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Biosynthesis of the Tunicamycins: A Review

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Abstract Tunicamycins are nucleotide sugar analogs produced by several *Streptomyces* species. In eukaryotes, tunicamycins inhibit UDP-*N*-acetylglucosamine: dolichol phosphate GlcNAc-1-P transferase (GPT) that catalyzes the first step in protein glycosylation. In bacteria they inhibit UDP-*N*-acetylmuramoyl-pentapeptide: undecaprenol phosphate MurNAc-pentapeptide-1-P transtransferase (MraY) that catalyzes an early stage in peptidoglycan cell wall assembly. Tunicamycins are substrate analog of GPT and MraY, such that the $\alpha\beta$-1″,11′-linked GlcNAc residue of the tunicamycins mimics the transferred GlcNAc-1-phosphate. The unusual structure of tunicamycins, particularly the unique 11-carbon sugar, tunicamine, and the $\alpha\beta$-1″,11′-*O*-glycosidic linkage, suggest its biosynthesis to be unique. This review discusses potential biosyntheses for tunicamycins *via* the synthesis and conjugation of uridine-5′-aldehyde and UDP-4-keto-*N*-acetylgalactosamine-5,6-ene and the subsequent formation of the α,β-1″,11′ glycosidic linkage.

Keywords tunicamycin, biosynthesis, glycosylation, carbohydrate, peptidoglycan

Introduction

Tunicamycins are nucleotide antibiotics produced by several *Streptomyces* species. The structures are highly unusual but well characterized [1～4] and are composed of uracil, *N*-acetylglucosamine (GlcNAc), an amide-linked fatty acid, and a unique 11-carbon 2-aminodialdose sugar called tunicamine (Fig. 1). The naturally-occurring tunicamycin exists as a mixture of ten or more individual components with different *N*-linked acyl chains [2]. Mycospocidins (from *Streptomyces bobiliae*) [5], streptovirudins (*S. griseoflavus* subsp. *thuringiensis*) [6, 7], antibiotics MM 19290 (*S. clavuligerus*) and 24010 (from an unidentified streptomycete) [8, 9], and corynetoxins (*Clavibacter toxicus*) [10] are structurally akin to the tunicamycins, differing only in the *N*-acyl moiety and/or substitution of 5,6-dihydrouracil for the uracil group.

The biological activities of the tunicamycins have been the subject of over 5000 citations, although until recently much less was known about their biosynthesis. In eukaryotes, tunicamycin blocks the first step of protein *N*-glycosylation by inhibiting the enzyme UDP-*N*-acetylglucosamine: dolichol phosphate GlcNAc-1-P transferase (GlcNAc-1-P translocase, GPT) [11]. The mammalian GPT catalyses the first step in protein glycosylation, accounting for the extreme toxicity of tunicamycin in mammals. Tunicamycin is a transition state analog of GPT, such that the $\alpha\beta$-1″,11′-glycosidically-linked GlcNAc residue of the tunicamycin mimics the transferred GlcNAc-1-phosphate residue in the transition state [12]. By contrast, in bacteria tunicamycin inhibits the enzyme UDP-*N*-acetylmuramoyl-pentapeptide: undecaprenol phosphate MurNAc-pentapeptide-1-P transferase (MurNAcpp-1-P translocase, MraY) [13, 14].

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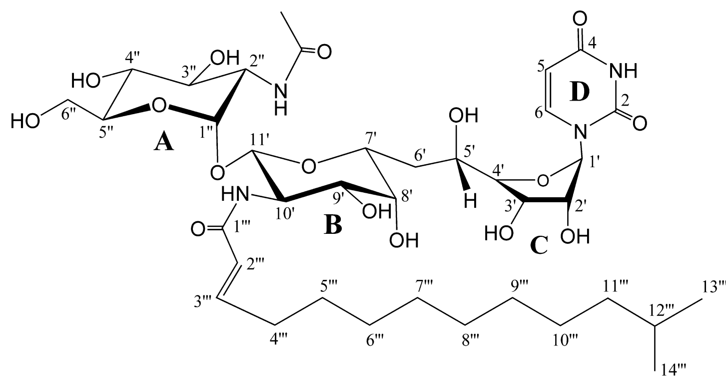

Fig. 1 Structure and numbering system for the tunicamycins.

