Letter

Chemoenzymatic Synthesis of Novel C-Ribosylated Naphthoquinones

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Title

Chemoenzymatic Synthesis of Novel C-Ribosylated Naphthoquinones

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Abstract

The biological activity of many natural products is dependent on the presence of carbohydrate units, which are usually attached via an O-glycosidic linkage by glycosyltransferases. Recently, an exceptional C-ribosylation event was discovered in the biosynthesis of the polyketide antibiotic alnumycin A. The two-step process involves initial attachment of D-ribose-5-phosphate to the polyaromatic aglycone by the C-glycosynthase AlnA and subsequent dephosphorylation by AlnB, an enzyme of the haloacid dehalogenase family. Here we tested 23 unnatural substrates to probe the C-ribosylation reaction. The chemoenzymatic synthesis of C-ribosylated juglone, 7-methyl juglone, monomethyl naphthazarin, 8-chloro-7-methyl juglone and 9-hydroxy-1,4-anthraquinone revealed the importance of a 1,4-quinoid system with an adjacent phenolic ring in order for reaction to occur. To further rationalize the molecular basis for reactivity, factors governing substrate recognition were investigated by NMR binding experiments. Additionally, the suitability of substrates for nucleophilic substitution was assessed by molecular modeling using density functional theory (DFT) calculations.

Many medically important natural products, such as the anticancer agent doxorubicin,[1] the antibiotic erythromycin[2] and the anthelmintic drug avermectin,[3] owe their activity to the carbohydrate moieties attached to the polyketide framework.[4] The formation of these units has been investigated in detail and the studies have clarified that the 6-deoxy sugars are modified as nucleoside diphosphates prior to attachment by glycosyltransferases.[5,6] The most common means of attachment is via an O-glycosidic linkage, which unfortunately makes the metabolites susceptible to hydrolysis.[7] Chemically more robust C-glycosylation[8] has been reported, e.g. during the biosynthesis of urdamycin, granaticin and medermycin,[9,10] but this mode of attachment is comparatively rare.

The aromatic polyketide alnumycin A (**1**, Figure 1) contains an exceptional 4′-hydroxy-5′-hydroxymethyl-2′,7′-dioxane unit which mimics the C-glycosylated carbohydrates.[11,12] In fact, alnumycin A consists of six stereoisomeric compounds that differ in their configurations within the dioxane unit.[13] The pathway responsible for the biosynthesis of the dioxane moiety has previously been elucidated *in vivo*[14,15] and reconstructed *in vitro*,[15] which confirmed that the whole dioxane unit is derived from D-ribose-5-phosphate. In the initial step, AlnA catalyzes the attachment of D-ribose-5-phosphate to the prealnumycin aglycone (**2**, Figure 1) via a C_8–$C_{1'}$ bond. The enzyme is homologous only to pseudouridine glycosidases such as YeiN, which is responsible for the conversion of pseudouridine 5′-phosphate to uracil and D-ribose-5-phosphate.[16] The AlnA reaction products included an anomeric pair, denoted as alnumycin P1 (**3**, 84%, Figure 1) and P2 (16%), which differed only in the configuration at C-1′.[17] The overall C-ribosylation is completed by dephosphorylation of the intermediates by AlnB, an enzyme of the haloacid dehalogenase superfamily,[18] resulting in the formation of the anomers alnumycin C1 (**4**, 76%, Figure 1) and C2 (24%).

Structural and mechanistic studies of AlnA have recently suggested that the attachment of D-ribose-5-phosphate proceeds through a Michael-type 1,4-addition.[17] The spacious active site of AlnA is composed of two compartments which are separated by a triad of catalytically important residues: E29, K86 and K159 (Figure 1). Crystallization experiments have further revealed that the phosphosugar co-substrate binds in a linear configuration in one of the clefts, while the other compartment, which is predominantly hydrophobic, is the expected binding site of **2**. The reaction is likely to proceed through initial deprotonation of D-ribose-5-phosphate by E29 and formation of an enolate, which enables the nucleophilic 1,4-addition to **2** (Figure S1). The conversion to **3** then occurs via hemi-ketal formation and restoration of the chromophore system. In contrast to the mechanistic proposal for AlnA, crystallographic snapshots of reaction intermediates of the pseudouridine glycosidase YeiN identified a lysine adduct during catalysis (Figure S1),[19] but the 100-fold disparity in activities of lysine mutants of YeiN[19] and AlnA[17] alludes that the two enzymes may differ in their respective mechanisms. The observation that AlnA is able to utilize D-ribulose-5-phosphate as an alternative substrate (Figure S1) also yields support to the mechanism involving a Michael-type 1,4-addition.

