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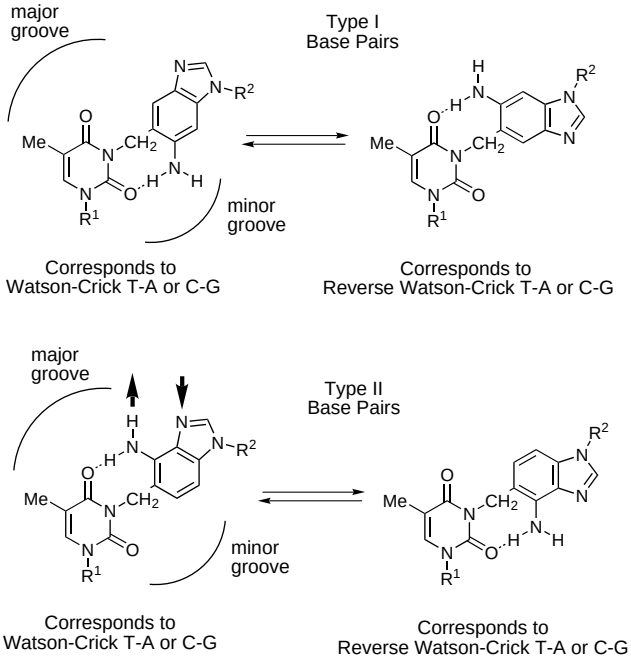
Synthesis of DNA Oligomers Possessing a Covalently Cross-Linked Watson–Crick Base Pair Model**

Hong-Yu Li, Yao-Ling Qiu, Elisabeth Moyroud, and Yoshito Kishi*

The concept of covalently cross-linked sections with molecular architecture similar to Watson–Crick hydrogen-bonded base pairs was introduced by Devadas and Leonard in the mid-1980's.[1] Since then, several types of covalently linked systems have been developed. However, these systems[2, 3] were generated from preformed double helices, as seen in the seminal work of Ferentz and Verdine. The Leonard system may offer unique opportunities to address questions regarding the chemistry of DNA and RNA, but this system has several drawbacks, including difficulty in attempted duplex formation and lack of conformational flexibility between the base pairs.[1]

We have recognized the possibility that CH_2-bridged base pair models may be uniquely suited to the chemical exploration of covalently cross-linked nucleosides/nucleotides. In addition to their increased chemical stability, these base pair models are expected to adopt only Watson–Crick or reverse Watson–Crick base pairings while maintaining conformational flexibility along the CH_2 bridge. We have focused on two specific types (types I and II) of base pair models (Scheme 1), with the anticipation that they might exhibit



Scheme 1. Covalently cross-linked Watson–Crick base pair models. A = 2′-deoxyadenosine, C = 2′-deoxycytidine, G = 2′-deoxyguanosine, T = 2′-deoxythymidine.

differing structural characteristics. Although both the type I and type II models adopt only Watson–Crick or reverse Watson–Crick base pairings in the sense of primary hydrogen-bonded base pairing, only the type II model provides a structural motif for formation of Hoogsteen triplets such as T–AT and C–GC: see the two bold arrows in Scheme 1 which indicate possible hydrogen-bonding sites in a major groove. We recently reported the synthesis and structural properties of type I and II base pairs.[4] In this paper, we present a method for incorporating these base pair models into DNA oligomers.

A priori, we considered that phosphoramidite-based solid-phase synthesis[3, 5] would best meet with our future needs, and we studied its applicability to three types of oligomers incorporating a covalently cross-linked base pair, the *n*, *h*, and *H* types. Our synthetic plan is schematically depicted in Scheme 2, with the order of chain elongation being indicated by a circled number. First, all of the proposed chain elongations take place in the 3′→5′ direction for *n*- and *h*-type oligomers, but the last chain elongation takes place in the 5′→3′ direction for *H*-type oligomers. Second, the order of the

