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Description

Title of Invention: A NEW KANAMYCIN COMPOUND, KANAMYCIN-PRODUCING STREPTOMYCES SPECIES BACTERIUM, AND METHOD OF PRODUCING KANAMYCIN

Technical Field

- [1] The present invention relates to a new kanamycin compound, a kanamycin-producing *Streptomyces species* bacterium, and a method of producing kanamycin using the bacterium.

Background Art

- [2] Kanamycin produced by *Streptomyces kanamyceticus* is a 4,6-disubstituted 2-deoxystreptamine-containing aminoglycoside antibiotic as are gentamicins and tobramycin, whereas neomycins and butirosins are 4,5-disubstituted aminoglycosides.
- [3] These types of antibiotics are produced mainly by actinomycetes and have been widely used for a long time. Like other antibiotics, the appearance of aminoglycoside-resistant bacteria, which produce aminoglycoside-modifying enzymes, cause serious problems. The issue of resistance has been addressed by the semi-synthetic variants of naturally-occurring aminoglycosides such as amikacin, dibekacin, and arbekacin. The central 2-deoxystreptamine (2-DOS) and pseudodisaccharides 2'-*N*-acetylparomamine, paromamine, and neamine are common biosynthetic intermediates of most aminoglycosides. The biosynthetic route to neamine via 2-deoxystreptamine and paromamine was elucidated using recombinant enzymes involved in butiroidin and neomycin biosynthesis.

Disclosure of Invention

Technical Problem

- [4] It has been reported a glycosyltransferase (GT) KanE (also called KanM2) from the kanamycin cluster, catalyzes the glycosylation of paromamine to produce 3"-deamino-3"-hydroxykanamycin C using uridine 5'-diphospho-D-glucose (UDP-Glc) as a cosubstrate. Alternatively, it is considered that kanamycin C and kanamycin B are produced as kanosamine (Kns: D-3-glucosamine) is transferred to paromamine and neamine, respectively, by KanE. Otherwise, it is considered that 3"-deamino-3"-hydroxykanamycin C is a precursor of kanamycin C, which can produce kanamycin B as position C-6' of kanamycin C is aminated. It is also considered that kanamycin A can be synthesized by either KanE-catalyzed addition of kanosamine to 2'-deamino-2'-hydroxynamine or by deamination of the position C-2' of kanamycin B. However, the biosynthesis of kanamycin from pseudodisaccharides or

3"-deamino-3"-hydroxykanamycin C has not been well known. The reason for this is that it is difficult to engineer aminoglycoside-producing actinomycetes genetically and obtain functional, soluble biosynthetic enzymes.

[5] The inventors of the present invention have constructed an effective heterologous expression system based on an engineered strain of *S. venezuelae* and elucidated the biosynthesis of gentamicin A₂.

[6] Therefore, an object of the present invention is to elucidate the biosynthetic pathway of kanamycins and provide a method of producing kanamycin compounds and a new antibiotic produced by the same.

Solution to Problem

[7] Recently, the inventors of the present invention isolated the kanamycin biosynthetic gene cluster from *S. kanamyceticus*. The production of kanamycin A by expression of this entire cluster in an aminoglycoside non-producing *S. venezuelae* indicated it contains all of the genes sufficient for the biosynthesis of the kanamycin complex.

[8] Therefore, the object of the present invention can be achieved by a vector comprising *kanA-kanB-kanK* and other kanamycin biosynthetic genes, a *Streptomyces species* bacterium transformed with the vector, a method for production of kanamycin by the bacterium, and a new kanamycin compound produced by the bacterium.

Advantageous Effects of Invention

[9] According to the present invention, a kanamycin-producing *Streptomyces species* bacterium, a method for effective production of kanamycin, and a new kanamycin compound produced by the same are provided.

Brief Description of Drawings

[10] The above and other features and advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawing, in which:

[11] FIG. 1A shows the structures of 2-DOS-containing aminoglycosides and FIG. 1B shows the previously proposed biosynthetic pathway of kanamycins. In FIG. 1A, kanamycin, tobramycin, and gentamicin are 4,6-disubstituted aminoglycosides, whereas butirosins and neomycins are 4,5-disubstituted aminoglycosides. They are all naturally-occurring products, whereas amikacin, dibekacin, and arbekacin are semi-synthetic aminoglycosides derived from kanamycins. AHBA is *S*-4-amino-2-hydroxybutyric acid. FIG. 1B shows the biosynthesis of compound 5 (solid lines). However, a complete biosynthetic pathway (dotted lines) for compound 8 as a final product has not been discovered. 2-DOI: 2-deoxy-scylo-inosose; 2-DOIA: 2-deoxy-scylo-inosamine; UDP-GlcNAc: uridine 5'-diphospho-D-2-*N*-acetylglucosamine; and UDP-Glc: UDP-D-glucose.

- [12] FIGS. 2A to 2H show the results of HPLC-ESI-MS analysis of kanamycin biosynthetic intermediates obtained *in vitro* expression of putative kanamycin gene sets in recombinant *S. venezuelae* hosts and *in vitro* reactions using cell-free extracts of recombinants. FIG. 2A shows the chromatograms of extracts from the recombinants (2-DOS, DOSf, and PAR) expressing *kanA-kanB-kanK* (2-DOS biosynthetic genes), *kanA-kanB-kanK-kanF* (*kanF* is a gene encoding the first glycosyltransferase), and *kanA-kanB-kanK-kanF-kacA* (*kacA* is a gene encoding deacetylase). Each colored block represents an annotated gene. FIG. 2B shows the chromatograms of compounds 2 and 9 produced by the first glycosyltransferase KanF using three kinds of UDP sugars such as UDP-glucose (Glc), UDP-2-*N*-acetylglucosamine (GlcNAc), and UDP-2-glucosamine (GlcN) as substrates. Each box represents the cell-free extract obtained from the recombinant host expressing *kanF*, and black-framed boxes indicate the boiled cell-free extracts as controls. FIG. 2C shows the chromatograms of extracts from the recombinant (PARcd) expressing *kanA-kanB-kanK-kanF-kacA* and *kanC-kanD* at the same time and the recombinant (PARil) expressing *kanA-kanB-kanK-kanF-kacA* and *kanI-kacL* at the same time. FIG. 2D shows the chromatograms of compounds 4 and 10 produced by KanI-KacL-catalyzed reaction using paromamine and 2'-deamino-2'-hydroxyparomamine as substrates. FIG. 2E shows the chromatograms of the extracts from the recombinant (KCXΔcΔd) expressing *kanA-kanB-kanK-kanF-kacA-kanE*, the recombinant (KCX) expressing *kanA-kanB-kanK-kanF-kacA-kanE-kanC-kanD*, the recombinant (KABΔcΔd) expressing *kanA-kanB-kanK-kanF-kacA-kanE-kanI-kacL*, and the recombinant (KAB) expressing *kanA-kanB-kanK-kanF-kacA-kanE-kanC-kanD-kanI-kacL*. FIG. 2F shows the chromatograms of KanC-KanD and KanE-catalyzed production of compounds 5 and 6, supplemented with paromamine and UDP-Glc. Arrows represent quenching of sequential reactions. FIG. 2G shows the chromatograms of KanC-KanD and KanE-catalyzed production of compounds 7 and 13, supplemented with paromamine and UDP-Glc. FIG. 2H shows the chromatograms of the KanI-KacL-catalyzed production of compounds 7 and 8.
- [13] FIG. 3 shows a decalcomania-like kanamycin biosynthetic pathway, in which each colored block represents an annotated gene and its product has been proven to catalyze the biosynthesis of kanamycin intermediates. Each colored-circle represents the functional group formed by the product of the gene shown by the same colored block.
- [14] FIGS. 4A to 4C show the results of HPLC-ESI-MS/MS analysis of kanamycin biosynthetic intermediates obtained from the recombinant strains in which the first glycosyltransferase-encoding gene has been changed. FIG. 4A shows the chromatograms of extracts from the recombinants expressing *kanA-kanB-kanK* and *kanF* (DOSf), *nemD* (DOSn), *tobMI* (DOS_{t1}) or *gtmG* (DOSg) at the same time, in which the bar

graph on the right side shows the yield of kanamycin pseudodisaccharide intermediates produced by each recombinant strain. The white bar indicates compound 2 and the gray bar indicates compound 9. Data were obtained from triplicate analyses. FIG. 4B shows the chromatograms of extracts from recombinants (KCX, KCX Δ fn, KCX Δ ft₁, and KCX Δ fg) expressing one of *kanA-kanB-kanK-kacA-kanE-kanC-kanD*, together with *kanF*, *nemD*, *tobM1*, or *gtmG*. The bar graph shows the yield of kanamycin pseudodisaccharides produced by each recombinant strain. The white bar indicates compounds 5 and 6, which are the congeners of compound 7 in the left-hand cycle of the decalcomania-like biosynthetic pathway. The gray bars indicate yields of compounds 11 and 12, which are the congeners of compound 8 in the right-hand cycle. FIG. 4C shows the chromatograms of extracts from recombinants (KAB and KAB Δ fn) expressing one of *kanA-kanB-kanK-kacA-kanE-kanC-kanD-kanI-kacL* together with *kanE* or *nemD*, in which the bar graph shows the yield of kanamycin produced by each recombinant. The white bars indicate the production of compounds 6 and 7, and the gray bar represents the production of compounds 12 and 8.

- [15] FIGS. 5A to 5C show the results of HPLC-ESI-MS/MS analysis of compounds 17, 19, and 20 produced by the recombinant strains. FIG. 5A shows the chromatograms of the products from the recombinant (KCXb) expressing a compound 12-producing gene set (*kanA-kanB-kanK-kacA-kanE-kanC-kanD*) together with seven butirosin biosynthetic genes (*btrG-btrH-btrI-btrJ-btrK-btrO-btrV*) or the recombinant (KABb) expressing a compound 8-producing gene set (*kanA-kanB-kanK-kacA-kanE-kanC-kanD-kanI-kacL*). Compound 15 is the AHBA-conjugated kanamycin derivative amikacin and compound 17 is the previously uncharacterized AHBA-conjugated kanamycin derivative 1-*N*-AHBA-kanamycin X. FIG. 5B shows the chromatograms of the products from the recombinants (KAB Δ fna and KAB Δ fn Δ et₂a) expressing *aprD3-aprD4* and a compound 8-biosynthetic gene set such as *kanA-kanB-kanK-nemD-kacA-kanE-kanC-kanD-kanI-kacL* or *kanA-kanB-kanK-nemD-kacA-tobM2-kanC-kanD-kanI-kacL* at the same time. Compounds 16 and 18 are tobramycin and nebramine as a tobramycin biosynthetic intermediate. FIG. 5C shows the structures of kanamycin analogs produced by the recombinants expressing *aprD3-aprD4* and *tobM2* genes. Each colored-circle represents the functional group formed by the product of the gene with annotation information shown by the same colored block.

- [16] FIG. 6A and 6B show the results of HPLC-ESI-MS/MS analysis of kanamycin pseudodisaccharides obtained from *in vitro* reactions using cell-free extracts of recombinant *S. venezuelae* strains expressing *kanE* and *kanC-kanD*, respectively. FIG. 6A shows the chromatograms of the production of compounds 11 and 12. Each box represents the cell-free extract obtained from the recombinant host expressing *kanE* or *kanC-kanD*,

black-framed boxes indicate the boiled cell-free extracts as controls, and arrows represent quenching of sequential reactions. FIG. 6B shows the chromatograms of the production of compounds 8 and 14.

- [17] FIG. 7 shows the results of HPLC-ESI-MS/MS analysis of compounds 16 and 18 produced from *in vitro* reactions using cell-free extracts of recombinant *S. venezuelae* strains expressing *aprD3-aprD4* gene pair. Each box represents the cell-free extract obtained from the recombinant host expressing *aprD3-aprD4* gene pair, and black-framed boxes indicate boiled cell-free extracts as controls.
- [18] FIG. 8 shows the results of HPLC-ESI-MS/MS analysis of compounds 16 produced from *in vitro* reactions using cell-free extracts of wild strains of *S. kanamyceticus* and *Streptomyces tenebrarius*. Each box represents the cell-free extract obtained from *S. kanamyceticus* ATCC 12853 (SK CFE) and *S. tenebrarius* ATCC 17920 (ST CFE), and black-framed boxes indicate boiled cell-free extracts as controls.
- [19] FIG. 9 shows the results of HPLC-ESI-MS/MS analysis of kanamycin pseudodisaccharides obtained from *in vitro* reactions using cell-free extracts of wild-type *S. kanamyceticus* strain. Each box represents the cell-free extract obtained from the *S. kanamyceticus* ATCC 12853 (SK CFE), and black-framed boxes indicate boiled cell-free extracts as controls.