The example shown is tunicamycin Tun 14:1. The ring systems are designated as follows: A Ring, α-linked D-GlcNAc; B Ring, β-linked pseudo-GalNAc; C Ring, pseudo-D-ribosyl; D Ring; *N*-linked uracil.

More recently, tunicamycin has also been shown to block *N*-palmitoylation of acyl-proteins by direct inhibition of the protein palmitoyltransferase [15, 16].

1. Biosynthesis of Uridine-5′-Aldehyde and the 5,6-ene Sugar Nucleotide Intermediate, UDP-4-Keto-*N*-acetylgalactosamine-5,6-ene

For biosynthetic purposes, it is useful to consider the tunicamycin core molecule as four ring systems (A, B, C, and D; Fig. 1). This consists of uracil (Ring D) *N*-glycosidically linked to D-pseudoribose (Ring C), which is C-5′–C-6′ linked to D-*N*-acyl-pseudogalactosamine (Ring B). Ring B is then *O*-glycosidically-linked to D-*N*-acetylglucosamine (Ring A). The known tunicamycins differ only by the 10′-*N*-acyl substituent on Ring B. The numbering system shown in Fig. 1 follows that proposed by Tsvetanova and Price [1, 17].

Two unique structural features of the tunicamycins are important when considering biosynthesis: 1. the 11-carbon bicyclic dialdose sugar, tunicamine (Rings BC); and 2. the αβ-1″,11′ glycosidic-to-glycosidic linkage between Rings B and A (Fig. 1). A biosynthetic pathway for the tunicamycins is outlined in Fig. 2. The first 11-carbon intermediate in the pathway, *N*-acetyl-tunicamine-uracil (Rings BCD) is potentially synthesized by ligation of uridine-5′-aldehyde (I) with an unsaturated 5,6-ene sugar nucleotide, UDP-4-keto-Glc(Gal)NAc-5,6-ene (II) (Fig. 2). Uridine-5′-aldehyde (I) does not occur in primary metabolism but may arise from oxidation of uridine *via* a 5′-dehydrogenase or oxidase activity. Uridine-5′-aldehyde can also be postulated as an early intermediate in the biosynthesis of the *Streptomyces*-produced antibiotics liposidomycins and mureidomycins that are structurally analogous to tunicamycin [18, 19].

A role for uridine-5′-aldehyde in the tunicamycin biosynthetic pathway is preferred rather than bond formation between the 5,6-ene sugar nucleotide (II) and ribose-5-aldehyde. Metabolic labeling studies with [*uracil*-5-^{2}H]uridine and [2-^{14}C]uridine have shown that uridine is directly incorporated into the tunicamycins [17]. If ribose-5-aldehyde were the precursor then a subsequent addition of uracil would be implied. During the *de novo* biosynthesis of uridine monophosphate (UMP), for example, uracil reacts with 5-phosphoribosyl-1-pyrophosphate (PRPP), catalyzed by the enzyme uracil phosphoribosyltransferase, (UPRTase) [20]. An analogous addition of uracil (Ring D) to *N*-acetyltunicamine (Ring BC) implicates *N*-acetyltunicamine-1-pyrophosphate as an intermediate and the involvement of a tunicamycin-specific UPRTase-like protein. In this respect, streptomycetes involved in nikkomycin biosynthesis have a pathway specific UPRTase activity, NikR, that selectively utilizes PRPP and imidazolone to form the imidazolone-containing nikkomycins [19, 21]. Moreover, if uridine is utilized at the nucleotide level, then an up-regulation of uridine biosynthesis or salvage pathways might be expected to support the additional requirement of tunicamycin production.

