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Synthesis and biophysical evaluation of 3′-Me-α-L-LNA – Substitution in the minor groove of α-L-LNA duplexes

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ABSTRACT

The synthesis and biophysical evaluation of 3′-Me-α-L-LNA is reported. The synthesis of the nucleoside building block phosphoramidite was accomplished starting from diacetone glucose. The 3′-Me group was introduced in the desired configuration by hydride mediated opening of an exocyclic epoxide. Inversion of the 2′-hydroxyl group was achieved by means of an oxidation/reduction sequence followed by cyclization onto a 5′-leaving group to assemble the [2.2.1] ring system. Biophysical evaluation of 3′-Me-α-L-LNA modified oligonucleotides showed good duplex thermal stabilizing properties which were similar to α-L-LNA. Mismatch discrimination experiments revealed that 3′-Me-α-L-LNA possess slightly enhanced discrimination properties for the GU wobble base-pair as compared to related nucleic acid analogs.

Locked nucleic acid (β-D-LNA or LNA, **1**) modified oligonucleotides provide unprecedented increases in the thermal stability of oligonucleotide duplexes.[1] As a result, LNA and related analogs have been extensively used for RNase H antisense,[2–7] splice modulation,[8] siRNA and microRNA[9] based therapeutic antisense applications. In addition, the high affinity recognition of cognate nucleic acids seen with LNA has resulted in the widespread use of this modification for oligonucleotide based diagnostic applications.[10] Wengel showed that the alpha anomer of enantio-LNA (α-L-LNA, **5**) also exhibits LNA-like high affinity recognition of complementary nucleic acids despite the structural differences between the nucleoside monomers.[11] Subsequent structural studies showed that the 2′,4′-bridge in LNA duplexes lies in the minor groove while this bridge is located inside the major groove for α-L-LNA modified duplexes.[12,13] As a result of these differences, introducing substitution along the 2′,4′-bridge in LNA orients them into the minor groove of the duplex, where they are generally well tolerated (Fig. 1).[4,14] On the other hand, introducing substitution at the 3′-position of LNA projects the appended group into the major groove where it negatively impacts duplex stability.[15] In contrast to the β-D-LNA series, introducing substitution on the 2′,4′-bridge in α-L-LNA generally disrupts hybridization. In a series of elegant studies,[16–18] Hrdlicka showed that alkyl groups on the 2′-nitrogen atom of 2′-amino-α-L-LNA were poorly tolerated except when the 2′-nitrogen had a pyrenyl moiety attached. In that case, very large increases in binding towards RNA strands containing abasic sites (ΔT_m/unit +33 °C) were observed due to stacking of the pyrenyl moiety at the abasic site within the duplex structure.

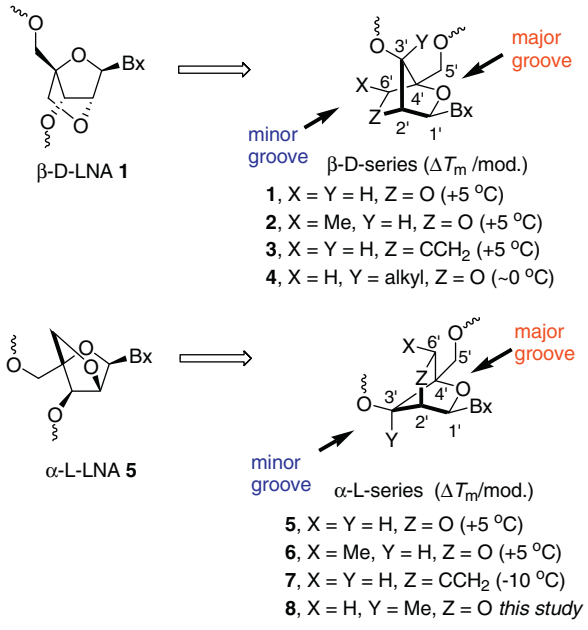

Figure 1. Structures of β-D-LNA, α-L-LNA and related analogs.

