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Increasing carbohydrate diversity via amine oxidation: Aminosugar, hydroxyaminosugar, nitrososugar and nitrosugar biosynthesis in bacteria

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Abstract

Bacterial secondary metabolites often contain attached carbohydrates that play a significant role in conferring biological activity. A small proportion of these bioactive sugars are derived from aminosugar oxidation to ultimately provide hydroxyaminosugars, nitrososugars, and nitrosugars. Recent advances in the elucidation of hydroxyaminosugar-, nitrososugar-, and nitrosugar-containing natural product gene clusters have enabled the proposal of biosynthetic pathways, the *in vitro* characterization of aminosugar oxidases, and the structure determination of key enzymes. This article focuses upon the key enzymatic transformations in aminosugar, hydroxyaminosugar, nitrososugar and nitrosugar biosynthesis, as well as the potential unique chemical reactivity of alkoxyaminosugars, with a particular focus upon developments within the last two years.

Introduction

Glycosylated secondary metabolites continue to serve as an important source for drug discovery. Key to these natural pharmacophores, the attached sugars are often critical for biological activity and subtle alterations in natural product glycosylation can transform a secondary metabolite's pharmacological properties, molecular and cellular specificity, and even mechanism of action [1,2]. In terms of the carbohydrate structural diversity within glycosylated secondary metabolites, variations upon deoxy- and aminosugars are the most prevalent, the biosyntheses of which have been recently reviewed [3-6]. Although less common, aminosugar oxidation – specifically, hydroxyaminosugars, nitrososugars and nitrosugars – uniquely extends nature's glycochemical diversity. These exotic sugars are distributed among various natural product scaffolds including anthracyclines - arugomycin, viriplanins (Fig. 1, **1** and **2**), cororubicin, and respinomycins (Fig. 1, **3** and **4**); enediynes – calicheamicin (Fig. 1, **5**) and esperamicin; spirotetronate antibiotics - tetracarcin A, kijanimicin, lobophorins, and arisostatins (Fig. 1, **6** and **7**); ansamycins – rubradirins (Fig. 1, **9** and **10**); and orthosomycins – evernimicins (Fig. 1, **11**-**14**). Although strictly not a nitrososugar, the natural monosaccharide streptozocin (Fig. 1, **8**) has also been included within this chemically diverse set of natural products. The attached carbohydrates of this unique natural product set are important to a broad range of biological effects exhibited by these

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compounds, including antibacterial, antitumor, antimalaria, anticholesterolemic, antiviral and antidiabetic activities. This article focuses upon the key enzymatic transformations in aminosugar, hydroxyaminosugar, nitrososugar and nitrosugar biosynthesis, as well as the potential unique chemical reactivity of alkoxyaminosugars, with a particular focus upon developments within the last two years.

Early discoveries and original biosynthetic hypotheses

The isolation of evernimicin (also referred to as everninomicin or Ziracin™) variants from *M. carbonacea* presented an early basis for studying the biosynthesis of hydroxyamino-, nitroso- and nitrosugars. Specifically, fermentation of *M. carbonacea* led to the isolation of the entire evernimicin series (amino-, hydroxyamino-, nitroso- and nitrosugar-containing) in addition to a novel alkoxyaminosugar metabolite Sch 49088 (Figure 1, **11**-**14**) [7,8]. Consistent with this, a series of respinomycins (most notably the amino- and nitrosugar variants, Fig. 1, **3** and **4**) were isolated from *S. xanthocidicus* [9], while a series of viriplanins (most notably the nitroso- and nitrosugar variants, Fig. 1, **1** and **2**) were isolated from *A. regularis* [10]. Interestingly, modification of the rubradirin isolation strategy from *S. achromogenes* also led to the discovery of the corresponding nitrososugar variant, which readily converted photo-oxidatively to rubradirin and thereby implicated nitrosugar variants to be artifacts derived from spontaneous oxidation of their nitrososugar precursors (Fig. 1, **9** and **10**) [11]. On the basis of these early studies, the biosynthesis of hydroxyamino-, nitroso-, and nitrosugars were anticipated to derive from successive oxidation of aminosugars, wherein some of the oxidative steps may be non-enzymatic, either before or after glycosyltransferase-catalyzed attachment to the natural product core structure (often referred to as the aglycon in such reactions).

