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Total Synthesis of Legionaminic Acid as Basis for Serological Studies

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Supporting Information

ABSTRACT: Legionaminic acid is a nine-carbon diamino monosaccharide that is found coating the surface of various bacterial human pathogens. Its unique structure makes it a valuable biological probe, but access via isolation is difficult and no practical synthesis has been reported. We describe a stereoselective synthesis that yields a legionaminic acid building block as well as linker-equipped conjugation-ready legionaminic acid starting from cheap D-threonine. To set the desired amino and hydroxyl group pattern of the target, we designed a concise sequence of stereoselective reactions. The key transformations rely on chelation-controlled organometallic additions and a Petasis multicomponent reaction. The legionaminic acid was synthesized in a form that enables attachment to surfaces. Glycan microarray containing legionaminic acid revealed that human antibodies bind the synthetic glycoside. The synthetic bacterial monosaccharide is a valuable probe to detect an immune response to bacterial pathogens such as *Legionella pneumophila*, the causative agent of Legionnaire's disease.

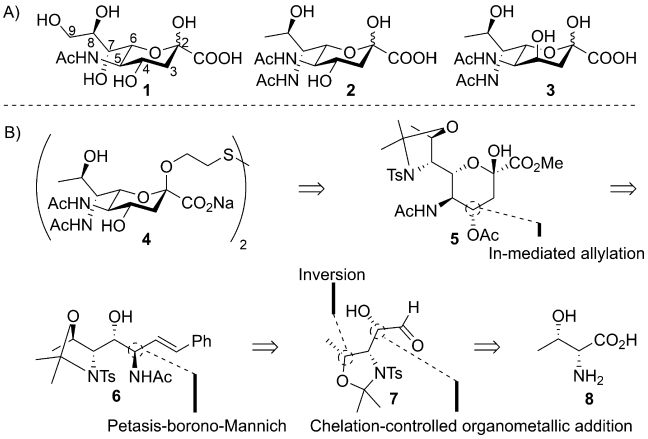
The human immune system can identify bacterial pathogens by interacting with specific carbohydrates on the outer membrane of the microbe.[1] In particular, homopolymers built from sialic acid monomers (**1**–**3**) are key players involved in recognition processes.[2,3] While *N*-acetylneuraminic acid **1** is ubiquitous in both mammalian and bacterial glycomes, legionaminic acids **2** and **3** are specific to bacteria.[3,4] Structurally, they differ from their closest mammalian relative, *N*-acetylneuraminic acid **1**, by lacking one oxygen at C9 and by exhibiting an amide in place of an hydroxyl group at C7 (Scheme 1).

A key virulence factor involved in Legionnaire's disease, a devastating form of pneumonia in humans, is the lipopolysaccharide (LPS) of *Legionella pneumophila*.[3,5] Legionaminic acid **2** is the major component of the LPS, a homopolymer of 5-*N*-acetimidoyl-7-*N*-acetyl legionaminic acids.[4,6−8]

The presence of legionaminic acids **2** and **3** (LegA) within glycoconjugates of bacterial pathogens[3,4] makes them ideal targets for pathogen detection and for the development of vaccines. Interestingly, legionaminic acid is also found in other prominent human pathogens such as *Acinetobacter baumannii*,[9] *Enterobacter cloacae*,[10] and *Campylobacter jejuni*.[3,11]

The structural complexity of legionaminic acids that renders them exquisite biological probes has a major drawback: the total synthesis of these compounds is notoriously difficult. So far, no synthetic strategy has been described to access fully functionalized legionaminic acid **2** and **3** glycosylating agents[3,8,12−14] for the construction of oligosaccharides or for the conjugation to slides or carrier proteins.[15,16]

Scheme 1. (A) Structures of *N*-Acetylneuraminic Acid 1, Legionaminic Acid 2, and 4-*Epi*-legionaminic Acid 3; (B) Retrosynthesis of Legionaminic Acid Building Block 5 as Well as Conjugation-Ready LegA 4



Here, we provide a versatile synthetic route to legionaminic acid building block **5** and linker-equipped legionaminic acid **4**. We describe a de novo strategy[17−27] starting from inexpensive and commercially available D-threonine **8**.

