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SPECIAL FEATURE: REVIEW ARTICLE

Discovery and applications of nucleoside antibiotics beyond polyoxin

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Abstract

Nucleoside antibiotics possess various biological activities such as antibacterial, antifungal, anticancer, and herbicidal activities. RIKEN scientists contributed to this area of research with two representative antifungal nucleoside antibiotics, blasticidin S and polyoxin. Blasticidin S was the first antibiotic exploited in agriculture worldwide. Meanwhile, the polyoxins discovered by Isono and Suzuki are still used globally as an agricultural antibiotic. In this review article, the research on nucleoside antibiotics mainly done by Isono and his collaborators is summarized from the discovery of polyoxin to subsequent investigations.

Introduction

Pesticides containing organic mercury were widely used to suppress plant pathogens until the 1950s especially against the rice blast disease. But the use of mercury-containing agrochemicals was banned around 1960 when it became clear that the cause of the Minamata disease was the cumulative toxicity of organic mercury. Thus, the development of a safe pesticide to replace mercury was sought, and antibiotics such as blasticidin S [1, 2] and kasugamycin [3, 4] were discovered (Fig. 1).

Polyoxin

Researchers of RIKEN's Antibiotics Laboratory led by Yusuke Sumiki and subsequently by Saburo Suzuki started the screening for antifungal antibiotics for the prevention of rice plant pathogens. A strong antifungal activity was detected in the culture broth of an actinomycete named *Streptomyces cacaoi* var. *asoensis* isolated from a soil sample collected at Mt. Aso, Kumamoto Prefecture [5]. The active substance was water-soluble and multicomponent. Although the isolation and purification were very difficult, they succeeded in obtaining the main component as a colorless crystal. With a structural feature containing many oxygen atoms, it was named polyoxin A (Fig. 2). Moreover, this compound appeared to have a nucleoside skeleton containing a hydroxymethyluracil moiety. At that time, there was no high-performance liquid chromatography (HPLC) so the numerous components were separated by an open cellulose column. By 1968, they had isolated and identified the chemical structures of all the components, polyoxins A–L (Fig. 2a) [6]. Isono pointed out at an international conference that the basic structure of polyoxin was similar to uridine diphosphate-*N*-acetylglucosamine which is a substrate of chitin synthetase. In 1970, Endo and Misato clearly demonstrated that polyoxin inhibited the enzymatic activity

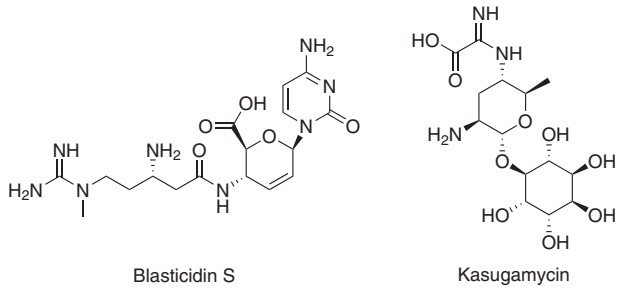

Fig. 1 Chemical structures of blasticidin S and kasugamycin

This article is dedicated to Dr Kiyoshi Isono on the occasion of his 88th birthday with respect and admiration for his great achievements in nucleoside antibiotics research

