The final stages of this intricate biosynthesis pathway involve *Rif15*, a two-subunit transketolase, and *Rif16*, a cytochrome P450 enzyme. These enzymes are essential for converting rifamycin SV back into rifamycin B [31].

### 3 Staurosporine

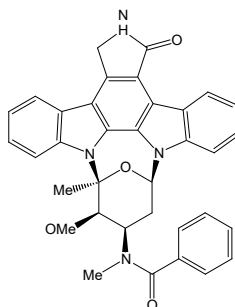
Staurosporine (Figure 2) was first discovered in 1977 during the screening of microbial alkaloids using chemical detection methods in a culture of *Streptomyces staurosporeus* strain AM-2282, which has undergone multiple taxonomy revisions and is now known as *Lentzea albida* AM-2282 [32]. Initially, it was primarily studied as a potential treatment for neurological disorders and other non-cancerous conditions [33].

Staurosporine has been found in various other actinomycetes, myxomycetes (slime molds), and cyanobacteria. Also in marine invertebrates like sponges, tunicates, bryozoans, and mollusks. However, it remains uncertain whether these invertebrates possess genes responsible for indolocarbazole biosynthesis, as many natural products from marine invertebrates are produced by associated microorganisms [34].

*Micromonospora*, for instance, yielded staurosporine along with 4'-N-methyl-5'-hydroxystaurosporine and 5'-hydroxystaurosporine from *Micromonospora* sp strain L-31-CLCO-002, isolated from the sponge *Clathrina coriacea* [35]. The bacterium was cultured at 28 °C for 96 h in the fermentation medium consisted of dextrose 0.5%, dextrin 2%, soybean meal 0.3%, yeast extract 0.5%, peptone 0.1%, calcium carbonate 0.4%, sodium chloride 0.2%, sodium sulphate 0.25%, potassium chloride 0.05%, magnesium chloride 0.05%, ammonium sulphate 0.05% in the distilled water with the pH adjusted to 7 before sterilization. This discovery sparked excitement about staurosporine's potential as an anti-cancer agent. Given that more than 90 tyrosine kinases are associated with malignant transformation and tumor angiogenesis, tyrosine kinase inhibitors (TKIs) gained prominence in cancer treatment. TKIs, capable of targeting both receptor and cytoplasmic kinases, offer promise by controlling kinase activation in cancer cells [36]. However, despite being one of the most potent inhibitors known, staurosporine exhibited high promiscuity, interacting with over 250 kinases, including those in blood plasma, at concentrations below 3 µM, rendering it unsuitable for therapeutic use [37].

To address this limitation, researchers modified staurosporine's structure to create midostaurin (figure 3). These structural changes were aimed at enhancing its specificity for particular kinases, notably FLT3 (FMS-like tyrosine kinase 3), a crucial target in the treatment of conditions such as acute myeloid leukemia (AML) and systemic mastocytosis. This modification paved the way for midostaurin to be used as a more targeted therapy in these diseases [38]. In the process of staurosporine biosynthesis, the enzyme *staO* initiates the synthesis by facilitating the conversion of L-tryptophan into the imine form of indole-3-pyruvic acid (IPA imine). Subsequently, *staD* catalyzes the coupling of two IPA imines, leading to the formation of chromopyrrolic acid. The formation of the indolocarbazole core of staurosporine is then guided by *staP*, which converts chromopyrrolic acid into three distinct indolocarbazole compounds: staurosporine aglycone (K252c), 7-hydroxy-K252c, and acryriaflavin A. This conversion involves intramolecular C–C bond formation and oxidative decarboxylation. Crystallography studies of the P450 enzyme *staP* have revealed that a heme group within *staP* removes two electrons from the indole ring, generating an indole cation radical. Subsequently, intramolecular radical coupling occurs, resulting in the formation of the C–C bond that shapes the indolocarbazole core [37].