Aminosugar biosynthesis – fundamentals and recent advances

As discussed above, aminosugar nucleotides, or possibly the corresponding aminosugar glycosides, are the key starting materials for aminosugar *N*-oxidation. C2-, C3-, C4-, and/or C6-aminosugars are well-represented among glycosylated secondary metabolites wherein the C2- and/or C6-aminosugars are more common to aminoglycosides [12], while C3- and/or C4-aminosugars are more common to a wide range of metabolites including aromatic and macrolide polyketides, non-ribosomal peptides, polyenes, enediynes, ansamycins, indolocarbazoles, nucleosides, orthosomycins and oligosaccharides [3,6]. In all cases, amine installation is catalyzed by pyridoxal phosphate (PLP)-dependent enzymes known as aminotransferases (E.C. 2.6.1.16) using an amino acid (typically L-Gln) as the amino donor and a C2-, C3-, C4-, or C6-ketosugar nucleotide as the amino acceptor (Fig. 2a, **16**). Unlike the biosynthesis of 2-aminosugars in primary metabolism, which derive from D-glucosamine-6-phosphate synthase-catalyzed amination of D-fructose-6-phosphate [13], the biosynthesis of aminosugars in secondary metabolism (including C2) derive from sugar nucleotide processes [3,6,12].

In terms of biosynthetic strategies, sugar amination is commonly also associated with C2, C3, C4 and/or C6 deoxygenation reactions. The most common of these, C6 deoxygenation, is catalyzed by a NAD^+-dependent NDP-hexose-4,6-dehydratase to provide a biosynthetic intermediate common to most novel sugars - an NDP-4-keto-6-deoxyhexose [5,6]. Enzymatic deoxygenation at the remaining three positions proceeds via drastically different mechanisms [5]. Deoxygenation at C2 occurs via β-elimination followed by hydride reduction, while C3 deoxygenation occurs via an unprecedented PLP-dependent radical-based mechanism [5]. In contrast, Liu and co-workers recently revealed C4 deoxygenation to proceed via an aminosugar intermediate (Fig. 2b). Specifically, amino sugar **19**, formed via DesI-mediated amination of **18**, was converted by a newly characterized SAM-dependent deaminase DesII [14] to the corresponding 4,6-dideoxyhexose (**20**). To complete the biosynthesis of TDP-D-desosamine

(**22**), a sugar precursor commonly employed in the biosynthesis of macrolide antibiotics such as erythromycin, a second aminotransferase-catalyzed reaction (DesV) is also required. In the absence of C4 deoxygenation, the alternative route to C3 aminosugars requires an isomerization to the requisite C3 ketosugar nucleotide precursor (Fig. 2c, **23**) as elucidated in recent studies on TDP-D-rubranitrose (the nitrosugar precursor to rubradirin, Fig. 1, **10**) and TDP-D-mycaminose (a precursor to the macrolide antibiotic tylosin) [15,16] (Fig. 2c). Using purified enzymes, both the D-rubranitrose and D-mycaminose studies revealed *in vitro* conversion of 4-keto species **23** or **18** to 3-amino species **24** or **26** in the presence of an isomerase/aminotransferase pair (RubN6/N4 or Tyl1a/TylB, respectively), while the latter study revealed Tyl1a was capable of generating the distinct 3-keto intermediate **25**.

Additional advances in the last two years relevant to aminosugar biosynthesis include the reported gene clusters for the aminohexose-bearing enediynes neocarzinostatin and maduropeptin [17,18] and the aminopentose-containing indolocarbazole AT2433 [19], the *in vitro* characterization of the polyene (nystatin) sugar C3-aminotransferase NysDII [20] and the structure determination of sugar aminotransferases DesI and DesV [21,22].

