The synthesis of novel chromogenic enzyme substrates for detection of bacterial glycosidases and their applications in diagnostic microbiology

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Abstract: The preparation and evaluation of chromogenic substrates for detecting bacterial glycosidase enzymes is reported. These substrates are monoglycoside derivatives of the metal chelators catechol, 2,3-dihydroxynaphthalene (DHN) and 6,7-dibromo-2,3-dihydroxynaphthalene (6,7-dibromo-DHN). When hydrolysed by appropriate bacterial enzymes these substrates produced coloured chelates in the presence of ammonium iron(III) citrate, thus enabling bacterial detection. A β-D-riboside of DHN and a β-D-glucuronide derivative of 6,7-dibromo-DHN were particularly effective for the detection of *S. aureus* and *E. coli* respectively.

Key words: enzyme substrates, chromogenic substrates, glycosidase, pathogenic microorganisms, bacterial detection.

**Introduction**

Synthetic enzyme substrates are utilised extensively in diagnostic clinical microbiology for the purpose of detecting and identifying pathogenic microorganisms.1-3 These substrates are designed to target microbiological species of interest (or groups of species) based upon their enzyme activity. An important sub-class of synthetic enzyme substrates are the chromogenic sugar-based enzyme substrates in which hydrolytic cleavage of the sugar moiety from the aglycone is mediated by an appropriate enzyme resulting in the liberation of a hydroxyaryl derivative, as shown by the representative examples in Scheme 1. The hydroxyaryl derivative can be coloured, thus allowing direct visualisation of the hydrolytic reaction as illustrated by the transformation of the colourless *ortho*-nitrophenyl β-D-galactopyranoside **1** into the yellow-coloured *ortho*-nitrophenol (ONP) (Eqn 1).4 Alternatively, if the liberated hydroxyaryl derivative is colourless, a subsequent chemical reaction can be employed to produce a coloured product. Thus, the β-galactoside derivative of 5-bromo-4-chloro-3-hydroxyindole (‘X-gal’) **2** is hydrolysed to produce 5-bromo-4-chloro-3-hydroxyindole which then undergoes an oxidative dimerization in air producing the blue-coloured indigo derivative **3** (Eqn 2).5 The indole-derived substrate, ALDOLTM 455 **4**,6 similarly generates a reactive 3-hydroxyindole intermediate which participates in a subsequent non-oxidative intramolecular aldol condensation yielding the yellow chromophore **5** (Eqn 3). Sugar-based chromogenic substrates have also been designed around a glycosidated catechol moiety (Figure 1). After enzymatic hydrolysis of the substrate, the resulting catechol aglycone undergoes chelation with metal ions that have been incorporated into the medium, therefore producing coloured metal-chelates. The hydrolysis of esculin **6** by a β-glucosidase enzyme yields D-glucose and esculetin (6,7-dihydroxycoumarin) which, in the presence of iron salts, produced a brown/black complex.7 Cyclohexenoesculetin-β-D-glucoside **7**8,9 (and also its β-D-galactoside derivative)10 similarly generated a black complex in the presence of iron salts. Alizarin β-D-glucoside **8**9 (and also its β-D-galactoside derivative)11 yielded a purple-coloured chelate in the presence of iron salts and a pink-coloured chelate with aluminium salts. Hydrolysis of 3’,4’-dihydroxyflavone β-D-ribofuranoside12 gave black colonies in the presence of iron and yellow colonies in the presence of aluminium. Other sugar-based substrates, which after enzymatic hydrolysis produce aglycones capable of chelation with metal ions, have also been prepared from non-catechol cores including glycosides of 8-hydroxyquinoline13 and 3-hydroxyflavone.9

In this paper, we report the synthesis and microbiological evaluation of a series of novel chromogenic sugar-based enzyme substrates based upon catechol, 2,3-dihydroxynaphthalene and 6,7-dibromo-2,3-dihydroxynaphthalene **9** cores (Figure 1).14,15 2,3-Dihydroxynaphthalene is inexpensive and available in large quantities (> 100 g) from several commercial suppliers thus making this an ideal starting material for the synthesis of enzyme substrates. Additionally, halogen atoms can be introduced into this ring-system remote from the hydroxyl-groups, *i.e.* at the 6,7-positions, whereas the introduction of halogen atoms into known substrates such as compounds **6** and **7** would only be possible adjacent to the hydroxy-groups which may have a detrimental effect on glycosidase activity. We anticipated that the introduction of halogen atoms would be beneficial for reducing diffusion of chelates in solid (agar) media. We envisaged that catechol-derived substrates would have potential applications in liquid media (where the resulting metal chelates would require appreciable aqueous solubility) and that the increased size of the naphthalene-derived substrates **9** would potentially generate a more insoluble end-point better suited for use in solid (agar) media, where diffusion of the chelate must be localised within colonies of microorganisms. The sugar components of structures **9** have been chosen to target a broad range of enzymatic activities across a range of clinically important pathogenic microorganisms. The sugar moieties together with illustrative applications in diagnostic microbiology include: (i) β-D-glucopyranosides (for the detection of enterococci and *Listeria monocytogenes*), (ii) β-D-galactopyranosides (for the detection of coliforms), β-D-glucuronides (for the detection of *Escherichia coli*), *N*-acetylhexosaminides (for the detection of the pathogenic yeast, *Candida albicans*) and β-D-ribofuranosides (for the detection of *Staphylococcus aureus*, including MRSA). Catechol β-D-ribofuranoside16 has previously shown efficacy for *S. aureus* detection in liquid media.17



Scheme 1. Hydrolysis of chromogenic enzyme substrates by β-galactosidase giving coloured products.



Figure 1. Chromogenic enzyme substrates possessing a catechol moiety.

**Synthesis of substrates**

Catechol 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside **10**18-21 was prepared from catechol in low yield using a Michael-type glycosidation procedure (Scheme 2). A Zemplén deprotection of compound **10** gave the required β-glucosidase substrate **11**. The proton-NMR spectral data of compounds **10** and **11** were consistent with those reported in the literature with large anomeric coupling constants confirming the β-configurations at the anomeric centres.20, 22 The direct reaction of glucose and catechol has been reported to give a 95:5 ratio of α:β anomers in low (11%) overall yield.23 2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranoside **12** was prepared using a Michel-type glycosidation reaction and after deprotection, the β-galactosidase substrate **13** was obtained. The coupling constant for the anomeric proton (7.7 Hz, d6-DMSO) and the chemical shift of C-1 (104.0 ppm, d6-DMSO) in the proton and carbon NMR spectra of compound **13** respectively, confirmed the presence of a β-glycoside. The tetraacetyl derivative **12** has been described previously in the literature and was reported as comprising a mixture of both α- and β-anomers.24 Somewhat surprisingly, the substrate **13** appears to be novel although the synthesis of its isomer with the α-configuration has been claimed but no NMR-spectral data was disclosed to support this assignment.24 Additionally, the large negative optical rotation (-33o in DMSO) reported for this proposed α-anomer structure is more aligned to the value expected from a β-D-galactopyranoside. The protected glucuronide derivative **14**25, 26 was prepared from catechol following a similar procedure to that reported in the literature.25 Deprotection of compound **14** under basic conditions, followed by acidification using an ion-exchange resin, afforded the required β-glucuronic acid derivative which was conveniently isolated as the cyclohexylamine salt **15**. The impure glucuronic acid has been proposed as a catechol metabolite produced from rabbits27 but this compound was not characterised directly. Methylation, per-acylation and finally hydrolysis of the metabolite gave 2-methoxyphenol (guaiacol) which suggested the rabbit metabolite was a mono-glucuronide.



