Biosynthesis of Tunicamycin and Metabolic Origin of the 11-Carbon Dialdose Sugar, Tunicamine*

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Tunicamycin is a reversible inhibitor of polyproline-phosphate: N-acetylhexosamine-1-phosphate translocases and is produced by several Streptomyces species. We have examined tunicamycin biosynthesis, an important but poorly characterized biosynthetic pathway. Biosynthetic precursors have been identified by incorporating radioactive and stable isotopes, and by determining the labeling pattern using electrospray ionization-collision induced dissociation-mass spectrometry (ESI-CID-MS), and proton, deuterium, and C-13 nuclear magnetic resonance (NMR) spectroscopy. Preparation and analysis of [uracil-5-2H]-labeled tunicamycin established the complete ESI-CID-MS fragmentation pathway for the major components of the tunicamycin complex. Competitive metabolic experiments indicate that 7 deuteriums incorporate into tunicamycin from [6,6-2H,2H]-labeled D-glucose, 6 of which arise from D-GlcNAc and 1 from uridine and/or D-ribose. Inverse correlation NMR experiments (heteronuclear single-quantum coherence (HSQC)) of 13C-labeled tunicamycin enriched from [1-[13C]glucose suggest that the unique tunicamine 11-carbon dialdose sugar backbone arises from a 5-carbon furanose precursor derived from uridine and a 6-carbon N-acetylamino-pyranose precursor derived from UDP-D-N-acetylglucosamine. The equivalent incorporation of 13C into both the α-1′ and β-11′ anomeric carbons of tunicamycin supports a direct biosynthesis via 6-carbon metabolism. It also indicates that the tunicamycin motif and the α-1′-linked GlcNAc residue are both derived from the same metabolic pool of UDP-GlcNAc, without significant differential metabolic processing. A biosynthetic pathway is therefore proposed for tunicamycin for the first time: an initial formation of the 11-carbon tunicamycin sugar motif from uridine and UDP-GlcNAc via uridine-5′-aldehyde and UDP-4-keto-6-ene-N-acetylhexitolose, respectively, and subsequent formation of the anomer-to-anomer α, β-1′,11′-glycosidic bond.

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1 The abbreviations used are: PEP, phosphoenolpyruvate; ESI-CID-MS, electrospray ionization-collision induced dissociation-mass spectrometry; HSQC, heteronuclear single-quantum coherence; LC-ESI-CID-MS, liquid chromatography-ESI-CID-MS; pseudoGalN, pseudoaminogalactopyranosyl; HPLC, high pressure liquid chromatography; CID, collision-induced dissociations; GlcN, [1-13C]glucosamine; amu, atomic mass units.
**EXPERIMENTAL PROCEDURES**

**Materials**—Tunicamycin was obtained commercially from Sigma or by methanol extraction from mycelia of *S. chartreusis* NRRL 3882. [1-14C]Glucose, [6-14C]glucose, [1-14C]H-glucose, and [1-13C]glucose were purchased from Sigma. 12-14C-Uridine was purchased from American Radiolabeled Chemicals, St. Louis, MO. Other chemicals, substituted uridine analogs, solvents, TLC plates, and cultural materials were supplied by Sigma.

**Bacterial Strains and Culturing**—Putative tunicamycin- or coryne-toxin-producing strains were obtained from the American Type Culture Collection (Streptomyces lysozymaticus, ATCC 31396; Clavibacter toxicus, ATCC 49908 and 49909) and ARS Northern Region Research Laboratory (S. chartreusis, NRRL 3882 and 12338; and Streptomyces clavuligerus, NRRL 3565). They were maintained on solid TYD agar (1.5%) and grown aerobically in liquid TYD medium at 37°C. The medium contains tryptone (2 g l−1), yeast extract (2 g l−1), glucose (0.3 g l−1) and MgCl2·6H2O (0.3 g l−1) (4). The optimal time for tunicamycin production in liquid TYD culture by *S. chartreusis* was 5–7 days.