Best Mode for Carrying out the Invention

- [20] The present invention provides a vector comprising at least one gene set selected from the group consisting of:
- [21] (1) *kanA*, *kanB*, and *kanK* (DOS strain);
- [22] (2) *kanA*, *kanB*, *kanK*, and *kanF* (DOSf strain);
- [23] (3) *kanA*, *kanB*, *kanK*, *kanF*, and *kacA* (PAR strain);
- [24] (4) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanC*, and *kanD* (PARcd strain);
- [25] (5) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanI*, and *kacL* (PARil strain);
- [26] (6) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, and *kanE* (KCXΔcΔd strain);
- [27] (7) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, and *kanD* (KCX strain);
- [28] (8) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanI*, and *kacL* (KABΔcΔd strain);
- [29] (9) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, and *kacL* (KAB strain);
- [30] (10) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, *kanD*, *btrG*, *btrH*, *btrI*, *btrJ*, *btrK*, *btrO*, and *btrV* (KCXb strain);
- [31] (11) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, *kacL*, *btrG*, *btrH*, *btrI*, *btrJ*, *btrK*, *btrO*, and *btrV* (KABb strain);
- [32] (12) *kanA*, *kanB*, *kanK*, and *nemD* (DOSn strain);
- [33] (13) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *kanE*, *kanC*, and *kanD* (KCXΔfn strain);
- [34] (14) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, and *kacL* (KABΔfn

- strain);
- [35] (15) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, *kacL*, *aprD3*, and *aprD4* (KAB Δ fna strain);
- [36] (16) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *tobM2*, *kanC*, *kanD*, *kanI*, *kacL*, *aprD3*, and *aprD4* (KAB Δ fn Δ et₂a strain);
- [37] (17) *kanA*, *kanB*, *kanK*, and *tobM1* (DOST₁ strain);
- [38] (18) *kanA*, *kanB*, *kanK*, *tobM1*, *kacA*, *kanE*, *kanC*, and *kanD* (KCX Δ ft₁ strain);
- [39] (19) *kanA*, *kanB*, *kanK*, and *gtmG* (DOSg strain); and
- [40] (20) *kanA*, *kanB*, *kanK*, *gtmG*, *kacA*, *kanE*, *kanC*, and *kanD* (KCX Δ fg strain), and
- [41] a kanamycin-producing recombinant *Streptomyces species* bacterium transformed with the vector.
- [42] Preferably, the *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, and *kacL* are derived from *Streptomyces kanamyceticus* and have sequence numbers 5 to 14.
- [43] The *aprD3*, *aprD4*, *tobM1*, and *tobM2* are derived from *Streptomyces tenebrarius* and have sequence numbers 15 to 18.
- [44] The *btrG*, *btrH*, *btrI*, *btrJ*, *btrK*, *btrO*, and *btrV* are derived from *Bacillus circulans* and have sequence numbers 19 to 25.
- [45] The *nemD* is derived from *Streptomyces fradiae* and has sequence number 26.
- [46] The *gtmG* is derived from *Micromonospora echinospora* and has sequence number 4.
- [47] Preferably, the *Streptomyces species* bacterium further expresses a kanamycin-resistant gene. Preferably, the kanamycin-resistant gene comprises *gtmF*, *gtmK*, and *gtmL* derived from *Micromonospora echinospora*, which have sequence numbers 1 to 3.
- [48] The *Streptomyces species* bacterium is *Streptomyces venezuelae*, but not limited thereto. Preferably, the *Streptomyces species* bacterium is *Streptomyces venezuelae* KCTC11725BP and, more preferably, the *Streptomyces species* bacterium is *Streptomyces venezuelae* KCTC11726BP. The *Streptomyces species* bacterium is *Streptomyces venezuelae* KCTC11727BP and, preferably, the *Streptomyces species* bacterium is *Streptomyces venezuelae* KCTC11728BP.
- [49] Moreover, the present invention provides a method of producing a kanamycin antibiotic using the *Streptomyces species* bacterium, and the kanamycin antibiotic produced by the present invention comprises the following compounds 1 to 20:
- [50] Compound 1: 2-deoxystreptamine (2-DOS)
- [51] Compound 2: 2'-N-acetylparomamine;
- [52] Compound 3: paromamine;
- [53] Compound 4: neamine;
- [54] Compound 5: 3"-deamino-3"-hydroxykanamycin C;
- [55] Compound 6: kanamycin C;

- [56] Compound 7: kanamycin B;
- [57] Compound 8: kanamycin A;
- [58] Compound 9: 2'-deamino-2'-hydroxyparomamine;
- [59] Compound 10: 2'-deamino-2'-hydroxyneamine
- [60] Compound 11: 3"-deamino-3"-hydroxykanamycin X;
- [61] Compound 12: kanamycin X;
- [62] Compound 13: 3"-deamino-3"-hydroxykanamycin B;
- [63] Compound 14: kanamycin D;
- [64] Compound 15: amikacin;
- [65] Compound 16: tobramycin;
- [66] Compound 17: 1-*N*-AHBA-kanamycin X;
- [67] Compound 18: nebramine;
- [68] Compound 19: 3'-deoxykanamycin C; and
- [69] Compound 20: 3'-deoxykanamycin A.
- [70] Moreover, the products obtained from the recombinant bacteria are as follows:
- [71] DOS: compound 1;
- [72] DOSf: compounds 2 and 9;
- [73] PAR: compounds 3 and 9;
- [74] PARcd: compounds 3 and 9;
- [75] PARil: compounds 4 and 10;
- [76] KCX Δ c Δ d: compounds 5 and 11;
- [77] KCX: compounds 6 and 12;
- [78] KAB Δ c Δ d: compounds 13 and 14;
- [79] KAB: compounds 7 and 8;
- [80] KCXb: compound 17;
- [81] KABb: compound 15;
- [82] DOSn: compounds 2 and 9;
- [83] KCX Δ fn: compounds 6 and 12;
- [84] KAB Δ fn: compounds 7 and 8;
- [85] KAB Δ fna: compounds 18, 19 and 20;
- [86] KAB Δ fn Δ et₂a: compound 16;
- [87] DOST₁: compounds 2 and 9;
- [88] KCX Δ ft₁: compounds 6 and 12;
- [89] DOSg: compounds 2 and 9; and
- [90] KCX Δ fg: compounds 6 and 12.
- [91] When the kanamycin is produced by the gene recombinant bacteria of the present invention, especially KAB Δ fn strain, the yield of kanamycin B as a precursor of arbekacin, which is one of the semi-synthetic aminoglycosides, is improved compared

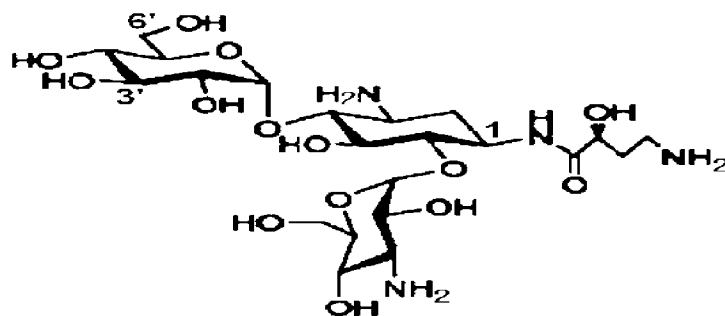
to wild-type strains.

[92] Moreover, with the use of the gene recombinant bacteria of the present invention, especially KABb and KAB Δ fn Δ et₂a strains, it is possible to produce amikacin and tobramycin by direct fermentation, not by semi-fermentation and semi-synthesis.

[93] Furthermore, the present invention provides a new compound represented by the following formula 1:

[94] [Formular 1]

[95]



[96] The new compound is compound 17, 1-*N*-AHBA-kanamycin X.

[97] The kanamycin compound of the present invention produced by the bacterium of the present invention has an antibacterial effect. More particularly, the compound of the present invention has an antibacterial effect on Gram-negative bacteria. The compound has an antibacterial effect on *Pseudomonas aeruginosa* and *Escherichia coli*, but not limited thereto. Especially, the new compound, 1-*N*-AHBA-kanamycin X, has an antibacterial effect on amikacin-resistant *P. aeruginosa*.

[98] Recently, various studies have reported that aminoglycoside antibiotics containing the 2-DOS have antiviral effects on various viruses such as bovine viral diarrhea virus (BVDV), dengue virus (DENV), etc. Moreover, many studies have reported that the aminoglycoside antibiotics, which induce mammalian ribosomes to readthrough premature stop codon mutations, can treat and improve hereditary diseases such as cystic fibrosis, muscular atrophy, Hurler syndrome, telangiectasis, and Usher syndrome. The kanamycin compound of the present invention also contains the 2-DOS as a fundamental structure, and thus it is considered that the kanamycin compound of the present invention has an antiviral effect and can treat and improve such hereditary diseases.

[99] Therefore, the present invention provides an antibacterial composition comprising the kanamycin compound as an effective ingredient.

[100] Moreover, the present invention provides an antiviral composition comprising the kanamycin compound as an effective ingredient.

[101] Furthermore, the present invention provides a pharmaceutical composition comprising the kanamycin compound as an effective ingredient for the treatment of at least one disease selected from the group consisting of cystic fibrosis, muscular

atrophy, Hurler syndrome, telangiectasis, and Usher syndrome.

- [102] The composition may be administered orally, rectally, vaginally, topically, transdermally, intravenously, intramuscularly, intraperitoneally, or subcutaneously. The effective dose of the active compound may vary depending on the minimum inhibitory concentration (MIC), the age and condition of the patient, particular disease, the severity of disease or condition, the route of administration, and administrator's decision.
- [103] The pharmaceutical composition may be medicated in the form of solid, semi-solid, or liquid depending on an administration pattern. It may include tablets, powders, suppositories, granules, creams, lotions, ointments, patches, aqueous solutions, suspensions, dispersions, emulsions, syrups, but are not limited to them. Active ingredients can be encapsulated in liposomes, nanoparticles, microcapsules, etc. Solid compositions such as tablets, pills, granules, etc. may be coated for convenience.
- [104] The composition of the present invention may contain pharmaceutically acceptable excipients such as diluents, carriers, isotoners, stabilizers, antioxidants, binders, colorants, flavorings, preservatives, thickening agents, etc. Such excipients can be selected by one skilled in the art according to the administration routes and formulations. Moreover, the composition of the present invention may contain a small amount of a non-toxic adjuvant such as a wetting agent, emulsifier, pH buffer agent, etc. The non-toxic adjuvant may include, but not limited to, acetate, sorbitan monolaurate, triethanolamine, and triethanolamine oleate. The composition for intravenous administration may be a solution in sterile isotonic aqueous buffer solution and may contain a local anesthetic for relief of pain in the injection area.