Hydroxyaminosugar biosynthesis

The unique conformation of the calicheamicin aryltetrasaccharide, which predominately derives from the hydroxyamino glycosidic bond (Fig. 1, **5**), is essential for **5**-DNA affinity and ultimately, the metabolite's remarkable ability to induce oxidative DNA strand scission [23-25]. The availability of the gene clusters encoding for the biosyntheses of the 10-membered enediynes calicheamicin [26] (Fig. 1 and 3a, **5**), esperamicin (accession number AY267372) (Fig. 3a, **28**), and dynemicin (Fig. 3a, **29**) [27], as well as the indolocarbazole AT2433 (Fig. 3a, **27**) [19] presented a genomic basis for proposing the biosynthetic pathway for the hydroxyaminosugar precursor TDP-4-hydroxyamino-6-deoxy-α-D-glucose (Fig. 3b, **30**). Specifically, comparison of the three enediyne loci helped eliminate genes common to the enediyne core biosynthesis (a common element of all three enediynes), while a comparison of the calicheamicin, esperamicin, and AT2433 loci facilitated the elimination of genes involved in the biosynthesis of the aminopentose moiety common to these three metabolites. In conjunction with the known routes to aminosugar biosynthesis described in the previous paragraph, this information led to a proposed pathway wherein two P450-dependent enzymes (CalO2 and CalE10) were identified as candidates for the putative aminosugar *N*-oxygenase (Fig. 3b, **19**→**30**). *In vivo* studies revealed *calS13* (also known as *calH*) could complement a Δ*desV S. venezuelae* disruption mutant and this complementation led to the production of a hybrid macrolide in which 4-amino-4,6-dideoxy-D-glucose was substituted for D-desosamine [28]. Based upon this *in vivo* study, CalS13 was assigned as the requisite C-4-ketosugar aminotransferase (Fig. 3b, **18**→**19**), reminiscent of DesI (Fig. 2b, **18**→**19**). Subsequent *in vitro* studies with CalO2 and CalE10 revealed only CalE10 could catalyze aminosugar *N*-oxidation and demonstrated, for the first time, the oxidation to occur at the sugar nucleotide stage to provide **30** (Fig. 3b) [29]. Furthermore, substrate specificity studies revealed CalE10-catalyzed oxidation to be both stereo- and regiospecific with only trace amounts of the corresponding nitrosugar detected. It should be noted that, while this was the first aminosugar *N*-oxygenase to be characterized, a related P450 enzyme (NocL, 41% identity) was recently characterized as the oxidase involved in nocardicin oxime formation [30].

Nitrososugar and nitrosugar biosynthesis

The elucidation of the biosynthetic gene clusters encoding the nitrosugar-containing bacterial secondary metabolites kijanimicin (a spirotetronate structurally related to the arisostatins, Fig. 1, **7**) [31] and rubradirins (Fig. 1, **10**) [32] presented a basis from which to propose the corresponding nitrosugar biosynthetic pathways. While the sequence of proposed events differ