**Isolation of Tunicamycin**—After growing *S. chartreusis*, NRRL 3882 in liquid TYD for 5 days, acid insoluble tunicamycin complex was precipitated by acidifying the culture with 0.2 M HCl. Mycelia and the acid-precipitate were harvested by centrifugation (4000 × g, 10 min), washed twice by resuspending in dilute HCl (0.2 M), and extracted by vortexing with methanol. Following centrifugation to remove cell debris, the methanolic supernatant was evaporated to dryness (40°C) by rotary evaporation. The methanolic residue was suspended in dilute HCl (0.2 M) and washed twice by resuspending in dilute HCl (0.2 M), and extracted by vortexing with methanol. Following centrifugation to remove cell debris, the methanolic supernatant was evaporated to dryness (40°C) and then redissolved in fresh methanol (200 μl). Preparative thin layer chromatography (Silica 60 plates with a fluorescent indicator) was used to purify the tunicamycin complex (butanol:ethanol:water, 5:2:3 by volume). Where necessary, further purification was achieved by reversed-phase HPLC as previously described (4).

**Metabolic Labeling Studies**—Streptomycetes were cultured in liquid TYD medium for 5 days prior to the addition of radiolabeled precursors. The labeled tunicamycin complex was acid precipitated after another 2 days of growth and analyzed by thin layer chromatography-autoradiography. *D-[1-14C]glucosamine-labeled tunicamycin* was selectively deacylated with 2M HCl at 100°C and shown. As reported earlier, neither *S. lysosuperificus* nor *S. clavuligerus* produced detectable amounts of tunicamycin when grown in liquid TYD culture (4).

**Stable Isotope Studies and Electrospray Mass Spectrometry**—We have previously developed a reverse-phase, positive-ion detected LC-ESI-MS assay for tunicamycins (4). Here, collision-induced dissociations (CID) were used to promote further MS fragmentations (LC-ESI-CID-MS) and were applied to localize the metabolic incorporation of stable isotopes into the tunicamycins (Fig. 2). To establish fragmentation pathways [(uracil-5-2H]tunicamycins were prepared by a novel application of Holler's deuterium exchange procedure (10). Tunicamycin complex (0.5 mg) was isotopically exchanged in D2O in the presence of triethylamine as a non-reactive volatile base. After incubation (7 days, 60°C) 100% exchange of deuterium at position 5 in the uracil ring was confirmed by LC-MS (Fig. 2) and proton NMR (data not shown).

LC-ESI-CID-MS analyses of unlabeled tunicamycins and [(uracil-5-2H]tunicamycins are shown in Fig. 2B. Molecular ions were observed as associated sodium adducts [M + Na]+ (4) plus a diagnostically useful [MH − 221]− fragment ion. The latter arises from fragmentation across the α(1′)-11′-glycosidic bond which generates oxonium ions for the N-acyl uracil-tunicamycin moieties of each tunicamycin component. The parent ions, de-glycosylated oxonium ions ([MH-221]−) and associated [MH-221-2H2O]− fragments retain the uracil moiety and are observed at 1 mass unit higher in the spectrum of the [uracil-5-2H]tunicamycins (Fig. 1B). The uracil moiety is subsequently lost as a neutral fragment, and the remaining fragment ions are unaffected by the 5-deuterium label, confirming the selective incorporation. The uracil neutral loss is also evident as a protonated ion at m/z 113 and 114 for the [(uracil-5-2H]tunicamycin.