The presence of *staC* plays a significant role in directing the formation of K252c as the primary product. Additionally, *staG* facilitates the formation of an N-glycosidic bond between N-13 and C-6', followed by the action of *staN*, a P450 homolog enzyme, which catalyzes an additional C–N bond formation between N-12 and C-2'. These two enzymes collectively convert K252c into 3'-O-demethyl, 4'-N-demethyl-staurosporine through intermediates known as holyrine A and holyrine B. To complete the biosynthesis process, *staMA* catalyzes N-methylation of 3'-O-demethyl, 4'-N-demethyl-staurosporine, and *staMB* catalyzes O-methylation, ultimately resulting in the formation of staurosporine [37].



**Fig. 3.** Midostaurin.

## 4 Tetrodotoxin

In Japan, fugu or puffer fish is a long-established delicacy, despite its well-known potential for toxicity. The primary culprit behind this risk to consumers is tetrodotoxin (TTX), a naturally occurring toxin found in over 20 species of puffer fish [39]. TTX is unique in that it is both water-soluble and heat-stable, meaning that cooking does not neutralize its toxicity; in fact, it can make it more potent [40]. Therefore, specially trained chefs must meticulously remove the hazardous parts of the fish, including the liver, ovaries, and skin, before serving. However, despite these precautions, there have been cases of human intoxications and even fatalities associated with the consumption of puffer fish [41].

TTX acts as a sodium channel blocker, binding to the sodium channels in the victim's excitable tissues, such as muscles and nerves, effectively immobilizing them. Initial symptoms include tingling sensations in the tongue and lips, followed by or concurrent with headaches and vomiting, which may progress to muscle weakness and ataxia. In severe cases, death can occur due to respiratory and/or heart failure [42].

Tetrodotoxin (TTX), one of the most potent nonprotein neurotoxins, was isolated from *Nocardiopsis dassonvillei* RG-33B, found in the ovaries of pufferfish *Fugu rubripes* in the Bohai Sea of China [43]. Culture condition of the bacterium cells was at 28°C for 5-7 days in the liquid medium containing 5.0 g of Bacto peptone, 1 g of yeast extract, 2.5 g of NaCl, and 10 g of glucose per L with pH adjustment to 7. TTX has been widely used in clinical settings as an analgesic, sedative, antispasmodic, and local anesthetic. Additionally, there have been clinical studies (Phase 3) evaluating the safety and efficacy of subcutaneous TTX for the treatment of moderate to severe inadequately controlled cancer-related pain [44].

TTX is not exclusive to puffer fish; it has been isolated from various marine animals, including the goby *Gobius criniger*, trumpet shellfish *Charonia sauliae*, xanthid crab *Atergatis floridus*, the blue-ringed octopus *Octopus maculosus*, starfish *Astropecten polyacanthus* and *Astropecten scoparius*. However, it's essential to note that not all puffer fish of the same species, whether wild or cultured, contain TTX [43].

The distribution of TTX among genetically unrelated animals, coupled with significant individual, regional, and seasonal variability in toxin concentration, has made the origin of TTX a highly controversial subject. Further research has indicated that cultured puffer fish are non-toxic but can become toxic when they ingest toxic livers from wild puffers [45]. This led to the postulation that all TTX-bearing animals might be infected by TTX-producing microorganisms living symbiotically within their bodies, a hypothesis later confirmed by the isolation of TTX-producing bacteria from different TTX-bearing animals [42].

## 5 Concluding remarks

In conclusion, the diverse world of Marine Actinomycetes, with a particular focus on rare species beyond *Streptomyces*, represents an invaluable reservoir of bioactive compounds. While *Streptomyces* has been extensively studied, the exclusive emphasis on this genus has led to the rediscovery of known compounds. Our review highlights the chemical composition, biological activity, and biosynthetic pathways of compounds sourced from marine rare Actinomycetes, especially those associated with marine invertebrates, a relatively underexplored area in comparison to sediment and water isolates. Notable compounds like rifamycin SV, staurosporine, and tetrodotoxin, produced by *Salinospora*, *Micromonospora*, and *Nocardopsis* genera, have shown promise in clinical trials for infection, pain relief, and anticancer treatments. This overview underscores the untapped potential of marine invertebrate-associated Marine Actinomycetes as a source of intriguing compounds with significant bioactivities. Exploring these bacteria's natural products holds the exciting prospect of unearthing novel compounds that could lead to the development of groundbreaking drugs in the future.

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