between the two proposed pathways, both conceptually build upon well-established precedent for ketosugar nucleotide amination (Fig. 3c, **23**→**32**) and *C*-methylation (Fig. 3c, **32**→**33**) to provide a reasonable, potentially common, *N*-oxidase substrate (Fig. 3c, **33**). The key *N*-oxidation step in these respective pathways (Fig. 3c, **33**→**34**) was proposed to be catalyzed by FAD-dependent oxidoreductases RubN8 and KijD3. Consistent with this postulation, KijD3 shares high sequence identity with both RubN8 (57% identity, 68% similarity) and the evernimicin EvdC (61% identity, 72% similarity) [31]. At some point, the pathways diverge with subsequent elaboration, ultimately differentiating the core sugar architecture in rubradirins (Fig. 3c, **9** or **10**) and kijanimicin (Fig. 3c, **35**). It is important to note that, with the exception of the RubN6/N4 *in vitro* study discussed in the previous aminosugar section of this review, no additional biochemical support exists for either proposed route. With this in mind, a few key distinguishing features of the current postulations should be noted. First, in contrast to the established precedent of methyltransferase-catalyzed ketosugar-α-*C*-methylation [33, 34], the formally proposed rubranitrose pathway lacks a critical C4-carbonyl for activation of the putative C3 carbon nucleophile. Second, *N*-oxidation in the rubranitrose pathway was formally proposed to occur after glycosyltransfer while the corresponding kijanimicin *N*-oxidation was suggested to occur at the sugar nucleotide stage. Third, on the basis of the previous isolation of a nitrososugar variant (Fig. 1, **9**) [11], enzyme-catalyzed *N*-oxidation in the rubranitrose pathway was postulated to provide the nitrososugar, followed by spontaneous auto-oxidation to the nitrosugar. In contrast, kijanimicin *N*-oxidation was proposed to lead directly to the nitrosugar. Finally, although the absolute stereochemistry of D-rubranitrose has been a subject of some controversy, the chemical synthesis of both enantiomers and the absence of a 5-epimerase in the rubradirin biosynthetic gene cluster are consistent with the original D-sugar assignment [32,35].

Amino-, hydroxyamino-, nitroso- and nitrosugar glycosyltransferases

Although most glycosyltransferases are single polypeptides, it was recently discovered that the macrolide desosaminyltransferase (an aminosugar glycosyltransferase) DesVII required an ‘auxiliary’ or ‘accessory’ protein, Des VIII, for *in vitro* and *in vivo* activity [36,37]. Since this seminal discovery, other aminosugar glycosyltransferase/activating protein pairs have been discovered, such as AknS/AknT (aclacinomycin) [38], TylM2/TylM3 (tylosin) [39], MycB/MydC (mycinamycin) [39], EryCIII/EryCII (erythromycin) [40], and additional putative homologs [41]. While the ‘auxiliary/accessory’ protein was initially proposed to serve as an aminosugar nucleotide chaperone [36], *in vitro* characterization of a variety of auxiliary/accessory protein-independent aminosugar glycosyltransferases, including the vancomycin and teichoplanin GtfA and GtfE [42,43], vicenistatin VinC [44], amphotericin and nystatin AmphDI and NysDI [45], and the calicheamicin aminopentosyltransferase CalG4 [46], is inconsistent with this early hypothesis. More recent studies suggest a glycosyltransferase-activating role for these auxiliary/accessory proteins [40].

With respect to glycosyltransferases associated with *N*-oxidized sugars, the *in vitro* characterization and crystal structure of the calicheamicin hydroxyaminosugartransferase CalG3 (which transfers 4,6-dideoxy-4-hydroxyamino-α-D-glucose to the enediyne warhead) was recently reported [47]. In this same study, CalG2 was demonstrated to form the corresponding hydroxyamino-glycosidic bond to the adjacent thiosugar in the aryltetrasaccharide. Although biochemical studies pertaining to nitroso- or nitrosugar glycosyltransferases are lacking, disruption of *rubG2* (the putative rudradirin nitrososugar glycosyltransferase) led to the production of the rubradirin aglycon *in vivo*, consistent with RubG2 as the requisite glycosyltransferase in this pathway [32].

The utility of alkoxyamine-appended natural products

As highlighted in the previous paragraphs, nature has devised ingenious methods for expanding the chemical diversity of sugars to provide natural products appended with nitroso-, nitro- and hydroxy/alkoxyaminosugars, the latter of which offer unique opportunities for additional natural product diversification. Specifically, Peri and co-workers first revealed that simple methoxyamine-appended model compounds could readily react, under slightly acidic conditions, with free sugars to form 'neoglycosides' [48]. This reaction, which is particularly advantageous over classical glycosylation strategies as it does not require protecting groups or anomeric activation, has subsequently been exploited for the diversification (referred to 'neoglycorandomization' [49,50]) of a variety of natural product scaffolds including the cardenolide digitoxin [51], the alkaloid colchicine [52], and the nonribosomal peptide vancomycin [53]. In each of these cases, differentially-glycosylated natural product analogs with improved activity and/or selectivity were discovered. More recently, Langenhan and co-workers revealed that the neoglycosylation reaction is not simply limited to methoxyamine-appended molecules but, in fact, tolerates a variety of alkoxy substituents [54]. In this context, it is intriguing to consider the potential reactivity of naturally-occurring alkoxyamine-appended metabolites such as calicheamicin (Fig. 1, **5**), esperamicin (Fig. 3a, **28**), or the evernimicin analog Sch 49088 (Fig. 1, **15**) in such chemoselective glycosylation reactions.