In turn, determination of the crystal structure of AlnB (Figure 1) revealed that the two-domain enzyme is composed of a core domain, which provides the catalytically important residues D15 and D17, and a cap domain that is utilized for substrate recognition.[17] Structure/function studies confirmed that the dephosphorylation reaction is likely to follow a nucleophilic course in tandem with acid/base catalysis, a fact well established for the HAD enzyme superfamily.[17,18]

In this paper, we have continued the characterization of the unusual two-step C-ribosylation process by probing the reaction using 23 potential substrates. The results show a strict requirement for a 1,4-quinoid system with an additional adjacent phenolic ring, which was the common denominator in the five converted substrates. Binding experiments by NMR suggest that the phenolic hydroxyl group may be required for correct orientation of the substrate in the active site of AlnA, while computational chemistry calculations highlight the importance of a suitable charge at the reacting carbon of the substrate amenable to nucleophilic substitution. Finally, the data demonstrated that AlnB also appeared to exhibit promiscuity towards alternative substrates as the enzyme was able to convert all of the phosphorylated AlnA reaction products to the final C-ribosylated compounds.

We initially tested various α,β-unsaturated carbonyl compounds (Figure 2), which should be suitable Michael acceptor-type molecules, as substrates. The set included linear molecules and compounds containing a single six-membered ring (**5**–**10**), naphthalene derivatives (**11**–**21**) with various hydroxy, dehydro, halo and dioxo substituents, 7-ethoxy coumarin (**22**) and several 9,10- and 1,4-anthraquinones (**23**–**27**). Recombinant AlnA and AlnB were produced in *E. coli* and purified as described previously[15] and incubated in the presence of the substrate analogs and D-ribose-5-phosphate. While none of the single ring compounds (**5**–**9**), including uracil (**10**), were accepted as substrates, analysis by HPLC indicated that juglone (**15**) was converted to a product with a distinctly different retention time but a similar UV-vis spectrum. Scaling up the reaction provided sufficient material for structure elucidation by ^{1}H and ^{13}C NMR and HR-ESI-MS (see Supporting Information). NMR indicated the sample to actually consist of a pair of novel stereoisomeric compounds differing in their configuration at C-1′, denoted as 3-(*β*-ribosyl) juglone (**28a**, 96.5%, Figure 2) and its *α* anomer (**28b**, 3.5%). These initial experiments revealed the importance of a 1,4-quinone substructure fused

laterally to another ring for reaction to occur since none of the non-quinoid two-ring compounds (**11**, **12** and **22**) nor the 9,10-anthraquinones (**23**–**26**) were accepted as substrates.

After these initial experiments, we probed the boundaries of substrate recognition of AlnA and AlnB in more detail by focusing on compounds resembling **15**. Firstly, substitutions at C-2 next to the reactive carbon of the polyaromatic substrates appeared to be detrimental to the activity as neither plumbagin (**21**) nor 2,3-dichloro-1,4-naphthoquinone (**14**) could be converted to C-ribosylates. Secondly, the native substrate **2** contains a heterocyclic *2H*-pyran ring as the third six-membered ring. In order to test the tolerance of AlnA and AlnB for variation in this part of the substrate, we tested 7-methyl juglone (**16**) and 9-hydroxy-1,4-anthraquinone (**27**). Both of these were found to be suitable substrates and were converted into the expected novel C-ribosylates **29** and **30** (Figure 2), respectively. NMR analysis (Supporting Information) confirmed that in the case of **29**, both C-1′ anomers were present, with the β anomer again predominating (96.5%). For **30**, the low sample amount precluded the observation of a minor anomer, but the one present was the β anomer. Thirdly, a free phenolic ring adjacent to the quinone ring was estimated to be requisite for the reaction, since surprisingly neither 1,4-naphthoquinone (**13**) nor *O*-methyl juglone (**20**) were converted. In addition, naphthazarin (**18**), possessing a benzene-1,4-diol substructure, was also found not to be a suitable substrate for AlnA/AlnB. However, other compounds with substitutions in the 8-position of the phenolic ring appeared to be tolerated to some extent, since new reaction products could be observed when monomethyl naphthazarin (**19**) and 8-chloro-7-methyl juglone (**17**) were used as substrates. However, the conversion rate of these two compounds was drastically reduced in comparison to **15** as estimated by HPLC (Table 1), which limited characterization of the reaction products to ^{1}H NMR (Supporting Information) and only