The retrosynthetic disconnection of linker-LegA **4** is shown in Scheme 1 with hemiketal **5** as universal building block. The latter is accessible by a sequence of an oxidation and an indium-mediated allylation of aminol **6**. Acetamide **6** is derived from α-hydroxy aldehyde **7** via a Petasis borono−Mannich reaction. Aldehyde **7** in turn originates from D-threonine **8** via an intramolecular inversion and a chelation-controlled organometallic addition reaction.

The total synthesis of legionaminic acid commenced with preparation of aldehyde **9**, which set the required stereogenic information at C7 and C8 in the final building block (Scheme 2). Since D-*allo*-threonine methyl ester is prohibitively expensive, aldehyde **9** was synthesized from D-threonine **8** via a slightly modified literature procedure.[28] The necessary C3-epimerization was achieved by anchimeric assistance. Methyl ester formation, *N*-benzoylation, followed by thionyl chloride-induced cyclization, acidic hydrolysis of the intermediate

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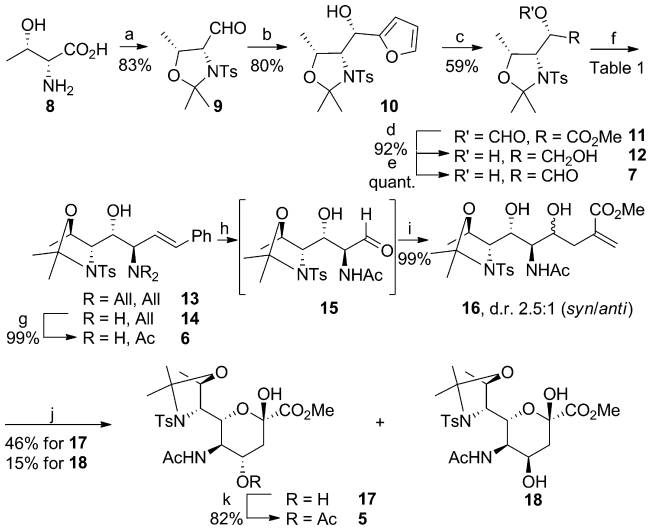
Scheme 2. Synthesis of Hemiketal Donor 5[a]



[a]Reagents and conditions: (a) $SOCl_2$, MeOH, reflux, 1 h, quant.; BzCl, NEt_3, MeOH, 0 °C, 4 h, quant.; $SOCl_2$, 0 °C, 5 days, 99%; 6 N HCl, reflux, 5 h; $SOCl_2$, MeOH, reflux, 1 h, 99%; TsCl, NEt_3, DCM, 0 °C, 4 h, 99%; 2,2-DMP, *p*-TsOH, toluene, 120 °C, 4 h, 98%; DIBAL-H, toluene, −78 °C, 2 h, 87%; (b) *n*-BuLi, furan, $MgBr_2{\cdot}OEt_2$, DME, −78 °C, 4 h, d.r. = 5:1 (*syn*/*anti*), 80% (*syn*); (c) O_3, DCM, MeOH, −78 °C, 1 h, then PPh_3 and $TMSCHN_2$, 0 °C, 59%; (d) $LiBH_4$, THF, 0 °C → rt, 12 h, 92%; (e) NaOCl, cat. TEMPO, KBr, DCM, sat. aq. $NaHCO_3$, 0 °C, 10 min, quant.; (f) (*E*)-styrylboronic acid, HNR_2, d.r. > 19:1 (*anti*/*syn*), yield and conditions see Table 1; (g) DMBA, $Pd(PPh_3)_4$, DCM, 35 °C, 2 h, then Ac_2O, $NaHCO_3$, MeOH, rt, 5 h, 99%; (h) O_3, PPh_3 (resin, cross-linked), then (i) In, methyl 2-(bromomethyl)-acrylate, EtOH, sat. aq. NH_4Cl, rt, sonication, 30 min, 99%, d.r. = 2.5:1 (*syn*/*anti*); (j) O_3, MeOH, −78 °C, 15 min, then Me_2S, rt, 4 h, 46% (*syn*), 15% (*anti*); (k) Ac_2O, pyr, DMAP, DCM, 0 °C → rt, 12 h, 82%. *p*-TsOH = *p*-toluenesulfonic acid, Bz = benzoyl, DMBA = 1,3-dimethylbarbituric acid, DMP = dimethoxypropane, Ts = Tosyl.