Concluding remarks

Given the importance of sugar attachments in mediating natural product bioactivity, advances in the study of novel sugar biosynthetic pathways will continue to provide new opportunities for *in vivo* pathway engineering [6]. In particular, the elucidation of the genes responsible for the key oxidation of aminosugar nucleotides to ultimately provide hydroxyamino-, nitroso-, and nitrosugars, clearly present a new set of genes to increase the sugar structural diversity of a vast array of natural products via *in vivo* pathway engineering. Although aminotransferase-catalyzed amine installation proceeds via a highly conserved mechanism, it is interesting to note that, like the variant routes to sugar deoxygenation, aminosugar nucleotide oxidation can be mediated via distinct oxidative enzymes (flavin-containing oxidases or P450s). However, the scope of naturally occurring sugar *N*-oxidation elucidated to date is limited to the C3 or C4 of the sugar (Fig. 1) and consistent with this, the first *N*-oxygenase to be characterized *in vitro* (CalE10) displayed notable regio- and stereospecificity. Thus, expanding sugar *N*-oxidation toward C2- or C6-aminosugar may ultimately depend upon structure-based engineering or enzyme evolution strategies, which will clearly be enabled via structural elucidation of aminosugar nucleotide oxidases. Finally, these unique aminosugar oxidases also potentially present enzymatic routes to the installation of chemoselective handles for differential glycosylation. Continued study of the structure and function of hydroxyamino-, nitroso-, and nitrosugar biosynthetic enzymes, as well as the many potential utilities of the corresponding novel sugars, are anticipated to offer many exciting opportunities.