[105]

[106] Table 1

[Table 1]

[Table]

Deoxyoligonucleotide primers used for PCR amplification of genes

Gene	Primer sequence(5' to 3', restriction site shown with underline)	Portion of gene	Enzyme site
<i>kanA-kanB</i>	cccggatccgaatcccccttcgtgacg	5'	<i>Bam</i> HI
	gtgactagtgttcgtcgaccaccgcgtcga	3'	<i>Spe</i> I
<i>kanK</i>	gcttctagaactccggagcacccgtgca	5'	<i>Xba</i> I
	gtgaagcttcgtggtgccggacagggcccta	3'	<i>Hind</i> III
<i>kanC</i>	gggacctctagaacgcgggtggtcgacgaac	5'	<i>Xba</i> I
	tcttccaagcttactagtgtgcggcggtcgccccga	3'	<i>Spe</i> I/ <i>Hind</i> III
<i>kanD</i>	gaccgctctagacacctccgaggtctctc	5'	<i>Xba</i> I
	tgaaaagcttactagtgggtgacgagacgccggg	3'	<i>Spe</i> I/ <i>Hind</i> III
<i>kanE</i>	acatctagaggctccggaagaccgccgacgcca	5'	<i>Xba</i> I
	tcgaagcttactagttagacgaggaggaccctt	3'	<i>Spe</i> I/ <i>Hind</i> III
<i>kanF</i>	cgaggctctagagccggaccagaaccatt	5'	<i>Xba</i> I
	tagaacaagcttactagtacgtcgggtgtcgtacgg	3'	<i>Spe</i> I/ <i>Hind</i> III
<i>kanI-kacL</i>	gatcgtctagacacctggttctggtccc	5'	<i>Xba</i> I
	aggggaaagcttactagtgtcaggagatgccgaccg	3'	<i>Spe</i> I/ <i>Hind</i> III
<i>kacA</i>	ggcgtctagataccgggagatcgggctgtg	5'	<i>Xba</i> I
	acaaagcttactagttgtctatagcgactccttgt	3'	<i>Spe</i> I/ <i>Hind</i> III
<i>nemD</i>	cactctagacatgccgtctctccccgt	5'	<i>Xba</i> I
	tcaaagcttactagtggcgcagatacggcgcac	3'	<i>Spe</i> I/ <i>Hind</i> III
<i>tobM1</i>	ccgtctagaccgccctttccccgcaac	5'	<i>Xba</i> I
	gataagcttactagtacacaccgtctattcctg	3'	<i>Spe</i> I/ <i>Hind</i> III
<i>tobM2</i>	cgatctagaccgggaggcgaccgtgt	5'	<i>Xba</i> I
	gcgaagcttactagttcagacgcatacggcccag	3'	<i>Spe</i> I/ <i>Hind</i> III
<i>gtmG</i>	tgtcctctagagctgccccgtcacttcccgc	5'	<i>Xba</i> I
	aaaaagcttactagtctactttccggaagaatc	3'	<i>Spe</i> I/ <i>Hind</i> III

<i>btrG-btrH</i>	ttctctagatctaggaaccgcacgcc	5'	<i>XbaI</i>
	ggctacgtaaaactagtgggttatccgcttttgc	3'	<i>SpeI/SnaBI</i>
<i>btrI-btrJ</i>	acctctagacggaaaccgccatcccat	5'	<i>XbaI</i>
	tcctacgtaaaactagttagttaatgaacagccgt	3'	<i>SpeI/SnaBI</i>
<i>btrK-btrO</i>	agctctagaaagcccgaagcctgcttg	5'	<i>XbaI</i>
	gtttacgtaaaactagttccctagaccggattcga	3'	<i>SpeI/SnaBI</i>
<i>btrV</i>	tattctagaattgacttataactcaat	5'	<i>XbaI</i>
	cattacgtaaaactagtatccgaacgtcacataag	3'	<i>SpeI/SnaBI</i>
<i>aprD3</i>	gggtctagatgatggacgtggccgcga	5'	<i>XbaI</i>
	cttaagcttactagtgggccgtcggtcgtcctg	3'	<i>SpeI/HindIII</i>
<i>aprD4</i>	ggctctagaaacccaccctcaccatccag	5'	<i>XbaI</i>
	cgcaagcttactagtatcgtgtggtctccggct	3'	<i>SpeI/HindIII</i>

[107]

[108] The inventors of the present invention have reconstructed a complete biosynthesis of kanamycin by integrating the plasmid containing various candidate genes isolated from *S. kanamyceticus* into *S. venezuelae* strain, which is deficient in biosynthesis of the endogenous deoxysugar thymidine 5'-diphospho (TDP)-D-desosamine. The inventors have amplified putative kanamycin biosynthetic genes by PCR with specific primers shown in table 1.

[109] The inventors have modified the kanamycin biosynthetic flux to produce kanamycin B (compound 7) as a main product. The reason for this is that the production of compound 7 leads to the production of 3'-deoxykanamycin and 1-*N*-AHBA kanamycin by direct fermentation, which will be valuable substitutes for the chemical synthesis of dibekacin and arbekacin.

[110] After examining the kanamycin analogs obtained from the recombinants and their structures, it has been confirmed that the kanamycin antibiotic, which is known as one of the compounds produced from the recombinant bacteria of the present invention, has the same properties as the commercially available standard kanamycin analog or the kanamycin analog derived from *S. kanamyceticus*. However, it has been ascertained that 1-*N*-AHBA-kanamycin X, a new kanamycin analog, has been produced by the recombinant heterologous strain of the present invention in addition to the conventional kanamycin antibiotics.

[111] First, compound 3 was biosynthesized from Glc-6-phosphate as shown in FIG. 1B. As expected, the recombinant heterologous strain expressing *kanA-kanB-kanK* (DOS strain) produced compound 1 (21.4 μ M) (FIG. 2A). To confer resistance to kanamycin

in *S. venezuelae* without modifying the aminoglycoside structure, three resistance genes *gtmF-gtmK-gtmL* from the gentamicin gene cluster were introduced into the recombinant heterologous strain. Transformants expressing these genes were resistant to >10.0 mg/ml of the commercially available kanamycin compared to 1.0 μ g/ml in control strains. These resistance genes were expressed together with the kanamycin biosynthetic genes in the heterologous host in subsequent experiments.

[112] Compound 2 (1.3 μ M) was produced by expressing DOS biosynthetic genes plus *kanF* encoding the first glycosyltransferase. Interestingly, a greater amount of compound 9 (7.1 μ M) was produced at the same time. Moreover, The cell-free extract of *S. venezuelae* expressing *kanF* supplemented with compound 1 as a glycosyl acceptor and UDP-Glc or UDP-GlcNAc as a glycosyl donor produced 9-fold greater amount of compound 9 than that of compound 2 (FIG. 2B). Therefore, it indicates that KanF accepts both UDP-Glc and UDP-GlcNAc as cosubstrates but preferentially transfers UDP-Glc to compound 1. Compound 3 (2.2 μ M) was produced by the recombinant strain (PAR strain) expressing *kacA* as 2'-N-acetylparomamine deacetylase together with compound 2/9 biosynthetic genes (FIG. 2A).

[113] It is necessary to convert compound 3 into compound 4 for the kanamycin biosynthesis. It was estimated that KanI and KacL would participate in the conversion of compound 3 into compound 4 based on the *in silico* analysis of the aminoglycoside gene cluster. Meanwhile, it is considered that KanC and KanD are responsible for the formation of UDP-kns by aminating the C-3 hydroxyl group of UDP-Glc. Otherwise it is considered that KanC and KanD participate in the production of compound 6 from compound 5.

[114] In one example of the present invention, *kanC-kanD* genes were first expressed in PAR strain (PARcd strain) to identify the functions of KanI-KacL and KanC-KanD. However, expression of *kanC-kanD* in the strain PAR (strain PARcd) produced only 3 and 9 (FIG. 2C), indicating that KanC-KanD does not participate in amination of the pseudodisaccharides. On the contrary, the recombinant strain (PARil strain) expressing *kanI-kacL* produced compound 10 (4.3 μ M) as well as compound 4 (2.2 μ M) (FIG. 2C). Incubation of the cell-free extracts of the recombinant expressing *kanI-kacL* with compounds 3 and 9 resulted in the production of compounds 4 and 10 (FIG. 2D).

[115] In one example of the present invention, pseudotrisaccharide kanamycins were biosynthesized. Compound 5 (3.2 μ M) and compound 11 (4.5 μ M), which was not known as a biosynthetic intermediate, were produced by the recombinant (KCX Δ c Δ d strain) expressing *kanE* encoding a second glycosyltransferase together with genes for biosynthesis of compound 3/9 (FIG. 2E). Additional expression of *kanC-kanD* in KCX Δ c Δ d strain (KCX strain) resulted in the production of compound 6 (1.6 μ M) and compound 12 (6.0 M), C-3" amination. The strain (KAB Δ c Δ d strain) obtained by ex-

pressing *kanI-kacL* in KAB Δ c Δ d strain produced compound 13 (2.0 μ M) and compound 14 (3.2 μ M). Moreover, the strain (KAB strain) obtained by expressing *kanC-kanD* in KAB Δ c Δ d strain produced compound 7 of 1.0 mg/l (2.1 μ M) and compound 8 of 3.1 mg/l (6.3 μ M) (FIG. 2E).

[116] In one example of the present invention, cell-free extracts of recombinant *S. venezuelae* strains expressing *kanE* or *kanC-kanD* were prepared to determine whether UDP-kns biosynthesized from UDP-Glc is linked at the C-6 position of pseudodisaccharide or whether a Glc moiety of pseudodisaccharide is converted into kanosamine. Compounds 3 and 4 were incubated with UDP-Glc to produce compounds 5/6 and 13/7, respectively. When compounds 3 and 4 and UDP-Glc were added to the cell-free extracts of the recombinant strain expressing *kanE*, compounds 5 and 13 were produced, respectively. When these reactions were quenched and the resulting mixture was incubated together with the cell-free extracts of the strain expressing *kanC-kanD*, compounds 5 and 13 remained and further conversion was not observed. However, when compounds 3 and 4 were reacted with UDP-Glc and the cell-free extracts of the strain expressing *kanC-kanD*, compounds 6 and 7 were produced, respectively (FIGS. 2F and 2G). The same results were observed when compounds 9 and 10 were used as substrates (FIG. 6). These results showed that the hydroxyl group of UDP-Glc was aminated before the glycosyl transfer reaction by the enzyme pair during the biosynthesis of kanosamine. This example can be observed in the biosynthesis of kanosamine in *Amycolatopsis mediterranei*, a rifamycin-producing strain.

[117] Moreover, when the cell-free extracts of the strain expressing *kanI-kacL* were incubated together with compounds 6 and 12, compounds 7 and 8 were produced, respectively. It is considered that KanI and KacL participate in the biosynthesis of compounds 4 and 10 as well as compounds 7 and 8 (FIGS. 2D and 2H), which shows their substrate flexibility toward pseudodi- and tri-saccharides. In summary, a decalcomania-like kanamycin biosynthetic pathway of the kanamycin complex dominated by KanF and KanE glycosyltransferases was identified (FIG. 3).

[118] In one example of the present invention, the biosynthesis of kanamycin was modified. Compound 8 is the major fermentation product in both wild-type kanamycin producer *S. kanamyceticus* and the heterologous host *S. venezuelae*. It was shown that KanF glycosyltransferase preferred UDP-Glc to UDP-GlcNAc in the previous experiment. KanI-KacL, which preferred compound 12 to compound 6, was involved in the yield of compounds 8 and 7 (FIG. 2H). While amikacin (compound 15) can be synthesized from compound 8, the arbekacin, a latest semi-synthetic aminoglycoside having antibacterial activity against resistant bacteria, can be produced by removing the 3',4'-hydroxyl group followed by 1-*N*-acylation with AHBA. It was supposed that

the biosynthesis of kanamycin tends to increase the yield of compound 7, if the *kanF* gene encoding the first glycosyltransferase was substituted with another gene encoding the glycosyltransferase which prefers UDP-GlcNAc to UDP-Glc as a glycosyl donor in the genetic construct for the biosynthesis of compounds 7 and 8. First, the inventors replaced *kanF* in the DOS strain with *nemD*, *tobM1*, and *gtmG* encoding three different glycosyltransferases from neomycin, tobramycin, and gentamicin. The DOSf strain as a control expressing *kanF* produced compound 2 (1.4 μ M; 14% of the total pseudodisaccharide) and compound 9 (7.1 μ M; 79%). On the contrary, the strain (DOSn strain) expressing *nemD* produced compound 2 (4.4 μ M; 57%) and compound 9 (3.4 μ M; 36%), from which it seemed that NemD use UDP-GlcNAc preferentially in contrast to KanF. When *tobM1* and *gtmG* replaced *kanF* (DOS_{t1} and DOS_g strains), there was no significant change in the ratio of compounds 2 and 9 produced (FIG. 4A).

