Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 951–954

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESIS OF β-*D*-ARABINOFURANOSYL-1-MONOPHOSPHORYL POLYPRENOLS: EXAMINATION OF THEIR FUNCTION AS MYCOBACTERIAL ARABINOSYL TRANSFERASE DONORS

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Received 17 December 1997; accepted 12 March 1998

Abstract: A convenient synthetic strategy has been developed to produce libraries of β-D-arabinofuranosyl-monophosphorylpolyprenol. Those containing C_{50} and C_{55} polyprenols were the most active as donors for the cell-free synthesis of the arabinans of mycobacterial cell walls.

The need to develop new and improved therapies for the treatment of tuberculosis has lead us to explore the biosynthesis of the key polysaccharides arabinogalactan (AG) and arabinomannan (AM) in the mycobacterial cell wall.[1-3] The immediate donor for the majority of arabinose units in AG and AM appears to be β-*D*-arabinofuranosyl-1-monophosphoryldecaprenol (DPA).[4,5] DPA was originally isolated from the alkali stable extracts of *M. smegmatis*.[4] However, its low natural abundance made it difficult to isolate sufficient quantities of the labeled natural material to explore its role in cell wall biosynthesis. As a consequence [1-C^{14}]-DPA was synthesized from [1-C^{14}]-arabinose and ficaprenol-C_{50} and was shown to be active in mycobacterial cell free extracts as an arabinose donor to the cell wall polysaccharides AG and AM.[5] Importantly, it was shown that the site of action of the antituberculosis drug ethambutol affected the arabinosyl transferases responsible for the synthesis of arabinan, particularly that of AG.[5,6] More recently, an assay using simple synthetic arabinanoside-based acceptors was developed, whereby, [1-C^{14}]-DPA proved to be an active donor of 2- and 5- linked Ara*f* units, that is to be the substrate for the β-*D*-arabinofuranosyl-1-monophosphoryldecaprenol: arabinan α(1→5) and β-*D*-arabinofuranosyl-1-monophosphoryldecaprenol: arabinan β(1→2) arabinosyl transferases.[7] Whether or not it is the donor of 3 linked Ara*f* was not established. Since these assays are now being developed to screen compound libraries for novel anti-mycobacterial agents, an assessment of the activity of the synthetic [1-C^{14}]-DPA is an important criteria. Ficaprenol C50 which was used in the synthesis of [1-C^{14}]-DPA, is only a minor component of the commercially available polyprenols isolated from *Fica elastica*, adding significantly to the cost of the assay and hindering its usage. It would be advantageous to use smaller, aqueous soluble donors or more abundant larger polyprenol mixtures and a synthetic strategy that would allow the synthesis of a variety of donors towards developing structure activity relationships which may be useful in the design of structurally related antagonists.

Previously, [1-C^{14}]-DPA was synthesized using a phoshoramidite coupling of ficaprenol and [1-C^{14}]-2,3,5-tri(*tert*-butyldimethylsilyl) arabinose to create the crucial β-phosphodiester linkage.[5] The phosphiting agent 2-cyanoethyl *N,N*-diisopropylchlorophosphorylamidite reacted with decaprenol, and then coupled to the protected arabinoside using tetrazole activation creating a mixture of anomers which after oxidation and base deprotection were separated by chromatography. This approach yielded sufficient amounts of the β-anomer for many assays despite the α-anomer being favored within the synthesis. Use of the same coupling conditions and reacting the protected arabinoside firstly with 2-cyanoethyl *N,N*-diisopropylchlorophosphorylamidite at 0°C yielded the α-anomer only. It was noted that glycosyl phosphites are highly activated and can be used as

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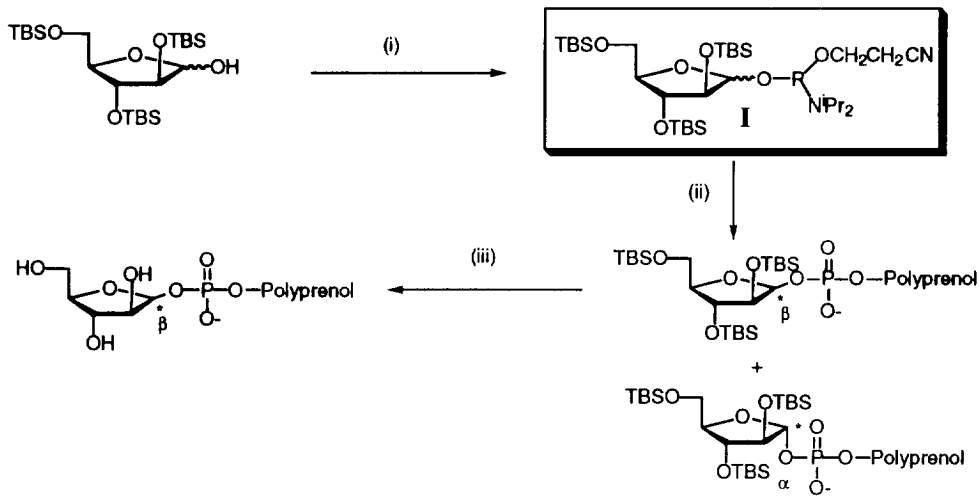
0960-894X/98/$19.00
PII: S0960-894X(98)00147-4

glycosyl donors under very mild conditions.[8] Thus, the β-*D*-2,3,5-tri(*tert*-butyldimethylsilyl) arabinophosphoramidite **I** may have eliminated or rearranged giving solely the α-anomer.



