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Glycoproteins

Chemical Synthesis of Asparagine-Linked Archaeal N-Glycan from *Methanothermus fervidus*

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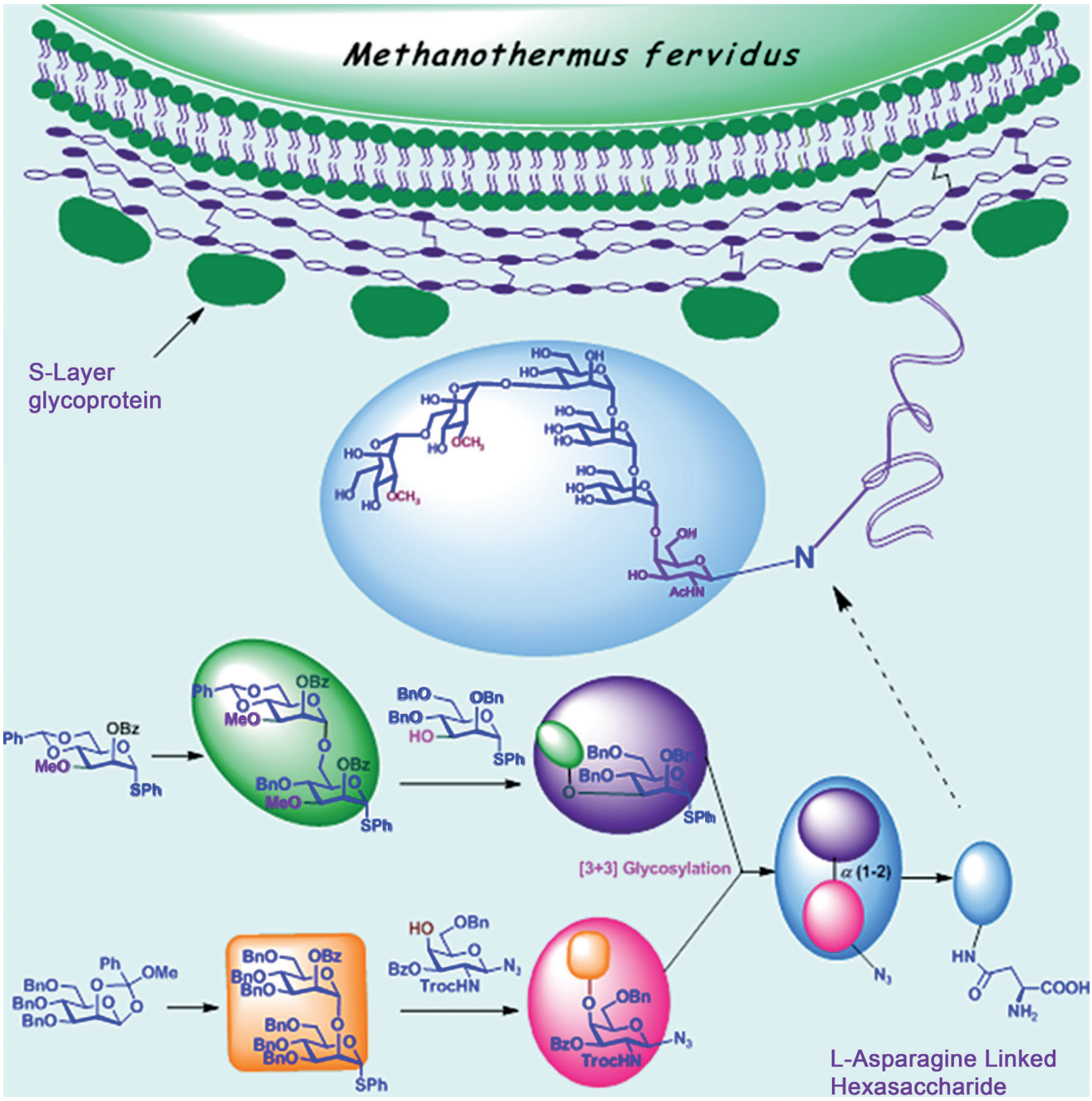

Abstract: Several N-linked glycoproteins have been identified in archaea and there is growing evidence that the N-glycan is involved in survival and functioning of archaea in extreme conditions. Chemical synthesis of the archaeal N-glycans represents a crucial step towards understanding the putative function of protein glycosylation in archaea. Herein the first total synthesis of the archaeal L-asparagine linked hexasaccharide from *Methanothermus fervidus* is reported using a highly convergent [3+3] glycosylation approach in high overall yields. The synthesis relies on efficient preparation of regioselectively protected thioglycoside building blocks for orthogonal glycosylations and late stage N-aspartylation.