The ESI-CID-MS fragmentation pathway for tunicamycin *Tun 16:1* is shown in Fig. 2A, B, and C. A 1,2-elimination of the uracil base generates a [C5H11O2NAcyl]− ion (m/z 512) followed by 3,4-elimination of the 3'-OH group, similar to that observed for oligonucleotides by McLuckey and Habibi-Gouderz (11, 12). A second loss of H2O then occurs from the pseudo-aminogalactopyranosyl motif of the tunicamycin backbone to generate [C5H11O2NAcyl]− (m/z 476). Fragmentation across the C-4′–C-5′ bond results in a neutral loss of the pseudo-arabino moiety, [C5H10O2NAcyl]− (m/z 392), across the C-7′–C-8′ bond. The ESI-CID-MS fragmentation pathway for tunicamycin *Tun 16:1* is shown in Fig. 2A, B, and C. A 1,2-elimination of the uracil base generates a [C5H11O2NAcyl]− ion (m/z 512) followed by 3,4-elimination of the 3'-OH group, similar to that observed for oligonucleotides by McLuckey and Habibi-Gouderz (11, 12). A second loss of H2O then occurs from the pseudo-aminogalactopyranosyl motif of the tunicamycin backbone to generate [C5H11O2NAcyl]− (m/z 476). Fragmentation across the C-4′–C-5′ bond results in a neutral loss of the pseudo-arabino moiety, [C5H10O2NAcyl]− (m/z 392), across the C-7′–C-8′ bond.
**Biosynthesis of Tunicamycin**

The biosynthesis of tunicamycin involves the incorporation of 14C from uridine into the tunicamycin molecule. Labeled tunicamycin was isolated from cultures of *S. chartreusis* and analyzed using mass spectrometry and thin-layer chromatography (TLC). The MS fragmentation analysis showed molecular ions for tunicamycin that were consistent with the expected structure.

**Stable Isotope and Competitive Metabolic Experiments**

Stable isotope and competitive metabolic experiments were performed to identify the pathway of carbon incorporation into tunicamycin. Labeled uridine was used as a substrate for tunicamycin biosynthesis, and the incorporation of 14C was monitored using ESI-CID-MS and LC-ESI-CID-MS. The results indicated that uracil is the primary substrate for tunicamycin biosynthesis.

**Fig. 1. Tunicamycin production and metabolic labeling from [2-14C]uridine.** A. *S. clavigulerus* (lane 1), *S. lysosuperificus* (lane 2), and *S. chartreusis* strains NRRL 3882 (lane 3) and 12338 (lane 4) were cultured in liquid TYD for 5 days prior to addition of [2-14C]uridine (0.1 μCi). Labeled tunicamycins were precipitated with acid (0.2 M HCl) 2 days later, washed, and redissolved in methanol. Thin layer chromatography against [2-14C]uridine standard (lane 6) was detected by autoradiography, and against tunicamycin standard (lane 5) by fluorescence quenching. B. preparative scale TLC of [2-14C]uridine-labeled tunicamycin. C. tunicamycins from the *S. chartreusis* strains were grown in TYD liquid culture (400 ml, 28 °C, 200 rpm) for 5 days. The mycelia were filtered off, acid-washed, and extracted with methanol. The culture supernatants were acidified to precipitate tunicamycins that were recovered by filtration. After redissolving in methanol, the extracts were assayed by HPLC on a RP-18 column eluted with MeCN:1% aq. acetic acid (40:60 v/v.). Eluents were monitored with a diode array detector at 360 nm, and assignments were confirmed by electrospray-MS (data not shown).
The oxonium ion generated from the GlcNAc residue, typically observed at \( m/z \) 204 in standard tunicamycin, was present at \( m/z \) 206 in the deuterated tunicamycins. Hence, although three deuteriums (D1, D2, D3) are associated with the initial loss of the GlcNAc neutral fragment, only two (D1 and D2) remain on the GlcNAc oxonium ion. Deuteriums D1 and D2 presumably incorporate into C-6 of the GlcNAc residue via the following pathway: [6,6-\(^2\)H,\(^2\)H]Glc-6-P \( \rightarrow \) [6,6-\(^2\)H,\(^2\)H]Fru-6-P \( \rightarrow \) [6,6-\(^2\)H,\(^2\)H]GlcN-6-P \( \rightarrow \) UDP-[6,6-\(^2\)H,\(^2\)H]GlcNAc. The remaining deuterium, D3, is assigned as the exchangeable anomeric proton and is presumably incorporated from [6,6-\(^2\)H,\(^2\)H]glucose via 3-carbon metabolism.