The 5,6-ene sugar nucleotide, UDP-4-keto*N*-acetylglucosamine-5,6-ene (II, Fig. 2), is likely biosynthesized from UDP-GlcNAc, in reactions catalyzed by 4-epimerase and 5,6-dehydratase activities. This is similar to the formation of sugar nucleotide intermediates in 6-deoxysugar metabolism [22]. A bifunctional UDP-GlcNAc C-6 dehydratase/C-4 epimerase from *Helicobacter pylori*, FlaA1, catalyzes the sequential conversion of UDP-

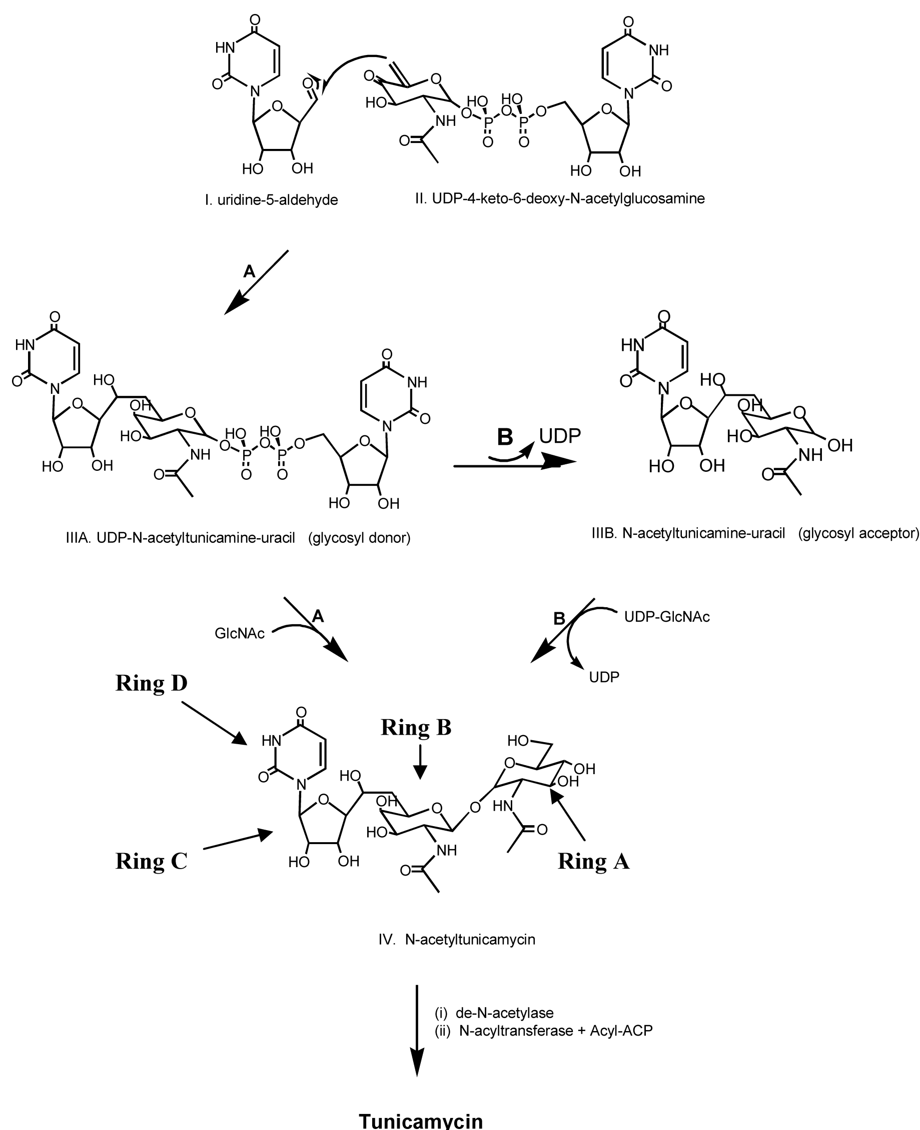

Fig. 2 Biosynthetic schemes leading to the tunicamycins.