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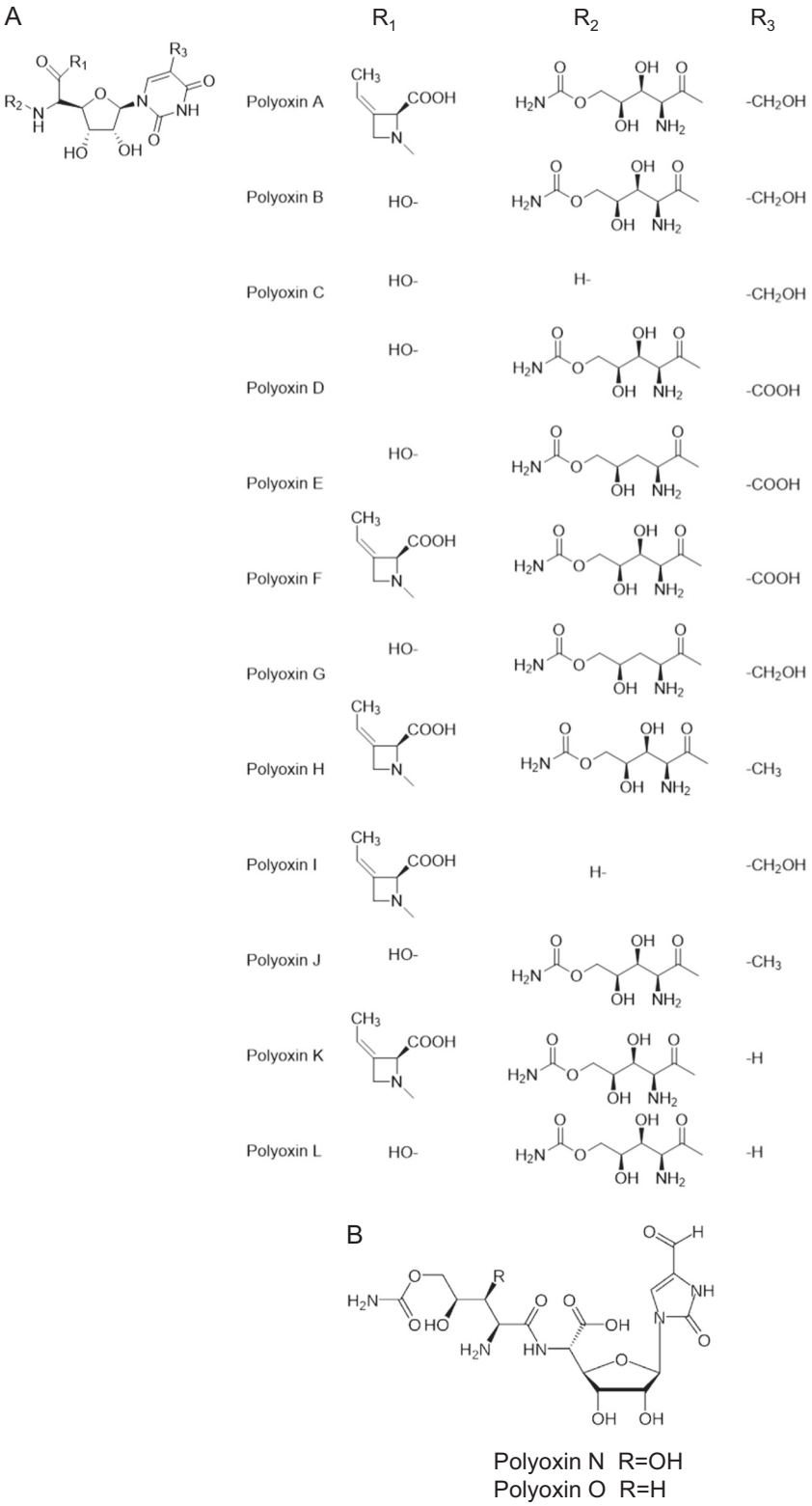


Fig. 2 **a** Chemical structures of polyoxins A–L. **b** Chemical structures of polyoxins N and O

of chitin synthetase [7]. The RIKEN researchers called the polyoxins "fungus penicillin" because they inhibit the cell wall synthesis of fungi. They further isolated new derivatives, polyoxins N and O (Fig. 2b). They studied the structure–activity relationship and made derivatives by interconversion between components via sulfite decarboxylation. It was also found that the producer strain, *S. cacaoi*, can synthesize unnatural polyoxins by the incorporation of 5-fluoro-, 5-bromo-, and 6-azauracil-uracil into the polyoxins.

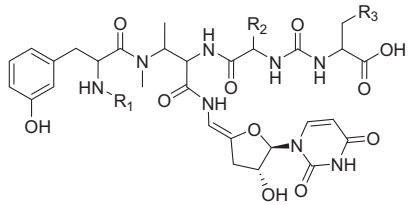
The polyoxins are effective not only for rice fungal diseases (*Pyricularia oryzae*, *Cochliobolus miyabeanus*) but also for various diseases caused by phytopathogenic filamentous fungi such as the gray mold disease of fruits (*Botrytis cinerea*) and the black spot disease of Japanese pear (*Alternaria kikuchiana*). Being a specific inhibitor of cell wall chitin, the polyoxins have no toxicity to animals and plants. In addition, they are easily degraded in the environment making them ideal green pesticides. To date, the polyoxins are still widely used as an agricultural antibiotic.