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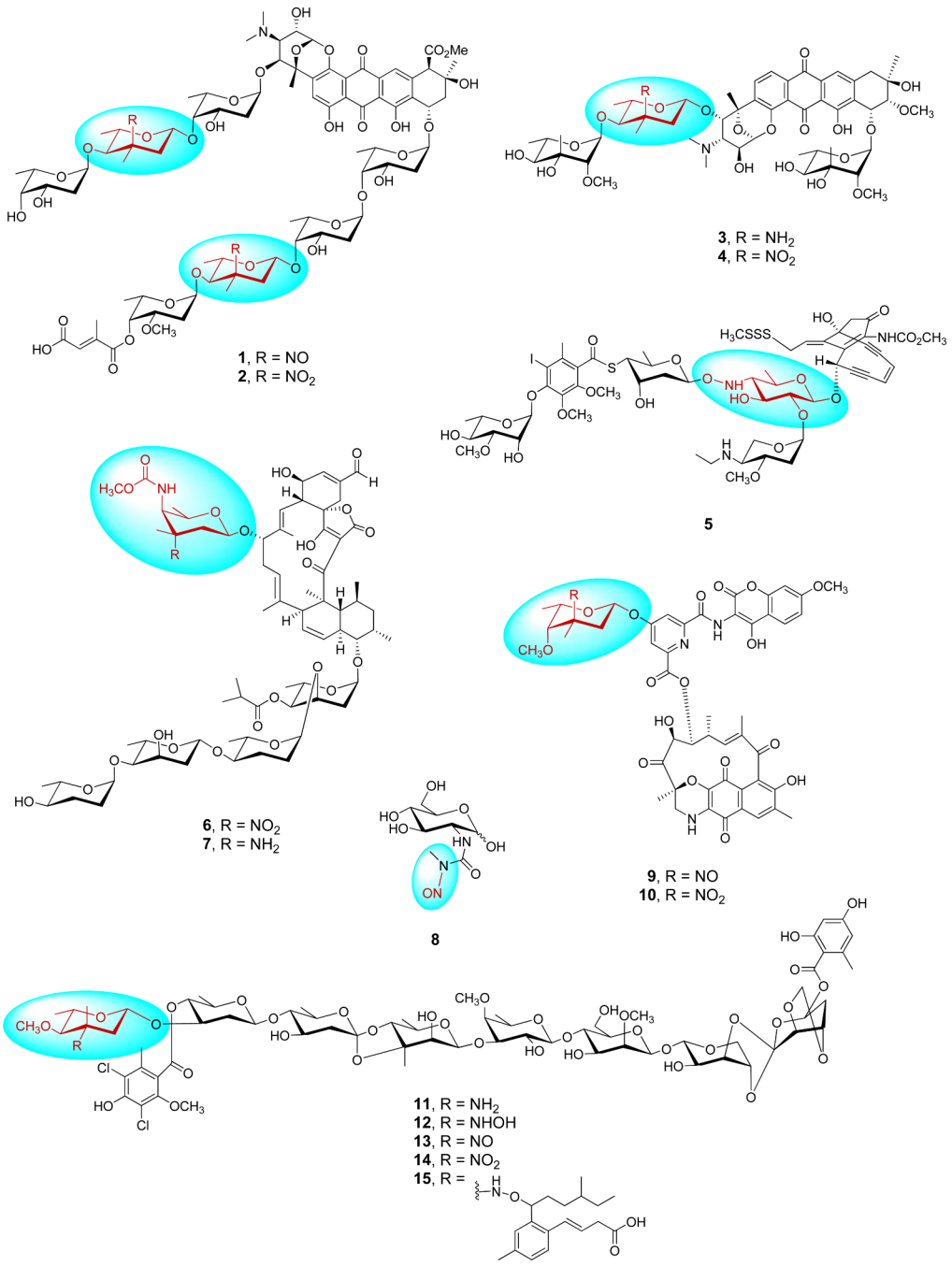

Fig. 1.

Representative amino- hydroxyamino-, nitroso-, and nitrosugar-containing bacterial secondary metabolites - viriplanins A (**1**) and D (**2**) from *Ampullariella regularis*, respinomycins A (**3**) and D (**4**) from *Streptomyces xanthocidicus*, calicheamicin γ^{I}_{1} (**5**) from *Micromonospora echinospora*, arisostatins A (**6**) and B (**7**) from *Micromonospora* sp. TP-AO316, streptozotocin (**8**) from *Streptomyces achromogenes*, and evernimicin variants - aminosugar analog (**11**), hydroxylaminosugar analog (**12**), nitrososugar analog (**13**), everninomicin D (**14**) and Sch 49088 (**15**) from *Micromonospora carbonacea*.