GlcNAc to UDP-4-keto-6-methyl-GlcNAc, which is stereospecifically reduced to UDP-QuiNAc [23]. Similarly, dTDP-glucose 4,6-dehydratase (RffG) from *E. coli* catalyzes the conversion of dTDP-glucose into dTDP-4-keto-6-deoxyglucose, but unlike the FlaA1 protein does not reduce the 4-keto group to 4-hydroxy group. The mechanism of the RffG protein has been studied in detail [23, 24]. Oxidation at C-4 to a keto group is critical in activating the deprotonation of the adjacent H-5, thus bringing its pK_a into the range 18～19 [23]. The generation of an internal C-4～C-5 enolate ion would then promote stepwise β-elimination of the hydroxy group from C-6. Indeed, the wild-type RffG does not act *via* a C-4–enolate, but rather by a concerted elimination of H-5 and 6-OH [23]. Importantly, this enzyme generates dTDP-4-keto-glucose-5,6-ene as an intermediate, as determined by rapid mix-quench MALDI-TOF mass spectrometry [24]. This intermediate is analogous to the UDP-4-keto-GlcNAc-5,6-ene intermediate (II) proposed for the tunicamycin pathway (Fig. 2).

2. Biosynthesis of the First 11-Carbon Sugar Intermediate, *N*-Acetyl-tunicamine Uracil

The carbon–carbon bond formation most likely occurs by nucleophilic attack of the 6′-ene group of II on the 5′-carbonyl of uridine-5-aldehyde (I, Fig. 2). Other reactions leading to carbon–carbon bond formation (such as those that utilize thiamine pyrophosphate) are considerably

less likely because they would lead to incorrect placement of the hydroxy group found at C-5′ of tunicamine. The condensation of the UDP-4-keto-GlcNAc-5,6-ene intermediate with uridine-5′-aldehyde requires that it be first converted to a nucleophile. This may occur by hydride addition at C-5 to form a carbanion at C-6. Alternatively, pyranose ring opening may occur generating a 5,6-enolate ion resonance-stabilized with the 5-keto-6-carbanion. Nucleophilic attack can then occur on uridine-5′-aldehyde to generate the new carbon–carbon bond. Pseudopyranosyl ring closure can only occur after reduction of the *de novo* tunicaminyl C-7′ keto group to a 7′-OH group with a subsequent reductive epimerization of the 4-keto into the galactopyranosyl configuration. Thus, the 5,6-ene sugar nucleotide intermediate potentially undergoes an aldolase-catalyzed attack on uridine-5′-aldehyde to form the tunicamine-uracil core (Rings BCD). Moreover, this sequence of events also suggests that *N*-acetyltunicamine-uracil acts as the glycosidic acceptor and UDP-GlcNAc as the glycosidic donor during the biosynthesis of the $\alpha\beta$-1″,11′ glycosidic linkage [17].

The sequence of reaction described above is formally similar to that catalyzed by dehydroquinate (DHQ) synthase [25], which converts 2-keto-3-deoxyarabino-heptulosonate-7-phosphate (DHAP) to dehydroquinate, the second step in aromatic amino acid biosynthesis [26, 27]. DHQ synthase acts on DHAP in its pyranose form and catalyzes four steps: 1) oxidation at C-5 by NAD^+ to give the 5-keto sugar; 2) β-elimination of 7-P to generate a 5-keto-6,7-ene sugar analogous to UDP-4-keto-GlcNAc-5,6-ene; 3) pyranose ring opening and intramolecular condensation between the 5,6-enolate ion and the 2-keto carboxyl group, which forms the quinate ring, and 4) reduction of the ketone at C-5. It can be postulated that the 11-carbon intermediate tunicamine-uracil (Rings BCD) is generated from UDP-GlcNAc by a similar series of reactions (Fig. 3).