Cell wall synthesis inhibitors

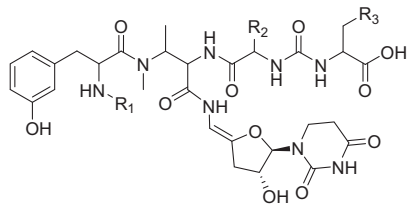
The excellent selective toxicity of the polyoxins was attributed to the inhibition of cell wall synthesis; therefore, Nishii and Isono designed several new screening systems targeting enzymes for cell wall synthesis in collaboration with Kazuo Izaki of Tohoku University. They established in vitro assay systems targeting bacterial peptidoglycan synthesis, yeast chitin synthesis, and fungal beta-glucan/mannan synthesis. In these screenings, lipopeptin [8], neopeptin [9], neopolyoxin [10, 11], and phosphazomycin [12] were discovered. In 1979, mucopeptin was isolated as an inhibitor of bacterial cell wall synthesis by Kusano et al. (reported only in Japanese, Japanese Patent Application 56-139499, 1981), however, the chemical structure could not be determined due to its complexity. In 1987, Lederle Laboratories isolated BO2964 and its analogs (US Patent 4,677,071, 1987), which might be similar compounds to mucopeptin. Moreover, chemically and biologically similar compounds, pacidamycins and mureidomycins (Fig. 3), were isolated by Abbott Laboratories [13–15] and Sankyo Co. Ltd. [16, 17], respectively, and the structures of these nucleoside peptides were revealed. Later, the stereochemistry of these compounds and their derivatives was elucidated by chemical synthesis [18].

Nikkomycin and neopolyoxin

Zahner et al. at the University of Tubingen reported the isolation and structure determination of the nikkomycin



Pacidamycins and Mureidomycins (except Mur B and D)



Mureidomycins B and D have a dihydrouracil instead of a uracil.

	R_1	R_2	R_3
Mur A	H	$(CH_2)_2SCH_3$	*m*-(HOPh)
Mur B	H	$(CH_2)_2SCH_3$	*m*-(HOPh)
Mur C	glycyl	$(CH_2)_2SCH_3$	*m*-(HOPh)
Mur D	glycyl	$(CH_2)_2SCH_3$	*m*-(HOPh)
Pac 1	alanyl	Me	3-indolyl
Pac 2	alanyl	Me	Ph
Pac 3	alanyl	Me	*m*-(HOPh)
Pac 4	H	Me	3-indolyl
Pac 5	H	Me	Ph

Fig. 3 Chemical structures of the pacidamycins (Pac) and mureidomycins (Mur)

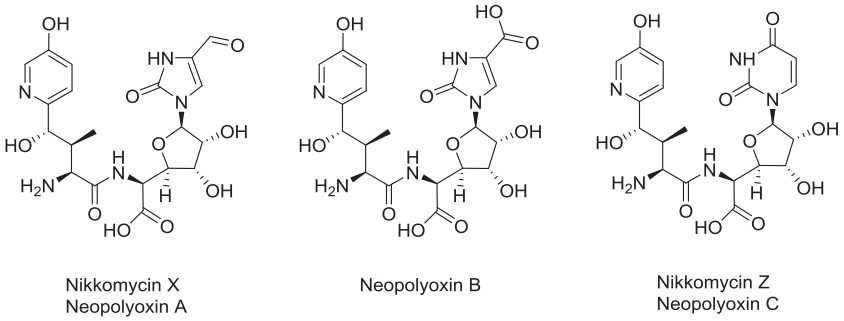


Fig. 4 Chemical structures of nikkomycins and neopolyoxins

group as a chitin synthesis inhibitor in 1976 [19]. Subsequently, the Isono group isolated neopolyoxins A–C from the polyoxin-producing strain [10, 11]. Coincidentally, neopolyoxins A and C were structurally identical to nikkomycin X and Z, respectively (Fig. 4) [10]. Isono et al. wondered how these derivatives, which differed only at the nucleobase moiety, were biosynthesized. Their effort revealed that pyrimidine undergoes a ring contraction reaction to form oxo-imidazoline [11]. On the other hand, the Zahner group continued to isolate many derivatives of nikkomycin [20, 21] and tried to develop these compounds as therapeutic agents but to no avail.