Subsequent fragmentations showed that deuterium is not incorporated into the uracil ring (\( m/z \) 113) (see also Fig. 2), so D4, D5, D6, and D7 are presumably all localized to the N-acyltunicamine motif. Deuteriums D4 and D5 are incorporated at tunicamine C-6' by the direct metabolism of [6,6-\(^2\)H,\(^2\)H]Glc via [6,6-\(^2\)H,\(^2\)H]GlcNAc, verifying the earlier finding of \([14C]\) GlcNAc incorporating into the tunicamicin (Fig. 4A). Hence, D4 and D5 are on fragments larger than \([C\(_6\)H\(_5\)ONAcyl]^-\) but are lost with the 72 amu neutral fragmentation across the C-7,8' bond. The \( \beta-11' \)-anomeric deuterium D6 is analogous to D3, and incorporation again occurs following metabolism of [6,6-\(^2\)H,\(^2\)H]glucose to 3-carbon compounds.

Competitive Metabolic Experiments—To further assign the localizations described above, competitive metabolic experiments were conducted. *S. chartreusis* cells were grown on TYD containing [6,6-\(^2\)H,\(^2\)H]glucose, as described, but were supplemented with a 3-fold equivalent excess of an unlabeled competitive metabolite: GlcNAc, uridine, ribose, glycerol, or succinate (Fig. 4B, Table I). These unlabeled precursors compete metabolically for the incorporation of labeled [6,6-\(^2\)H,\(^2\)H]glucose into the tunicamycin, which resulted in selective editing of deuterium atoms. The competed [6,6-\(^2\)H,\(^2\)H]glucose-labeled tunicamycins were analyzed by LC-CID-ESI-MS as described above. Unlabeled GlcNAc competed with the [6,6-\(^2\)H,\(^2\)H]glucose label so that the observed tunicamycin molecular ions are only 1 mass unit greater than corresponding unlabeled controls, and 6 mass units less than a labeled control experiment (Fig. 4B). Fragmentation analysis indicates that this remaining deuterium was located in the tunicaminyl moiety rather than in the N-acetyltunicamine group. Hence, unlabeled GlcNAc competes for the incorporation of three deuteriums into the \( \alpha-1' \)-GlcNAc and three into tunicamine. These data support the concept that the tunicamycin pseudoGalN pyranose ring is metabolically derived from GlcNAc (presumably via UDP-GlcNAc).

Unlabeled uridine or ribose competed for the incorporation of [6,6-\(^2\)H,\(^2\)H]glucose and gave tunicamycins with six deuteriums (Table I). MS fragmentation analysis indicated that the deuterium whose incorporation was blocked, D7, was located in the

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**Fig. 2. Tunicamycin ESI-CID-MS assignments.** A, generalized scheme for the positive ion ESI-CID-MS fragmentation of tunicamycin Tun 16:1A. B, ESI-CID-MS spectra of tunicamycin (top) and chemically exchanged [uracil-5-\(^2\)H]tunicamycin (bottom). Both spectra are of Tun 16:1A that was eluted from the LC at 11.3 min. C, detailed pathway for the fragmentation of tunicamycin Tun 16:1A. These assignments aid localization of the metabolic stable isotope incorporation.
Biosynthesis of Tunicamycin

The deuterium label was also sometimes observed in the \([C_4H_5ONAcyl]\) fragments and the acyloxy ions (Table I). This was confirmed by gas chromatography-mass spectrometry (GC-MS) analysis of the fatty acid component following methanolysis (data not shown) and by NMR analysis (see later). This is presumably because D7 occupies two locations (D7a and D7b), but the label is retained at the tunicamine pseudo-GalN moiety. Hence, assuming stereoselectivity, only one deuterium should be lost from \([5,5-2H,2H]\)uridine prior to incorporation into tunicamycins.