Scheme 2. Preparation of catechol-derived substrates. Reagents and conditions: (i) α-D-acetobromoglucopyranoside, acetone, aq. NaOH, rt, overnight; (ii) MeOH, NaOMe, rt, overnight; (iii) α-D-acetobromogalactopyranoside, acetone, aq. NaOH, rt, overnight; (iv) 1,2,3,4-tetra-*O*-acetyl α-D-glucuronic acid methyl ester,25 4-toluenesulfonic acid (cat.), reduced pressure, 70-75 oC, 50 min; (v) (a) aq. NaOH, acetone, rt, 2 h, (b) Amberlite IR 120 H+ ion exchange resin, (c) cyclohexylamine.

The synthetic routes to the substrates based on the 2,3-dihydroxynaphthalene and 6,7-dibromo-2,3-dihydroxynaphthalene cores are depicted in Scheme 3. A Michael-type glycosylation of 2,3-dihydroxynaphthalene **16a** gave the acetylated sugar **17a** which was deprotected giving the required β-glucosidase substrate **18a**.28-30 A photoluminescence approach for the detection of β-D-glucosidase activity (but not in microorganisms) using compound **18a** has recently been reported.31 The physical and spectral data of compounds **17a** and **18a** were in good accord with published data.28 Similarly prepared were the novel dibrominated analogues **17b** and **18b**, both of which were associated with the large anomeric proton coupling constants and negative optical rotations confirming the presence of the β-isomers. The novel β-galactosidase substrates **20a** and **20b** were prepared from compounds **19a** and **19b** respectively by analogous procedures. The β-glucosaminidase substrate **22a** was synthesised from the reaction of 2,3-dihydroxynaphthalene **16a** and α-acetochloroglucosamine under basic conditions (giving the intermediate **21a**) followed by deprotection. The β-glucuronidase substrate **24a** was synthesised using a similar procedure to that shown in Scheme 2 for the preparation of substrate **15**. The intermediate **23b** was produced from the reaction of naphthalene **16b** with a glucuronide trichloroacetimidate32 in the presence of boron trifluoride etherate. Deprotection of compound **23b**, followed by treatment with cyclohexylamine then afforded the substrate **24b**. The reaction of either α-D-ribofuranosyl trichloroacetimidate33 or 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose with naphthalenes **16a** and **16b** in the presence of boron trifluoride etherate produced the β-anomers of the acetylated intermediates **25a** and **25b**. Deprotection of these two compounds afforded the required ribofuranoside substrates **26a** and **26b** respectively**.**



Scheme 3. Preparation of 2,3-dihydroxynaphthalene and 6,7-dibromo-2,3-dihydroxynaphthalene derived substrates. Reagents and conditions: (i) α-D-acetobromoglucopyranoside or α-D-acetobromogalactopyranoside, acetone, aq. NaOH, rt, overnight; (ii) MeOH, NaOMe, 4 oC, overnight; (iii) α-acetochloroglucosamine, acetone, K2CO3, heat on water bath, 15 min.; (iv) 1,2,3,4-tetra-*O*-acetyl-α-D-glucuronic acid methyl ester, 4-toluenesulfonic acid (cat.), AcOH, Ac2O, reduced pressure, 120 oC, 1 h (compound **23a**) or α-D-glucuronide trichloroacetimidate, BF3•Et2O, CH2Cl2, rt, 30 min. (compound **23b**); (v) (a) aq. NaOH, acetone, rt, 2 h, (b) Amberlite IR 120 H+ ion exchange resin, (c) cyclohexylamine; (vi) α-D-ribofuranosyl trichloroacetimidate, BF3•Et2O, CH2Cl2, 5 min., rt (compound **25a**); (vii) 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose, BF3•Et2O, 3 Å mol. sieves, CH2Cl2, 15 min., rt (compound **25b**).

**Evaluation of substrates**

In order to simplify the microbiological evaluation of the substrates, each assay comprised a representative panel of 20 clinically important microorganisms which were inoculated simultaneously onto a single Columbia agar plate. A standardised inoculum of approximately 108 colony forming units (CFU)/mL was prepared using a densitometer and 1 µL was delivered onto the agar surface using a multipoint inoculation device (final inoculum: approximately 106 CFU/spot). All assays were conducted at 37oC in air for 18 hours. For each assay, the panel of microorganisms comprised 10 Gram-negative bacteria, 8 Gram-positive bacteria and 2 yeasts. Each substrate was incorporated into the agar medium at a concentration of 300 mgL-1 in the presence of ammonium iron(III) citrate (500 mgL-1). The substrates were added to molten agar at 50°C, after the agar had been sterilised by autoclaving.

Microorganism growth was compared to control plates in which no substrate or metal salt was present. Control plates were also prepared containing metal ions (500 mgL-1) in the absence of substrates. All of the collection of microorganisms exhibited good growth on the substrate-free control plates and no growth inhibition was apparent in the presence of the metal salt.

For illustrative purposes, selected substrates were subjected to additional testing in a broth-based medium. For this purpose, we selected tryptone soya broth (Oxoid) that was supplemented with ammonium iron(III) citrate (500 mgL-1) before sterilization by autoclaving. The broth was then supplemented aseptically with 300 mgL-1 of substrate and inoculated with 106 CFU of test organism before incubation for 18 h at 37°C.