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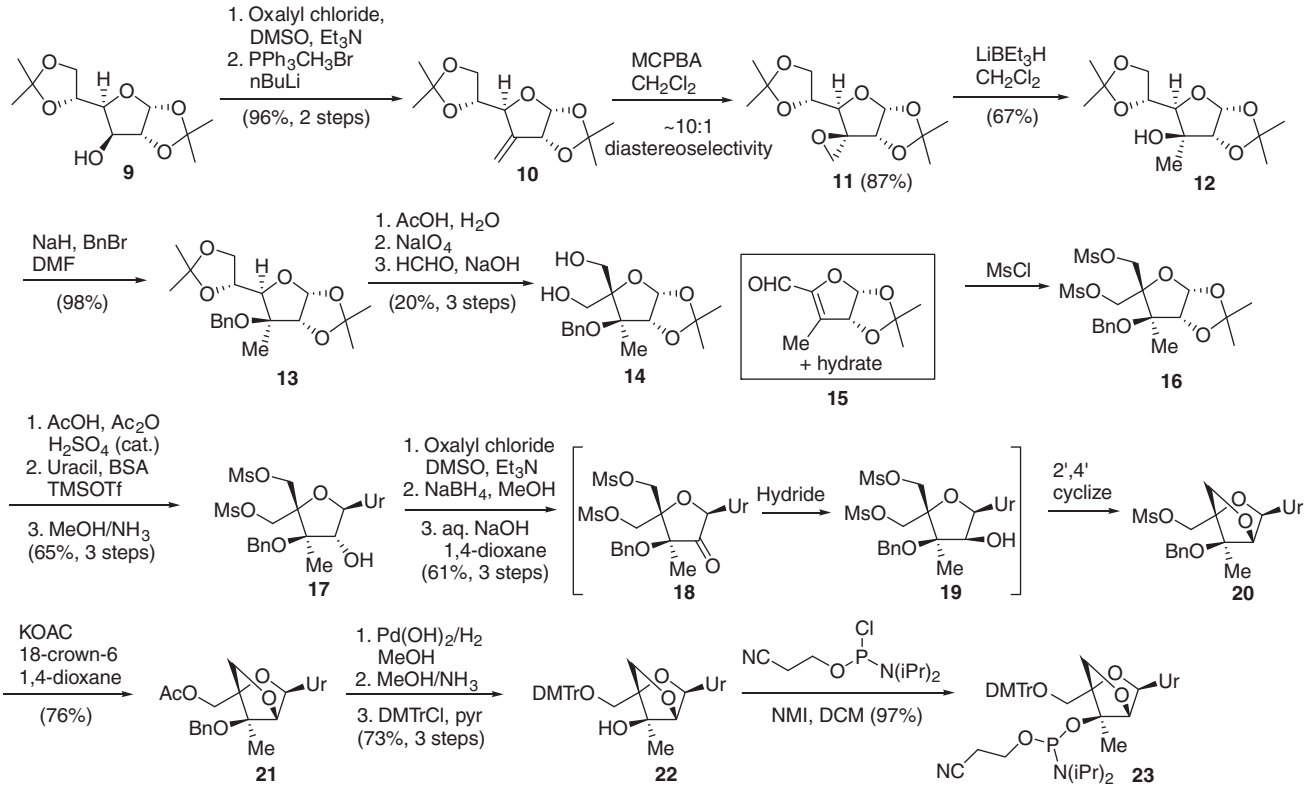
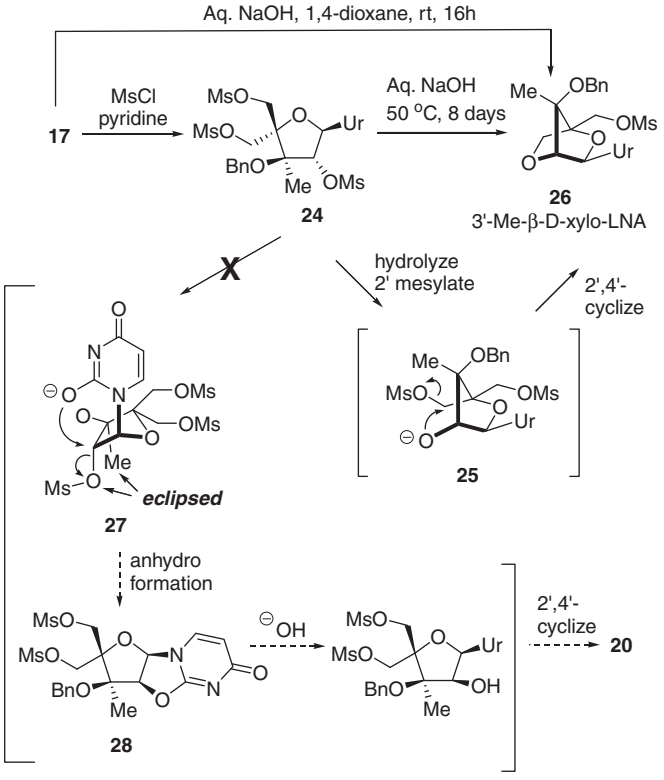