[119] Moreover, in another example of the present invention, *nemD*, *tobM1*, and *gtmG* were separately substituted for *kanF* in KCX strain. When comparing the ratio of compounds 6 and 12 (1.6 μ M, 16% and 6.0 μ M, 60%) produced by KCX strain, the ratio of pseudodisaccharides 6 and 12 (5.0 μ M, 65% and 0.4 μ M, 7%) produced by the recombinant strain (KCX Δ fn) expressing *nemD* was inverted. However, the ratio of pseudodisaccharide products 6 and 12 in the recombinant strain expressing either *tobM1* (KCX Δ ft₁) or *gtmG* (KCX Δ fg) were similar to each other (FIG. 4B). Moreover, while the yields of compounds 7 and 8 produced by KAB strain were 21% (2.3 μ M) and 61% (6.8 μ M), the yield of compound 7 in the strain (KAB Δ fn) obtained by substituting *kanF* with *nemD* was increased to 46% (FIG. 4C).

[120] In another example of the present invention, 1-*N*-AHBA-kanamycin and 3'-deoxykanamycin were produced by direct fermentation. The inventors have modified the kanamycin biosynthetic pathway for *in vivo* production of 1-*N*-acylated kanamycin containing AHBA such as compound 15 and 3'-deoxykanamycin such as compound 16. The AHBA molecular structure was observed in the naturally-occurring butirosin. Recently, it has been reported that the *btrG-btrH-btrI-btrJ-btrK-btrO-btrV* gene set is responsible for biosynthesis and introduction of the AHBA side chain into the amino group at C-1 of 2-DOS in butirosin. Compound 17 (0.6 mg/l, 1.0 μ M), a new aminoglycoside compound, was produced by introducing these genes into the KCX strain. Moreover, 0.5 mg/l (0.8 μ M) of compound 15 was successfully produced by expressing the *btr* gene set in the KAB strain (FIGS. 5A and 5C).

[121] Moreover, the inventors have analyzed the aminoglycoside gene cluster by bioinformatics analysis to find *aprD3-aprD4*, two putative apramycin genes, from *S. tenebrarius*, a tobramycin (compound 16)-producing strain. It seems that the *aprD3-aprD4* genes are responsible for C-3' deoxygenation of pseudodi- and/or tri-saccharides.

- [122] In one example of the present invention, the *aprD3-aprD4* genes were introduced into KAB Δ fn engineered for the increased production of compound 7. The resulting strain KAB Δ fna produced compound 18 as a major product and produced compounds 19 and 20 but did not produce compound 16 (FIGS. 5B and 5C). This result shows that the activity of AprD3-AprD4 pair removes the 3'-hydroxyl group in compounds 3, 10, and 4. It also indicates the second glycosyltransferase KanE selectively transfers kanosamine to compounds 3 and 10 but not to compound 18. To select the glycosyltransferase that transfers kanosamine to compound 18 to produce compound 16, cell-free extracts of *S. kanamyceticus* and *S. tenebrarius* were prepared and incubated together with compound 18. The cell-free extract of *S. tenebrarius* containing *tobM2* as a second glycosyltransferase encoding gene converted compound 18 into compound 16, but the cell-free extract of *S. kanamyceticus* did not (FIG. 7). Therefore, the inventor have constructed the glycosyltransferase encoding genes *nemD*, *tobM2*, and *aprD3-aprD4* and a new strain (KAB Δ fn Δ et₂a) expressing compound 7 biosynthetic gene and produced compound 16 (0.4 mg/l, 0.8 μ M) (FIGS. 5B and 5C). The cell-free extracts of the strain expressing *aprD3-aprD4* converted compound 4 into compound 18, but did not convert compound 7 into compound 16, which means that these enzymes are active only to pseudodisaccharides (FIG. 7).
- [123] In one embodiment of the present invention, antibacterial spectra against kanamycin intermediates and analogs produced by the present invention were measured. Typical gram-negative strains (*E. coli* and *Pseudomonas aeruginosa*) and four clinically isolated strains were employed to check the antibacterial spectra (See table 2).
- [124] Among the pseudodisaccharides, the 6'-amino compounds 4 and 10 were more active than the 6'-hydroxy derivatives 3 and 9 against kanamycin-sensitive *E. coli* strains. Compared with compounds 6 and 12 containing 6'-hydroxy group, their 6'-amino counterparts 7 and 8 were more active against kanamycin-sensitive test strains. In addition, the 3"-amino compounds 6, 7, 8, and 12 showed increased activity against the test strains when compared with the corresponding 3"-hydroxy derivatives 5, 13, 14, and 11. Therefore, when C-6' and/or C-3" hydroxyl group of kanamycin were aminated, the antibacterial activity was increased, which was consistent with the previous study.
- [125] In the test with the clinically isolated amikacin-sensitive *P. aeruginosa* strain, most kanamycin intermediates showed very low antibacterial with exception of the compounds 6, 7, 8, and 12. Interestingly, new compound 17 showed improved antibacterial activity against all the test strains compared with amikacin (compound 15). Especially, compound 15 showed no activity on the clinically isolated amikacin-resistant *P. aeruginosa* strain, whereas, compound 17 showed very strong activity (MIC ~64).

[126] Table 2

[Table 2]

[Table]

Antibacterial spectra against Gram-negative bacteria

Kanamycin-related aminoglycosides		MIC ($\mu\text{g/ml}$)					
		Typical strains		Clinically isolated strains			
		<i>E. coli</i> ATCC 25922(Kan ^S)	<i>P. aeruginosa</i> ATCC 27853(Kan ^R)	<i>E. coli</i> CCARM 1A020(Kan ^S)	<i>E. coli</i> CCARM 1A023(Kan ^R)	<i>P. aeruginosa</i> CCARM 2206(Amk ^S)	<i>P. aeruginosa</i> CCARM 2178(Amk ^R)
Pseudomonas saccharides	3	128	NA	128	NA	NA	NA
	9	128	NA	128	NA	NA	NA
	4	64	NA	64	NA	128	NA
	10	64	NA	64	NA	128	NA
Pseudomonas saccharides	6	16	NA	16	NA	64	NA
	12	16	NA	16	NA	64	NA
	5	128	NA	NA	NA	NA	NA
	11	NA	NA	NA	NA	NA	NA
	7	2	NA	4	NA	32	NA
	8	2	NA	4	NA	16	NA
	13	128	NA	128	NA	NA	NA
	14	128	NA	128	NA	NA	NA
AHBA-pseudomonas saccharides	15	128	64	128	32	≤0.25	NA
	17	16	16	32	16	0.25	64

[127]

- [128] MIC: minimum inhibitory concentration. Type strains and clinically isolated strains were obtained from ATCC (American Type Culture Collection, USA) and CCARM (Culture Collection of Antimicrobial Resistant Microbes, Republic of Korea), respectively.
- [129] Kan^S and Kan^R represent the kanamycin-sensitive strain and the kanamycin-resistant strain, respectively. Amk^S and Amk^R represent the amikacin-sensitive strain and the amikacin-resistant strain, respectively.
- [130] NA represents no activity at 128 $\mu\text{g/mL}$.
- [131] The inventors have constructed 3D models of glycosyltransferases such as KanF and NemD. The 3D models were generated by homology modeling using the crystal structure of the MshA as a template. These enzymes shared over 40% overall sequence similarity, respectively. Critical residues and their interactions were preserved in both strongly supporting conserved catalytic association mechanisms.
- [132] To gain insight into the catalytic mechanism, the simulations aimed at the preparation of KanF/NemD complex with glycosyl-donors/acceptor were repeated. First, the inventors performed a docking of the glycosyl-donors (UDP-Glc and UDP-GlcNAc) to the putative substrate binding site in both glycosyltransferases KanF and NemD using AutoDock, respectively. Secondly, the glycosyl-acceptor (compound 1) was docked into the putative glycosyl-acceptor binding site of each glycosyltransferase/ glycosyl-donors complex using CDOCKER and deeply docked into the Glc or GlcNAc area, thus making ideal interactions with their glycosyl-donors and the surrounding residues of each glycosyltransferase.
- [133] The distance between the C1 atom of the glycosyl-donors and O3 atom of compound 1 fluctuated only within very narrow limits ($\sim 3.0\text{\AA}$). The total energy of the [KanF or NemD/glycosyl-donors/compound 1] complex was stabilized and remained stable during the simulation. The radius of gyration for the complexes was found to be stable at roughly $20\text{\AA}$ throughout the simulation, suggesting that the presence of compound 1 in the complexes induce the protein to adopt more compact form compared to the proteins in the absence of compound 1. There is a large hydrophilic pocket between two domains of glycosyltransferase KanF (or NemD). The pocket is formed by glycosyl-donor neighboring hydrophilic residues such as Glu14 (Glu41), Gln19 (Gln46), Gln20 (Gln47), His88 (His115), Tyr110 (Tyr137), Thr111 (Thr138), Asp117 (Asp204), Lys218 (Lys245), Asn272 (Asn299), Glu292 (Glu319), Glu293 (Glu320), Ser297 (Ser324), and Glu300 (Glu327). Analysis of hydrophilic-hydrophilic contact in the docked structure showed that these residues are playing a crucial role in the interaction. The sugar groups of glycosyl-donors/compound 1 would interact with hydrophilic residues of the binding pocket. The uracil moiety of both glycosyl-donors forms hydrophobic interactions with Gly243 (Gly270), Phe269 (Phe296), and Ile275

(Ile302), whereas their ribose moieties build the hydrophilic interactions with Ser297 (Ser324), and Glu300 (Glu327). The hydrophilic contact between compound 1 and Tyr110 (Tyr137) is strong because the phenolic group of Tyr is moved somewhat inward or outward from its original position as compound 1 which is located above the phenolic group tries to get closer to Tyr110 (Tyr137). However, Leu89 (Leu116) make a strong hydrophobic interaction with compound 1 which doesn't move much at all. The amino group at C3 position in compound 1 interacts with the Asp177 (Asp204) which allows the dissipation of the delocalized negative charge of compound 1. The hydroxyl group at C6 position in compound 1 make hydrophilic interactions with His88 (His115), whereas the ring moiety of compound 1 makes hydrophobic interactions with Gly16 (Gly43) and Val17 (Val44). The Glc or GlcNAc part in the glycosyl-donor is anchored to a pocket formed by Glu292 (Glu319), Glu293 (Glu320), and Leu294 (Leu321), proposing that these residues might play a critical role in the stability of those complexes. The sugar parts of glycosyl-donors stack against between the carboxyl moieties of Glu292 (Glu319) and Glu293 (Glu320). Leu294 (Leu321) is located at almost the center of hydrophobic and hydrophilic interaction with sugar moiety.

- [134] The inventors have performed the molecular dynamics and binding free energy analysis. The glycosyl-donors have several hydrophilic groups as described above, thus their hydrogen bond interaction with glycosyltransferases as well as solvent molecules at the active site is important for them to bind tightly with both KanF and NemD. The number of hydrogen bonds between KanF and UDP-Glc (~5) is larger than that between KanF and UDP-GlcNAc (~2), whereas the number between NemD and UDP-Glc (~3) is smaller than that between NemD and UDP-GlcNAc (~6), indicating that UDP-Glc binds more tightly to KanF than does UDP-GlcNAc to NemD.
- [135] The calculated free energies of UDP-Glc and UDP-GlcNAc binding to KanF and NemD is -42.2, -47.3, -41.4, and -46.7 kJ/mol, respectively (See table 3).
- [136] Table 3

[Table 3]

Average binding free energies(kJ/mol) between glycosyl-donors and surrounding residues of KanF and NemD during molecular dynamics simulation.

	KanF+ UDP-GlcNAc	KanF+ UDP-Glc	NemD+ UDP-Glc	NemD+ UDP-GlcNAc
$\left\langle E_{enz}^{vdW} \right\rangle$	-7.98±0.59	-7.13±0.17	-8.07±0.24	-7.45±0.40
$\left\langle E_{enz+sub}^{vdW} \right\rangle$	-42.09±0.39	-35.04±0.84	-43.90±0.38	-41.95±0.08
$\left\langle E_{enz}^{Elec} \right\rangle$	-258.33±1.65	-284.48±1.47	-253.13±1.34	-277.01±1.83
$\left\langle E_{enz}^{vdW} \right\rangle$	-329.18±0.47	-367.88±0.48	-321.53±0.74	-356.63±0.18
$\left\langle E_{enz+sub}^{vdW} \right\rangle$	-42.25±1.15	-47.28±1.82	-41.37±1.18	-46.71±1.09

[137] * The binding free energy (ΔG) calculation was based on the molecular dynamics simulation;

$$[138] \quad \Delta G = 0.2 \left(\left\langle E_{enz-sub}^{vdW} \right\rangle - \left\langle E_{enz}^{vdW} \right\rangle \right) - 0.5 \left(\left\langle E_{enz-sub}^{Elec} \right\rangle - \left\langle E_{enz}^{Elec} \right\rangle \right)$$

[139] wherein $\langle \rangle$ denotes averages of the van der Waals (vdW) and electrostatic (Elec) interactions between the substrate (sub) such as UDP-Glc and its surrounding residues in enzymes (enz) such as KanF and NemD. 0.2 and 0.5 are scaling factor for vdW and Elec, respectively.