Strong growth was observed for the whole panel of microorganisms in the presence of the catechol-derived substrates **11**, **13** and **15** exemplifying the non-inhibitory nature of these substrates (Table 1). Where hydrolysis of the substrates had occurred and catechol was liberated, the brown colour of the resulting iron chelate was extensively dispersed around the border of the colonies and this was attributed to diffusion of the catechol into the surrounding medium. The microbial strains showed mostly expected activity with the glucopyranoside substrate **11** with the exception of an unexpected weak positive reaction with *Salmonella typhimurium*, which is not known to produce β-glucosidase. The weak reaction observed with *E. coli* is consistent with the low level of inducible enzyme known to be produced by this species.9, 34 For substrate **13**, coloration was only generated by known producers of β-galactosidase and hydrolysis of the glucuronide substrate **15** only occurred with *E. coli* as expected.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Substrate **11** | | Substrate **13** | | Substrate **15** | |
|  | **Microorganism / Referencea** | **Growthb** | **Colourc** | **Growthb** | **Colourc** | **Growthb** | **Colourc** |
|  | Gram-negative microorganisms |  |  |  |  |  |  |
| 1 | *Escherichia coli* NCTC 10418 | ++ | +/- brown | ++ | + brown | ++ | + brown |
| 2 | *Serratia marcescens* NCTC 10211 | ++ | + brown | ++ | +/- brown | ++ | - |
| 3 | *Pseudomonas aeruginosa* NCTC 10662 | ++ | - | ++ | - | ++ | - |
| 4 | *Burkholderia cepacia* ATCC 25416 | ++ | - | ++ | - | ++ | - |
| 5 | *Yersinia enterocolitica* NCTC 11176 | ++ | + brown | ++ | - | ++ | - |
| 6 | *Salmonella typhimurium* NCTC 74 | ++ | +/- brown | ++ | - | ++ | - |
| 7 | *Citrobacter freundii* 46262 (wild) | ++ | + brown | ++ | + brown | ++ | - |
| 8 | *Morganella morganii* 462403 (wild) | ++ | - | ++ | - | ++ | - |
| 9 | *Enterobacter cloacae* NCTC 11936 | ++ | + brown | ++ | + brown | ++ | - |
| 10 | *Providencia rettgeri* NCTC 7475 | ++ | + brown | ++ | - | ++ | - |
|  |  |  |  |  |  |  |  |
|  | Gram-positive microorganisms |  |  |  |  |  |  |
| 11 | *Bacillus subtilis* NCTC 9372 | ++ | - | ++ | - | ++ | - |
| 12 | *Enterococcus faecalis* NCTC 775 | ++ | + brown | ++ | - | ++ | - |
| 13 | *Enterococcus faecium* NCTC 7171 | ++ | + brown | ++ | +/- brown | ++ | - |
| 14 | *Staphylococcus epidermidis* NCTC 11047 | ++ | - | ++ | - | ++ | - |
| 15 | *Staphylococcus aureus* NCTC 6571 | ++ | + brown | ++ | - | ++ | - |
| 16 | *Staphylococcus aureus* (MRSA)NCTC 11939 | ++ | + brown | ++ | - | ++ | - |
| 17 | *Streptococcus pyogenes* NCTC 8306 | ++ | + brown | ++ | - | ++ | - |
| 18 | *Listeria monocytogenes* NCTC 11994 | ++ | + brown | ++ | - | ++ | - |
|  |  |  |  |  |  |  |  |
|  | Yeasts |  |  |  |  |  |  |
| 19 | *Candida albicans* ATCC 90028 | ++ | - | ++ | - | ++ | - |
| 20 | *Candida glabrata* NCPF 3943 | ++ | - | ++ | - | ++ | - |

a NCTC: National Collection of Type Cultures; ATCC: American Type Culture Collection; NCPF: National Collection of Pathogenic Fungi.

b ++ strong growth, + moderate growth, +/- weak growth.

c ++ strong colour, + moderate colour, +/- weak colour, - no noticeable colour. Diffusion of the colour into the medium was noted in all cases.

Table 1. Evaluation of the catechol substrates.

The 2,3-dihydroxynaphthalene-derived substrates **18a**, **20a** and **22a** (Table 2) allowed only moderate growth of all members of the panel of microorganisms suggesting that these substrates were inhibitory to some extent. Strong growth of the Gram-negative microorganisms and moderate growth of the Gram-positive microorganisms and the yeasts were seen with the glucuronide substrate **24a**. In contrast, strong growth of all 20 microorganisms was observed in the presence of the ribofuranoside substrate **26a**. Purple coloured colonies were produced with this series of substrates when appropriate enzymatic activity was present. As with the catechol-derived substrates, diffusion of colour into the surrounding medium occurred and hence these substrates would be better suited for use in liquid media. For substrate **18a**, coloration was generated only by known producers of β-glucosidase including enterococci and *Listeria monocytogenes*, suggesting this substrate could be exploited for detection of these species. Similarly, for substrate **20a** activity was only observed for known producers of β-galactosidase. Substrate **22a** is expected to detect β-hexosaminidase activity and several bacteria demonstrated hydrolysis of this substrate including known producers of this enzyme e.g. *Serratia marcescens*.35 Disappointingly, no coloration was generated by *Candida albicans* within the 18 h incubation period and this species is known to produce β-hexosaminidase.36 The glucuronide substrate **24a** was only hydrolysed by *E. coli,* as expected, producing strongly coloured colonies. Substrate **26a** effectively detected β-ribosidase activity in a number of Gram-negative species and activity was consistent with that previously reported for other β-ribosidase substrates.12, 37 Among Gram-positive bacteria, reactivity was restricted to *S. aureus* strains and there was clear differentiation from other Gram-positive species, suggesting that this may be a useful substrate for detection of *S. aureus*. Figure 2 illustrates the differentiation of *S. aureus* from other Gram-positive microorganisms in liquid media. The usefulness of β-ribosidase detection for the identification of *S. aureus* has previously been observed.16

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | Substrate **18a**b | Substrate **20a**b | Substrate **22a**b | Substrate **24a**c | Substrate **26a**d |
|  | **Microorganism / Referencea** | **Coloure** | **Coloure** | **Coloure** | **Coloure** | **Coloure** |
|  | Gram-negative microorganisms |  |  |  |  |  |
| 1 | *Escherichia coli* NCTC 10418 | Tr. purple | ++ purple | - | ++ purple | ++ purple |
| 2 | *Serratia marcescens* NCTC 10211 | ++ purple | + purple | ++ purple | - | ++ purple |
| 3 | *Pseudomonas aeruginosa* NCTC 10662 | - | - | - | - | - |
| 4 | *Burkholderia cepacia*  ATCC 25416 | - | - | - | - | - |
| 5 | *Yersinia enterocolitica* NCTC 11176 | - | - | + purple | - | - |
| 6 | *Salmonella typhimurium* NCTC 74 | - | - | - | - | ++ purple |
| 7 | *Citrobacter freundii* NCTC 9750 | NT | NT | - | - | ++ purplef |
| 8 | *Morganella morganii* 462403 (wild) | NT | NT | - | - | ++ purple |
| 9 | *Enterobacter cloacae* NCTC 11936 | + purple | ++ purple | +/- purple | - | ++ purple |
| 10 | *Providencia rettgeri* NCTC 7475 | ++ purple | - | - | - | ++ purple |
| 11 | *Klebsiella pnueumoniae* NCTC 10896 | ++ purple | ++ purple | NT | NT | NT |
| 12 | *Acinetobacter baumannii* ATCC 19606 | - | - | NT | NT | NT |
|  |  |  |  |  |  |  |
|  | Gram-positive microorganisms |  |  |  |  |  |
| 13 | *Bacillus subtilis* NCTC 9372 | + purple | - | - | - | - |
| 14 | *Enterococcus faecalis* NCTC 775 | + purple | - | ++ purple | - | - |
| 15 | *Enterococcus faecium* NCTC 7171 | + purple | Tr. purple | ++ purple | - | - |
| 16 | *Staphylococcus epidermidis* NCTC 11047 | - | - | - | - | - |
| 17 | *Staphylococcus aureus* NCTC 6571 | - | - | - | - | + purple |
| 18 | *Staphylococcus aureus* (MRSA)NCTC 11939 | - | - | - | - | + purple |
| 19 | *Streptococcus pyogenes* NCTC 8306 | - | - | ++ purple | - | - |
| 20 | *Listeria monocytogenes* NCTC 11994 | + purple | - | ++ purple | - | - |
|  |  |  |  |  |  |  |
|  | Yeasts |  |  |  |  |  |
| 21 | *Candida albicans* ATCC 90028 | - | - | - | - | - |
| 22 | *Candida glabrata* NCPF 3943 | - | - | - | - | - |