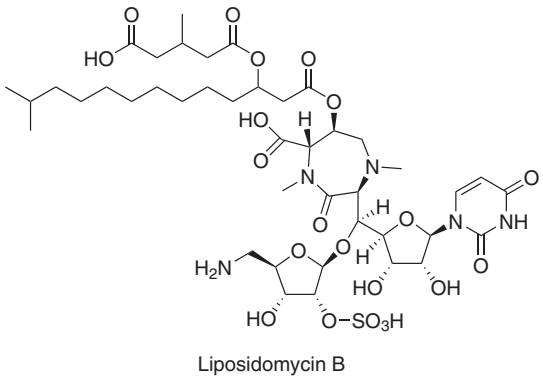


Fig. 5 Chemical structure of liposidomycin B

Liposidomycin and caprazamycin

The liposidomycin complex was isolated from *Streptomyces griseosporeus* as an inhibitor of bacterial peptidoglycan synthesis (Fig. 5). Despite the difficulty of isolating the more than 12 components of the mixture, Kimura et al. successfully accomplished the task [22, 23]. They were able to elucidate the complex nucleoside structure by collaborating with McCloskey who was in charge of the structure elucidation [24]. Both polyoxin and liposidomycin contain a uracil moiety and inhibit the fungal and bacterial cell wall synthesis, respectively, however, their mode of action is different from each other. The polyoxins inhibit fungal chitin synthesis unlike liposidomycin which inhibits bacterial peptidoglycan synthesis. Isono dubbed liposidomycin as a "bacterial polyoxin" because of their structural and target similarities.

Furthermore, the precise action mechanism of liposidomycin was shown to be as an inhibitor of UDP-*N*-acetylmuramoyl-pentapeptide: undecaprenyl-phosphate phospho-*N*-acetylmuramoyl-pentapeptide transferase (MraY), an essential membrane enzyme for bacterial cell wall biosynthesis (Fig. 6) [25]. Despite the remarkable activity of liposidomycin in the cell-free peptidoglycan biosynthesis, antibacterial activity was not strong and observed only in limited bacteria. Therefore, its development as a therapeutic agent was suspended.

In 2003, the Bikaken group isolated caprazamycin (Fig. 7) from the culture broth of *Streptomyces sp.* MK730-62F2, and found that it was active against mycobacteria [26]. The caprazamycins consisted of several members, and the structures were identified by extensive NMR analyses [27]. To examine the fragments of the structures, they subjected the caprazamycins to acid treatment. Subsequently, the core moiety of caprazamycin, caprazen (CPZEN) (Fig. 8), was obtained in high yield. Semisynthetic derivatives containing the CPZEN moiety with different side chains, were then obtained [28]. One of the derivatives, CPZEN-45, showed remarkable activity against *Mycobacterium tuberculosis* [29]. Moreover, CPZEN-45 showed antibacterial activity against caprazamycin-resistant strains, including a strain overexpressing MraY. Finally, it was revealed that the *N*-acetylglucosamine-1-phosphate transferase, WecA, is the target of the caprazamycins [30].

Liposidomycin and caprazamycin have more complex chemical structures compared with CPZEN-45. Liposidomycin and caprazamycin inhibited MraY activity which recognize UDP-MurNAc-pentapeptide as a substrate. CPZEN-45 has a simpler structure and inhibited WecA activity which uses UDP-GlcNAc as a substrate (Fig. 9).

Guanine 7-*N*-oxide

As described above, the Isono group were interested in isolating the inhibitors of microbial cell wall synthesis.