Stable Isotope Studies and NMR Spectroscopy—NMR provides a tool to comprehensively assign the localization of stable isotopes into tunicamycins. Key features are the 6-CH\(_2\) and 6'-CH\(_2\) groups of the tunicamine and \(\alpha-1'\)-GlcNAc residue; the origin of the C-5 pseudo-riboyl bridge; and the \(\beta-1'\)-N-, \(\beta-11'-O\), and \(\alpha-1'\)-O anomeric assignments. Isotopically-labeled tunicamycins were isolated from cultures of \(S.\ chartreusis\) grown on TYD enriched with \([1-13C]\)glucose. Following purification by preparative TLC, the isolates were analyzed by H-1 NMR, C-13 NMR, and HSQC 13C-1H correlation spectroscopy. The methylene groups were assigned as geminal pairs from a DEPT-HSQC correlation experiment (Fig. 5), and anomeric assignments were made from COSY and total correlation spectroscopy (TOCSY) spectra (data not shown) and HSQC data (Fig. 5). Quantitative isotopic enrichments were deduced by comparing the normalized integrated HSQC signals of the \(13C\)-enriched tunicamycin with those of the unlabelled control. Significant enhancement of 16 HSQC signals was observed in the HSQC experiment (Fig. 5 and Table II). The largest isotopic enrichments were at the anomeric carbons C-11' and C-1', 20- and 22-fold respectively, which were ~20% of the maximum theoretic isotopic incorporation. Significantly, both anomeric carbons are isotopically enhanced from \([1-13C]\)glucose to an equal extent, indicating a direct incorporation at the hexose level rather than prior metabolism to 2- or 3-carbon units. Hence, the tunicamycin pseudoGalN moiety and the \(\alpha-1'\)-GlcNAc residue are apparently derived directly from glucose, from the same metabolic pool of GlcNAc or UDP-GlcNAc. In addition, isotopic enrichment was observed at C-6 in both the tunicamine pseudoGalN and the \(\alpha-1'\)-GlcNAc...
FIG. 4. The pseudogalactopyranosyl ring of tunicamycin is metabolically derived from glucosamine. A, TLC autoradiography analysis of tunicamycin complex metabolically radiolabeled from D-[1-14C]glucosamine (Lane 3 from S. chartreusis, NRRL 3882; Lane 4 from S. chartreusis, NRRL 12338). Two labeled spots, free glucosamine and tunicamycin-uracil, are observed after acid hydrolysis (lane 1). The latter spot quenched fluorescence in the TLC plate because of the uracil ring. Lane 2, [1-14C]glucosamine. B.1, stable isotope incorporation analysis by LC-ESI-CID-MS. B.2, tunicamycin Tun 16:1A metabolically labeled from [6,6-2H]glucose. (Insert shows isopomer distribution.) B.3, [6,6-2H]glucose-labeled Tun 16:1A metabolically competed with unlabeled GlcNAc. Figures above the mass values indicate number of incorporations as detailed under “Results” and “Discussion” and shown schematically in C.

Table I

ESI-CID-MS analysis of competitive metabolic experiments

*S. chartreusis*, NRRL 3882 was cultured in TSY media containing isotopically-labeled glucose as the metabolic precursor and a 3-fold molar excess of an unlabeled competitive metabolite: GlcNAc, uridine, succinate, glycerol, or ribose as indicated. Labeled tunicamycins were acid precipitated and methanol extracted after 5 days and analyzed by LC-ESI-CID-MS. In each case, the MS fragment ion masses shown are for excess of an unlabeled competitive metabolite: GlcNAc, uridine, succinate, glycerol, or ribose as indicated. Labeled tunicamycins were acid precipitated and methanol extracted after 5 days and analyzed by LC-ESI-CID-MS. In each case, the MS fragment ion masses shown are for excess of an unlabeled competitive metabolite: GlcNAc, uridine, succinate, glycerol, or ribose as indicated.