oxazoline, and esterification yielded D-*allo*-threonine methyl ester in 98% over five steps without chromatographic purification. The latter was transformed to the corresponding *N*-toluenesulfonamide, followed by an acetalization and DIBAL-H reduction of the intermediate ester, which provided D-*allo*-threoninal **9** in 84% overall yield.[29]

With threoninal **9** in hand, a Cram-chelate organometallic addition reaction of 2-lithiofuran to threoninal **9** resulted in the formation of the desired *syn*-configured alcohol **10** (Scheme 2).[29] Crystallization gave diastereomerically pure **10** in 80% yield. Since the oxidation of the furan moiety failed using ruthenium-based protocols,[30] we treated alcohol **10** with ozone followed by methylation to give methyl ester **11** in 59% yield. The conversion of ester **11** to diol **12** employing lithium borohydride in THF[31] proceeded in 92% yield. X-ray crystallographic analysis of diol **12** confirmed the stereochemical assignment (see Supporting Information).[32]

α-Hydroxy aldehyde **7** was required as precursor to introduce a protected amine via a Petasis borono−Mannich reaction.[33,34] Careful optimization of the chemoselective oxidation of diol **12** to aldehyde **7** was crucial in circumventing the notorious propensity of α-hydroxy aldehydes to decompose or epimerize. Subjecting alcohol **12** to Dess−Martin oxidation[35] resulted in low conversion, while TEMPO-mediated oxidation employing trichloroisocyanuric acid as the stoichiometric oxidant[36] did not proceed reproducibly. Finally, when Anelli's modification of the TEMPO oxidation was used,[37] the desired aldehyde **7** was obtained in quantitative yield without loss of stereochemical purity.[38]

To access protected diamines **13** or **14**, a Petasis borono−Mannich reaction between aldehyde **7**, (*E*)-styrylboronic acid, and an amine component was used (Table 1). The newly formed stereocenter displays *anti*-configuration relative to the directing free hydroxyl group. The high degree of stereocontrol reported by Petasis and Zavialov[34] arises from two energetically different conformations of the intermediate complexes (not shown, for review see ref 33).

Table 1. Optimization of the Petasis Multicomponent Reaction

entry	amine	solvent	temperature	time	yield
1	$NHAll_2$[a]	EtOH	rt	36 h	49%
2	$NHAll_2$[b]	DCM	120 °C[f]	0.5 h	<10%
3	$NHAll_2$[b]	DCM/HFIP[c]	40 °C	24 h	46%
4	$NHAll_2$[b]	DCM/HFIP[d]	40 °C	24 h	57%
5	$NHAll_2$[b]	EtOH/water[e]	40 °C	2 h	23%
6	$NHAll_2$[b]	DCM	rt	24 h	0%[f]
7	NH_2All[a]	EtOH	rt	24 h	76%

[a]Equivalents (**7**:(*E*)-styrylboronic acid:amine) 1:1:1. [b]Equivalents (**7**:(*E*)-styrylboronic acid:amine) 1:1.5:1.5. [c]3:1. [d]9:1. [e]4:1. [f]Microwave irradiation. *N*,*O*-Isopropylidene hydrolyzed. HFIP = 1,1,1,3,3,3-hexafluoro-2-propanol.

Table 1 summarizes the optimization process of the key Petasis borono−Mannich reaction. Initially, we applied standard reaction conditions using ethanol as the solvent and equimolar amounts of all reactants as well as a reaction time of 36 h (entry 1).[34] Despite quantitative conversion of **7**, the isolated yield of aminol **13** was low (49%). Microwave irradiation is known to accelerate and facilitate Petasis reactions.[38,39] However, in our hands, it did not result in acceptable rates of conversion (entry 2). Polar, protic solvents such as hexafluoroisopropanol are known to increase reaction rates and improve yields for this transformation. Unfortunately, the yields were comparable to the initial trials (entries 3 and 4). Use of an ethanol−water mixture as well as dichloromethane as solvent resulted in *N*,*O*-isopropylidene cleavage rather than improved yields (entries 5 and 6). Eventually, the use of monoallyl amine provided aminol **14** in 76% yield (entry 7). As desired, the exclusive formation of the *anti*-diastereomers **13** and **14** with a d.r. > 19:1 was observed in all cases.