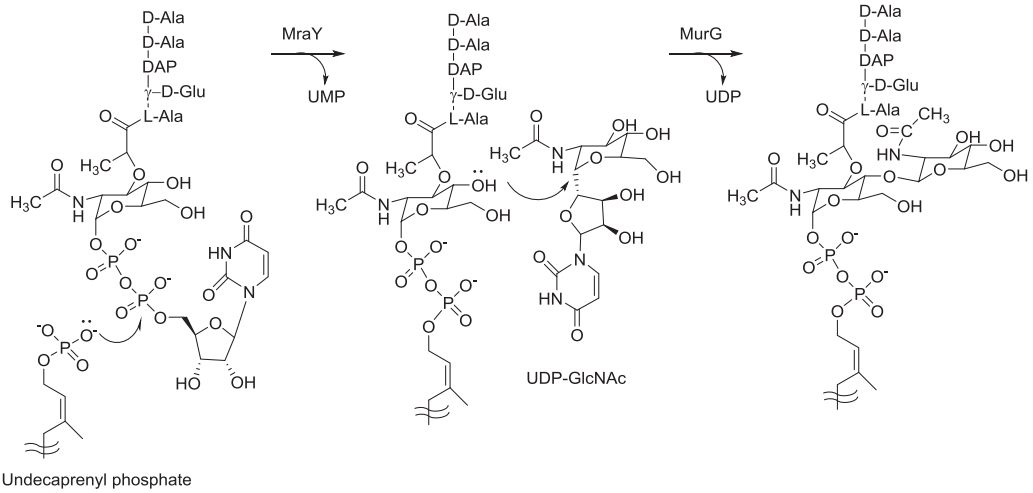


Fig. 6 Key enzymes, MraY, and MurG, for peptidoglycan biosynthesis in bacterial cell wall. MraY (PDB code: 4J72) is phospho-*N*-acetylmuramoyl-pentapeptide transferase and catalyzes the first step of bacterial peptidoglycan synthesis to form lipid I. MurG (PDB code: 1NLM) is UDP-*N*-acetylglucosamine-*N*-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol *N*-acetylglucosamine transferase, and converts lipid I to lipid II

Nishii came to RIKEN from a company and established the screening system of the inhibitors of cell wall synthesis. Although he went back to the company, he kept the collaboration with Isono and they discovered guanine 7-*N*-oxide (Fig. 10) [31]. It was an unusual nucleobase and exhibited remarkable anticancer and antiviral effects [32]. This simple compound had never been synthesized, but the microbes had taught us its existence. Later, a collaboration of RIKEN and Kanazawa University succeeded in its chemical synthesis [33]. It was expected to be developed as an antiviral agent but the development was unsuccessful. The Isono group expanded the screening target to antitumor compounds from the mid-1980s along with antimicrobial agents.

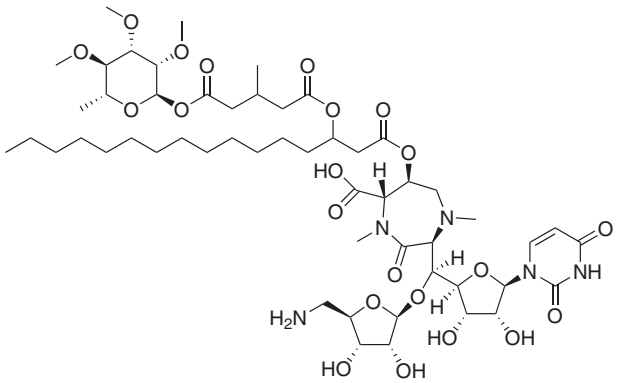


Fig. 7 Chemical structure of caprazamycin A

Ascamycin

The nucleoside antibiotic, ascamycin [34], was isolated from a *Streptomyces sp*. together with dealanylascamycin which was identical to AT-265 (Fig. 11) [35]. AT-265 (dealanylascamycin) showed a broad range of antibacterial activity against various Gram-negative and Gram-positive bacteria. On the contrary, ascamycin showed selective toxicity against plant pathogenic Gram-negative bacteria, *Xanthomonas campestris* pv. *citri* (*X. citri*) and *X. campestris* pv. *oryzae* (*X. oryzae*). When the inhibitory activity of ascamycin and dealanylascamycin was tested using a cell-free protein synthesis system, both compounds inhibited the protein synthesis of bacteria not only in *X. citri* but also in *Escherichia coli* [36]. These data suggested that the differential antibacterial activity of ascamycin and dealanylascamycin was dependent on the