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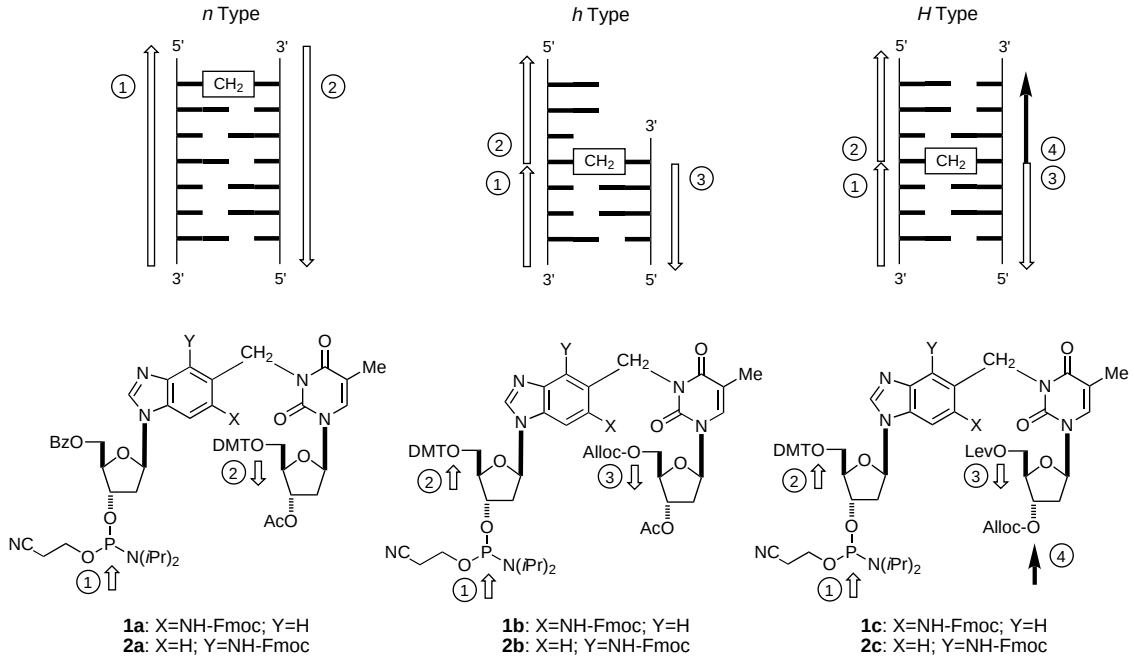
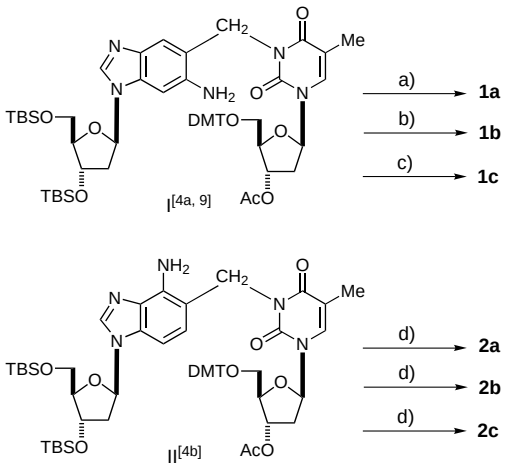


Scheme 2. Synthetic plan for *n*-, *h*-, and *H*-type oligomers. Ac = acetyl, Alloc = allyloxycarbonyl, Bz = benzoyl, DMT = 4,4′-dimethoxytrityl, Fmoc = 9-fluorenylmethoxycarbonyl, Lev = levulinoyl.

second and third chain elongations for the *h* and *H* types can be inverted simply by switching the DMT group with the Alloc or the Lev group in **1b** and **2b** or **1c** and **2c**, respectively. Third, an Fmoc group is chosen for protection of the amino group; this allows for simultaneous removal, along with deprotection of the protecting groups on the A, T, G, and C nucleotides, upon detachment of the oligomers from the solid support. Fourth, we plan to employ solid-phase synthesis with controlled-pore glass (CPG) supports.[6] Thus, alcoholic protecting groups compatible with this solid support are required; Alloc and Lev protecting groups are known to meet with this requirement.[7, 8]

To put this plan in practice, we first required base pairs **1a**–**c**[9] and **2a**–**c**, which were efficiently synthesized as summarized in Scheme 3. With the arbitrarily chosen base sequences shown in Scheme 4, the feasibility of this plan was then tested. Clearly, the crucial step in these syntheses entails adding a CH_2-bridged base pair model onto an elongating oligonucleotide chain, that is, step 8 for *n*-type oligomer syntheses, step 3 for *h*-type, and step 5 for *H*-type. The base pairs **1a**–**c** and **2a**–**c** could be incorporated into these oligomers approximately as efficiently as the nonmodified nucleotides (see Experimental Section for more details).[10] However, to achieve this efficiency, longer coupling times and higher concentrations of the modified bases were required.[11] Under these conditions, approximately 10–15 equivalents of a modified base were delivered to a DNA synthesizer. However, the unused modified base could be recovered almost quantitatively by employing the method recently reported.[12] In the case of *h*-type oligomer syntheses, the solid support was removed from the synthesizer after completion of the second chain elongation, subjected to Alloc deprotection on the solid support,[7] and reintroduced into the synthesizer to complete the third chain elongation, step 11. In the case of *H*-type