Scheme 1. Synthesis of 3′-Me-α-L-LNA uridine phosphoramidite.

We recently showed that that replacing the 2′-oxygen atom in α-L-LNA with an exocyclic methylene group (**7**) was detrimental to duplex thermal stability.[19] As a continuation of that exercise, we also showed strong duplex stabilization properties with 6′-Me-α-L-LNA (**6**) where the methyl group points towards the 3′-phosphodiester linkage and is situated towards the edge of the major groove of the modified duplex.[20] While the above SAR was informative with regard to the steric requirements for hybridization within the major groove, we also wanted to evaluate the effect of introducing steric bulk in the minor groove of α-L-LNA duplexes. The minor groove of oligonucleotide duplexes serves as an important recognition element for many nucleic acid binding proteins such as RNase H[21,22] and introducing substitution could have a major impact on the biological properties of ASOs containing such modifications. Moreover, identifying sites on the α-L-LNA scaffold for site-specific introduction of conjugates or fluorescent tags in the minor groove of the modified duplexes could be useful for oligonucleotide based diagnostic applications. In this letter, we present the synthesis and hybridization properties of 3′-Me-α-L-LNA (**8**) and show that introducing steric bulk in the minor groove of α-L-LNA modified duplexes maintains the high affinity recognition of complementary nucleic acids observed with α-L-LNA.

Synthesis of the 3′-Me-α-L-LNA uracil phosphoramidite **23** was initiated from diacetone glucose **9** (Scheme 1). In diacetone glucose, oxidation of the 3′-hydroxyl followed by addition of MeMgBr is known to provide almost exclusively the undesired epimeric (*R*) 3′-methyl sugar due to attack of the organometallic reagent from the less hindered concave face of the *cis*-fused bicyclic 1,2-acetonide.[23] To circumvent this, we first prepared the exocyclic olefin **10** from diacetone glucose by a Swern/Wittig sequence. Reaction of **10** with MCPBA resulted in conversion of the exocyclic olefin to a mixture of epoxide diastereomers (~10:1) from which the major and desired product **11** could be cleanly isolated after chromatography. Reduction of the epoxide **11** using super hydride provided the tertiary alcohol **12** with the methyl group now in the



Scheme 2. Rationale for formation of 3′-Me-β-D-xylo-LNA.

desired (*S*) configuration.[24] The tertiary alcohol in **12** was protected as the benzyl ether to provide **13** in excellent yield. Selective hydrolysis of the 5,6-acetonide, followed by oxidative cleavage of the alcohol with periodate and treatment with excess formaldehyde and sodium hydroxide provided diol **14** in low yield. The major product appeared to be compound **15** which is formed by beta-elimination of the 3′-benzyl ether upon treatment of the intermediate aldehyde with a strong base such as sodium hydroxide. Interestingly, no beta-elimination of the 3′-ether is reported with analogous sugar intermediates which do not have a 3′-methyl group of when the 3′-alkyl group is in the opposite configuration.[15,25] Efforts to suppress the beta-elimination reaction by cooling the reaction prior to adding sodium hydroxide, adding a large excess of formaldehyde to trap the anion prior to beta-elimination or slow addition of base were not successful. However, we were still able to prepare multi-gram quantities of diol **14** which were adequate to provide gram quantities of the final phosphoramidite **23**, due to the relative ease of preparing tertiary alcohol **13** (~50 g) early in the synthetic scheme. Next, diol **14** was reacted with methanesulfonyl chloride to provide the bis-mesylate **16**. Acetolysis of the 1,2-acetonide followed by a Vorbruggen reaction with per-silylated uracil to install the nucleobase provided nucleoside **17** after removal of the 2′-acetyl group with methanolic ammonia.