| Unlabeled tunicamycin | [5-2H-uracil] tunicamycin | [1,13C]-glucose-labeled tunicamycin | [6,6-2H,2H]-glucose-labeled tunicamycin | GlcNAc metabolic chase | Uridine metabolic chase | Succinate metabolic chase | Glycerol metabolic chase | Ribose metabolic chase |
|-----------------------|--------------------------|-------------------------------------|----------------------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| 853                   | 854 (+1)                 | 855 (+2)                            | 860 (+7)                               | 854 (+1)              | 859 (+6)              | 858 (+5)              | 858 (+5)               | 859 (+6)               |
| 610                   | 611 (+1)                 | 611 (+1)                            | 624 (+6)                               | 613 (+3)              | 613 (+3)              | 613 (+3)              | 613 (+3)               |
| 574                   | 575 (+1)                 | 575 (+1)                            | 580 (+5)                               | 577 (+3)              | 577 (+3)              | 577 (+3)              | 577 (+3)               | 577 (+3)               |
| 462                   | 462 (+0)                 | 463 (+1)                            | 466 (+4)                               | 466 (+3)              | 466 (+3)              | 466 (+3)              | 466 (+3)               | 466 (+3)               |
| 378                   | 378 (+0)                 | 379 (+1)                            | 382 (+4)                               | 381 (+3)              | 381 (+3)              | 381 (+3)              | 381 (+3)               | 381 (+3)               |
| 276                   | 276 (+0)                 | 277 (+1)                            | 280 (+4)                               | 280 (+3)              | 280 (+3)              | 280 (+3)              | 280 (+3)               | 280 (+3)               |
| 204                   | 204 (+0)                 | 205 (+1)                            | 206 (+2)                               | 206 (+2)              | 206 (+2)              | 206 (+2)              | 206 (+2)               | 206 (+2)               |
| 113                   | 114 (+1)                 | 113 (+0)                            | 113 (+0)                               | 113 (+0)              | 113 (+0)              | 113 (+0)              | 113 (+0)               | 113 (+0)               |

rings, 4-fold for the tunicamycin and 6-fold for the GlcNAc signals. Incorporation at the C-6 positions results from the metabolism of [1,13C]glucose via glycolysis to yield labeled 3-carbon compounds and their subsequent re-incorporation into [6,13C]glucose via gluconeogenesis. This is consistent with Embden-Meyerhoff-Parnas catabolism in these bacteria rather than the Entner-Doudoroff pathway (13, 14, 15). Importantly, 7- and 4-fold enrichment at C-1’ and C-5’ of the pseudo-riboyl motif is also observed, confirming that this part of the tunicamycine backbone originates from a ribose carbon skeleton. Known sugar metabolism supports the formation of [1, 5-13C]ribose from [1,13C]glucose via the pentose phosphate pathway (8, 16, 17). Isotopic enrichment of the N-acetyl methyl carbon of the α-1’-GlcNAc and the C-2’’ and C-4’’ carbons (but not C-3’’)
of the N-acyl chains occurs by 3-carbon metabolism, presumably via acetyl-CoA. Further isotopic incorporation into the ω-methyl groups of the fatty acid chains presumably originates from valine, a precursor in the branched-chain fatty acids biosynthesis (18, 19). Minor enrichment at C-5 and C-6 in the uracil ring of tunicamycin can be ascribed to the following pathway.

\[ 1-\text{^{13}C}\text{glucose} \rightarrow \text{pyruvate} \rightarrow \text{acetyl-CoA} \rightarrow \text{oxaloacetate} \rightarrow \text{aspartate} \rightarrow \text{uracil} \]