Scheme 3. Reagents and conditions. a) 1. FmocCl, DMAP, Pyr, RT, 92%; 2. nBu_4NF, AcOH, THF, RT, 93%; 3. BzCl, Pyr, CH_2Cl_2, 0°C, 63% of the product, along with 33% of starting material; 4. $(iPr)_2NP(Cl)O(CH_2)_2CN$, $Et(iPr)_2N$, CH_2Cl_2, RT, 93%; b) 1. FmocCl, DMAP, Pyr, RT, 92%; 2. TCA, CH_2Cl_2, RT, 96%; 3. Alloc-OBt, DMAP, Pyr, THF, RT, 95%; 4. nBu_4NF, AcOH, THF, RT, 89%; 5. DMTCl, DMAP, CH_2Cl_2, RT, 92%; 6. $(iPr)_2NP(Cl)O(CH_2)_2CN$, $Et(iPr)_2N$, CH_2Cl_2, RT, 95%; c) 1. NH_3, MeOH, RT, 100%; 2. FmocCl, DMAP, Pyr, RT, 95%; 3. Alloc-OBt, Pyr, DMAP, THF, RT 96%; 4. TCA, CH_2Cl_2, RT, 90%; 5. $(Lev)_2O$, Pyr, RT, 92%; 6. nBu_4NF, AcOH, THF, RT, 87%; 7. DMTCl, Pyr, CH_2Cl_2, RT 93%; 8. $(iPr)_2NP(Cl)O(CH_2)_2CN$, $Et(iPr)_2N$, CH_2Cl_2, RT, 100%; d) steps (h) 1–3, (h) 1–5, or (h) 1–7 of Scheme 1 in ref. [4b] as appropriate, followed by $(iPr)_2NP(Cl)O(CH_2)_2CN$, $Et(iPr)_2N$, CH_2Cl_2, RT, 94% for **2a**, 95% for **2b**, 97% for **2c**. Abbreviations: Bt = benzotriazol-1-yl, *n*Bu = normal butyl, DMAP = 4-dimethylaminopyridine, Et = ethyl, *i*Pr = isopropyl, Pyr = pyridine, TBS = *tert*-butyldimethylsilyl, TCA = trichloroacetic acid, THF = tetrahydrofuran.

oligomer syntheses, the column containing the solid support was removed from the synthesizer twice (after completion of the second and third chain elongations), subjected to Lev and

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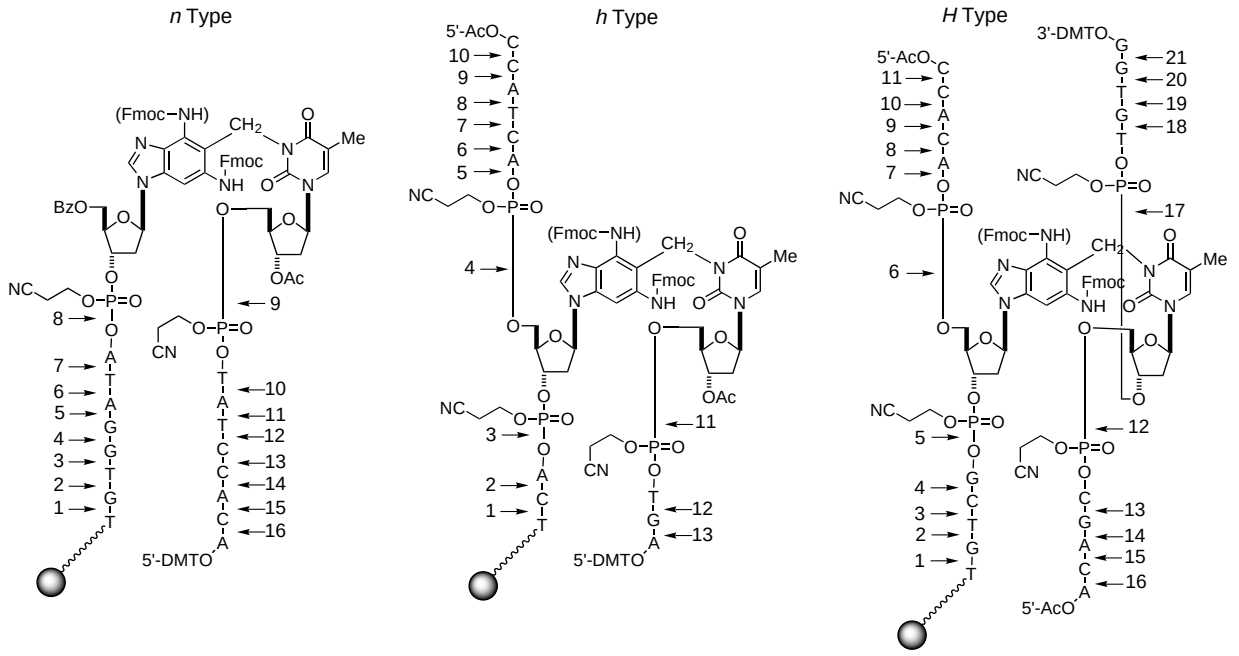