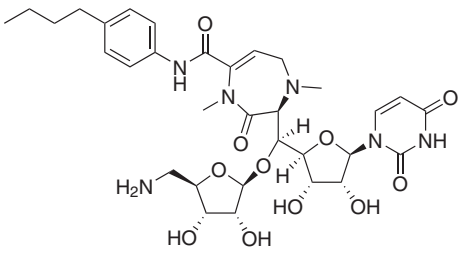


Fig. 8 Chemical structure of CPZEN-45

membrane permeability. Based on this hypothesis, a hydrolyzing enzyme of ascamycin was purified from the membrane fraction of *X. citri*. Because it was a new enzyme belonging to the proline iminopeptidase family, it was named Xc-aminopeptidase (Fig. 12) [37]. Later, the

gene encoding Xc-aminopeptidase was cloned from *X. citri* by our group [38].

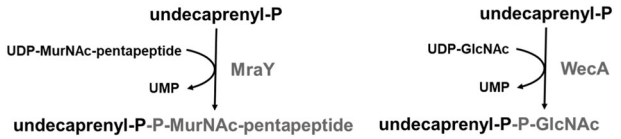
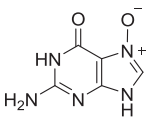
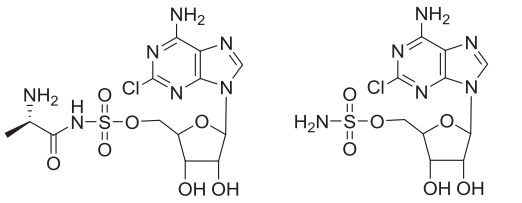


Fig. 9 Substrate difference of MraY and MurG. The target enzyme of liposidomycin and caprazamycin was MraY which used UDP-MurNAc-pentapeptide as a substrate. The target enzyme of CPZEN-45 was WecA which used UDP-GlcNAc as a substrate



Guanine 7-*N*-oxide

Fig. 10 Chemical structure of guanine 7-*N*-oxide



Ascamycin AT-265 (dealanylascamycin)

Fig. 11 Chemical structures of ascamycin and AT-265 (dealanylascamycin)

When ascamycin was isolated, it was considered as a lead compound against plant pathogenic bacteria. Moreover, the aminopeptidase gene was cloned from *X. citri*, and a homologous gene was found in *Neisseria gonorrhoeae*, a Gram-negative bacterium which causes the sexually transmitted genitourinary infection, gonorrhea. Then, the inhibitory activity of ascamycin against *N. gonorrhoeae* was examined, but the potency was very low. It was speculated that the localization of the aminopeptidase in *N. gonorrhoeae* might be different from that in *X. campestris*. At the same time, it was reported that the aminopeptidase activity in tumor cell lines is higher than that in normal cells. This observation suggested that ascamycin might act as a prodrug of an antitumor compound, and the structure-activity relationship of ascamycin derivatives against tumor cells was examined. Many ascamycin derivatives were synthesized by substituting the amino acid moiety [39, 40] in which the prolyl derivative of ascamycin was the most potent among the aminoacyl derivatives. This result was consistent with the observation that tumor cells expressed high aminopeptidase activity, especially proline iminopeptidase activity [41].

Protein kinase inhibitors

At the beginning of the 1980s, tumor biology research was rapidly advanced and protein kinases were targeted by the screeners as antitumor agents [42–44]. As a representative