**DISCUSSION**

The dialdose tunicamine has a configuration that resembles a d-ribofuranose 5-membered ring 5,6-linked to a d-aminogalactopyranosyl 6-membered ring. Although other hemi-acetal cyclic forms are possible, in practice they are not observed, suggesting that tunicamine is formed by ligation of cyclic ribofuranose and aminogalactopyranose structures that are already locked into these configurations as their closed cyclic furanose and aminogalactopyranose structures that are analogous nucleotide antibiotics. Incorporation studies revealed that the C-6′ of polyoxin and nikkomycin nucleosides arise from C-3 of PEP, by condensation of PEP with uridine-5′-aldehyde to give octofuranulosyluronic acid nucleoside as an intermediate (7, 8, 20). A putative enolpyruvate transferase gene (nikO) with homology to UDP-GlcNAc enolpyruvate

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**TABLE II**

Quantitative enrichments of 1-\text{^{13}C}\text{glucose} into tunicamycin as analyzed by HSQC inverse-correlation spectroscopy

| Structural motif | HSQC assignment | \text{^{13}C}-isotopic enrichment |
|-----------------|-----------------|---------------------------------|
| Uracil          | 5, 6            | 2                               |
| Tunicamine      | β1′(ribosyl) 4  |                                 |
|                 | 4′′′(ribosyl) 2  |                                 |
|                 | 2′(ribosyl) 2   |                                 |
|                 | 4′(ribosyl) 1   |                                 |
|                 | 5′(ribosyl) 7   |                                 |
|                 | 6′a 4           |                                 |
|                 | 6′b 4           |                                 |
|                 | 10′ 1           |                                 |
|                 | β1′′′ 20        |                                 |
| GlcNAc          | α1′′′ 22        |                                 |
|                 | 6′ 6            |                                 |
|                 | 3′ 6            |                                 |
|                 | 5′′′ 1          |                                 |
|                 | 6′b 6           |                                 |
|                 | (ω-2) CH3 13    |                                 |
|                 | (ω-1) CH 5      |                                 |
|                 | ω CH3 5         |                                 |

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\[ \beta1′(\text{ribosyl})\]

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\[ \beta1′′′ \]

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\[ \alpha1′′′ \]

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\[ \omega \]

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\[ \text{UDP-GlcNAc} \]
transferases (MurA) and 5-enolpyruvyl shikimate 3-phosphate synthases (EPSP), has been implicated in this biosynthesis (20). MurA and EPSP catalyze the ligation of PEP to the 3'-OH of UDP-GlcNAc in peptidoglycan biosynthesis or to the 5'-OH of shikimate-3-phosphate, respectively. In an analogous aldol-type reaction, NikO is suggested to catalyze the condensation of PEP with uridine-5'-aldehyde or ribofuranosyl-4-formyl-4-imidazolone-5'-aldehyde. This reaction is also similar to those catalyzed by the deoxyheptulose-7-phosphate, deoxyxylulosate-8-phosphate, and deoxyxynulosonate-9-phosphate synthases involved in the biosynthesis of 7-, 8-, and 9-carbon long-chain sugars, respectively (6).