Scheme 4. Synthesis of *n*-, *h*-, and *H*-type oligomers. The order in which the chains were elongated is indicated. For synthetic details and yields, see the Experimental Section.

Alloc deprotections on the solid support,[7, 8] and reintroduced into the synthesizer to complete the third and fourth chain elongations, steps 12 and 17. The overall efficiencies of these syntheses are summarized in the Experimental Section and demonstrate that the CH_2-bridged base pair models **1a**–**c** and **2a**–**c** could be efficiently incorporated into *n*-, *h*-, and *H*-type oligomers.[13]

After completion of the chain elongations, the oligomers were detached from the solid support, isolated, and purified under the conditions specified in the Experimental Section. The syntheses were carried out on 1-μmol scale and furnished approximately 0.45 μmol, 0.35 μmol, and 0.15 μmol of HPLC-purified *n*-, *h*-, and *H*-type oligomers, respectively (Figure 1).[14]

The molecular weight of each synthetic oligomer was established by mass spectrometry. In the early phase of these studies, we relied on electrospray ionization (ESI) mass spectrometry, but in later phases, we used the matrix-assisted laser desorption/ionization (MALDI) mass spectrometry for technical reasons.[15] By knowing both the order of nucleotides introduced on an elongating chain and the molecular weight of the products, we could secure the structure of the synthetic oligomers.

In conclusion, we have demonstrated the synthetic feasibility of *n*-, *h*-, and *H*-type DNA oligomers possessing a covalently cross-linked Watson–Crick base pair model. It should be noted that the current study is focused on the synthesis of *anti-parallel* DNA duplexes. However, with adjustments of the protecting groups in **1a**–**c** and **2a**–**c**, the plan depicted in Scheme 2 can be extended to the synthesis of *parallel* DNA duplexes. Circular dichroism and NMR spectroscopic studies, which demonstrate that the structural properties of DNA oligomers containing a covalently cross-linked Watson–Crick base pair model resemble those of the corresponding native duplex DNA, will be reported elsewhere.[16]

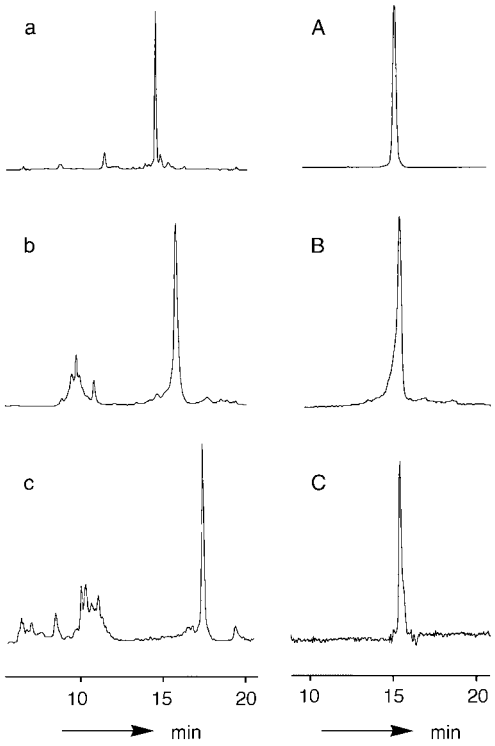

Figure 1. HPLC chromatograms of crude and purified DNA oligomers. Traces a)–c) indicate the purity of the crude products obtained after the detachment/deprotection step: a) type II, *n* type; b) type II, *h* type; c) type II, *H* type. Traces A)–C) indicate the purity of the isolated products after the final purification step: A) type II, *n* type; B) type II, *h* type; C) type II, *H* type. For further details, see the Experimental Section.