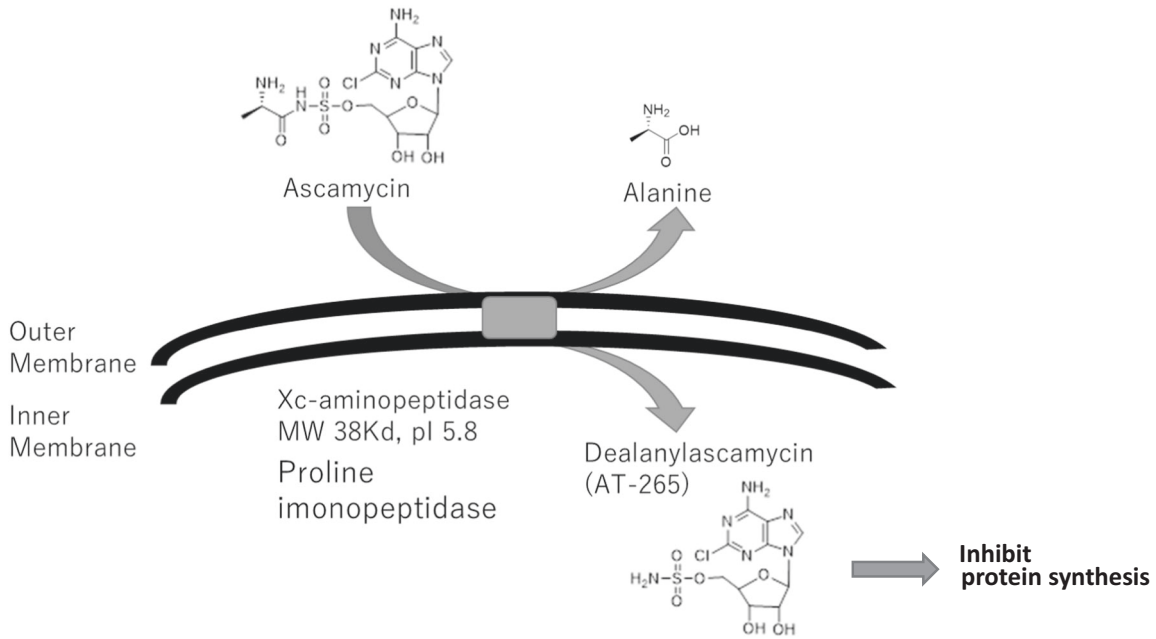


Fig. 12 Selective mode of action of ascamycin. Ascamycin was converted to AT-265 by Xc-aminopeptidase which is an iminopeptidase with molecular weight 38,000 Da

discovery in the field of the tumor biology, Nishizuka et al. revealed that protein kinase C (PKC) is a receptor protein of the tumor promoting compounds, phorbol esters [45, 46].

Responding to those backgrounds, the screening target of the Isono group was gradually shifted from agrochemical compounds to antitumor compounds [47, 48]. During the screening for PKC inhibitors using the bleb forming assay, a deazaadenosine-type nucleoside compound, sangivamycin (Fig. 13) was found and it showed strong inhibitory activity against PKC [49]. Although sangivamycin was a known compound, it was the first report regarding the PKC inhibition. Tubercidin, a deazaadenosine nucleoside antibiotic [50], was previously isolated by RIKEN Antibiotics Laboratory, and the mode of action was revealed to be via incorporation into DNA and RNA, and inhibition of their synthesis [51]. In those days, the mode of action of the deazaadenosine antibiotics was thought to be same. In other words, they were mimicking adenosine, but recently, new biological activities of the deazaadenosine antibiotics have been reported [52–54].