[140] The inventors have discovered that the substrate-flexible glycosyltransferase KanF synthesizes compounds 9 and 2 by taking both UDP-Glc and UDP-GlcNAc as cosubstrates for attachment to compound 1. The discovery of the structure of compound 9 indicated that KanF is a glucose transferase as well as an *N*-acetylglucosamine transferase. The activity of this glycosyltransferase has not been known in the biosynthesis of aminoglycoside. Moreover, compound 9 is converted into compound 10 in the same way as compound 3 is converted into compound 4 by KanI-KacL. KanI-KacL also convert compounds 6 and 12 into compounds 7 and 8, respectively. The second glycosyltransferase KanE also shows remarkable substrate flexibility toward the glycosyl-acceptor and produces compounds 6, 7, 12, and 8, respectively by transferring UDP-Kns to compounds 3, 4, 9, and 10. KanE also takes UDP-Glc as the glycosyl-donor and transfers UDP-Glc to the pseudodisaccharide, thereby producing

compounds 5, 13, 11, and 14 (FIGS. 2 and 3).

[141] The method of directly producing AHBA-binding kanamycin using the recombinant strain of the present invention is first discussed as the direct fermentative production of the semi-synthetic aminoglycoside.

[142] Moreover, compound 17 (1-*N*-AHBA-kanamycin X) as a new aminoglycoside compound produced by the present invention has strong antibacterial activity against all kanamycin and amikacin-resistant test strains. The unique structural difference between compound 15 and 17 is a functional group attached at C-6' position (FIG. 5C), which indicates that compound 17 is active against bacteria having resistance to compound 15 by removing the 6 -amino group which acts as a target of aminoglycoside 6 -acetyltransferase. Another advantage of compound 17 is the reduced toxicity due to the reduction in the number of amino groups.

[143] Furthermore, the *in vivo* production of compound 16 by a modified pathway is more economical than the conventional method using hydrolysis of 6"-*O* - carbamoyltobramycin which occupies 9% of the total nebramycin factors produced by *S. tenebrarius*.

Mode for the Invention

[144] Hereinafter, the present invention will be described by way of examples in detail. However, the following examples are provided to facilitate the understanding of the present invention, and the present invention is not limited to or by the following examples.

[145] **[Example 1]**

[146] **Preparation of materials**

[147] Standard kanamycin, kanamycin B, neomycin, paromomycin, tobramycin, and amikacin were purchased from Sigma (USA). 2-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), phenol/chloroform/isoamyl alcohol (25:24:1), uridine 5'-diphospho-D-glucose (UDP-Glc), and glass beads (150 to 212 μ m) were also purchased from Sigma. Heptafluorobutyric acid (HFBA) was obtained from Fluka, and HPLC-grade acetonitrile, methanol, and water were obtained from J. T. Baker. 2-deoxystreptamine (2-DOS, compound 1) and UDP-2-*N*-acetyl-D-glucosamine (UDP-GlcNAc) were purchased from GeneChem (Republic of Korea). Cation solid-phase exchanger (OASIS MXC SPE, 3 mL/60 mg) and vacuum manifold were purchased from Waters. The culture medium components, soybean meal, yeast extract, and malt extract were acquired from BD Science (USA).

[148] Paromamine and neamine were prepared from paromomycin and neomycin, respectively, by methanolysis. UDP-2-D-glucosamine (UDP-GlcN) was prepared by enzymatic reaction of UDP-D-glucose pyrophosphorylase with glucosamine-

1-phosphate and uridine 5'-triphosphate (UTP). *Escherichia coli* DH10B and plasmid Litmus 28 (New England Biolabs) were used for routine subcloning. High-copy number *E. coli-Streptomyces* shuttle vector pSE34 containing the strong *ermE** promoter plus a thiostrepton resistance marker was used as an expression plasmid.

[149] **[Example 2]**

[150] **Culture of strains**

[151] The recombinant strains of *S. venezuelae* were grown in liquid R2YE at 30°C for preparation of protoplasts, which were regenerated on R2YE agar medium supplemented with thiostrepton (30 µg/ml). The *E. coli* strains used for subcloning were grown in LB medium supplemented with ampicillin (50 µg/ml) to select for plasmids.

[152] For production of kanamycin biosynthetic intermediates and their analogs, *S. venezuelae* strains expressing the biosynthetic candidate genes were cultivated at 30°C for 4 days in one liter of baffled Erlenmeyer flasks containing 300 mL of R2YE medium supplemented with thiostrepton (25 µg/ml). *S. kanamyceticus* ATCC 12853 was grown at 30°C for 5 days in liquid ISP2 (0.4% yeast extract, 1.0% malt extract, and 0.4% glucose), and *S. tenebrarius* ATCC 17920 was grown at 30°C for 5 days in fermentation medium (2.0% glucose, 2.0% soluble starch, 4.0% soybean meal, 0.5% yeast extract, 0.5% CaCO₃, and 0.4% MgSO₄·7H₂O, pH 7.0).

[153] To check the antibacterial spectra of kanamycin biosynthetic intermediates and their analogs, a total six kinds of Gram-negative bacteria such as kanamycin-sensitive (Kan^S) *E. coli* ATCC 25922, kanamycin-resistant (Kan^R) *Pseudomonas aeruginosa* ATCC 27853, Kan^S *E. coli* CCARM (Culture Collection of Antimicrobial Resistant Microbes, Korea) 1A020, Kan^R *E. coli* CCARM 1A023, amikacin-sensitive (Amk^S) *P. aeruginosa* CCARM 2206, and amikacin-resistance (Amk^R) *P. aeruginosa* CCARM 2178 were used.

[154] **[Example 3]**

[155] **Cloning and construction of expression plasmids and recombinant *S. venezuelae* strains**

[156] An engineered strain of *S. venezuelae*, which is deficient in biosynthesis of the endogenous deoxysugar thymidine 5'-diphospho (TDP)-D-desosamine, was used as a heterologous host. A gene replacement plasmid, pYJ188, was introduced into protoplasts of *S. venezuelae* YJ003 mutant for deletion of the kanamycin modifying gene (*aphII*) by a replicative plasmid-mediated homologous recombination. Several double crossover mutants were identified on the basis of their phenotypes of kanamycin sensitivity and their genotypes by Southern hybridization.

[157] DNA fragments containing a variety of kanamycin biosynthetic genes were amplified from pSKC2 by PCR with specific deoxyoligonucleotide primers (See table 1). The DNA fragments encoding NemD, GtmG, AprD3, AprD4, TobM1, and TobM2 were

obtained by PCR-amplification using genomic DNA of *S. fradiae* ATCC 10745, *Micromonospora echinospora* ATCC 15385, and *S. tenebrarius* ATCC 17920, respectively. DNA fragments containing *btrI*, *btrJ*, *btrK*, *btrO*, *btrV*, *btrG*, and *btrH* required for S-4-amino-2-hydroxybutyric acid (AHBA) biosynthesis and transfer were PCR-amplified using genomic DNA of *Bacillus circulans* NR3312 as a template. Each pair of primers contained several restriction sites to facilitate subcloning of each DNA fragment.

- [158] PCR was performed using *Pfu* polymerase (Fermentas) under the manufacturer's recommended conditions. All PCR products were cloned into Litmus 28 and sequenced to confirm their authenticity.
- [159] For expression of 2-DOS biosynthetic genes, PCR-amplified fragments containing *kanA-kanB* and *kanK* were digested with *BamHI/SpeI* and *XbaI/HindIII*, respectively, and simultaneously ligated with pSE34 which had been cut with *BamHI* and *HindIII*, thus generating pDOS. Plasmid pDOSf (*kanA-kanB-kanK-kanF-gtmF-gtmK-gtmL*) was constructed to express *kanF* along with 2-DOS biosynthetic genes by ligating the *SpeI/HindIII*-digested DNA fragment of *kanF* with the *XbaI/HindIII* fragments of resistance gene set (*gtmF-gtmK-gtmL*) isolated from pYJ489, and inserting into pDOS as an *XbaI/HindIII* fragment.
- [160] All subsequent cloning steps for construction of plasmids pPAR, pPARcd, pPARil, pKCXΔcΔd, pKCX, pKABΔcΔd, pKAB, pDOSn, pDOS_t, pDOSg, pKCXΔfn, pKCXΔft₁, pKCXΔfg, and pKABΔfn were performed using the compatible cohesive ends of *SpeI* and *XbaI* sites.
- [161] For the direct fermentative production of 1-N-AHBA kanamycins, plasmid Litmus 28 containing *btrG-btrH* was digested by *SpeI/SnaBI* and ligated with the DNA fragment *btrI-btrJ* that had been cut by *XbaI/SnaBI*. The resulting plasmid carrying *btrG-btrH-btrI-btrJ* was subsequently ligated with the DNA fragment containing *btrK-btrO-btrV* in the same manner. Then, the plasmid containing *btrG-btrH-btrI-btrJ-btrK-btrO-btrV* was digested by *XbaI/SpeI* and transplanted into the *XbaI* site of pKCX and pKAB (See table 2) to generate pKCXb and pKABb, respectively. The correct orientations of the DNA fragments containing *btrG-btrH-btrI-btrJ-btrK-btrO-btrV* in the pKCXb and pKABb were confirmed by restriction fragment analyses.
- [162] Next, plasmid pKABΔfna and pKABΔfnΔet₂a were constructed in attempt to produce 3'-deoxykanamycins *in vivo*. The plasmid Litmus 28 containing *aprD3* was digested by *SpeI/HindIII* and combined with the *XbaI/HindIII* DNA fragment containing *aprD4*. The plasmid containing *aprD3-aprD4* gene set was ligated with the *XbaI/HindIII* DNA fragments containing *nemD-kanE-kanC-kanD-kanI-kacA-kacL-gtmF-gtmK-gtmL*. The resulting plasmid was moved into the *XbaI/HindIII* sites of pSE34 containing *kanA-kanB-kanK* (pDOS) to generate pKAB fna.

Plasmid pKAB Δ fn Δ et₂a was equivalent to pKAB Δ fna supplemented with *tobM2* instead of *kanE*.

- [163] To prepare the cell-free extract of *S. venezuelae* for functional assignment of each kanamycin biosynthetic gene products, DNA fragments containing *kanF*, *kanE*, *kanI-kacL*, *kanC-kanD*, and *aprD3-aprD4* were transplanted into pSE34 as *XbaI/HindIII* fragments, thus generating pKanF, pKanE, pKanI-KacL, pKanC-KanD, and pAprD3-AprD4, respectively. All resulting plasmids were then transformed into an engineered strain of *S. venezuelae*, thus yielding the corresponding recombinants.

- [164] **[Example 4]**

- [165] **Isolation and identification of kanamycin biosynthetic intermediates and their analogs**

- [166] HPLC was performed with a Spherisorb S5 ODS2 (250 x 20 mm, Waters) semi-prep column on the products of engineered recombinant strains in Example 3. The products were eluted with the same mobile phase used in HPLC-ESI-MS/MS analysis at a flow rate of 12 mL/min over a period of 150 min. The resulting eluent was fractionated into 3-mL portions that were monitored by HPLC-ESI-MS/MS to detect and characterize the presence of each kanamycin-related biosynthetic intermediate and analog.

Fractions containing products of interest were pooled and extracted using OASIS MCX SPE followed by freeze-drying. ¹H, ¹³C, and 2D ¹H-¹H COSY NMR spectra were acquired using a Varian INOVA 500 spectrometer at 298 K. Chemical shifts were reported in ppm using trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid (TSP) as an internal reference. The assignment of each compound was carried out by comparison with previously assigned ¹H and ¹³C NMR spectra, and NMR data processing was done using MESTREC (Magnetic Resonance Companion) software.