Attempts to invert the 2′-hydroxyl group by means of an anhydro-formation/ring-opening sequence were unsuccessful due to very slow formation of the anhydro-nucleoside intermediate **28** (Scheme 2). Instead, under the reaction conditions employed, the 2′-mesylate was slowly hydrolyzed to generate the 2′-hydroxyl group (**25**) which cyclized into the 5′-mesylate to provide the 3′-Me-xylo nucleoside **26**. Presumably, formation of the anhydro-nucleoside has to proceed through a transition state where the 3′-methyl group eclipses the adjacent 2′-mesylate (**27**) and this disfavors S_N2 displacement of the 2′-mesylate by the 2-oxygen atom of the pyrimidine nucleobase. To further confirm the identity of the cyclized β-D-xylo nucleoside **26**, nucleoside **17** was treated with aqueous sodium hydroxide to provide **26** by direct displacement of the 5′-mesylate by the 2′-hydroxyl group. To avoid this problem, the 2′-hydroxyl group was inverted by first oxidation to the 2′-ketone **18**, followed by reduction with sodium borohydride to provide **19** almost exclusively. Presumably, attack of the hydride takes place from the relatively less hindered *re* face of the ketone in **18** to provide alcohol **19**. Further treatment of **19** with aqueous sodium hydroxide provided the 2′,4′-cyclized nucleoside **20**. The 5′-mesylate in **20** was next displaced by heating with potassium acetate and 18-crown-6 in 1,4-dioxane to provide the 5′-acetate **21**. For solubility reasons, we chose to remove the benzyl group first using catalytic hydrogenation, followed by removal of the 5′-acetate and reprotection of the 5′-hydroxyl group as the DMTr ether to provide nucleoside **22** in good yield. Finally, a phosphitylation reaction provided the desired phosphoramidite **23**.

The stereochemistry of the cyclized nucleosides **20** and **26** was confirmed by NMR spectroscopy (Fig. 2).[26] In nucleosides **20** and **26**, H1′ and H2′ appear as singlets indicative of locked [2.2.1] ring system. For nucleoside **20**, NOESY crosspeaks were visible between the 3′-Me group and the H1′ and between H6′ and H6 on the pyrimidine nucleobase. For nucleoside **26**, NOESY crosspeaks were visible between the 3′-Me group and H6′ and between H1′ and H6″.

The effect of 3′-Me-α-L-LNA modification on duplex thermal stability was measured using a single and two tandem incorporation of the modified nucleoside (Table 1). Oligonucleotides were synthesized on a 2 μmol scale using T-CPG support, 0.l M solutions of all phosphoramidites in acetonitrile, 0.5 M 1*H*-tetrazole as the activator and standard oxidizing and capping reagents. An extended coupling time of 8 min was used for incorporation of the modified nucleosides and the efficiency of incorporation was

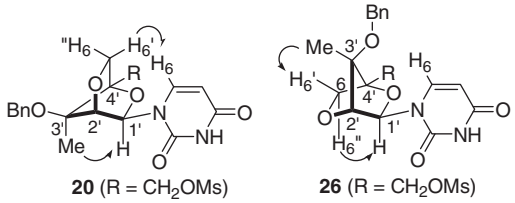


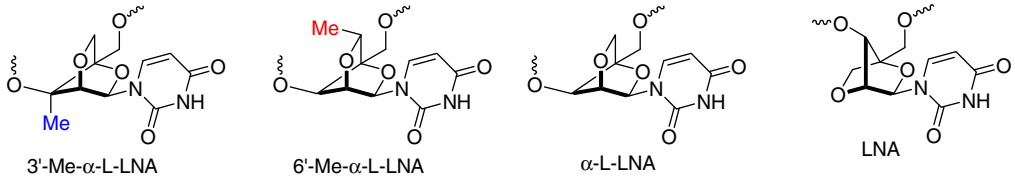
Figure 2. Stereochemical assignment for nucleosides **20** and **26** showing relevant NOESY crosspeaks.