a NCTC: National Collection of Type Cultures; ATCC: American Type Culture Collection; NCPF: National Collection of Pathogenic Fungi.

b all microorganisms exhibited moderate growth in the presence of these substrates.

c the Gram-negative microorganisms exhibited strong growth and the Gram-positive microorganisms and yeasts showed moderate growth in the presence of this substrate.

d all microorganisms exhibited strong growth in the presence of this substrate.

e ++ strong colour, + moderate colour, +/- weak colour, - no noticeable colour. NT, not tested. Diffusion of the colour into the medium was noted in all cases.

f *Citrobacter freundii* 4626 (wild).

Table 2. Evaluation of the 2,3-dihydroxynaphthalene-based substrates.

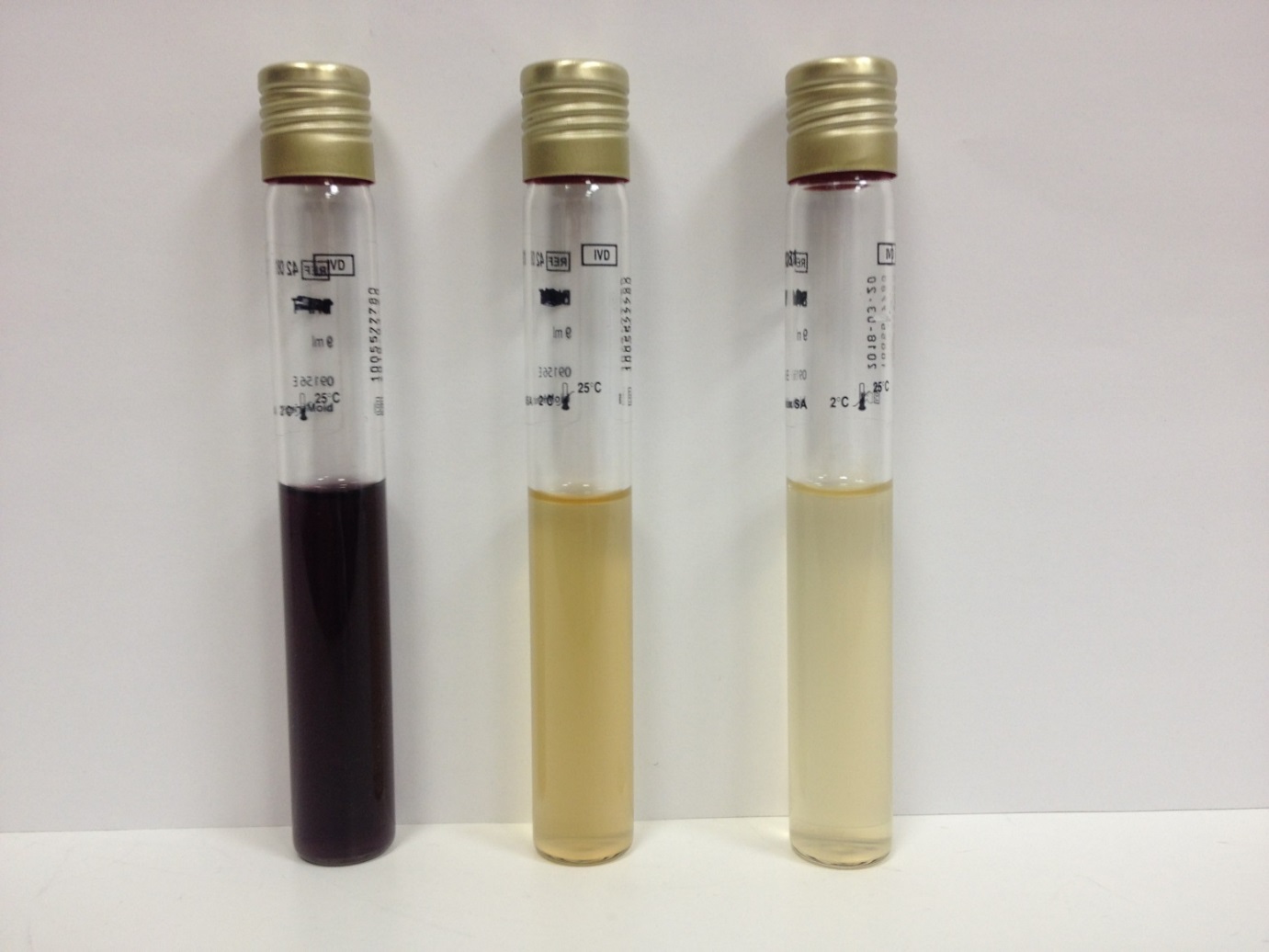


Figure 2. Detection of β-ribosidase activity using substrate **26a** in *Staphylococcus aureus* NCTC 6571 (left) and lack of reaction shown by *Staphylococcus epidermidis* NCTC 11047 (middle) and *Enterococcus faecalis* NCTC 775 (right).

In order to produce non-diffusible chelates that might be suited for use in solid (agar) media, the series of 6,7-dibromo-2,3-dihydroxynaphthalene substrates **18b**, **20b**, **24b** and **26b** were prepared (Table 3) with the expectation that the incorporation of the bromine substituents would reduce the solubility of the resulting chelates in this medium, thus limiting their diffusion out of bacterial colonies. Microorganism growth was generally moderate in the presence of substrates **18b** and **20b** and strong in the presence of substrate **24b** whereas substrate **26b** was inhibitory to Gram-positive bacteria. In agreement with expectation, the brown colours generated by enzymatic hydrolysis of these substrates were largely localised within the boundaries of the microorganism colonies and diffusion of colour into the surrounding medium was minimal.