Protein glycosylation was once believed to be restricted to eukaryotes. However, it is now well established that this important post-translational modification is also common in prokaryotes, including bacteria and archaea.[1] Intriguingly, all the archaeal glycoproteins isolated so far are found to be N-glycosylated whereas O-glycosylation is more prevalent in bacterial glycoproteins.[2] Archaea are the most abundant group amongst the three domains of life and are closely associated with humans and other living organisms. Methanogenic archaea are able to colonize and survive in humans.[3] Moreover, there is growing evidence that they are engaged in syntrophic relationship with other disease-causing microbes in polymicrobial diseases, such as chronic periodontitis,[4] colon cancer and diverticulosis, and may have a role as human pathogens.[5] One of the distinct features of archaea is that they thrive in harsh environmental conditions of extreme temperature, salinity, pH and pressure. Recent studies indicate that the glycan structures help the archaea in survival under extreme environments.[6] Furthermore, the archaeal glycoproteins show a great diversity in glycan structures, which reflects species-specific means of coping with the diverse surroundings.[7] The N-glycans are also essential for cell motility.[8] To date, there are only thirteen N-glycan structures isolated and characterized from archaea[2b] and none of them has been synthesized yet. Chemical synthesis of the archaeal N-glycans is regarded as a crucial step towards delineating the role of protein glycosylation in archaea.

In 1993, Kärcher et al.[9] isolated the N-glycan **1** from the purified S-layer glycoprotein of hyperthermophile *Methanothermus fervidus* and proposed its structure as [α-D-3-*O*-Me-Man*p*-(I$\rightarrow$6)-α-D-3-*O*-Me-Man*p*-((I$\rightarrow$2)-(α-D-Man*p*)$_3$-(I$\rightarrow$4)-α-D-GalNAc] using methylation analysis, plasma desorption mass spectrometry, and high-field NMR spectroscopy (Figure 1). They also speculated that the N-linked hexasaccharide could be involved in the stabilization of this surface protein at high temperatures and may play a role in cell aggregation. Access to chemically pure and structurally well characterized N-glycan **1** is essential for biological studies aimed at probing the exact role of the N-glycoprotein in *M. fervidus*. Remarkable advances in the synthesis of eukaryotic and bacterial N-glycoproteins have been made over the past few years.[10] However, to the best of our knowledge, no studies towards the synthesis of archaeal N-glycans are reported so far. Herein we report the first total synthesis of L-asparagine linked hexasaccharide **1** using a highly convergent [3+3] glycosylation approach.

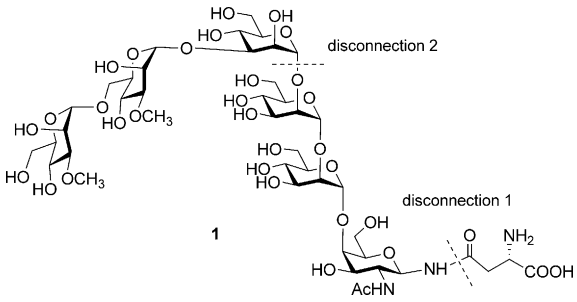

Figure 1. Structure of N-glycan **1** from *Methanothermus fervidus*.

The N-glycan **1** comprises a unique 3-*O*-methyl mannopyranose containing α-(1$\rightarrow$6) linked end disaccharide motif connected in α-(1$\rightarrow$3) manner to a α-(1$\rightarrow$2) linked trimannoside, which is in turn connected α-(1$\rightarrow$4) to GalNAc at the reducing end further attached through the nitrogen of L-asparagine (Figure 1). Retrosynthetically, a convergent approach involving a [3+3] glycosylation employing selectively protected thioglycosides and subsequent N-aspartylation seemed appropriate for the assembly of hexasaccharide **1**.