tentative assignment of the structures of the corresponding C-ribosylates **31** and **32**, respectively, could be conferred.

Thus, the experiments revealed a complex set of rules required for reactivity by AlnA and AlnB. In order to comprehend the underlying causes of these restrictions, we next experimentally assessed (i) the relative strength of substrate binding to AlnA by linewidth broadening ($\nu_{½}$) of the ^{1}H NMR signals due to the presence of the enzyme[20] and (ii) the binding epitope of the substrate to AlnA by waterLOGSY[20] NMR experiments. In addition, we utilized molecular modeling using density functional theory (DFT) calculations to (iii) quantify the atomic charge at the reactive site C-3 (Q_{C3}) and (iv) evaluate the energy difference ($\Delta\Delta G$) between the substrate and a postulated enol intermediate (Supporting Information).

Estimation of the strength of binding (Table 1) revealed that while **15** was bound strongly, this was not an absolute requirement for reactivity, since **16**, which could also be converted into the corresponding C-ribosylate **29**, was found to bind only weakly to AlnA. Perhaps more surprisingly, the non-reacting **18** appeared to bind to AlnA strongly, and even **13** and **20** were better in this respect than **16**. The waterLOGSY results (Table 1) indicated that **15** was wholly embedded in the active site as indicated by the even intensities for all of the proton resonances. A similar result was also obtained for two of the other accepted substrates that were suitable for the binding studies, **16** and **19**, although in the case of **19** the signal originating from the methoxy group was weaker. Of the non-reacting substrates, **21** displayed even binding for the whole molecule, except for the methyl group, suggesting that the binding mode of the substrate could be correct, especially since the H-3 resonance at the reacting

carbon yielded a clear signal. This was not the case with **13**, which gave weak signals particularly for H-2 and H-3 indicating an incorrect mode of binding, and with **20** where the signal from the methoxy group was weakened.

Since the binding studies could not comprehensively explain the non-reactivity of all of the compounds (e.g. **18** and to some extent **21**), we complemented the experimental results with DFT calculations in order to elucidate chemical properties that might prohibit the reaction. The suitability of the molecules to act as Michael acceptors was assessed by the atomic charge at C-3 (Table 1). For most of the substrates, Q_{C3} values were similar (−0.17 to −0.18), but a much higher electron density (−0.25) was obtained for **21** which could explain why the compound was not converted. In addition, the proposed reaction mechanism assumes the formation of a high-energy enol intermediate. This prerequisite was simulated in the DFT calculations in the absence of the enzyme by comparison of the reactant substrate relative to a C-3 methylated enol intermediate (see Supporting Information). The differences in the Gibbs' free energies ($\Delta\Delta G$) for the substrate reactions relative to **2** ($\Delta\Delta G$, Table 1) were then compared to evaluate the thermodynamic viabilities of each reaction. The calculations indicated that all reacting substrates had $\Delta\Delta G$ values in the range of −0.74–4.62 kcal mol^{-1}, as did many of the unreactive naphthoquinone substrates except for **18** and **21** which had values in excess of 7 kcal mol^{-1}. Interestingly, 2,3-dichloronaphthoquinone (**14**) yielded a reasonable $\Delta\Delta G$ value of 1.28 kcal mol^{-1}.