- [167] **[Example 5]**

- [168] **Analysis of kanamycin biosynthetic intermediates and analogs**

- [169] Kanamycin biosynthetic intermediates and their analogs produced by recombinant *S. venezuelae* strains expressing aminoglycoside biosynthetic genes were extracted from the fermentation medium using the OASIS MCX (Waters) SPE cleanup procedure and analyzed by HPLC-ESI-MS/MS.

- [170] Analytical HPLC-ESI-MS/MS was performed on the analytes in an XTerra MS C₁₈ column (50 x 2.1 mm, 3.5 μ m, Waters). The analytes were eluted with acetonitrile and 10 mM heptafluorobutyric acid (Fluka) for 45 minutes. Quantification of the analytes was conducted using MS/MS in the multiple reactions monitoring mode. This was done by selecting the two mass ions set to detect a transition of the parent ion to the product ion specific to the selected analytes: compound 1, 163 > 84; compound 2, 366 > 163; compounds 3 and 10, 324 > 163 ; compound 4, 323 > 163; compounds 5, 12 and 14, 486 > 163; compounds 6, 8 and 13, 485 > 163; compound 7, 484 > 163;

compound 9, 325 > 163; compound 11, 487 > 163; compound 15, 586 > 264; compound 16, 468 > 163; compound 17, 587 > 264; compound 18, 307 > 163; and compounds 19 and 20, 469 > 163. Three separate cultivations and extractions were performed.

[171] **[Example 6]**

[172] ***In vitro* reactions to analyze AprD3-AprD4 activity**

[173] *In vitro* reactions to analyze the activity of AprD3-AprD4 pair on 3'-deoxygenation in the pseudodi- or tri-saccharide were carried out by supplementing 100μM neamine or kanamycin B with the cell-free extracts from the recombinant host expressing *aprD3-aprD4*. After incubation at 30°C for 2 hours, the reaction was quenched. The resulting supernatant containing the product of interest was extracted using OASIS MCX SPE, and then subjected to HPLC-ESI-MS/MS analysis. Independent experiments were carried out in duplicate, and the results are shown in FIG. 7.

[174] **[Example 7]**

[175] ***In vitro* reactions to analyze glycosyltransfer activity of KanF**

[176] Cell-free extracts of *S. venezuelae* were prepared by glass-bead homogenization. These cell-free extracts were suspended in a Tris buffer solution containing 100 mM TrisHCl (pH 7.6), 10 mM MgCl₂, 6 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), and each protein concentration was calibrated. The glycosyltransfer reaction by KanF was initiated by supplementing 100μM 2-DOS together with 200 M UDP-Glc (Sigma), UDP-GlcNAc (GeneChem), or UDP-GlcN as cosubstrates to the cell-free extracts of the recombinant host expressing only *kanF*. 100 μM 6'-hydroxy pseudodi- and tri-saccharides 3, 6, 9, and 12 were mixed with the cell-free extracts of the recombinant host expressing *kanI-kacL* to measure the activity of KanI-KacL for C-6'-amination. The resulting cell-free extracts were incubated at 30°C for 2 hours before quenching with ice-cold phenol/chloroform/isoamyl alcohol (25:24:1, Sigma) and then subjected to centrifugation at 18,000 g for 5 minutes. The supernatant containing the product of interest was extracted using OASIS MCX SPE, mixed with 100 μM water, and then subjected to HPLC-ESI-MS/MS analysis.

[177] **[Example 8]**

[178] ***In vitro* reactions to analyze KanC-KanD activity**

[179] *In vitro* reactions to analyze the activity of KanC-KanD were carried out using cell-free extracts of two *S. venezuelae* strains expressing *kanE* or *kanC-kanD*. 200 μM UDP-Glc and 100 μM compound 3 or 4 were mixed with the cell-free extracts of the strains expressing *kanE* (or *kanC-kanD*) and incubated under the same conditions as Example 7 before quenching the reactions. After the resulting supernatant containing the product of interest was incubated together with the cell-free extracts of the strains expressing *kanC-kanD* (or *kanE*) at 30°C for 2 hours, the reaction was quenched. The

resulting supernatant was extracted in the above manner and then subjected to HPLC-ESI-MS/MS analysis.

[180] **[Example 9]**

[181] ***In vitro* reactions using cell-free extracts from wild-type strains of *S. kanamyceticus* and *S. tenebrarius***

[182] *In vitro* reactions to determine the glycosyltransfer activities of KanE from *S. kanamyceticus* and TobM2 from *S. tenebrarius* on nebramine, were performed by supplementing 100 μ M nebramine with the cell-free extract obtained from *S. kanamyceticus* (SK CFE) and TobM2 from *S. tenebrarius* (ST CFE). To minimize the presence of the kanamycin- or tobramycin-related congeners in the cell-free extracts of both wild-type strains, which might interfere with reactions, the cell-free extracts derived from glass-bead homogenization were further treated using OASIS MCX SPE pass-through as follows. At first, the cell-free extracts obtained from both wild-type strains were loaded onto each SPE cartridge, which was conditioned with methanol and water. Next, the pass-through from the cartridge was pooled, and the same step was repeated again. The above-mentioned cleanup procedures were all done in ice-cold conditions of about 4°C. The resulting cell-free extracts were incubated with compound 18 at 30°C for 2 hours, and then subjected to the OASIS MCX SPE cleanup and HPLC-ESI-MS/MS analysis. Independent experiments were carried out in duplicate, and the results are shown in FIG. 8.

[183] Reactions to determine the conversion of compound 6, 7, or 12 into compound 8 were performed by supplementing 100 μ M of compound 6, 7, or 12 with the cell-free extracts obtained from *S. kanamyceticus*. These cell-free extracts derived from glass-bead homogenization were further purified using OASIS MCX SPE pass-through as described above. After incubation at 30°C for 2 hours, the reaction was quenched. The resulting supernatant containing the product of interest was extracted using OASIS MCX SPE, and then subjected to HPLC-ESIMS/MS analysis. Independent experiments were carried out in duplicate, and the results are shown in FIG. 9.

[184] **[Example 10]**

[185] **Measurement of MIC of kanamycin biosynthetic intermediates and analogs**

[186] Minimum inhibitory concentrations (MICs) of various kanamycin-related pseudodi- and tri-saccharides and AHBA conjugated kanamycin analogs (except for compounds 16, 18, 19, and 20) were measured by broth microdilution according to the Clinical and Laboratory Standard Institute (CLSI, formerly NCCLS). As described in Example 2, gram-negative *E. coli* strains, *P. aeruginosa* strains, and clinically isolated strains were incubated in Mueller-Hinton broth (BD Science) at 30°C. Then, the aminoglycoside produced in the present invention was diluted 2-fold serially to give final concentration between 0.25 to 128 μ g/ml. An aliquot of water was used as a negative control group.

The growth of the strains was observed using the Labsystems Bioscreen C, and the minimum concentrations of the aminoglycoside diluted in the broth medium to inhibit the growth of the bacterial strains were measured.

[187] **[Example 11]**

[188] **3-dimensional (3D) implementation for glycosyltransferases such as KanF and NemD**

[189] Homology modeling of a couple of glycosyltransferases such as KanF and NemD was performed with MODELLER and optimized by FoldX using the atomic co-ordinates of MshA (PDB code: 3C48) as a template. The 3D structure models were assessed by VADAR program. Molecular docking was employed to determine the binding conformation of the glycosyl donors (UDP-Glc and UDP-GlcNAc) and acceptor (2-DOS), and energy-minimized and structure-optimized with Gaussian03 using HF/6-31G(d) basis set for molecular dynamics simulation.

[190] The docking of the glycosyl donors and 2-DOS to glycosyltransferases was initially conducted according to the predicted topological binding sites by several algorithms. The automated docking was carried out by CDOCKER program (Accelrys, Inc.) based on the MMFF force field and AutoDock 4.0 program suite. The active site was defined with 6 Å radius sphere from the putative catalytic center of KanF and NemD. Each complex model was solvated with TIP3 water molecules in a cube box and ensured the whole surface of KanF and NemD protein with their glycosyl donors/2-DOS to be covered by a water layer with a thickness more than 12Å. The energy minimization for each complex was performed using the steepest descent algorithm, followed by the conjugate gradient in the CHARMM. Then, a 500-ps position restrained molecular dynamics was performed with the proteins and glycosyl donors/2-DOS using CHARMM package. Finally, a 3 nanoseconds molecular dynamics was started by taking initial velocities from a Maxwellian distribution at 300 K. Solvent and glycosyl donors/2-DOS were independently, weakly coupled to a temperature bath with a relaxation time of 0.1 -ps. The system was also isotropically, weakly coupled to a pressure bath at 1.0 atm with a relaxation time of 0.5 picoseconds and an isothermal compressibility of $0.5 \cdot 10^{-3}$ /bar. Long-range electrostatics was calculated by the particle-mesh Ewald method. Short-range van der Waals and coulombic interactions were cut off at 0.1 Å. All bond lengths were constrained using the SHAKE algorithm, and the time step was set to 0.002 picoseconds. The binding free energies between the glycosyl donors and KanF (or NemD) were calculated using the linear interaction energy method using default parameters.

[191] While the invention has been shown and described with reference to certain exemplary embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the

spirit and scope of the invention as defined by the appended claims.

[192] **[Accession Nos.]**

[193] KAB Δ fn strain: KCTC11725BP

[194] KAB Δ fn Δ et₂a strain: KCTC11726BP

[195] KCXb strain: KCTC11727BP

[196] KABb strain: KCTC11728BP

Industrial Applicability

[197] According to the present invention, a kanamycin-producing *Streptomyces species* bacterium, a method of effectively producing kanamycin, and a new kanamycin compound produced by the same are provided.

[198]

Claims

[Claim 1]

A vector comprising at least one gene set selected from the group consisting of:

- (1) *kanA*, *kanB*, and *kanK*;
- (2) *kanA*, *kanB*, *kanK*, and *kanF*;
- (3) *kanA*, *kanB*, *kanK*, *kanF*, and *kacA*;
- (4) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanC*, and *kanD*;
- (5) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanI*, and *kacL*;
- (6) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, and *kanE*;
- (7) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, and *kanD*;
- (8) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanI*, and *kacL*;
- (9) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, and *kacL*;
- (10) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, *kanD*, *btrG*, *btrH*, *btrI*, *btrJ*, *btrK*, *btrO*, and *btrV*;
- (11) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, *kacL*, *btrG*, *btrH*, *btrI*, *btrJ*, *btrK*, *btrO*, and *btrV*;
- (12) *kanA*, *kanB*, *kanK*, and *nemD*;
- (13) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *kanE*, *kanC*, and *kanD*;
- (14) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, and *kacL*;
- ;
- (15) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, *kacL*, *aprD3*, and *aprD4*;
- (16) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *tobM2*, *kanC*, *kanD*, *kanI*, *kacL*, *aprD3*, and *aprD4*;
- (17) *kanA*, *kanB*, *kanK*, and *tobM1*;
- (18) *kanA*, *kanB*, *kanK*, *tobM1*, *kacA*, *kanE*, *kanC*, and *kanD*;
- (19) *kanA*, *kanB*, *kanK*, and *gtmG*; and
- (20) *kanA*, *kanB*, *kanK*, *gtmG*, *kacA*, *kanE*, *kanC*, and *kanD*.