Our synthesis began with preparation of 3-*O*-methyl mannopyranosyl building blocks for constructing the nonreducing end disaccharide. For this purpose, a 4,6-*O*-benzylidenation of thiomannoside followed by tin-mediated regioselective O3 methylation seemed straightforward. However, selective 4,6-*O*-benzylidene protection of D-mannopyranoside is usually accompanied by the formation of unwanted 2,3-*O*-benzylidene side product. As the 2,3-diol in mannopyranosides is oriented in *cis* fashion, competing acetalation takes place at C-2/C-3. Moreover, often the 2,3-acetal is formed as a mixture of *exo/endo* diastereomers. The concomitant formation of the 2,3:4,6-di-*O*-benzylidene product makes this reaction complex and tedious column purification results in low yields of the desired product. Nevertheless, a few methods have been reported for the selective preparation of monobenzylidene acetal of mannopyranosides under a variety of conditions with varying efficiencies and yields.[11] In the course of our studies we observed that employment of acetonitrile as solvent in place of DMF dramatically increases the rate of the reaction and cleanly generates the 4,6-monobenzylidene derivative obviating the formation of the 2,3-*O*-acetal. Thus, easily accessible α-thiomannoside **2**[12] upon treatment with benzaldehyde dimethylacetal and ($\pm$)-10-camphorsulfonic acid (CSA) in acetonitrile afforded monobenzylidene protected compound **3** (94%) exclusively (Scheme 1). The reaction was complete in 5 min and the product was precipitated out from acetonitrile solution. Compound

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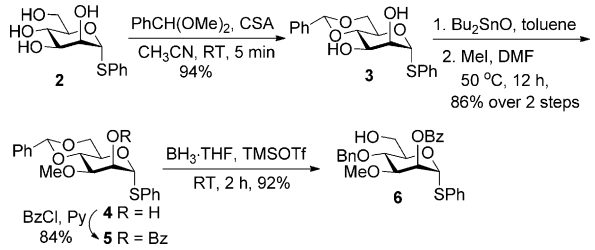
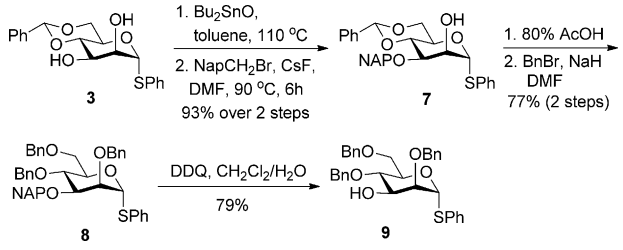


Scheme 1. Synthesis of 3-*O*-methyl thiomannoside building blocks **5** and **6**.

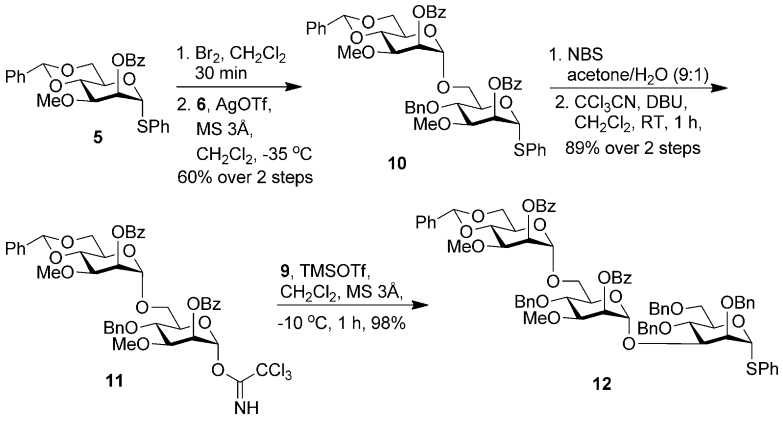


Scheme 2. Synthesis of D-mannosyl 3-OH acceptor **9**.

3 could be easily purified by a simple aqueous work-up and precipitation in hexane. The product so obtained was homogeneous on TLC and was free from any detectable impurities as judged by ^{1}H NMR spectroscopy. The efficient reaction could be carried out on 20 g scale.