~85% for single and ~75% for the tandem incorporations. In this sequence, 3′-Me-α-L-LNA showed good hybridization properties (**A2**, ΔT_m +4.0 °C/mod.), which were slightly lower than those observed for α-L-LNA (**A6**, ΔT_m +4.6 °C/mod.) and LNA (**A8**, ΔT_m +4.6 °C/mod.) modified oligonucleotides. Incorporation of two tandem 3′-Me-α-L-LNA (**A3**) or α-L-LNA (**A7**) nucleotides provided an almost identical increase in T_m per modification as compared to a single incorporation of the high affinity nucleotide. We also measured the mismatch discrimination properties of 3′-Me-α-L-LNA and the related 6′-Me-α-L-LNA (**A4** and **A5**) using mismatched RNA complements. For both, the single and tandem incorporation of 3′-Me-α-L-LNA, we observed excellent discrimination for the UC (ΔT_m −14.6 °C/mod.) and the UU (ΔT_m −15.1 °C/mod.) mismatched pairs which were similar to the discrimination observed with *R*-6′-α-L-LNA, α-L-LNA, LNA and DNA for the same mismatches. Interestingly, we observed slightly enhanced selectivity for the GU wobble base pair using 3′-Me-α-L-LNA (ΔT_m −6.7 °C/mod.) as compared to *R*-6′-Me-α-L-LNA (ΔT_m −5.3 °C/mod.), α-L-LNA (ΔT_m −4.7 °C/mod.), LNA (ΔT_m −5.1 °C/mod.) or DNA (ΔT_m −4.1 °C/mod.).

Lastly, we constructed a structural model of a modified duplex containing 3′-Me-α-L-LNA using a published structure of an α-L-LNA/RNA duplex as described previously (Fig. 3).[19] The model clearly shows that the 3′-Me group lies in the minor groove of the oligonucleotide duplex and does not experience any steric interactions with the sugar-phosphate backbone. The model also indicates that the 3′-position in α-L-LNA is a suitable site for appending additional substitution in the form of conjugates or reporter molecules which could be very useful for oligonucleotide based diagnostic applications.

In conclusion, we present that synthesis and biophysical characterization of 3′-Me-α-L-LNA modified oligonucleotides. The synthesis was accomplished starting from diacetone glucose and utilized hydride opening of an exocyclic epoxide to install the 3′-methyl group in the desired configuration. The 3′-protected ether was found to be extremely susceptible to beta-elimination during installation of the 4′-hydroxymethyl functionality. Interestingly, presence of the 3′-Me group almost completely abolished formation of the anhydro nucleoside, an extremely facile reaction of thymidine nucleosides. Instead, inversion of the 2′-hydroxyl group was accomplished by means of an oxidation reduction sequence with reduction of the 2′-ketone taking place away from the bulky nucleobase. Biophysical evaluation revealed that 3′-Me-α-L-LNA shows similar hybridization properties to α-L-LNA and LNA itself. Examination of 3′-Me-α-L-LNA in mismatch discrimination experiments indicated that this modification possesses slightly better discrimination for the GU wobble base-pair as compared to 6′-α-L-LNA, α-L-LNA, LNA and DNA. Lastly, a structural model showed that the 3′-Me-group resides in the minor groove of the modified duplex and could also serve as a site for introduction of other functional groups such as conjugates and reporter molecules. Further evaluation of this modification in biological experiments is underway and the results will be reported in due course.

Table 1
Thermal stability measurements of 3′-Me-α-L-LNA, *R*-6′-Me-α-L-LNA, α-L-LNA and LNA modified oligonucleotides versus RNA complements



Oligomer	Mass		% UV purity	Modification	Sequence (5′–3′)[a]	ΔT_m[b] (°C) versus RNA			
	Calcd	Found				X = A	X = G	X = C	X = U
A1	3633.4	3632.9	–	DNA	d(GCGTTTTTTGCT)	(45.6)	−4.1	−13.3	−13.8
A2	3661.4	3660.8	98.4	3′-Me-α-L-LNA	d(GCGTT**U**TTTGCT)	+4.1	−6.7	−14.6	−13.3
A3	3689.4	3689.0	96.3	3′-Me-α-L-LNA	d(GCGTT**UU**TTGCT)	+4.0	−6.3	−14.6	−15.1
A4	3661.4	3660.9	98.6	6′-Me-α-L-LNA	d(GCGTT**U**TTTGCT)	+5.5	−5.3	−15.5	−13.9
A5	3689.4	3688.9	97.9	6′-Me-α-L-LNA	d(GCGTT**UU**TTGCT)	+4.8	−5.3	−15.0	−15.4
A6	3647.4	3646.5	97.8	α-L-LNA	d(GCGTT**U**TTTGCT)	+4.6	−4.7	−15.4	−13.8
A7	3661.3	3660.8	97.9	α-L-LNA	d(GCGTT**UU**TTGCT)	+4.6	nd	nd	nd
A8	3647.4	3646.8	98.1	LNA	d(GCGTT**U**TTTGCT)	+4.6	−5.1	−14.9	−12.9