[Claim 2]

The vector of claim 1, wherein the *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, and *kacL* are derived from *Streptomyces kanamyceticus*,

wherein the *aprD3*, *aprD4*, *tobM1*, and *tobM2* are derived from *Streptomyces tenebrarius*,

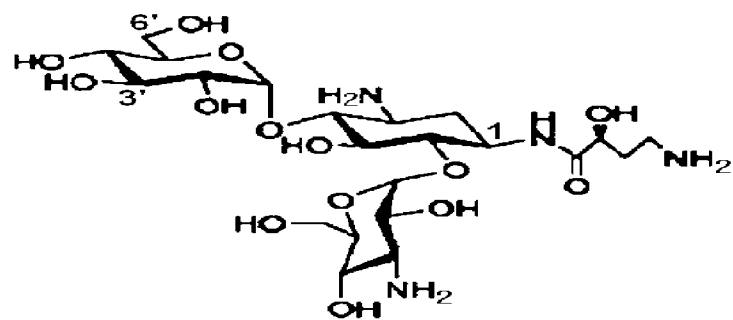
wherein the *btrG*, *btrH*, *btrI*, *btrJ*, *btrK*, *btrO*, and *btrV* are derived from *Bacillus circulans*,

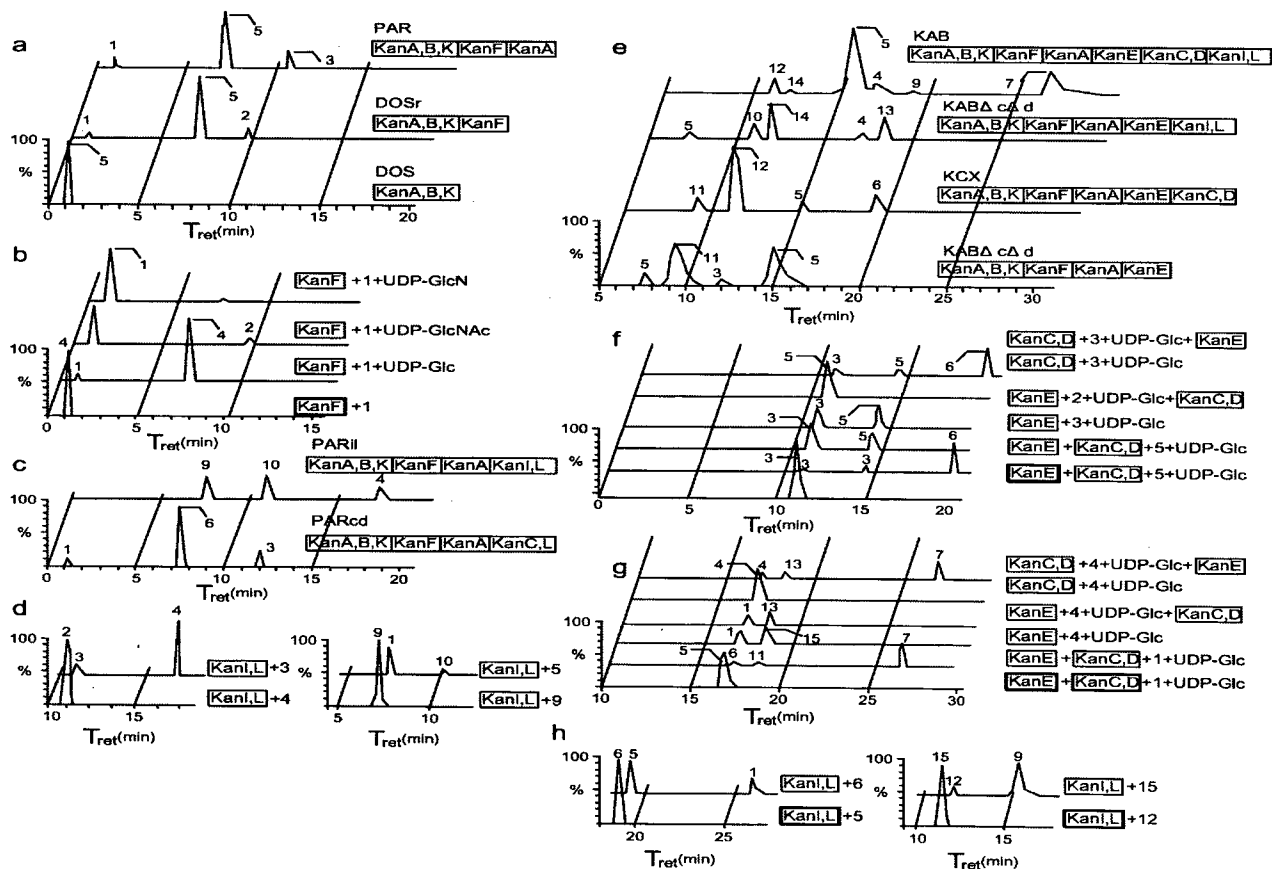
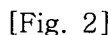
wherein the *nemD* is derived from *Streptomyces fradiae*, and

wherein the *gtmG* is derived from *Micromonospora echinospora*.

- [Claim 3] A *Streptomyces species* bacterium expressing at least one gene set selected from the group consisting of:
- (1) *kanA*, *kanB*, and *kanK*;
 - (2) *kanA*, *kanB*, *kanK*, and *kanF*;
 - (3) *kanA*, *kanB*, *kanK*, *kanF*, and *kacA*;
 - (4) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanC*, and *kanD*;
 - (5) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanI*, and *kacL*;
 - (6) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, and *kanE*;
 - (7) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, and *kanD*;
 - (8) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanI*, and *kacL*;
 - (9) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, and *kacL*;
 - (10) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, *kanD*, *btrG*, *btrH*, *btrI*, *btrJ*, *btrK*, *btrO*, and *btrV*;
 - (11) *kanA*, *kanB*, *kanK*, *kanF*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, *kacL*, *btrG*, *btrH*, *btrI*, *btrJ*, *btrK*, *btrO*, and *btrV*;
 - (12) *kanA*, *kanB*, *kanK*, and *nemD*;
 - (13) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *kanE*, *kanC*, and *kanD*;
 - (14) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, and *kacL*;
- ;
- (15) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *kanE*, *kanC*, *kanD*, *kanI*, *kacL*, *aprD3*, and *aprD4*;
 - (16) *kanA*, *kanB*, *kanK*, *nemD*, *kacA*, *tobM2*, *kanC*, *kanD*, *kanI*, *kacL*, *aprD3*, and *aprD4*;
 - (17) *kanA*, *kanB*, *kanK*, and *tobM1*;
 - (18) *kanA*, *kanB*, *kanK*, *tobM1*, *kacA*, *kanE*, *kanC*, and *kanD*;
 - (19) *kanA*, *kanB*, *kanK*, and *gtmG*; and
 - (20) *kanA*, *kanB*, *kanK*, *gtmG*, *kacA*, *kanE*, *kanC*, and *kanD*.
- [Claim 4] The *Streptomyces species* bacterium of claim 3, further expressing a kanamycin-resistant gene.
- [Claim 5] The *Streptomyces species* bacterium of claim 4, wherein the kanamycin-resistant gene comprises *gtmF*, *gtmK*, and *gtmL* derived from *Micromonospora echinospora*.
- [Claim 6] The *Streptomyces species* bacterium of claim 3, wherein the bacterium is *Streptomyces venezuelae*.
- [Claim 7] The *Streptomyces species* bacterium of claim 3, wherein the bacterium is *Streptomyces venezuelae* KCTC11727BP expressing the tenth gene set.
- [Claim 8] The *Streptomyces species* bacterium of claim 3, wherein the bacterium

- is *Streptomyces venezuelae* KCTC11728BP expressing the eleventh gene set.
- [Claim 9] The *Streptomyces species* bacterium of claim 3, wherein the bacterium is *Streptomyces venezuelae* KCTC11725BP expressing the fourteenth gene set.
- [Claim 10] The *Streptomyces species* bacterium of claim 3, wherein the bacterium is *Streptomyces venezuelae* KCTC11726BP expressing the sixteenth gene set.
- [Claim 11] A method of producing a kanamycin compound using the bacterium of claim 3.
- [Claim 12] A method of producing amikacin by direct fermentation of the bacterium of claim 8.
- [Claim 13] A method of producing tobramycin by direct fermentation of the bacterium of claim 10.
- [Claim 14] A method of improving the yield of kanamycin B using the bacterium of claim 9.
- [Claim 15] Kanamycin compounds produced by the method of claim 11.
- [Claim 16] An antibacterial composition comprising the compound of claim 15 as an effective ingredient.
- [Claim 17] The antibacterial composition of claim 16, wherein the antibacterial composition has an antibacterial activity against Gram-negative bacteria.
- [Claim 18] The antibacterial composition of claim 17, wherein the Gram-negative bacteria is *Escherichia coli* or *Pseudomonas aeruginosa*.
- [Claim 19] An antiviral composition comprising the compound of claim 15 as an effective ingredient.
- [Claim 20] A pharmaceutical composition comprising the compound of claim 15 as an effective ingredient for treatment of at least one disease selected from the group consisting of cystic fibrosis, muscular atrophy, Hurler syndrome, telangiectasis, and Usher syndrome.
- [Claim 21] A compound of formula 1 or a pharmaceutically acceptable salt thereof:
[화학식 1]