A β-D-ribofuranoside derived from 1,4-dibromo-2,3-dihydroxynaphthalene was also prepared (*i.e.* the isomer of compound **26b**) but this substrate produced a strong background coloration in agar media and no discernible enzyme activity could be observed against the background. This suggests that the location of bromine atoms adjacent to hydroxy-groups would not produce practical substrates as noted in the introduction.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Substrate **18b** | | Substrate **20b** | | Substrate **24b** | | Substrate **26b** | |
|  | **Microorganism / Referencea** | **Growthb** | **Colourc** | **Growthb** | **Colourc** | **Growthb** | **Colourc** | **Growthb** | **Colourc** |
|  | Gram-negative microorganisms |  |  |  |  |  |  |  |  |
| 1 | *Escherichia coli* NCTC 10418 | + | Tr. brown | + | ++ brown | ++ | ++ brown | - | - |
| 2 | *Serratia marcescens* NCTC 10211 | + | ++ brown | + | + brown | ++ | - | + | ++ brown |
| 3 | *Pseudomonas aeruginosa* NCTC 10662 | + | - | + | - | ++ | - | + | + brown |
| 4 | *Burkholderia cepacia* ATCC 25416 | + | + brown | + | +/- brown | ++ | - | + | +/- brown |
| 5 | *Yersinia enterocolitica* NCTC 11176 | + | - | + | - | ++ | - | + | - |
| 6 | *Salmonella typhimurium* NCTC 74 | + | - | + | - | ++ | - | +/- | ++ brown |
| 7 | *Citrobacter freundii* NCTC 9750 | + | + brown | + | + brown | ++ | - | + | ++ brown |
| 8 | *Morganella. morganii* 462403 (wild) | + | - | + | - | ++ | - | + | ++ brown |
| 9 | *Enterobacter cloacae* NCTC 11936 | + | - | +/- | - | ++ | - | + | ++ brown |
| 10 | *Providencia rettgeri* NCTC 7475 | + | ++ brown | + | - | ++ | - | - | - |
|  |  |  |  |  |  |  |  |  |  |
|  | Gram-positive microorganisms |  |  |  |  |  |  |  |  |
| 11 | *Bacillus subtilis* NCTC 9372 | + | + brown | + | - | ++ | - | - | - |
| 12 | *Enterococcus faecalis* NCTC 775 | + | Tr. brown | + | Tr. brown | ++ | - | - | - |
| 13 | *Enterococcus faecium* NCTC 7171 | + | +/- brown | +/- | +/- brown | ++ | - | - | - |
| 14 | *Staphylococcus epidermidis* NCTC 11047 | + | - | + | - | ++ | - | - | - |
| 15 | *Staphylococcus aureus* NCTC 6571 | + | Tr. brown | + | - | ++ | - | - | - |
| 16 | *Staphylococcus aureus* (MRSA) NCTC 11939 | + | Tr. brown | + | - | ++ | - | - | - |
| 17 | *Streptococcus pyogenes* NCTC 8306 | + | - | +/- | - | ++ | - | - | - |
| 18 | *Listeria monocytogenes* NCTC 11994 | + | +/- brown | + | +/- brown | ++ | - | - | - |
|  |  |  |  |  |  |  |  |  |  |
|  | Yeasts |  |  |  |  |  |  |  |  |
| 19 | *Candida albicans* ATCC 90028 | + | - | + | - | ++ | - | +/- | - |
| 20 | *Candida glabrata* NCPF 3943 | + | - | + | - | + | - | +/- | - |

a NCTC: National Collection of Type Cultures; ATCC: American Type Culture Collection; NCPF: National Collection of Pathogenic Fungi.

b ++ strong growth, + moderate growth, +/- weak growth, no noticeable growth.

c ++ strong colour, + moderate colour, +/- weak colour, Tr. trace of colour, - no noticeable colour.

Table 3. Evaluation of brominated substrates.

Substrate **18b** did not show potential as a suitable substrate for detection of β-glucosidase producing pathogens as weak reactions were observed for enterococci and *Listeria monocytogenes*. Some unexpected (albeit weak) positive reactions were also observed for other species such as *S. aureus*. β-Galactosidase is most often targeted as a useful marker for coliforms and moderate to strong positive reactions were obtained as expected for *E. coli*, *S. marcescens* and *C. freundii* with substrate **20b**. However, *E. cloacae* (a β-galactosidase producing coliform) did not produce coloration. It is likely that activity of this and other species could be improved by inclusion of an inducer of β-galactosidase such as isopropyl-β-D-thiogalactoside (IPTG). As anticipated, the glucuronide substrate **24b** was only hydrolysed by *E. coli* producing strongly coloured colonies and illustrative agar plates are depicted in Figure 3. In addition to the 20 microorganism plate which clearly shows a positive response for *E. coli*, four single microorganism plates which included two *E. coli* strains (expected to give positive responses) and *S. marcescens* and *S. typhimurium* (both expected to produce negative responses) were prepared. These four plates also showed a clear difference between positive and negative results and a good contrast between the colour of the colonies and the background. Growth inhibition was even more pronounced with substrate **26b** with inhibition observed for *E. coli*, *P. rettgeri* and all of the eight Gram-positive bacteria tested. This precluded its potential application for the detection of *S. aureus*.

|  |  |
| --- | --- |
|  |  |

Figure 3. Agar plates produced using substrate **24b**. Left frame: arrangement of 20 microorganisms on a single agar plate (blue spots represent Gram-negative bacteria, red spots represent Gram-positive bacteria and the two yellow spots are the yeast species). Numbers correspond to microorganisms in Table 3. Right frame: left, 20 selected microorganisms on a single agar plate; middle top, *E. coli* NCTC 10418; top right, *E. coli* NCTC 12241; middle bottom, *S. marcescens* NCTC 10211; bottom right, S. *typhimurium* NCTC 74.

**Conclusion**

We have reported the synthesis and evaluation of a novel series of chromogenic glycosidase substrates, some of which show potential for the detection of pathogenic bacteria. By introducing two bromine atoms at the 6,7-positions in the naphthalene-based substrates, diffusion of the resulting chromogens into agar media is minimised. The chromogens produced by the non-brominated substrates diffused from the microorganism colonies thus precluding their use in agar media, but these substrates have potential for use in liquid media.

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**Experimental**

NMR spectra were recorded on a Jeol spectrometer at either 270 or 400 MHz for 1H spectra and either 68 or 100 MHz for 13C spectra. All chemical shifts are quoted in ppm relative to tetramethylsilane. In the assignment of signals the abbreviation DHN is used for 2,3-dihydroxynaphthalene and CHA for cyclohexylamine. Optical rotations were measured on an Optical Activity AA10 polarimeter. High resolution mass spectra (HRMS) were obtained from the EPSRC Mass Spectrometry Facility (Swansea). Flash chromatography was performed using Fluorochem Ltd silica gel (60 Å). Mixed solvents are recorded as volumetric ratios. EtOH is methylated spirit. Water is deionised water. HPLC was performed using an Agilent 1260 modular HPLC instrument fitted with a multiple wavelength detector and a Phenomenex Kinetex 2.6 μ C18, 150 x 2.1 mm column. Data was acquired at 280 nm with a flow rate of 0.4 mL per minute at ambient temperature. Mobile phase A was water containing 0.1% v/v formic acid and mobile phase B was acetonitrile containing 0.1% v/v formic acid. Substrates were prepared in a mixture 95% A/ 5% B. The gradient used was: 0-10 mins 95% A/ 5% B; 10-13.5 mins 5% A/ 95% B; 13.5-18 mins 95% A/ 5% B. The HPLC traces of key substrates are shown in the supplementary information.