Hence, it would seem that various naphthoquinones can be utilized as substrates for AlnA and AlnB but that several conditions must be met for reaction to occur (Figure 3 and Table 1). Firstly, the substrate must bind to AlnA, and bind in the correct orientation though it appears that strong binding per se is not an absolute requirement based on the results of **16**. For these

conditions to be fulfilled, a hydroxyl group at C-5 (C-9 in **27**) seems obligatory as this provides an anchor point and may also be essential for placing the substrate in the correct orientation. Based on the crystal structure of AlnA and docking experiments with **2**, the contact point may be the backbone amide group of L32, which is within hydrogen bonding distance to the hydroxyl group and one of the quinone oxygens (Figure 1). A particularly illustrative example of this is **20**, which binds to AlnA and has suitable chemical characteristics for reactivity, but which appears to bind in an incorrect orientation due to the methoxy group at C-5. Secondly, the substrate must possess suitable reactivity at C-3 that is amenable to nucleophilic substitution, and high electron density may inhibit the reaction. For example, **21** has a much higher calculated atomic charge on C-3 than all of the reacting substrates. Thirdly, it would appear that some of the substrates tested would result in a high-energy intermediate, which may prohibit the reaction. Thus, substrates that were initially expected to react but did not despite having a hydroxyl present at C-5, e.g. **18** and **21** were found to have much higher $\Delta\Delta G$ values compared to substrates that did react, e.g. **2** and **15** (Table 1). In the case of **21**, a steric clash between the methyl group and K186 may be an alternative explanation for its non-reaction. However, in the case of **18**, a similar situation is unlikely since other substituents in the 8-position in the phenolic ring (e.g. 8-chloro and 8-methoxy in **17** and **19**, respectively) did not preclude reaction. Based on the crystal structure of AlnA, the reactivity of **19**, which contains a bulky 8-methoxy substituent can be considered surprising as there appears to be only a limited amount of space available in the area of the substituent due to a nearby loop comprising residues T123, A124 and G125 (Figure 1).

In this paper we have demonstrated that AlnA and AlnB can be utilized to attach a ribose moiety via a C–C bond to a variety of substrates. Such modifications are challenging to accomplish through chemical means and since glycosylation reactions have been noted to

have an important role in the biological activity of natural products,[4,7] this natural promiscuity of the enzymes is encouraging. In particular, the reaction of **27** indicates that the *2H*-pyran ring of the natural substrate **2** may be amenable for further exploration. In addition, establishing the principle requirements for substrate reactivity for the native enzymes paves the way for structure-based protein engineering attempts to expand the substrate acceptance of the enzymes for the generation of novel C-ribosylated metabolites. An attractive starting point for these studies would be the T123-A124-G125 loop region to allow more variation in the substitution pattern of the phenolic ring of **15**.

The results described above are in line with a Michael-type 1,4-addition reaction mechanism that has been proposed previously for AlnA,[17] but a mechanism involving Schiff base formation cannot be entirely excluded. A key difference between the two mechanisms proposed for AlnA[17] and YeiN[19] is that they theoretically prefer different atomic charges at the reacting carbon of the substrates prealnumycin and uracil, respectively (Figure S1). Of the compounds tested, **21** should be a good substrate for the Schiff-base mechanism, but a poor one for the Michael-addition due to the high electron density at C-3 (Table 2). The compound could not be converted into the corresponding C-ribosylate, which is consistent with the Michael-addition mechanism. However, the binding studies suggest that **21** binds very weakly to AlnA and might even be bound in the wrong orientation (Table 2), and therefore additional experiments are required in the future to conclusively verify the reaction mechanism of AlnA.

METHODS

Chemical Syntheses of Substrates 16, 17, 19, 20 and 27. The condensation of maleic anhydride separately with *m*-cresol, 4-chloro-3-methyl phenol and 1-naphthol under Friedl–

Crafts conditions afforded **16**, **17** and **27**, respectively.[21] General reaction conditions consisted of melting 25 g $AlCl_3$ and 5 g NaCl at 463 K under vigorous stirring followed by addition of the appropriate phenol (15 mmol) and maleic anhydride (15 mmol). After 2 minutes, the reaction mixture was poured into a mixture of ice and 12 M HCl and kept for one hour. The reaction product was extracted with toluene, washed with deionized water and isolated by column chromatography (Silica Gel Mesh 60, Merck) followed by semi-preperative HPLC. The O-methylation of juglone (**15**, 49.8 mg) and naphthazarin (**18**, 61 mg) was accomplished by their treatement with 0.56 mmol methyl iodide (MeI) and silver (I) oxide (**15**, 0.26 mmol; **18**, 0.24 mmol) in 0.9 mL dichloromethane at room temperature.[22] After 20 hours, additional charges of MeI (0.56 mM) and Ag_2O (**15**, 195 mmol; **18**, 205 mmol) were added and the reaction stirred for a further 3 hours. The methylated compounds **20** and **19**, respectively, were isolated by column chromatography (Silica Gel Mesh 60, Merck) and stored in $CHCl_3$. NMR spectroscopic analysis of the compounds is presented in the Supporting Information.