A similar mechanism may be involved in carbon-chain extension of the tunicamycin 11-carbon backbone. However, this does not seem to occur via PEP. If the pseudogalN part of tunicamycin (i.e. C6'-C11') arose from PEP, the major incorporation of label from [1-13C]glucose would be expected to occur at C-6 and would certainly be significantly greater that at C-11. This is because [1-13C]glucose is predominantly metabolized to [3-13C]PEP via glycolysis, which if condensed with uridine-5'-aldehyde, the major incorporation into tunicamycin should be expected at C-6. The evidence indicates the reverse; incorporation of label at C-11 is 5-fold greater than at C-6 (Table II). Hence, [1-13C]glucose incorporates into the pseudogalN ring mainly by 6-carbon metabolism, so the label ends up mainly in the anomeric position, i.e. C-11'. In addition, label from [6,6-2H,2H]glucose is incorporated into the 6'-position of the tunicamycin pseudogalN ring. If this enrichment occurred via PEP, it should be competed out by glycerol in the competitive metabolic experiments. The finding that this does not occur demonstrates that [6,6-2H,2H]glucose is incorporated into tunicamycin as a 6-carbon unit, not via PEP. 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 13C-label from [1-13C]glucose into both the α-C11' of the GlcNAc residue and the β-C11' anomeric carbon of the pseudogalN ring indicates that both are derived from the same metabolic pool without significant differential metabolic processing. Hence, the biosynthesis of both of these residues likely occurs from the same precursor, either UDP-GlcNAc or GlcNAc. This might implicate GlcNAc-α,β-1,1'-GlcNAc disaccharide as an intermediate during tunicamycin biosynthesis, analogous to trehalose or sucrose biosynthesis. The α,β-1,1'-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. In this case, however, one GlcNAc residue must first be activated as a sugar nucleotide, which would lead to non-equivalent incorporation of 13C label. This is contrary to what was actually observed, and it is therefore more likely that the tunicamycin α-1,1'-GlcNAc and the tunicamycin are both derived from the same metabolic pool of UDP-GlcNAc. One possibility is that UDP-GlcNAc is converted by 4-epimerase, and 5,6-dehydratase activities to UDP-4-keto-5,6-ene-N-acetylhexosamine, similar to intermediates formed during 6-deoxyxylulosi-9-carbon metabolism (21).

A bifunctional UDP-GlcNAc 6 dehydratase/C4 epimerase from Helicobacter pylori, FlaA1 catalyzes the sequential conversion of UDP-GlcNAc to UDP-4-keto-6-methyl-GlcNAc, which is stereospecifically reduced to UDP-QuinNAc (21). Similarly, dTDP-glucose 4,6-dehydratase (RffG protein) from Escherichia coli catalyzes the conversion of dTDP-glucose into dTDP-4-keto-6-deoxyglucose, but unlike the FlaA1 protein, dTDP-glucose 4,6-dehydratase does not reduce the 4-keto group to a 4-hydroxy group. The mechanism of the RffG protein has been studied in detail (22, 23). Oxidation at C4 to a keto group is critical in activating the deprotonation of the adjacent H5, thus bringing its pKa into the range 18–19 (23). Generation of an internal C4/C5 enolate ion would then promote stepwise β-elimination of the hydroxy group from C6. Indeed, the wild-type RffG does not act via a C4-enolate, but rather by a concerted elimination of H5 and 6-0H (23). Importantly, this enzyme generates dTDP-4-keto-glucose,5,6-ene as an intermediate, as determined by rapid mix-quench matrix-assisted laser desorption ionization time-of-flight mass spectrometry (22). This intermediate is analogous to the UDP-4-keto-N-acetylglucosamine-5,6-ene intermediate proposed for the tunicamycin pathway (Fig. 6).

The proposed condensation of 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 C5 to form a carbanion at C6. 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 C7 keto group to a 7-OH group. A subsequent reductive epimerization of the 4-keto gives the galactopyranosyl configuration. Thus, the 5,6-ene sugar nucleotide intermediate potentially undergoes an aldolase-catalyzed attack on uridine-5'-aldehyde to form the tunicamycin-uracil core. A second equivalent of UDP-GlcNAc is then used to add the α-1,1'-linked GlcNAc residue (Fig. 6). This sequence of events indicates that N-acetylglucosamine-uracil acts as the glycosidic acceptor and UDP-GlcNAc as the glycosidic donor during biosynthesis of the unique head-to-head glycosidic bond in a step catalyzed by an αβ-1,1 specific glycosyltransferase. Further experiments to establish the biosynthesis of tunicamycin at the enzymatic level are presently in progress.

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