6,7-Dibromo-2,3-dihydroxynaphthalene **16b**38 was prepared following a literature procedure.

The known acylated sugar derivatives **10**,20 **12**24 and **17a**28 were prepared by a Michael-type glycosidation procedure (420-503 mmol scale) in a solution of acetone and aqueous sodium hydroxide. The crude products were purified by column chromatography (eluent toluene: acetone 10:1) followed by trituration with EtOH (compounds **10** and **12**) or trituration (MeOH) followed by recrystallization from MeOH (compound **17a**). A Zemplén deprotection of compounds **10** and **17a** afforded the known glycosides **11**18,22,39 and **18a**28 respectively. The ester **14** was prepared by a similar method to that described in the literature, m.p. 128-130oC (lit. 136-137 oC).25 1H NMR: (DMSO-d6) 9.28 (1H, s, OH), 6.99 (1H, d, *J* 7.9 Hz, Ar-H), 6.93-6.83 (2H, m, Ar-H), 6.73 (1H, m, Ar-H), 5.44 (2H, m), 5.09-5.02 (2H, m), 4.62 (1H, d, *J*4,5 9.9 Hz, H-5), 3.35 (1H, s, OCH3), 2.01 (3H, s, OAc), 2.00 (3H, s, OAc), 1.99 (3H, s, OAc); 13C NMR: (DMSO-d6) 170.1 (Ac), 169.9 (Ac), 169.6 (Ac), 167.8 (C=O), 148.4, 145.0, 124.7, 119.7, 119.3, 117.2 (catechol), 99.4 (C-1), 71.7, 71.5, 71.3, 69.6 (C-2/3/4/5), 53.1 (OCH3), 21.0 (Ac), 20.9 (Ac), 20.8 (Ac).

The synthesis of the β-D-glucuronic acid derivatives and the β-D-ribofuranosides are described below. The preparation of all other compounds is described in the supplementary information.

Catechol-β-D-glucuronic acid, cyclohexylamine salt **15**.

Compound **14** (5.46 g, 12.8 mmol) was dissolved in acetone (75 mL) and a solution of NaOH (2.81 g, 70.3 mmol) in H2O (37.5 mL) was added. The reaction mixture was stirred at room temperature for 2 hours and then passed down a column of Amberlite IR 120 H+ ion exchange resin (50 g). Fractions containing the product were combined and basified with cyclohexylamine (5 mL). The solution was kept at 4 oC overnight and then evaporated, giving an amber oil which was triturated with EtOH (100 mL). A white precipitate formed and the mixture was left at 4 oC overnight. The precipitate was then collected affording compound **15** (3.23 g, 65%) as a white solid, m.p. 208-210 oC, [α]D22 -51o (*c* 0.504 in H2O). 1H NMR: (DMSO-d6) 7.05, (1H, dd, J 1.5 and 7.9 Hz, Ar-H), 6.90-6.69 (3H, m, Ar-H), 4.60 (1H, d, *J*1,2 7.9 Hz, H-1), 3.40 (1H, d, *J*4,5 10.0 Hz, H-5), 3.26-3.13 (2H, m), 2.85 (1H, m), 1.81 (2H, m, CHA), 1.64 (2H, m, CHA), 1.51 (1H, m, CHA), 1.25-1.18 (5H, m, CHA). 13C NMR: (DMSO-d6) 172.8 (C=O), 147.8, 146.1, 123.7, 119.8, 118.3, 116.7, 103.2 (C-1), 76.4, 74.4, 73.7, 72.7 (C-2/3/4/5), 49.7 (C-NH2, CHA), 31.1 (CHA), 25.2 (CHA), 24.3 (CHA). HRMS (ESI) for C12H13O8-: m/z calcd 285.0616; measured: 285.0616. The HPLC of this substrate showed a single compound (see supplementary data).

6′-Methyl (3-hydroxynaphthalen-2-yl)-2′,3′,4′-tri-*O*-acetyl-β-D-glucuronate **23a**.

A mixture of compound **16a** (34.4 g, 215 mmol) and 1,2,3,4-tetra-*O*-acetyl-α-D-glucuronide 6-methyl ester (MTAG) (40 g, 106 mmol) were heated in an oil bath to 120 oC on a rotary evaporator under reduced pressure until a homogeneous melt was obtained. 4-Toluenesulphonic acid (PTSA) (150 mg, 0.8 mmol) in acetic acid/acetic anhydride (1 mL) was added and the mixture was stirred at 120 oC on a rotary evaporator under reduced pressure for 1 hour. A TLC of the reaction mixture showed some remaining MTAG and a further quantity of PTSA (150 mg, 0.8 mmol) in acetic acid/acetic anhydride (1 mL) was added and the mixture stirred at 120 oC under reduced pressure for a further 30 min. The dark oil was allowed to cool to room temperature and after standing overnight was dissolved in CH2Cl2 (300 mL). The solution was washed sequentially with sat. aq. NaHCO3 (4 x 50 mL), H2O (500 mL), brine (500 mL) and the organic fraction was then separated, dried (MgSO4) and evaporated giving a brown foaming oil (59.1 g). The foam was purified by flash chromatography over silica gel (1 Kg) (eluent; toluene: acetone 10:1) and fractions of approximately 200 mL were collected. Fractions 19-26 were combined and evaporated giving a red solid (29.66 g). The red solid was triturated with EtOH (150 mL) and kept at 4 oC overnight. The resulting pale yellow, fluffy solid was collected affording compound **23a** (12.6 g, 25%), m.p. 191-192 oC, [α]D23 -26o (*c* 0.5 in CHCl3). 1H NMR (DMSO-d6): 9.80 (1H, s, OH), 9.67 (2H, dd, *J* 8.0 and 12 Hz, Ar-H), 7.45 (1H, s, Ar-H), 7.28 (2H, m, Ar-H), 7.18 (1H, s, Ar-H), 5.69 (1H, d, *J*1,2 8 Hz, H-1), 5.48 (1H, t, *J*3,4=3,2 10 Hz, H-3), 5.18-5.04 (2H, m, H-2/4), 4.71 (1H, d, *J* 10 Hz, H-5), 3.63 (3H, s, CO2CH3), 2.01 (3H, s, Ac), 2.00 (3H, s, Ac), 1.99 (3H, s, Ac); 13C NMR (DMSO-d6): 170.1 (Ac), 169.9 (Ac), 169.6 (Ac), 167.8 (C-6) 148.2, 146.4, 131.2, 128.2, 127.3, 126.2, 125.2, 123.9, 114.1, 111.0 (10 x DHN), 98.7 (C-1), 71.8, 71.6, 71.3, 69.6 (C-2/3/4/5), 53.2 (CH3), 21.0 (Ac), 20.9 (Ac), 20.8, (Ac). HRMS (ESI) for C23H28NO11 [M+NH4]+: m/z calcd 494.1657; measured: 494.1646.