***In vitro* Reaction Assays.** The production and purification of the recombinant AlnA and AlnB was done according to the protocol reported previously.[15] The enzymes were stored in phosphate buffer (50 mM Na_2HPO_4, 150 mM NaCl, 5 mM $MgCl_2$, 50 % glycerol, pH 6.85) at −20 °C. A typical reaction was done in a buffered solution (50 mM Na_2HPO_4, 100 mM NaCl, pH 6.85) with 50 μM AlnA, 20 μM AlnB, 10 mM D-ribose-5-phosphate and 250 μM of the screened compound together with 5 or 10% DMSO. Oxygen-reduced conditions were effected with 100 nM glucoseoxidase and 70 mM D-glucose in the presence of 5 μM catalase. The reactions were incubated at 288 K for 4 hours and quenched by lowering the pH either by addition of formic acid (1% final concentration) or ammonium acetate buffer (pH 3.6). Products were isolated either by solvent extraction using either $CHCl_3$ or ethyl acetate, or,

alternatively, SPE-column extraction (C18 or phenyl phase, Sulpelco) using MeOH or ACN as eluent.

Analysis of Metabolites. The reactions were analyzed by HPLC (Shimadzu SCL-10Avp) with a C-18 column (Sulpelco Discovery C18, 4.6 × 50 mm, 5 μm) using UV-vis detection (250–600 nm). A gradient from 15% methanol in 60 mM ammonium acetate buffer (pH 3.6) to 100% MeOH was used with a flow rate of 0.5 mL min^{-1}. The ribosylated compounds were isolated from the reaction extract by semi-preparative HPLC (Merck Hitachi L-6200A) with a reverse phase column (SunFire Prep C18, 10 × 250 mm, 5 μm) using a gradient run similar to the analytical reactions. The fractions collected were extracted with $CHCl_3$ or, alternatively, by SPE-column extraction (C-18, Sulpelco), and stored in MeOH.

LC-MS and NMR Measurements. HPLC-ESI-MS were acquired in either negative or positive mode using a MicroTOF-Q mass spectrometer (Bruker Daltonics) linked to a HPLC-system (Agilent Technologies 1200 series) using the Discovery column and similar conditions as in the analytical HPLC runs. NMR spectra were acquired at 25 °C with a Bruker Avance 500 MHz NMR spectrometer in $CDCl_3$ (D_2O/H_2O for the binding experiments). A full description of the NMR methodology is presented in the Supporting Information.

Molecular Modeling. DFT quantum chemical calculations were performed using *Gaussian09* (version A.01) and analyzed using *GaussView* (version 3.07). Structures were preliminarily geometry-optimized as per previously described.[13] A full description of the molecular modeling is presented in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Details of NMR methodology, molecular modeling methodology and compound characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Table 1. Evaluation of the Most Important Substrates in Regards to Binding to AlnA by NMR and Potential Reactivity by Molecular Modeling. The Results Are Color-Coded Based on Good (Green), Tolerated (Orange) and Unsuitable (Red) Characteristics

compound	rel. binding from $\nu_{½}$	binding orientation by waterLOGSY	model. pred. Q_{C3}	model. pred. $\Delta\Delta G$ / kcal mol^{-1}	rel. reactivity by HPLC / %
2	n/a[a]	n/a[a]	–0.175	0.00	81
13	medium to weak	weak, especially H-2 and H-3	–0.186	3.85	no reaction
15	strong	signals even	–0.176	4.62	77
16	weak[b]	signals even	–0.175	0.17	100
17	n/a[a or c]	n/a[a or c]	–0.172	2.43	5.4
18	strong	n/a[d]	–0.173	7.09	no reaction
19	strong	signals even except for OMe (slightly low)	–0.174	–0.74	11
20	medium to weak	H-6 low, H-8 high, OMe low	–0.188	4.56	no reaction
21	very weak	signals even except for Me (low)	–0.249	7.23	no reaction
27	n/a[c]	n/a[c]	–0.175	3.41	99

Legend: n/a, not applicable. [a]Not accessible for binding studies due to micelle formation. [b]Difficult to assess as the compound only sparingly soluble. [c]Compound solubility too low. [d]Only one signal for the non-exchangeable Hs due to symmetry and exchange, therefore waterLOGSY unable to provide binding orientation information.