The major difference between the formation of DHQ and that proposed for tunicamine-uracil is that the intramolecular condensation catalyzed by DHQ synthase is replaced by an intermolecular reaction between the UDP-GlcNAc-5,6-enolate ion and the aldehyde group of uridine 5′-aldehyde. The 4-keto group is formed as a part of this mechanism to promote de-protonation at H-5, and is then stereoselectively reduced to an axial-OH group to give the galactopyranose configurataion. Hence, the GlcN to GalN 4-epimerization might be an integral part of the events catalyzed by the proposed DHQ synthase-like mechanism.

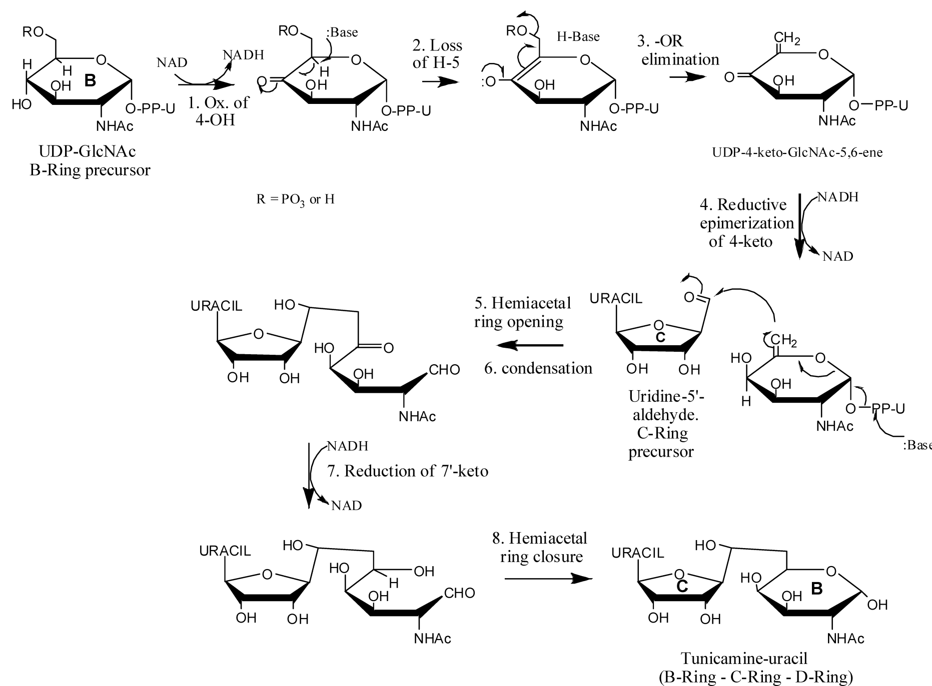

Fig. 3 Mechanism for the biosynthesis of *N*-acetyltunicamine-uracil (Rings BCD) similar to the sequence of reactions catalyzed by dehydroquinate synthase in the shikimate pathway.

1. B Ring precursor, UDP-GlcNAc, is oxidized at C4; 2. Deprotonated at C5; 3. β-elimination of the 6-OH (or 6-PO4) to form the 5,6-ene; 4. Reductive epimerization of 4-keto; 4. and 5. Hydrolysis drives enolate formation *via* ring opening; 6. 5′,6-Condensation with uridine-5′-aldehyde; 7. Reduction of tunicaminyl 7′-keto to 7′-OH; and 8. Pseudopyranose ring closure.