6′-Methyl (6,7-dibromo-3-hydroxynaphthalen-2-yl)-2′,3′,4′-tri-*O*-acetyl-β-D-glucuronate **23b**.

To a stirred solution of α-D-glucuronide trichloroacetimidate (10 g, 21 mmol) and compound **16b** (6 g, 18.9 mmol) in CH2Cl2 (100 mL) was added BF3•OEt2 (10 drops, ~ 20 μL). The reaction mixture was stirred at room temperature for 30 min. and then poured into a mixture of CH2Cl2 (500 mL) and sat. aq. NaHCO3 (500 mL). The organic layer was separated and washed with sat. NaHCO3 (4 x 500 mL), H2O (500 mL) and then dried (MgSO4) and evaporated. The residue was triturated with EtOH (100 mL) and the mixture kept at 4 oC overnight. The solid was collected giving compound **23b** (2.89 g, 24%) as a white solid, m.p. 176-177 oC, [α]D25 -16o (*c* 0.25 in acetone). 1H NMR (DMSO-d6): 10.22 (1H, broad s, OH), 8.19 (1H, s, Ar-H), 8.17 (1H, s, Ar-H), 7.51 (1H, s, Ar-H), 7.20 (1H, s, Ar-H), 5.66 (1H, d, *J*1,2 8 Hz, H-1), 5.51 (1H, t, *J*3,4=3,2 9 Hz, H-3), 5.19-5.05 (2H, m, H-2/4), 4.70 (1H, d, *J*4,5 9 Hz, H-5), 3.64 (3H, s, CO2CH3), 2.03 (3H, s, Ac), 2.01 (3H, s, Ac), 2.00 (3H, s, Ac); 13C NMR (DMSO-d6): 170.1 (Ac), 170.0 (Ac), 169.6 (Ac), 167.7 (C-6) 149.5, 147.6, 131.6, 131.3, 130.6, 128.3, 120.1, 118.1, 112.9, 110.0 (10 x DHN), 98.5 (C-1), 71.7, 71.7, 71.2, 69.7 (C-2/3/4/5), 53.1 (CH3) 21.0 (Ac), 20.9 (Ac), 20.8, (Ac). HRMS (ESI) for C23H26Br2NO11 [M+NH4]+: m/z calcd 649.9867; measured: 649.9868.

(3-Hydroxynaphthalen-2-yl)-β-D-glucuronic acid, cyclohexylamine salt **24a**.

Compound **23a** (6.1 g, 12.8 mmol) was dissolved in acetone (75 mL) and a solution of NaOH (2.81 g, 70.25 mmol) in H2O (37.5 mL) was added. The mixture was stirred at room temperature for 2 hours and then passed down an Amberlite IR120 H+ ion exchange resin column (50 g). The eluent containing the product was basified using cyclohexylamine (CHA) (5 mL) producing a white precipitate. The mixture was kept at 4 oC overnight and the white fluffy solid was collected, washed with H2O and then acetone affording compound **24a**  (2.8 g, 53%), m.p. 223-224 oC, [α]D19 -96o (*c* 0.5 in H2O). 1H NMR (DMSO-d6): 7.64 (2H, m, Ar-H), 7.46 (1H, s, Ar-H), 7.28 (2H, m, J 6.93 Hz, Ar-H), 7.20 (1H, s, Ar-H), 4.95 (1H, d, *J*1,2 7 Hz, H-1), 3.58 (1H, d, *J*3,4=4,5 9 Hz, H-4), 3.35 (2H, m, H-2/3), 3.24 (1H, m, H-5), 2.83 (1H, m, CHA), 1.92-1.48 (5H, m, CHA), 1.23-1.05 (5H, m, CHA); 13C NMR (DMSO-d6): 172.7 (C-6), 147.8, 147.1, 130.7, 128.7, 127.3, 126.3, 124.9, 123.7, 112.1, 110.7 (10 x DHN), 100.0 (C-1), 76.6, 74.8, 73.6, 72.8 (C-2/3/4/5), 49.6 (CHA), 31.0 (CHA), 25.1 (CHA), 24.3 (CHA). HRMS (ESI) for C16H16NO8 [M+H]+: m/z calcd 335.0772; measured: 335.0767. The HPLC of this substrate showed a single compound (see supplementary data).

(6,7-Dibromo-3-hydroxynaphthalen-2-yl)-β-D-glucuronic acid, cyclohexylamine salt **24b**.

Following the procedure described above for the preparation of compound **24a**, compound **23b** (2.5 g, 3.9 mmol) gave compound **24b** (1.32 g) and a second crop (840 mg) from concentration of the filtrate. Compound **24b** (2.16 g, 95%) was obtained as a white solid, m.p. 221-224 oC, [α]D25 -159o (*c* 0.25 in H2O). 1H NMR (DMSO-d6): 8.12 (1H, s, Ar-H), 8.09 (1H, s, Ar-H), 7.47 (1H, s, Ar-H), 7.20 (1H, s, Ar-H), 4.96 (1H, d, *J*1,2 6.9 Hz, H-1), 3.58 (1H, d, *J*4,5=4,3 9.4 Hz, H-4), 3.39-3.18 (m, H-2/3/5/H2O), 2.86 (1H, m, CHA), 1.85-1.50 (5H, m, CHA), 1.20-1.00 (5H, m, CHA); 13C NMR (DMSO-d6): 172.9 (C-6) 149.3, 148.3, 131.4, 130.6, 130.5, 128.7, 119.2, 117.2, 110.6, 109.7 (10 x DHN), 101.4 (C-1), 76.6, 74.7, 73.5, 72.7 (C-2/3/4/5), 49.7 (CHA), 31.0 (CHA), 25.1 (CHA), 24.3 (CHA). HRMS (ESI) for C16H13Br2O8 [M+H]+: m/z calcd 490.8983; measured: 490.8976. The HPLC of this substrate showed a single compound (see supplementary data).

(3-Hydroxynaphthalen-2-yl)-2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranoside **25a**.