Figures and Legends to the Figures

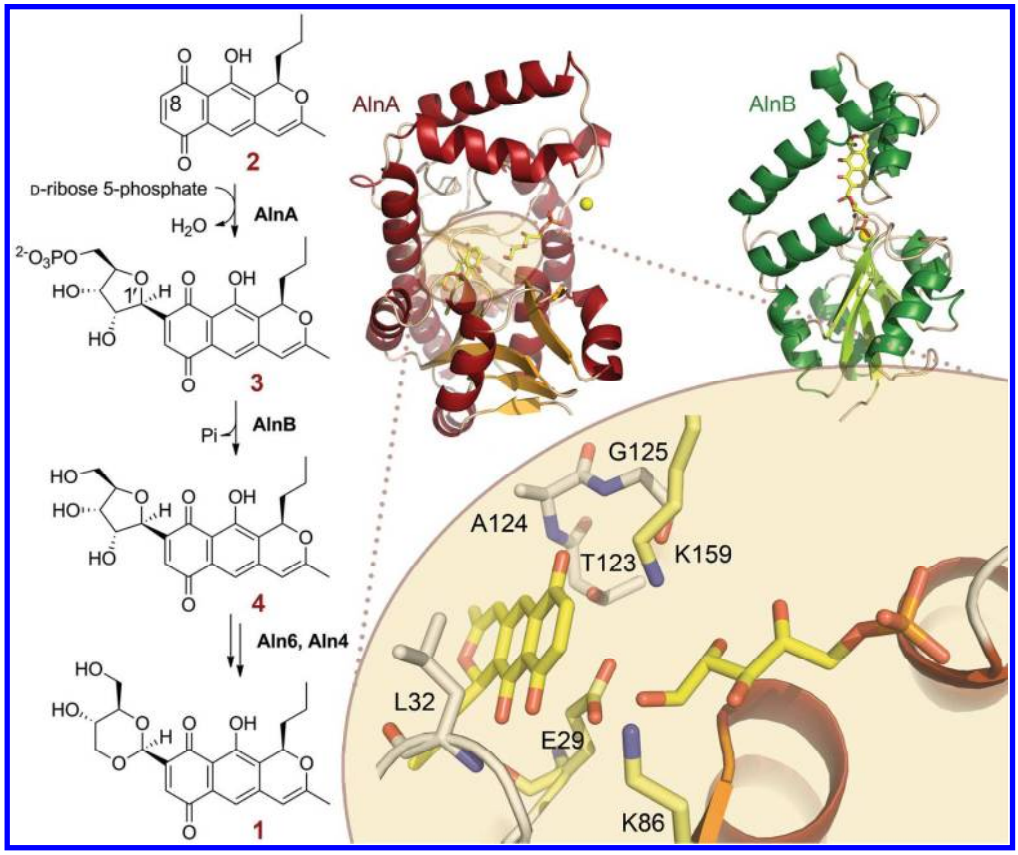

Figure 1. Model for the biosynthesis of alnumycin A (**1**)[15,17] and the structures of the AlnA (red) and AlnB (green) proteins that are responsible for the overall C-ribosylation reaction.[17] The active site of AlnA containing the reacting molecules is derived from a crystal structure of AlnA in complex with D-ribose-5-phosphate onto which prealnumycin (**2**) has been docked.[17] The active site of AlnB containing alnumycin P (**3**) is derived from a crystal structure of AlnB onto which **3** has been docked.[17]