Scheme 1: (i) 2-cyanoethyl *N,N*-diisopropylchlorophosphorylamidite, EtN^iPr_2, CH_2Cl_2, -78°C (ii)(a) Polyprenol, tetrazole (b) H_2O_2, THF (c) KOH, MeOH (d) separate SiO_2 (iii) NH_4OH, MeOH

The synthesis of **I** was achieved by cooling the reaction mixture during the addition of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite to -78 °C and then slowly warming the mixture to 0 °C which allowed for the formation of **I** as a mixture of epimers (Scheme 1). **I** was then coupled to a polyprenol using tetrazole activation which yielded a mixture of α and β epimers of a similar ratio (5:1, α:β) to that obtained with the previous coupling technique.[5]

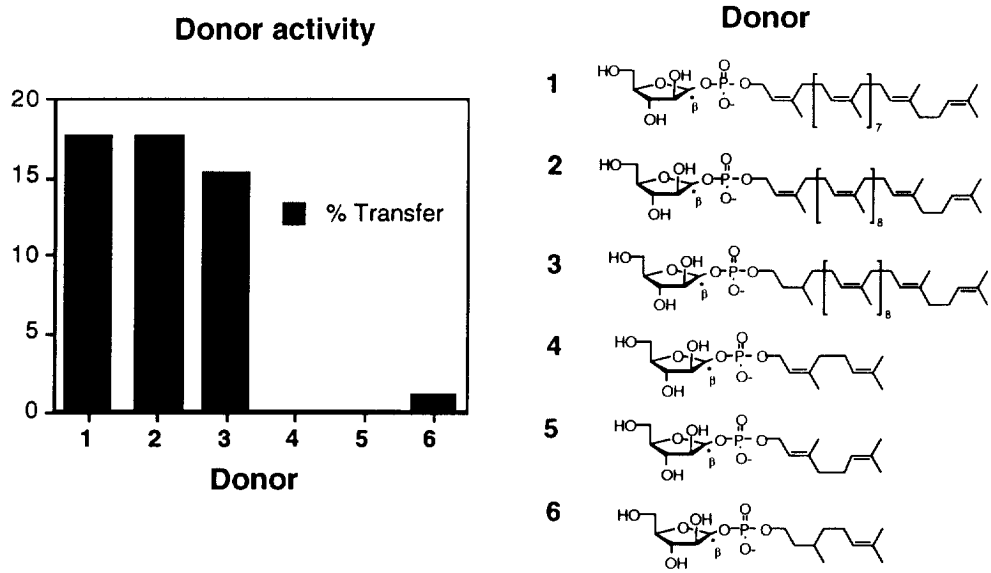


Figure 1: Activities of the donors within the neoglycolipid based arabinosyl transferase assay

The new synthetic procedure allows for the subsequent splitting of the arabinophosphoramidite intermediate **I** and its addition to a variety of polyprenols or alcohols to create a library of β-*D*-arabinofuranosyl-monophosphorylpolyprenols following separation of the α/β anomeric mixtures and final deprotection.[9] We had shown previously that the α-anomer of DPA was inactive as a donor,[5] and, as a result, the β-anomers were assayed only for arabinosyl transferase activity (Figure 1).[10] In the present context, it was evident that the long chain polyprenol derivatives C_{50} (**1**), C_{55} (**2**) and dolichol C_{55} (**3**) were active within this mycobacterial arabinosyl transferase assay as sugar donors. Importantly, the high transferase activity associated with the dolichol derivative (**3**) is in accord with other bacterial activities for which a variety of polyprenyl donors have been studied extensively studied, particularly the *O*-antigen biosynthesis in *Salmonella anatum*, where the polyprenyl based donors showed a lack of specificity for the saturation state of the first isoprene unit.[11,12] The C_{10} prenol derivatives nerol (**4**) , geraniol (**5**) and citronellol (**6**) were all found to be inactive as sugar donors within the arabinosyl transferase assay system.

In summary, we have described a simple process that allows for the synthesis of β-arabinosyl-phosphorylpolyprenol libraries from an arabinosylphoramidite intermediate **I** which works well for both polyprenols and dolichols. In addition we demonstrated that the long chain prenols (**1** – **3**) were all active in the mycobacterial arabinosyl transfer assay described in this communication. Notably, there was little difference in the activity between C_{50} and C_{55} polyprenols, which is beneficial as this may enable the useage of crude ficaprenol in further large scale syntheses and incorporation experiments. As a consequence, this strategy may also help us to further understand the structure activity relationships of mycobacterial arabinosyl transferase donors and allow for the synthesis of a wide range of potential donors and antagonists.