Exerimental Section

Synthesis of *n*-, *h*-, or *H*-type DNA oligomers was carried out on 1-μmol scale with an Applied Biosystems 392 DNA/RNA synthesizer (coupling time: 25 seconds for 3′-CE phosphoramidites; 10 minutes for the modified

dinucleoside phosphoramidites and for 5′-CE phosphoramidites) or with a PerSeptive Expedite 8909 (coupling time: 90 seconds for 3′-CE phosphoramidites; 15 minutes for the modified dinucleoside phosphoramidites and for 5′-CE phosphoramidites). CE = β-cyanoethyl.

The following reagents were used: deblocking mix: TCA/CH_2Cl_2; activator: 1*H*-tetrazole/MeCN; cap mix A: Ac_2O/THF; cap mix B: 1-methylimidazole/Pyr/THF; oxidizing solution: I_2/H_2O/Pyr/THF. All the reagents and the dA-, dT-, dC-, or dG-3′/5′-CE phosphoramidites were purchased from Glen Research, Virginia. A 0.1 M solution of dA-, dT-, dC-, or dG-3′/5′-CE phosphoramidites was used with the Applied Biosystems 392 DNA/RNA synthesizer, whereas a 0.06 M solution was used with PerSeptive Biosystems Expedite 8909 DNA/RNA synthesizer. A 0.08 – 0.1 M solution was used for coupling **1a – c** or **2a – c** onto a chain. Removal of the Alloc protecting group was performed with $[Pd(PPh_3)_4]$ and HCO_2NH_4 in THF at RT over 24 h and removal of the Lev protecting group was required $NH_2NH_2 \cdot H_2O$ (0.5 M) in Pyr/HOAc at RT for 10 min.

Based on the amount of DMT released in a coupling cycle over that in the preceding coupling cycle, the coupling yield was estimated to be 97 – 100 % for step 8 of the *n*-type, steps 3 and 11 of the *h*-type, and steps 5, 12, and 17 of the *H*-type oligomer syntheses, and 98 – 100 % for all the other steps. The overall yields, estimated from the amount of DMT released in the second-to-last coupling cycle over that in the first coupling cycle, were 70 – 80 %, 60 – 70 %, and 50 – 60 % for the *n*-, *h*-, or *H*-type oligomers, respectively.

Purification/isolation of an oligomer was achieved through a five-step procedure: 1) 28 % NH_4OH, 55 °C, 24 h (detachment/deprotection of an oligomer from solid support); 2) HPLC purification under the conditions indicated below; 3) 10 % AcOH (deprotection of DMT); 4) C18 cartridge filtration; 5) lyophilization. The HPLC chromatograms (Figure 1) were recorded on a Vydac C4 column (10 × 250 mm); solvent system: MeCN/100 mM $AcONH_4$, 3 % – 50 % over 20 min for a) – c) and 2 % – 15 % over 20 min for A) – C); flow rate: 3 mL min^{-1}. Traces a – c indicate the purity of the crude products obtained after step 1 of the purification. Retention times: a) type II, *n* type: 15.0 min; b) type II, *h* type: 16.1 min; c) type II, *H* type: 17.2 min. Traces A – C indicate the purity of the products after step 5 of the purification. Retention times: A) type II, *n* type: 15.4 min); B) type II, *h* type: 15.9 min; C) type II, *H* type: 16.1 min.