Conversion of the *N*-allyl group of **14** into the corresponding *N*-acetate was achieved by Pd(0)-catalyzed deallylation followed by chemoselective *N*-acetylation to give amide **6** in 99% yield (Scheme 2).[38] Oxidative cleavage of *N*-cinnamylacetamide **6** to give aldehyde **15** required careful optimization of the ozonolytic conditions to prevent epimerization and decomposition of the labile α-aminoaldehyde moiety.[27,40] Zinc and dimethyl sulfide reduced the ozonide, but the transformation required long reaction times.[27] The use of resin-bound triphenyl phosphine[41] resulted in complete reduction of the intermediate ozonide without epimerization. Thus, quantitative conversion of hydroxylamine **6** to *N*-acetamide aldehyde **15** was achieved. Indium-mediated allylation of aldehyde **15** to enoate **16** is an established method to introduce a masked pyruvate unit.[42−44] While other potential methods such as the Cornforth synthesis[45] are performed using harsh

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conditions, the In-mediated allylation offers mild reaction conditions and can be performed in aqueous media.[43] Quantitative conversion of aldehyde **15** to the desired allylic alcohol **16** was achieved using sonication and a solvent mixture consisting of ethanol and aqueous ammonium chloride. Ammonium chloride was used as alternative to aqueous hydrogen chloride,[44] which would hydrolyze the *N*,*O*-isopropylidene acetal. Labile enoate **16** was isolated in high yield (99%) in favor of the *syn*-configuration (d.r. = 2.5:1 (*syn*/*anti*)). Oxidative cleavage of the double bond on enoate **16** proved problematic and initially led to decomposition or low yields. The use of a reductive workup (dimethyl sulfide) after ozonolytic cleavage of the alkene moiety in methanol provided legionaminic acid **17** and 4-epilegionaminic acid derivative **18** in good yield.

To obtain linker-equipped legionaminic acid **4**, the installation of an anomeric linker via glycosidation of a suitable legionaminic acid building block was required. Screening of protocols for the glycosidation of sialic acid building blocks[46] (e.g., anomeric chlorides, acetates and phosphites, thioglycosides, and *N*-phenyl-trifluoroacetimidate, results not shown) was irreproducible. Finally, we found that a dehydrative glycosylation protocol[47] furnished the desired linker-conjugate. Thus, diol **17** yet had to be elaborated into C4-*O*Ac hemiketal donor **5** by selective acylation. Careful optimization allowed for selective C4-*O*-acetylation when using 2 equiv of acetic anhydride and pyridine in the presence of catalytic amounts of DMAP to yield monoacetylated hemiketal **5** in high yield.[44] When starting from D-threonine, the overall yield of acetate **5** was 10%. To confirm the stereochemical configuration of diol **17**, the corresponding C2/C4-*O*Ac derivate was characterized (see Supporting Information).

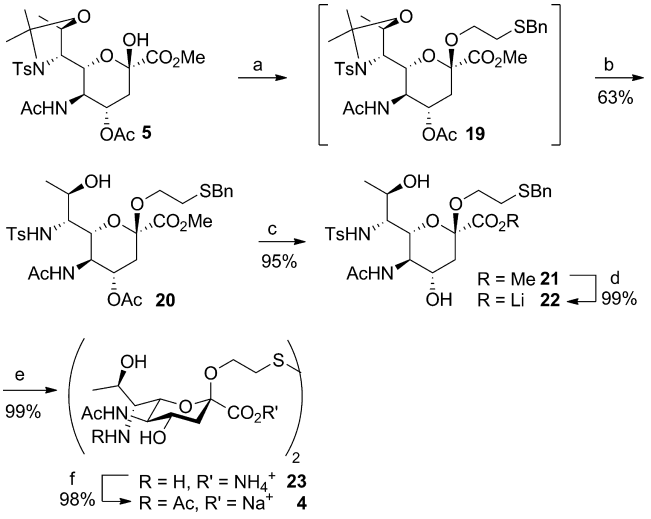
Dehydrative glycosylation of 4Ac5*N*AcLeg donor **5** provided crude thioether **19** (Scheme 3).[47] Surprisingly, attempts to purify crude **19** all failed due to decomposition of the material. Thus, acetal hydrolysis using aqueous TFA was performed prior to purification to provide glycoside **20** (63% β-anomer). Although the α-anomer was formed, it proved to be inseparable from minor decomposition products by means of silica gel chromatography. A 1D heteronuclear correlation experiment (selective, coupled, 1D $^{1}H-^{13}C$-HMBC) was performed to determine the heteronuclear coupling constant. A $^{3}J_{C\text{-}1,H\text{-}3ax}$ of 1.9 Hz confirmed the β-configuration of the anomeric center.[46] Starting from thioether **20**, acetate removal gave alcohol **21** in close to quantitative yield. Hydrolysis of the methyl ester, by using 20 equiv of lithium hydroxide to prevent the formation of elimination byproducts, gave carboxylate **22** in quantitative yield.