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9. Synthesis of arabinofuranosyl-monophosphorylpolyprenoids: [1-C^{14}]-2,3,5-tri(*tert*-butyl dimethylsilyl) arabinose (0.4 mmol, 450 μCi) prepared as described previously,[7] was placed in an oven dried flask under argon and to which was added dichloromethane (20 mL) and diisopropylethylamine (142 μL, 0.55 mmol). The flask was then cooled to -78°C and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (125 μL, 0.55 mmol) was added dropwise. The flask was then allowed to warm slowly to 0 °C over 1 h yielding the arabinosyl phosphoramidite Solution A. Six **1**–**6** predried screw capped tubes containing stir bars were prepared containing: **1**, ficaprenol C_{55} (34 mg, 44 μmol), tetrazole (4.0 mg, 54 μmol); **2**, ficaprenol C_{50} (34 mg, 48 μmol), tetrazole (1.6 mg, 21 μmol); **3**, dolichol C_{55} (14 mg, 18 μmol), tetrazole (4.4 mg, 59 μmol); **4**, nerol (17 mg, 110 μmol) tetrazole (10.0 mg, 136 μmol); **5**, geraniol (17 mg, 110 μmol) tetrazole (10.0 mg, 136 μmol); and **6**, citronellol (17 mg, 110 μmol), tetrazole (10.0 mg, 136 μmol). The tubes were then cooled to 0 °C and solution A: **1**, 2.0 mL; **2**, 0.8 mL; **3**, 2.2 mL; **4**, 5 mL; **5**, 5 mL; and **6**, 5 mL was added under a stream of argon to each tube. The tubes were then capped, stirred at

0 °C for 1 h, and then allowed to warm gradually to room temperature, and stirred for 4 h. The solvent was removed by evaporation under a stream of argon and the residue titurated with hexane (3 x 1 mL). The washings were then filtered through a plug of glass wool into tubes containing stirrer bars and concentrated to dryness under a stream of argon. To the tubes was added THF (**1–3**, 1 mL; **4–6**, 2 mL) and the contents were stirred rapidly before the addition of hydrogen peroxide (**1–3**, 10 μL; **4–6**, 20 μL) and stirring was continued for 1 min before the addition of 5% potassium hydroxide in methanol (**1–3**, 2 mL; **4–6**, 4 mL). The tubes were then left to stir for 30 min before the addition of chloroform (**1–3**, 6mL; **4–6**, 12 mL) and water (**1–3**, 3 mL; **4–6**, 6 mL) the mixture was then shaken and left to form a biphase. The lower organic fraction was then recovered and chromatographed by silica gel chromatography.[7] The separated anomers were then deprotected using ammonium flouride in methanol,[7] to afford the α and β-arabinofuranosyl-monophosphorylpolyprenols (cpm): **1α** (28,176,900), **1β** (4,312,410); **2α** (30,925,000), **2β**(7,462,940); **3α** (10,730,800), **3β** (2,106,890); **4α** (79,245,000), **4β** (16,595,000); **5α** (20,676,600), **5β** (5,141,250); **6α** (62,660,000); **6β** (10,112,500). All samples co-migrated with cold synthetic standards and were assessed as pure using TLC autoradiography.

10. Neoglycolipid Based Acceptor Assay: Each donor (**1–6**, 80,000 cpm) stored in $CHCl_3$:MeOH:NH_4OH (65:25:4) was added to of labeled microcentrifuge tubes (0.6 mL) and dried under a stream of argon. The tubes were then placed in a vacuum desiccator for 15 min to remove any residual solvent. The acceptor α-*D*-Ara*f*-(1→5)-α-*D*-Ara*f*-1-O-$(CH_2)_7CH_3$ (50mM, 12.8μL) was added to the tubes and the mixture sonicated to ensure a homogeneous solution. To each tube was added buffer containing 50mM MOPS pH 7.9, 5mM 2-mercaptoethanol and 10mM $MgCl_2$ (167.2 μL), ATP (1mM, 20 μL) and *M. smegmatis* membranes resuspended in buffer (120 μL) and incubated at 37 °C for 1 h, followed by the addition of $CHCl_3$:CH_3OH (1:1, 1066 μL), mixed thoroughly, and the supernatant recovered and evaporated to dryness. The contents of the tubes were resuspended in 50% aqueous ethanol loaded directly onto an Whatman Strong Anion Exchange (SAX) column and elueted with 50% aqueous ethanol (3 mL) and counted. Control tubes contained the same ingredients; however they were quenched by the addition of $CHCl_3$:CH_3OH prior to the start of the incubation. The incorporation of [^{14}C]Ara*f* was determined by subtracting the radioactivity in control tubes from that of the test tubes.

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