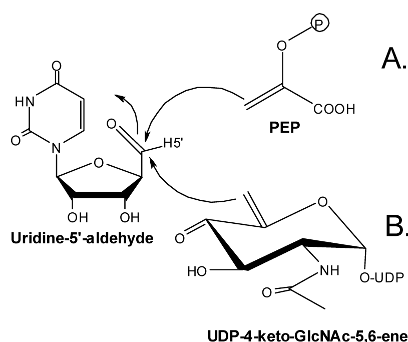

Fig. 4 Possible condensations of uridine-5′-aldehyde with A. phosphoenolpyruvate; or B. UDP-4-keto-GlcNAc-5,6-ene.

The former reaction is implicated in nikkomycin and polyoxin biosynthesis. The latter is proposed as a mechanism for the formation of the 11-carbon sugar backbone, tunicamine, as detailed in Fig. 3.

An alternative biosynthesis of the 11-carbon intermediate may occur by sequential addition of smaller units, either *via* a polyketide-type mechanism, or by ligation of uridine-5′-aldehyde to phosphoenolpyruvate (PEP). This latter reaction has been implicated in the biosynthesis of other nucleotide antibiotics, *e.g.*, polyoxins and nikkomycin, where the 8-carbon aminohexuronic acid moiety originates from PEP and uridine-5-aldehyde [18, 19, 21]. The nikkomycin-producing *S. tendae*, for example, produces an aldolase (NikO) that catalyzes the condensation of uridine-5-aldehyde with phosphoenolpyruvate (PEP) [19]. This reaction is similar to those catalyzed by the deoxyheptulose-7-phosphate, deoxyoctulosonate-8-phosphate, and deoxynonulosonate-9-phosphate synthases involved in the biosynthesis of 7-, 8- and 9-carbon long-chain sugars, respectively [28].

A similar aldolase-catalyzed reaction could be involved in carbon chain extension of the tunicamine (Rings BC) backbone (Fig. 4). However, this does not seem to occur *via* PEP. If the Ring B part of tunicamine (*i.e.*, C6′ to C11′) arose from PEP the major incorporation of label from [1-^{13}C]Glc would be expected to occur at C-6′ of Ring B, and would certainly be significantly greater that at C-11′. This is because [1-^{13}C]Glc is predominantly metabolized to [3-^{13}C]PEP *via* glycolysis. If this [3-^{13}C]PEP was condensed with uridine-5′-aldehyde (Fig. 4), the major incorporation into tunicamine should be expected at positon 6′ in the B ring. The evidence indicates the reverse: incorporation of label at C-11′ is 5-fold greater than at C-6′ [17]. Hence, [1-^{13}C]Glc incorporates into Ring B mainly by 6-carbon metabolism, so the that Glc C-1 ends up mainly in the anomeric position of Ring B, *i.e.* C-11′. In addition, deuterium label from [6,6-^{2}H,^{2}H]glucose is incorporated into the 6′-position of the tunicamine Ring B [17]. If this enrichment occurred *via* PEP, glycerol should compete for its incorporation. The finding that this does not occur demonstrates that [6,6-^{2}H,^{2}H]glucose is incorporated into tunicamine as a 6-carbon unit, not *via* PEP [14, 17]. Moreover, any alternative 2-carbon extension mechanism is also excluded because incorporation would be expected at every second position rather than the selective incorporations observed.

The equivalent incorporation of ^{13}C-label from [1-^{13}C]glucose into both the α-C1″ of Ring A and the β-C11′ anomeric carbon of Ring B indicates that both are derived from the same metabolic pool without significant isotopic dilution [17]. Hence, the biosynthesis of both of these residues (Rings A and B) likely occurs from the same precursor, either UDP-GlcNAc or GlcNAc. This might implicate GlcNAc-$\alpha\beta$-1″,11′-GlcNAc disaccharide (Ring AB) as an intermediate during tunicamycin biosynthesis, analogous to trehalose or sucrose biosynthesis. The $\alpha\beta$-1″,11′-linked disaccharide could be selectively 4-epimerized and 5,6-dehydrated on the β-linked residue prior to coupling to the uridine/ribose moiety to generate the 11-carbon sugar (Rings BCD). In this case, however, one GlcNAc residue must first be activated as a sugar nucleotide, which would lead to nonequivalent incorporation of ^{13}C. This is contrary to what was actually observed [17], and it is therefore more likely that the tunicamycin α-1″-GlcNAc (Ring A) and the tunicamine sugar (Rings BC) are both derived from the same metabolic pool of UDP-GlcNAc.