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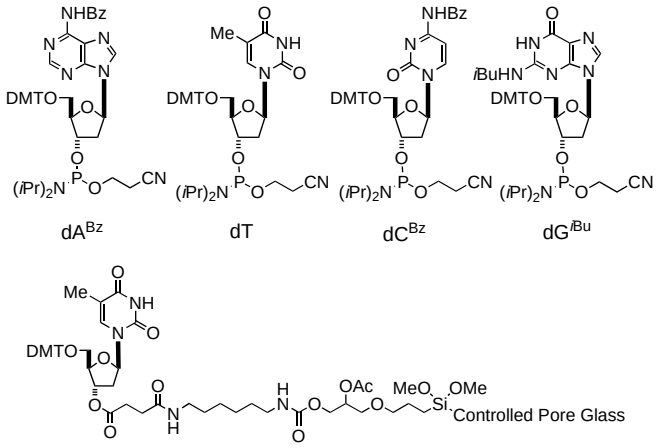
[6] The following phosphoramidites and dT-lcaa-CPG (500 Å; loading capacity: 40 μmol g^{-1}) from Glen Research, Virginia, were used for the current studies. *i*Bu = isobutyryl, lcaa = long chain alkylamino.



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[9] Two modifications have been made on the original synthesis of I.[4a] These are: 1) $NaBH_4$ reduction of the mixed anhydride, that is, step (b) 5. in Scheme 1 of ref. [4a], is better performed at – 78 → – 30 °C, and 2) separation of N1- and N3-glycosides can most practically be achieved after the $NaBH_4$ reduction. With these improvements, a 20-gram scale synthesis of I can be carried out, with one silica filtration. The overall yield of **5** from **1** in Scheme 1 in ref. [4a] is approximately 26 %.

[10] At an earlier stage of this study, the solid-phase synthesis was carried out on an Applied Biosystems 392 DNA/RNA Synthesizer in the Verdine group. We thank Professor G. Verdine for his generosity and help. In later stages, the synthesis was performed on a PerSeptive Biosystems Expedite 8909. No obvious difference in synthetic efficiency was noticed between the two instruments.

[11] Using a PerSeptive Biosystems Expedite 8909, optimal conditions were briefly studied for the case of **1a** and **2a**. To achieve the efficient attachment of **1a** and **2a** onto the elongating chain, their concentration should be at least 0.08 M and the coupling time for attaching them on the elongating chain should be longer than the coupling time (90 seconds) for the nonmodified nucleotides.

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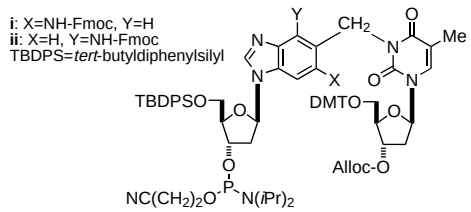
[13] We tested the feasibility of *H*-type oligomer syntheses with polystyrene-based solid support. According to the procedure reported by Strazewski (S. Gunzenhauser, E. Biala, P. Strazewski, *Tetrahedron Lett.* **1998**, *39*, 6277), the polystyrene-based solid support loaded with T (loading capacity: 25 μmol g^{-1}) was prepared and used for the solid-phase synthesis. Two modified base pairs **i** and **ii** were successfully incorporated into *H*-type oligomers with an efficiency similar to that outlined in the Experimental Section. We thank Dr. Peter Strazewski, University of Basel, for a generous gift of polystyrene polymer.



[14] The low yields of *H*-type oligomers, relative to the estimated overall yields (see the Experimental Section), were largely due to the difficulties encountered during HPLC purification.

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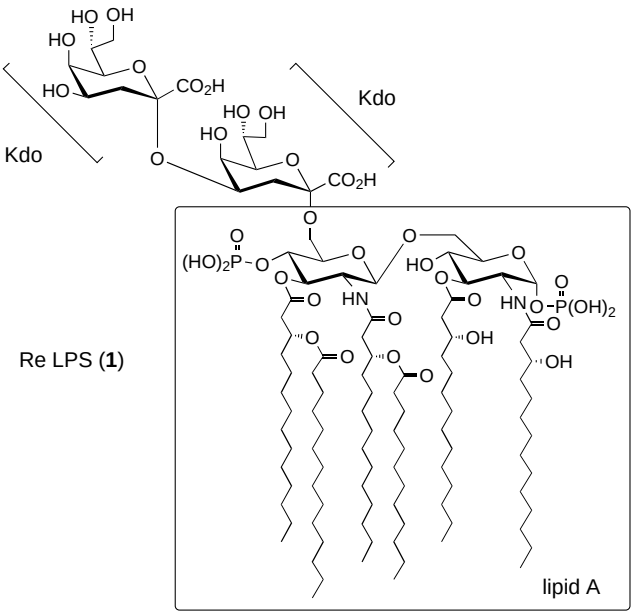
[15] *n* Type: *m*/*z* calcd: 5445; type I found: 5444 (negative ion ESI-MS); type II found: 5440 (MALDI-MS, matrix: 3-hydroxypicolinic acid/ammonium citrate). *h* Type: *m*/*z* calcd: 4154; type I found: 4151 (MALDI-MS, matrix: 2,6-dihydroxyacetophenone/ammonium citrate); type II found: 4127 (MALDI-MS, matrix: 2,6-dihydroxyacetophenone/ammonium citrate). *H* Type: *m*/*z* calcd: 6683; type I found: 6669 (MALDI-MS, matrix: 2,6-dihydroxyacetophenone/ammonium citrate); type II found: 6671 (MALDI-MS, matrix: 3-hydroxypicolinic acid/ammonium citrate).
[16] H.-Y. Li, Y.-L. Qiu, Y. Kishi, *ChemBioChem*, in press.