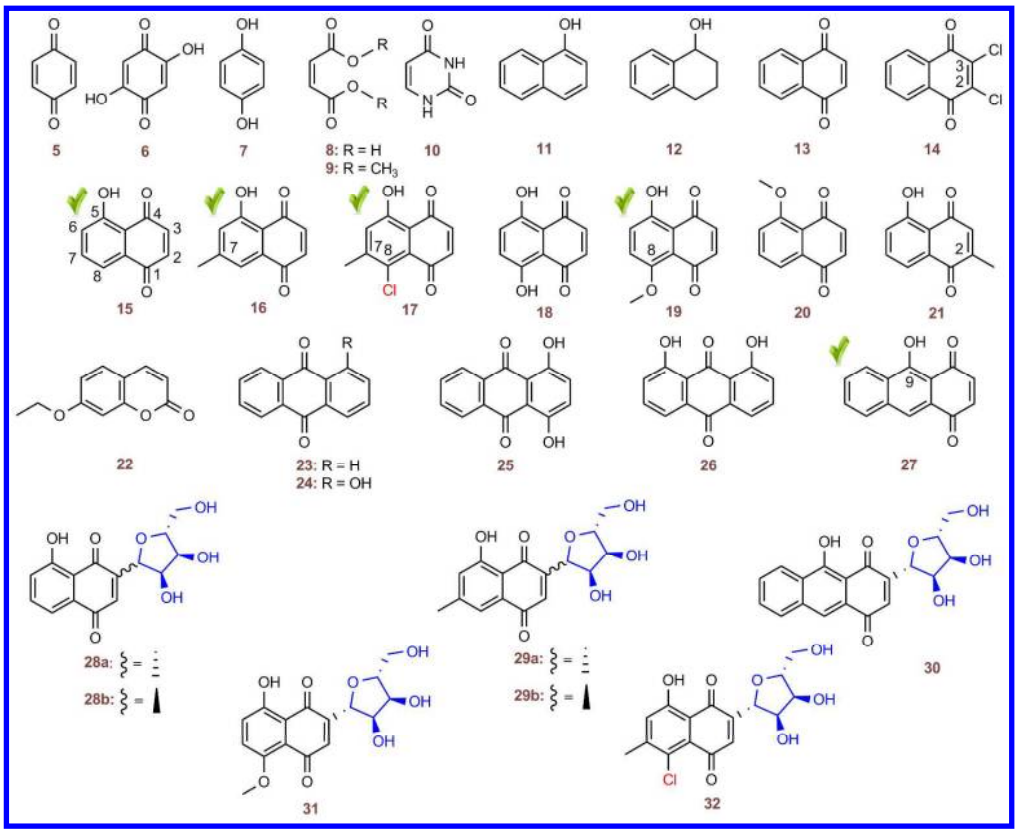

Figure 2. Structures of the 23 substrates, **5**–**27**, tested in this work and the novel C-ribosylated reaction products **28**–**32**. Tick marks designate accepted substrates.

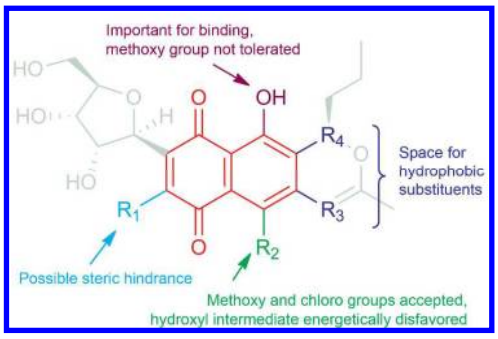

Figure 3. Design rules for the C-ribosylation of naphthoquinones.

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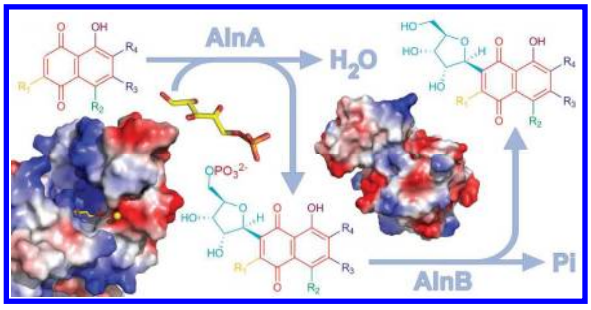
Bastian Blauenburg, Terhi Oja, Karel D. Klika and Mikko Metsä-Ketelä



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