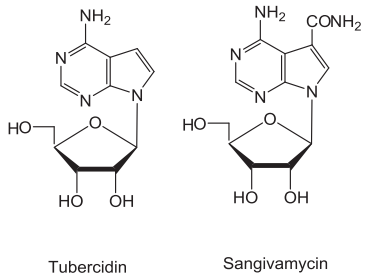


Fig. 13 Chemical structures of tubercidin and sangivamycin

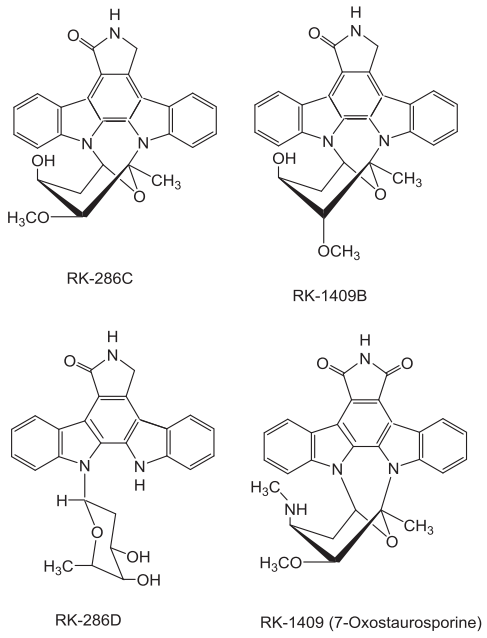


Fig. 14 Chemical structures of derivatives of staurosporine

As the bleb-forming assay was very rapid and sensitive to detect PKC inhibitors [48], indolocarbazole-type PKC inhibitors besides sangivamycin were effectively identified. Indolocarbazole-type compounds, staurosporine and K-252a, were reported as PKC inhibitors by the Kyowa Hakko group. The RIKEN group found new derivatives of the indolocarbazoles such as RK-286C, RK-1409A and B. (Fig. 14) [55–61]. As the chemical structure of the indolocarbazole is partially similar to adenosine, many kinase inhibitors have been developed based on the structure of indolocarbazole [62].

Phosmidosine

Phosmidosine (Fig. 15) was isolated by Uramoto et al. as a weak antifungal antibiotic from the culture of *Streptomyces* sp. RK-16 [63]. Later, Matsuura et al. isolated phosmidosine and its derivative, phosmidosine B, as detransforming compounds showing tumor morphology reversion activity in *src*-oncogene-transformed NRK (tsNRK) cells (Fig. 16) [64]. As these tsNRK cells contain the temperature sensitive v-*src* gene, the cell morphology was transformed at 32 °C and reverted to the normal morphology at 39 °C. As shown in Fig. 16, phosmidosine reverted the transformed morphology to the normal morphology at 32 °C. Moreover, phosmidosine arrested the cell-cycle progression at the G1

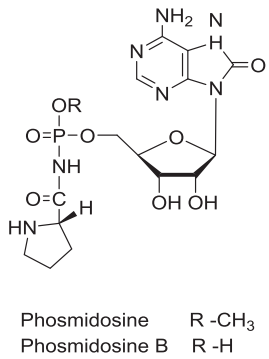


Fig. 15 Chemical structures of phosmidosine and phosmidosine B

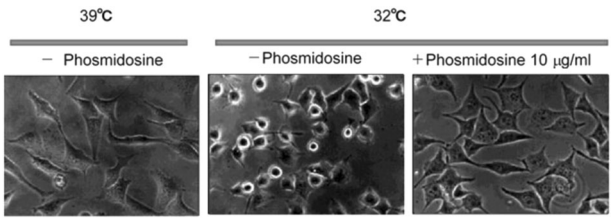


Fig. 16 Morphology of tsNRK cells. Left panel: tsNRK cells were cultured at 39 °C without phosmidosine. Middle and right panels: tsNRK cells were cultured at 32 °C, without phosmidosine (middle panel) or with 10 μg/ml phosmidosine (right panel)

phase through the inhibition of cyclin D1 expression in cancer cells [65].

Conclusion and future direction

Many important nucleoside/nucleotide compounds such as zidovudine (AZT) [66] and lamivudine (3TC) [67] have been synthesized and developed as antiviral agents, particularly as anti-HIV agents [67, 68]. However, in this review, the antibiotics are mainly focused on those isolated as microbial metabolites.

During the golden age of antibiotic discovery, there were a lot of chances to discover novel antibiotics. Moreover, the newly discovered antibiotics were launched to the market within a few years. Nowadays, it has become too difficult to explore novel antibiotics which could be exploited as therapeutic agents. Therefore, a new approach is needed to overcome these difficulties to find a new compound from microbial fermentation broths. One strategy is the construction of a broth library and further, a semipurified (fraction) library with HPLC/MS data [69]. As high-throughput screening (HTS) requires a large number of the screening samples, most pharmaceutical companies gave up on natural products and shifted to use the chemical library prepared by combinatorial synthesis. However, the semipurified (fraction) library is applicable to HTS. In addition, HPLC/MS data of the fraction library enables a certain degree of dereplication of known compounds from the microbial fermentation broths [70, 71]. Moreover, recent advances in biosynthesis enable production of a large amount of the targeted antibiotics [72–74].

Microbial metabolites are still thought to be an important source for drug screening because it offers a wider chemical space (diversity) and stronger observed biological activity compared with the compounds synthesized by combinatorial chemistry. Among the antibiotics, the nucleoside antibiotics are fascinating because of their broad biological activities [75–77].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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