Sequence of RNA complement 5′-r(AGCAAAXAACGC)-3′, X = A represents the perfectly matched complement, while X = G, C or U represent the mis-matched complements.
[a] Bold and underlined alphabet indicates modified residue.
[b] T_m values were measured in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 0.1 mM EDTA, ΔT_m values were calculated by subtracting the T_m measured for the duplexes of modified oligomers versus matched and mis-matched RNA complements from the T_m of the perfectly matched **A1**/RNA (45.6 °C) duplex.

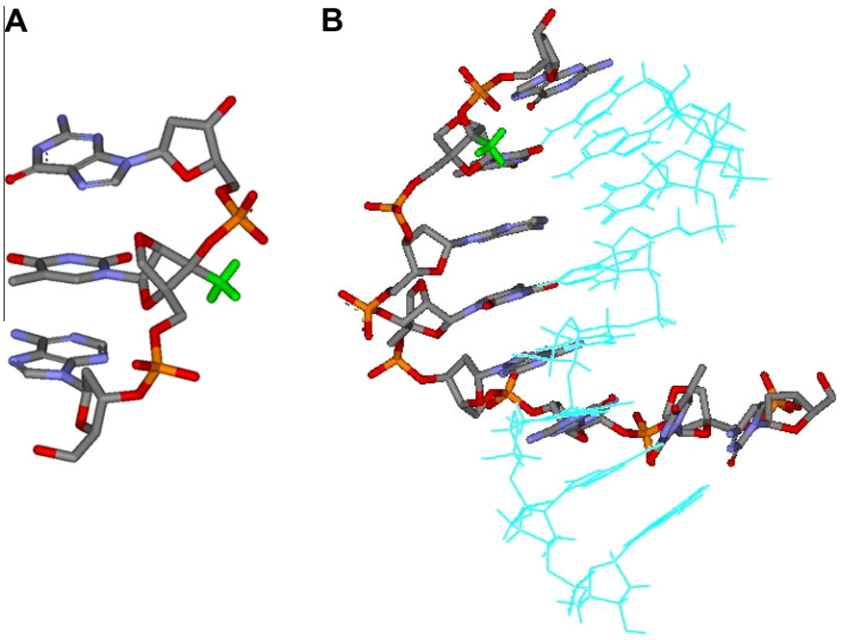


Figure 3. Hypothetical model of a 3′-Me-α-L-LNA modified duplex with RNA showing (a) the 3′-Methyl group highlighted in green and (b) the 3′-Me group located in the minor groove of the modified duplex. The α-L-LNA nucleobase in the published report (Ref.[13]) is thymine while the nucleobase for 3′-Me-α-L-LNA described in the present Letter is uracil.

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26. Compound **20**: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.64 (s, 3H), 3.06 (s, 3H), 4.06 (d, J = 8.5 Hz, 1H), 4.41 (d, J = 8.5 Hz, 1H), 4.46–4.65 (m, 4H), 4.69 (s, 1H), 5.80 (d, J = 8.1 Hz, 1H), 5.94 (s, 1H), 7.24–7.47 (m, 5H), 7.75 (d, J = 8.1 Hz, 1H), 9.29 (br s, 1H). Compound **26**: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.57 (s, 3H), 3.11 (s, 3H), 3.96 (d, J = 11.1 Hz, 1H), 4.04 (d, J = 9.0 Hz, 1H), 4.11 (d, J = 8.9 Hz, 1H), 4.58 (m, 2H), 4.62 (s, 1H), 4.72 (d, J = 11.9 Hz, 1H), 5.21 (d, J = 8.1 Hz, 1H), 5.63 (s, 1H), 7.11 (m, 2H), 7.22–7.38 (m, 3H), 7.47 (d, J = 8.1 Hz, 1H), 9.15 (br s, 1H).