Global deprotection by reductive cleavage of the thioether and the *N*-sulfonamide moiety was achieved by Birch reduction (Scheme 3). Carboxylate **22** was treated with sodium in liquid ammonia without co-solvent to provide the desired amine **23** as the disulfide in quantitative yield. Amide **4** was finally obtained by treatment of amine **23** with sodium hydroxide and acetic anhydride. Starting from D-threonine, the overall yield of *N*-acetamide **4** was 6%.

To evaluate whether a legionaminic acid monosaccharide is a sufficient epitope for antibody recognition, human sera were screened against the synthetic structures using glycan microarrays (Scheme 4).[48−51] Carbohydrates **4** and **23** were immobilized via their thiol linker on maleimide-coated glass slides. Treatment with pooled human sera revealed that legionaminic acid **4** was recognized, presumably as a result of previous immune responses to bacterial pathogens displaying a legionaminic acid motif. IgG serum antibody levels against acetamide **4** were substantially higher than against amine **23** (Scheme 4). This side chain modification appears to be crucial for the specificity of antibody recognition. Since no bacterial glycans isolated so far display free amino groups on C7, we chose amine **23** as negative control to exclude unspecific binding. We further confirmed the preferential binding of human antibodies to antigen **4** using two other pooled human antisera (CDC1992[53] and anti-Hib human reference serum,[54] results not shown). Legionaminic acid-binding antibodies in human sera show that this rare carbohydrate from bacterial pathogen origin is recognized by the human immune system. Conjugate **4** is a valuable tool for the production of novel pathogen detection systems or vaccines.

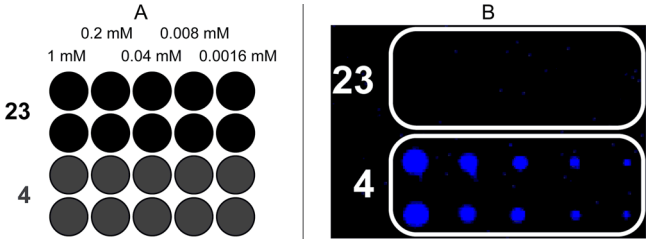
In summary, we have completed the de novo synthesis of orthogonally protected legionaminic acid **5**. This building block served as a glycosylating agent in the synthesis of linker-equipped legionaminic acid **4**. Glycan arrays containing synthetic antigen **4** showed that this epitope is recognized by

Scheme 3. Synthesis of Glycoside 4[a]



[a]Reagents and conditions: (a) Ph_2SO, Tf_2O, HOC_2H_4SBn, 4 Å MS, −78 °C → −50 °C, 2.5 h; then (b) DCM, TFA, H_2O (10:3:1 v/v), 0 °C, 5 min 63%; (c) NaOMe, MeOH, rt, 12 h, 95%; (d) 20 eq. LiOH, MeOH/H_2O (1:1 v/v), rt, 15 h, 99%; (e) $Na_{(s)}$, $NH_{3(l)}$, −78 °C, 45 min, then O_2 atmosphere, 99%; (f) NaOH, Ac_2O, H_2O, 0 °C, 60 min, then MeOH, 2 h, rt, 98%.

Scheme 4. Microarray Analysis of Synthetic LegA[a]



[a](A) Printing pattern. Legionaminic acid structures (**4** and **23**) printed in concentration from 1−0.0016 mM. (B) Representative microarray scan representing IgG antibodies to LegA **4** and **23** after incubation with Human Reference Serum 007sp.[52]

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human antibodies present in blood sera. Immunization studies and additional binding studies are ongoing.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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