To a stirred mixture of compound **16a** (14.9 g, 93 mmol) in CH2Cl2 (200 mL) was added BF3•OEt2 (3 mL) followed by a solution of α-D-ribofuranosyl trichloroacetimidate (13 g, 30.9 mmol) in CH2Cl2 (100 mL). After approximately 5 min. the reaction mixture was poured into a mixture of sat. aq. NaHCO3 (300 mL) and CH2Cl2 (200 mL). The organic layer was separated and washed with sat. aq. NaHCO3 solution (4 x 500 mL). A TLC showed the organic fraction still contained a significant amount of unreacted compound **16a**, therefore it was washed with sat. aq. Na2CO3 solution (2 x 500 mL) (no remaining compound **16a** by TLC) and then with H2O (500 mL), dried (MgSO4) and evaporated giving an amber foam. The foam was triturated with MeOH (50 mL) and the resulting white solid collected yielding compound **25a** (6.24 g, 54%), m.p. 142-144 oC. [α]D22 -69o (*c* 0.99 in acetone). 1H NMR: (DMSO-d6) 9.61 (1H, s, OH), 7.64 (2H, t, *J* 9 Hz, Ar-H), 7.39 (1H, s, Ar-H), 7.26 (2H, m, Ar-H), 7.17 (1H, s, Ar-H), 5.94 (1H, s, H-1), 5.53 (2H, m, H-2/3), 4.35 (2H, m, H-5a/5b), 4.00 (1H, dd, *J* 11 and 5 Hz, H-4), 2.12 (3H, s, Ac), 2.06 (3H, s, Ac), 1.86 (3H, s, Ac); 13C NMR: (DMSO-d6) 170.6 (Ac), 170.2 (Ac), 170.1 (Ac) 147.7, 145.3, 130.7, 128.4, 127.2, 125.0, 126.2, 123.8, 111.6, 110.7 (10 x DHN), 103.3 (C-1), 79.9, 74.8, 71.2, 63.9 (C-2/3/4/5), 21.0 (Ac), 20.9 (Ac), 20.8 (Ac). HRMS (ESI) for C21H26NO9 [M+NH4]+: m/z calcd 436.1602; measured: 436.1609.

(6,7-Dibromo-3-hydroxynaphthalen-2-yl)-2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranoside **25b**.

Compound **16b** (3 g, 9.4 mmol), 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (3.3 g, 10.3 mmol) and 3Å molecular sieves (1 g) were stirred in CH2Cl2 (30 mL) for 10 min. and then BF3•OEt2 (3 mL, 24 mmol) was added. The reaction mixture gradually formed a clear amber solution after approximately 5 min. of stirring at room temperature. After 15 min., a thick white precipitate had formed and the reaction mixture became difficult to stir. The reaction mixture was poured into a mixture of CH2Cl2 (200 mL) and sat. NaHCO3 (200 mL). The organic layer was separated, washed sequentially with sat. aq. NaHCO3 solution (2 x 200 mL), H2O (200 mL) and then dried (MgSO4) and evaporated giving a white solid. The solid was triturated with EtOH (50 mL) and then collected affording compound **25b** (4.42 g, 81%), m.p. 185-186 oC, [α]D23 -51o (*c* 1 in CHCl3). 1H NMR (DMSO-d6): 10.01 (1H, broad s, OH), 8.15 (1H, s, Ar-H), 8.14 (1H, s, Ar-H), 7.42 (1H, s, Ar-H), 7.20 (2H, m, Ar-H), 5.95 (1H, s, H-1), 5.53 (2H, m, H-2/3), 4.42-4.31 (2H, m, H-4/5a), 4.00 (1H, dd, *J*4,5b 5.0 and *J*5a,5b 11 Hz, H-5b), 2.14 (3H, s, Ac), 2.07 (3H, s, Ac), 1.85 (3H, s, Ac); 13C NMR (DMSO-d6): 170.5 (Ac), 170.1 (Ac), 170.0 (Ac), 149.1, 146.3, 131.4, 130.8, 130.6, 128.5, 119.5, 118.1, 110.3, 109.8 (10 x DHN), 103.1 (C-1), 80.0, 74.8, 71.1, 63.6 (C-2/3/4/5), 21.0 (Ac), 20.9 (Ac), 20.8 (Ac). HRMS (ESI) for C21H24Br2NO9 [M+NH4]+: m/z calcd 591.9812; measured: 591.9805.

(3-Hydroxynaphthalen-2-yl)-β-D-ribofuranoside **26a**.

Deprotection of compound **25a** (1.0 g, 1.7 mmol) by a similar procedure to that described above for the preparation of compound **18b** afforded compound **26a** (542 mg, 39%) as a white solid, m.p. 181-182 oC, [α]D22-143o (*c* 0.55 in acetone/H2O). 1H NMR: (DMSO-d6) 7.63 (2H, m, Ar-H), 7.39 (1H, s, Ar-H), 7.25 (2H, m, Ar-H), 7.14 (1H, s, Ar-H), 5.53 (1H, s, H-1), 4.14 (2H, broad m, H-2/3), 3.92 (1H, m, H-4), 3.59 (1H, dd, *J*5a,4 4 Hz and *J*5a,5b 11 Hz, H-5a), 3.41 (1H, m, H-5b and H2O); 13C NMR: (DMSO-d6) 148.2, 146.5, 130.5, 128.7, 127.1, 126.1, 124.8, 123.7, 112.4, 110.5 (10 x DHN), 106.9 (C-1), 85.1, 75.1, 70.9, 63.0 (C-2/3/4/5). HRMS (ESI) for C15H16NaO6 [M+Na]+: m/z calcd 315.0839; measured: 315.0844. The HPLC of this substrate showed a single compound (see supplementary data).

(6,7-Dibromo-3-hydroxynaphthalen-2-yl)-β-D-ribofuranoside **26b**.

Deprotection of compound **25b** (1.0 g, 1.7 mmol) by a similar procedure to that described above for the preparation of compound **18b** afforded compound **26b** (697 mg, 89%) as a white solid, m.p. >240 oC (decomp.), [α]D23 -90o (*c* 0.5 in MeOH). 1H NMR (DMSO-d6): 7.73 (1H, s, Ar-H), 7.53 (1H, s, Ar-H), 7.04 (1H, s, Ar-H), 6.30 (1H, s, Ar-H), 5.25 (1H, d, *J*1,2 2.0 Hz, H-1), 4.32 (1H, t, *J*3,4=2,3 5.0 Hz, H-3), 4.05 (1H, dd, *J*1,2 2.0 Hz and *J*2,3 5 Hz H-2), 3.79 (1H, m, H-4), 3.52 (1H, dd, *J*4,5a 3.0 Hz and *J*5a,5b 12 Hz, H-5a), 3.41 (1H, H-5b and H2O); 13C NMR (DMSO-d6): 163.3, 153.4, 134.3, 130.7, 127.1, 124.3, 117.7, 111.8, 110.6, 108.6 (10 x DHN), 108.4 (C-1), 85.5, 75.0, 70.2, 61.7 (C-2/3/4/5). HRMS (ESI) for C15H18Br2NO6 [M+H]+: m/z calcd 465.9495; measured: 465.9495. The HPLC of this substrate showed a single compound (see supplementary data).

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