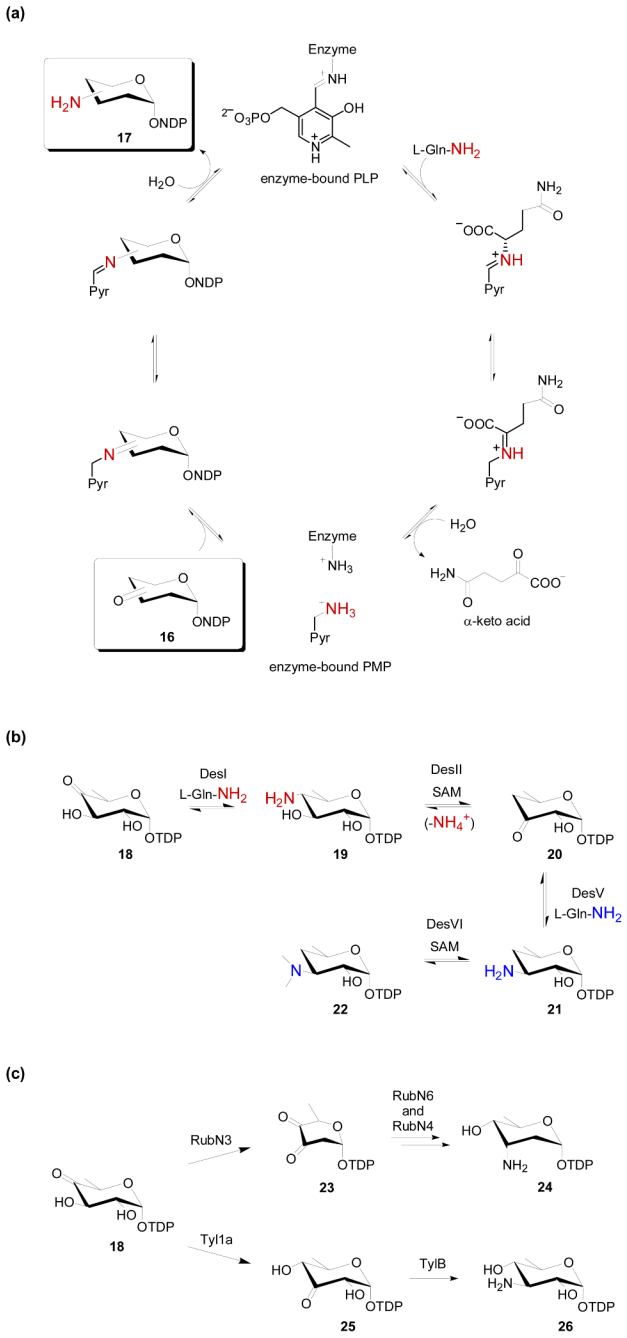

Fig. 2.

Fundamentals and recent advances relevant to aminosugar biosynthesis. **(a)** General catalytic mechanism of an aminotransferase. **(b)** The biosynthesis of TDP-D-desosamine. An unusual amination-deamination in this pathway contributes to C4 deoxygenation. **(c)** Aminotransferase-catalyzed transformation *en route* to TDP-D-rubranitrose (**23**→**24**) and TDP-D-mycaminose (**18**→**26**). PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; NDP, nucleoside diphosphate; TDP, deoxythymidine diphosphate; SAM, (*S*)-adenosylmethionine.