a

100 %

$T_{ret}(\text{min})$

KanA,B,K

KanF

DOSr

DOSn

DOS_{t1}

DOS_{t1}

2 4 6 8

b

100 %

$T_{ret}(\text{min})$

KanA,B,K

KanF

KCX

KCX Δ frn

KCX Δ ft1

KCX Δ fg

KanA|KanE|KanC,D

5 6 11 12

2 4 6 8

c

100 %

$T_{ret}(\text{min})$

KanA,B,K

KanF

KAB

KAB Δ frn

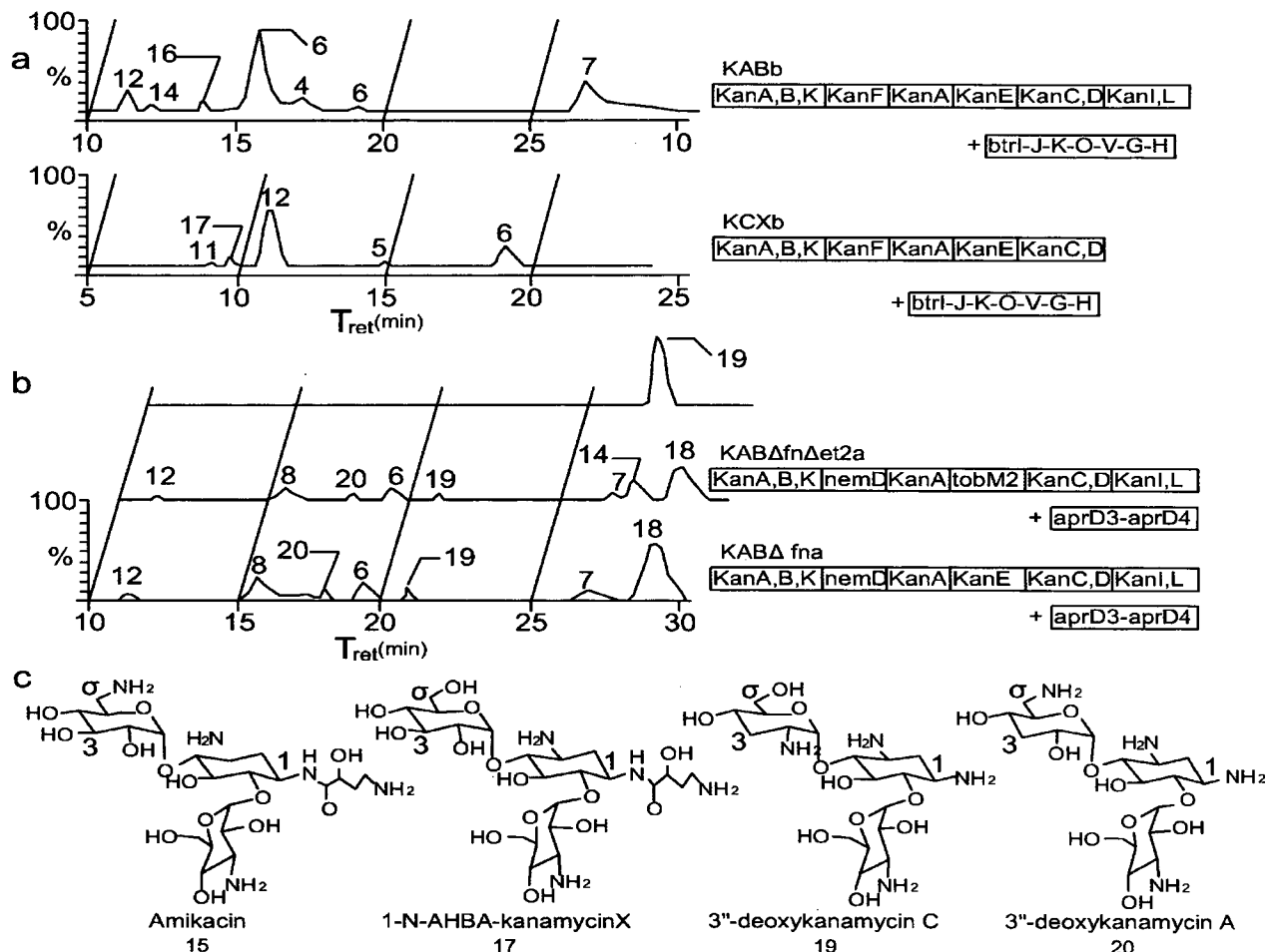
KanA|KanE|KanC,D|KanI,L

4 6 7 8 12 14

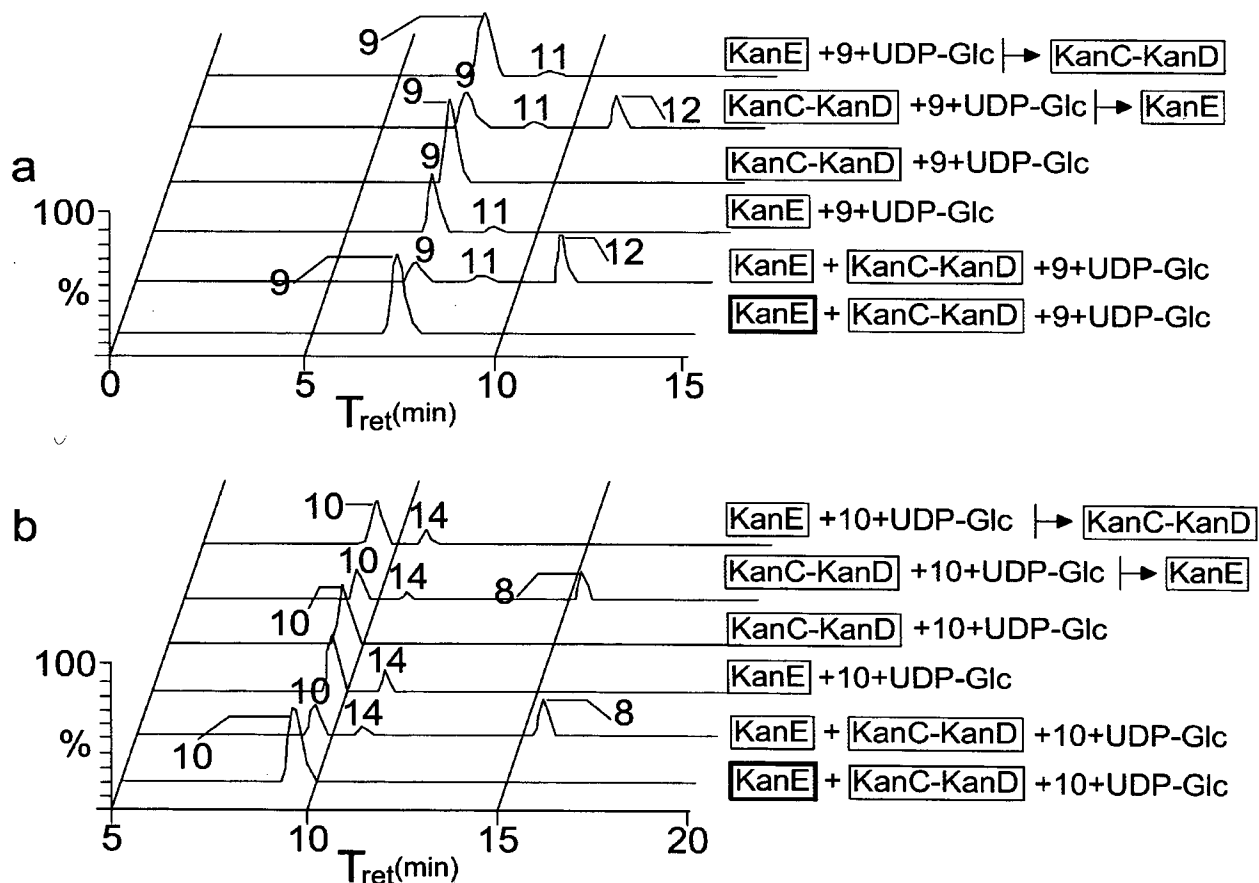
2 4 6 8

Yield(μM)

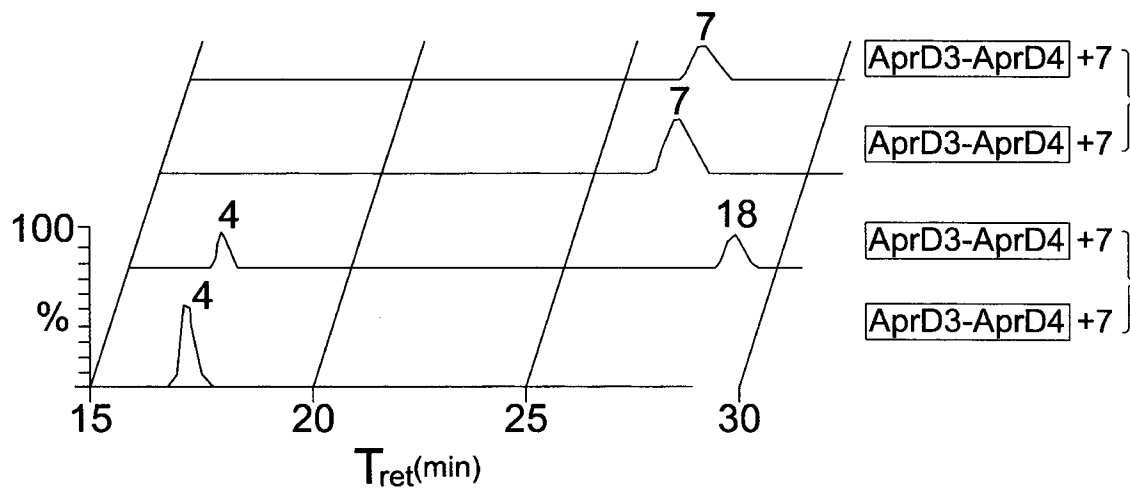
[Fig. 5]



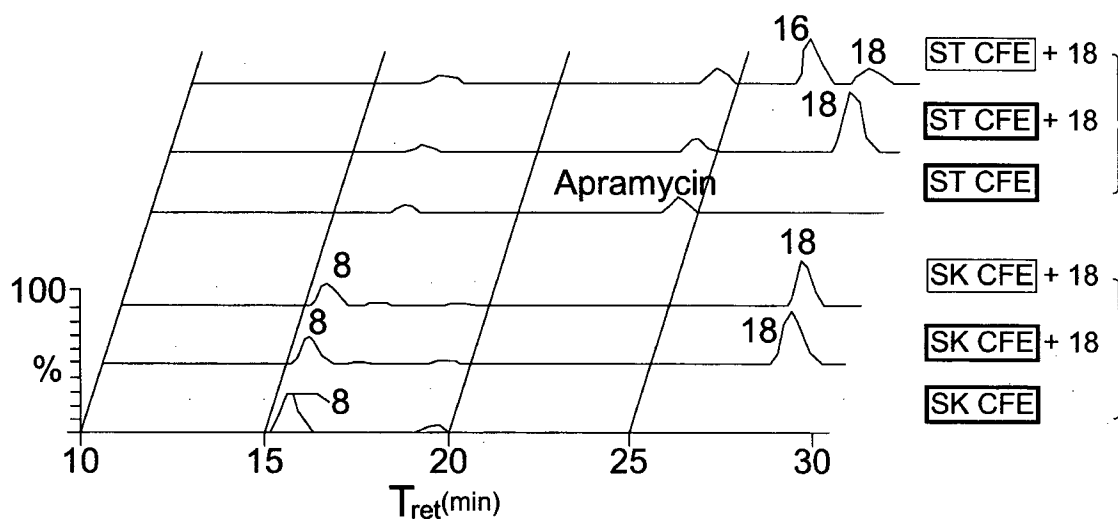
[Fig. 6]



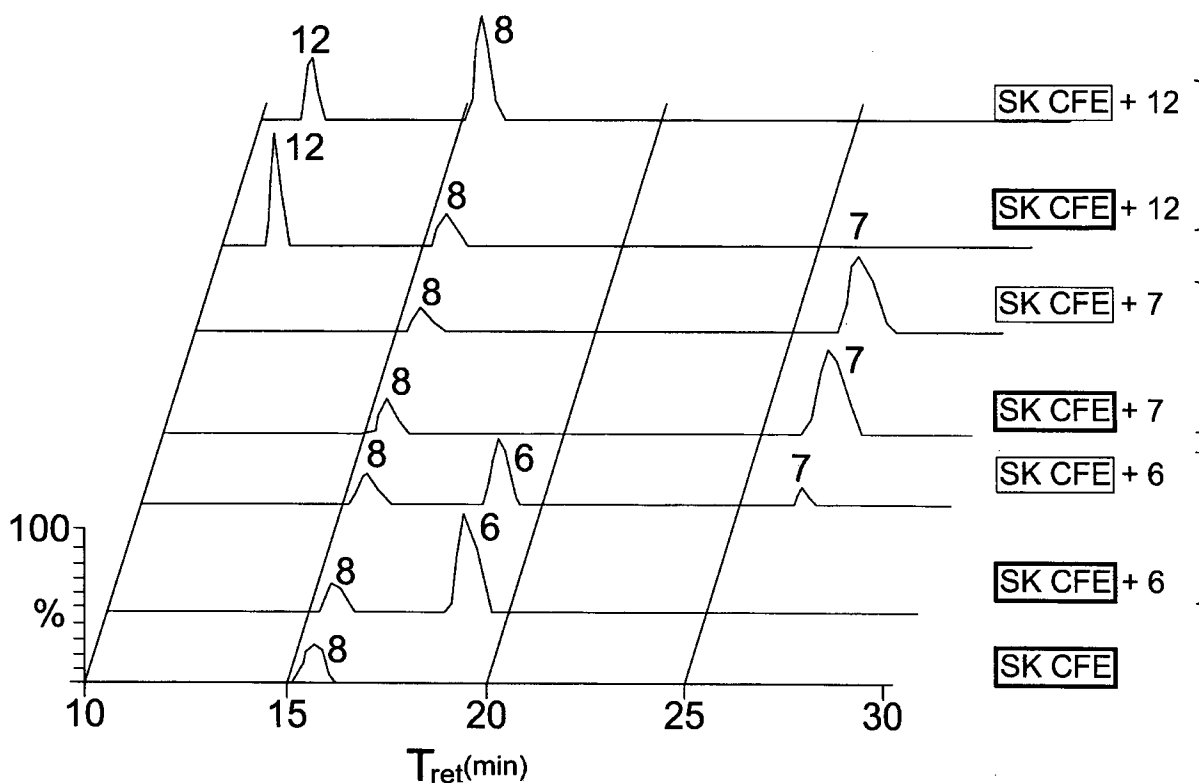
[Fig. 7]



[Fig. 8]



[Fig. 9]



A. CLASSIFICATION OF SUBJECT MATTER*C12N 15/76(2006.01)i, C12N 15/31(2006.01)i, C12N 1/21(2006.01)i, C12P 1/06(2006.01)i, C12R 1/465(2006.01)n*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/76; C12N 15/00; C12N 15/31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: kanamycin, streptomyces, kanamyceticus, venezuelae, vector, fermentation, amikacin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PARK, 'Characterization of the biosynthetic route to 4,6-disubstituted amino glycosides using heterologous expression system', Ph.D. Dissertation, EWha U niv. 28 February 2010 See abstract, pp.38,45,53,139,142,143, fig.5	1-21
A	NEPAL et al., 'Heterologous production of paromomycin in streptomyces lividans TK24 using kanamycin biosynthetic genes from streptomyces kanamyceticus ATC C12853', Molecules and Cell, Vol.27, pp.601-608 31 May 2009 See whole document	1-21
A	KR 10-1991-0003096 A (KAN-JIN CORPORATION) 26 February 1991 See abstract, claims	1-21
A	KR 10-2003-0013450 A (ID BIOMEDICAL CORPORATION) 14 February 2003 See abstract, claims	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 JUNE 2011 (22.06.2011)

Date of mailing of the international search report

23 JUNE 2011 (23.06.2011)

Name and mailing address of the ISA/KR

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Facsimile No. 82-42-472-7140

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2010/007041

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. a sequence listing filed or furnished

☐

on paper

☒

in electronic form

b. time of filing or furnishing

☐

contained in the international application as filed

☒

filed together with the international application in electronic form

☐

furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2010/007041

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