3. Formation of the α,β-1″,11′ Glycosidic Linkage

A second key question arises concerning the stereoselective formation of the α,β-1″,11′-glycosidic linkage between tunicamine-uracil (Rings BCD) and the *N*-acetylglucosamine residue (Ring A) (Fig. 1). This might occur directly by pathway A (Fig. 2) in which UDP-*N*-acetyltunicamine-uracil (IIIA) acts as the glycosidic donor, or may involve hydrolysis of IIIA to *N*-acetyltunicamine-uracil (IIIB, pathway B in Fig. 2). This additional step is analogous to the hydrolysis of UDP-diacylglucosamine to lipid X during the biosynthesis of bacterial lipid A [29]. In this scheme, *N*-acetyltunicamine-uracil (IIIB) is the glycosidic acceptor and UDP-GlcNAc would presumably act as the donor molecule. If the DHQ synthase-like hypothesis (Fig. 3) is valid, the hydrolysis of the UDP is integral to the mechanism, and we must accept that the glycosylation acceptor is, indeed, *N*-acetyltunicamine-uracil (Ring ABC) and that UDP-GlcNAc is the donor substrate.

Table 1 Enzymes potentially involved in the biosynthesis of tunicamycin

Tunicamycin biosynthetic enzymes	Enzyme function	Enzyme product
1. Uridine 5′-dehydrogenase	Oxidation of uridine	Uridine 5′-aldehyde
2. Dehydroquinate (DHQ) synthase-like activity	As described in Fig. 3	*N*-Acetyltunicamine-uracil
3. α-*N*-Acetylglucosaminyl transferase	Transfer of GlcNAc from UDP-GlcNAc to *N*-acetyltunicamine-uracil	*N*-Acetyltunicamycin
4. De-*N*-acetylase	Removes the *N*-acetyl group from *N*-acetyltunicamycin	Deacetylated tunicamycin
5. *N*-Acyltransferase	Acylates the free amino group at C-10′ in the deacetylated tunicamycin	Tunicamycins

Conclusion

A biosynthetic pathway is proposed for tunicamycin from uridine and UDP-GlcNAc, that potentially uses just 5 enzymes (Table 1). Uridine is initially oxidized to uridine-5-aldehyde by a uridine dehydrogenase or oxidase (Enzyme 1). UDP-GlcNAc is converted to UDP-4-keto-GlcNAc-5,6-ene, possibly by a mechanism similar to that catalyzed by DHQ synthase (Enzyme 2). This mechanism also accounts for the GlcN to GalN epimerization step, and the enolate anion-directed condensation with the 5′-carboxyl group of the uridine-5′-aldehyde. The intermediate, *N*-acetyltunicamine-uracil (Rings BCD), acts as the acceptor substrate for addition of the $\alpha\beta$-1″,11′-linked GlcNAc residue (Ring A) from the donor substrate UDP-GlcNAc. This is catalyzed by an α-directing $\alpha\beta$-1,1-glycosyltransferase (Enzyme 3). Finally, a de-*N*-acetylase (Enzyme 4) is postulated that cleaves the *N*-acetyl group from tunicamine, and an *N*-acyltransferase (Enzyme 5) that *N*-acylates the amino group. The latter enzyme is predicted to have a relatively broad selectivity with respect to the acyl group leading to the multiple *N*-acylated forms of tunicamycin that occur naturally. The identification, over-expression, and mutation of the genes that encode for the biosynthetic enzymes in *Streptomyces chartreusis* and other tunicamycin-producing microorganisms will undoubtedly help to validate these biosynthetic pathways.

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