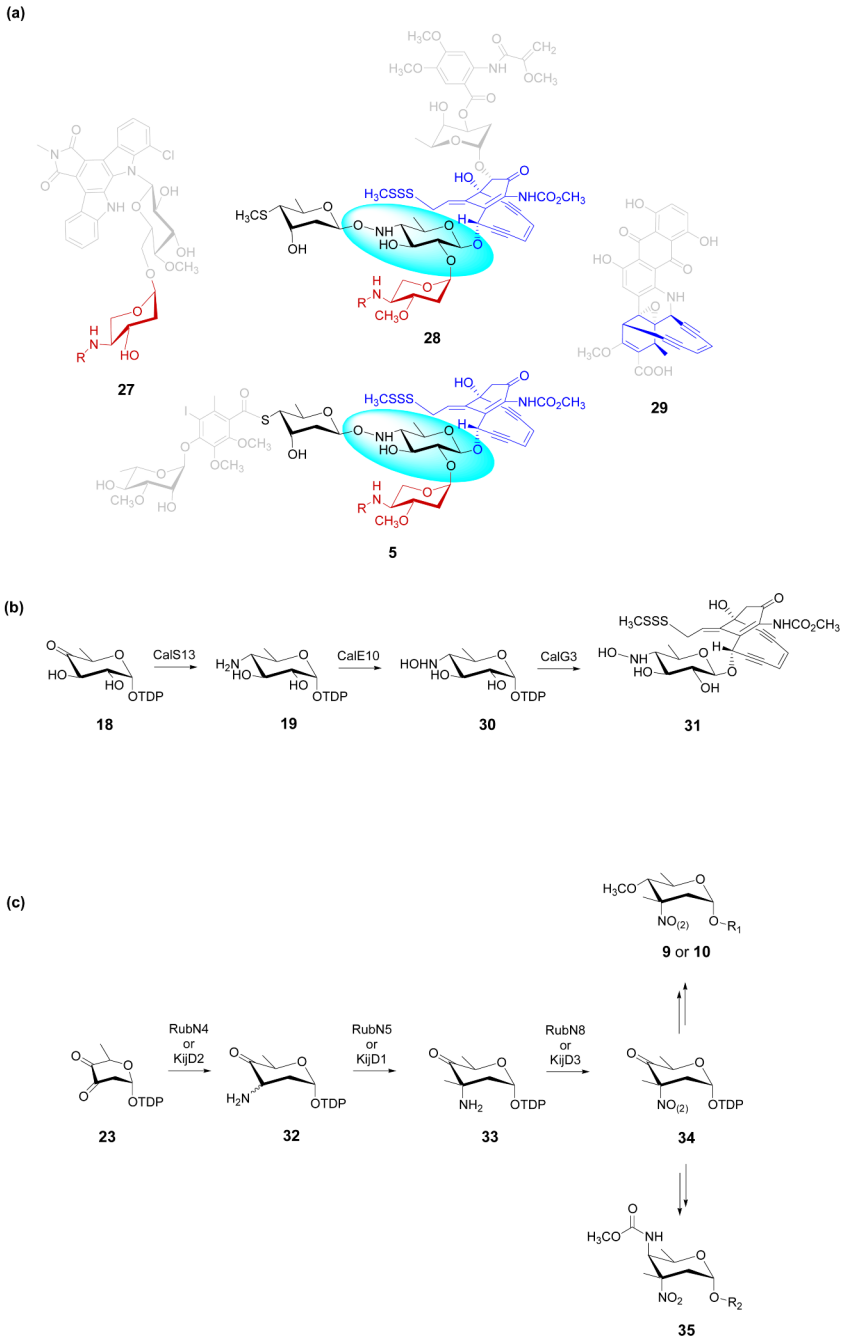

Figure 3.

(a) Schematic representation of comparative genomics analysis for delineation of the genes involved in hydroxyaminosugar biosynthesis. Specifically, genes common to the esperamicin (**28**), calicheamicin (**5**) and dynemicin (**29**) loci are anticipated to be involved in the biosynthesis of the enediyne core (highlighted in blue), while genes common to the esperamicin (**28**), calicheamicin (**5**) and AT2433 (**27**) loci are anticipated to be involved in the biosynthesis of the common aminopentose moiety (highlighted in red). The remaining genes common to the esperamicin and calicheamicin loci are anticipated to be involved in thiosugar and hydroxyaminosugar biosynthesis. **(b)** The biosynthesis and attachment of 4,6-dideoxy-4-hydroxyamino-D-glucose in the calicheamicin-producer *M. echinospora*. All steps have been

confirmed via *in vitro* biochemical studies. **(c)** Proposed biosynthetic pathways for the rubradirin nitroso/nitrosugar (rubranitrose) (**9** or **10**) and the kijanimicin nitrosugar (kijanose) (**35**). TDP, deoxythymidine diphosphate.

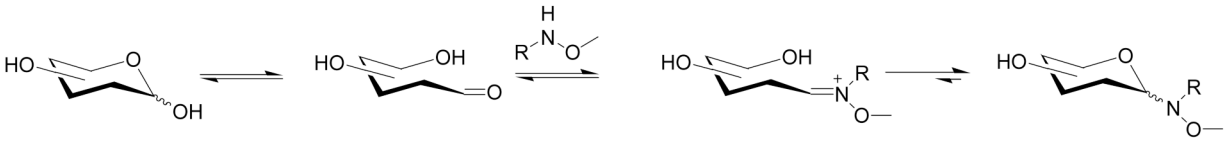

Figure 4.
General schematic of a neoglycosylation reaction. In ‘neoglycorandomization’, R can be any complex natural product scaffold.