First Total Synthesis of the Re-Type Lipopolysaccharide**

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Lipopolysaccharides (LPS) are ubiquitous glycoconjugates located on the surface of Gram-negative bacteria such as *Escherichia coli*, and exhibit multiple and potent biological activity, both toxic and beneficial, towards higher animals.[1] LPS consist of a phosphorylated acyl glucosamine disaccharide covalently bonded to a polysaccharide. The former glycolipid, lipid A, is the entity responsible for most of the biological activity of LPS.[2, 3] Lipid A is therefore never present on living bacterial cells in the free form; it is an artificial product obtained only by the acid hydrolysis of LPS. The simplest LPS molecule that is known to be present on bacterial cells and that comes into contact with animal cells is the so-called Re-type LPS produced by the *Escherichia coli* Re mutant (Re LPS, **1**).[4] This LPS derivative is composed of lipid A and two molar equivalents of 3-deoxy-D-*manno*-2-octurosonic acid (formerly called 2-keto-3-deoxy-D-*manno*-octonic acid (Kdo)). The chemical synthesis of Re LPS is not only a highly challenging goal, but would also contribute towards the elucidation of the biological and physicochemical role of the sugar moieties linked to lipid A. It has often been implied that the polysaccharide portion enhances or modifies the activity of Re LPS.[5] In fact, Re LPS that only has two units of Kdo was reported to exhibit more potent antitumor and cytokine-inducing activity than lipid A.[6, 7] However, these observations have never been confirmed owing to the inherent heterogeneity and the difficult purification of natural specimens. An efficient synthesis of Re LPS would also provide easy access to various structural congeners required for the study of structure–activity relationships. Such a strategy based on chemical synthesis has been effective in our studies of the role of acyl and phosphoryl moieties in the bioactivity of lipid A.[8]

Until now, only partial syntheses of Re LPS have been reported, for example, our synthesis of the 1-*O*-dephospho analogue.[9] The total synthesis of Re LPS has not yet been accomplished because of the difficulty in synthesizing the complex structure, which contains highly acid-labile glycosyl phosphate, as well as base-labile ester functional groups in the amphiphilic structure. A high-yielding and stereoselective formation of α-Kdo glycosides[10–13] is a key step towards the synthesis of Re LPS. We have recently completed an efficient synthesis of lipid A[8c, 14] and its labeled analogues[15] by means of an optimized synthetic pathway coupled with a high-yielding efficient purification method for the final products based on liquid–liquid partition.[16] These achievements prompted us to undertake the synthesis of Re LPS (Scheme 1). Herein we describe an efficient stereoselective glycosylation method with Kdo donors, and the first total synthesis of Re LPS. A direct comparison of the biological activities of synthetic and natural specimens is also reported.

Based on our experience with the synthesis of lipid A and its analogues,[8, 9, 15, 16] we employed benzyl-type groups that are removable by neutral hydrogenolysis, as they are resilient protecting groups for the hydroxy, carboxy, and phosphate groups in **2**. The 4-(trifluoromethyl)benzyl group, which was found to be resistant to oxidation but readily removable by hydrogenolysis,[17] was used especially for the protection of the hydroxy groups on the 3-hydroxyacyl residues. Unsubstituted benzyl groups at these positions are prone to air-oxidation and gradually are transformed into the corresponding benzoyl groups during storage, even at $-5\,^{\circ}C$.[17, 18] The tetrasaccharide backbone of **2** was formed by the successive coupling of two

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