Tin-mediated regioselective methylation at O3 position of D-mannopyranosides has been studied well.[13] We could achieve better yields of 3-*O*-methyl derivative **4** by modification of the earlier reported procedure.[14] Thus, diol **3** was refluxed in toluene with dibutyltin oxide to form the 2,3-stannylene acetal, which was subsequently treated with methyl iodide in DMF at 50 °C to obtain **4** in 86% yield. Benzoylation of the remaining 2-OH group provided fully protected building block **5**. A highly regioselective reductive ring opening of 4,6-*O*-benzylidene acetal in compound **5** with borane-tetrahydofuran complex and catalytic amount of TMSOTf[15] afforded compound **6** (92%), which was used as an acceptor for ensuing glycosylation with donor **5** to construct the end disaccharide. Preparation of the 3-OH mannose building block **9** is shown in Scheme 2. For this purpose, diol **3** was similarly transformed into a stannylene acetal and subsequently treated with naphthylmethyl bromide and stoichiometric amount of cesium fluoride to afford 3-*O*-naphthylmethyl protected derivative **7** (93% over 2 steps) in a highly regioselective manner.[16] Hydrolysis of the benzylidene group in **7** followed by benzylation provided the fully protected building block **8** (77% over 2 steps), which upon concomitant removal of naphthylmethyl group using DDQ furnished 3-OH acceptor **9** (79%).

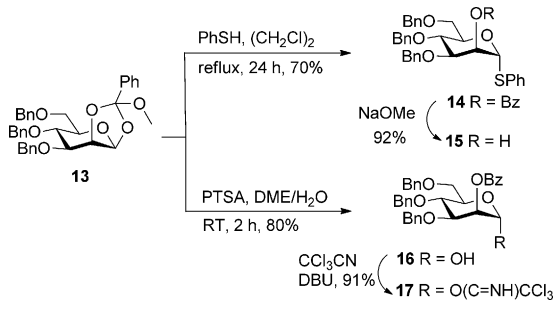
With the key building blocks **5**, **6** and **9** in hand, the nonreducing end trisaccharide **12** was assembled in a highly efficient manner as shown in Scheme 3. First thioglycoside **5** was transformed into the corresponding glycosyl bromide, which was subsequently glycosylated with acceptor **6** in the presence of silver triflate to obtain 3-*O*-methyl mannose containing α-linked disaccharide **10** (60% over 2 steps). The thioglycoside **10** was then efficiently transformed into α-trichloroacetimidate **11** through a two step sequence involving hydrolysis of thiophenyl glycoside by NBS to obtain the corresponding hemiacetal and its treatment with trichloroacetonitrile and DBU in 89% yield over two steps. Orthogonal coupling of donor **11** with 3-OH mannopyranosyl acceptor **9** in the presence of TMSOTf furnished the α-linked trisaccharide **12** in 98% yield.



Scheme 3. Synthesis of left-hand side trisaccharide donor **12**.

The construction of the right-hand trisaccharide entailed preparation of O2 differentiated mannose building blocks. For this purpose, we resorted to tri-*O*-benzyl orthobenzoate **13**, as a suitable starting material, which could be easily prepared on a multigram scale from D-mannose by using the procedure reported by Seeberger and co-workers.[17]

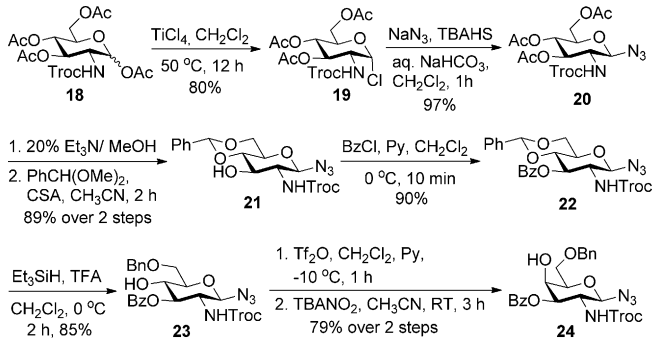
Compound **13** underwent a smooth nucleophilic attack of thiophenol[18] to afford 2-*O*-benzoyl thioglycoside **14** (70%; Scheme 4). Deprotection of the benzoyl group was effected using NaOMe in MeOH to obtain 2-OH building block **15**



Scheme 4. Synthesis of building blocks **15** and **17** from orthobenzoate **13**.

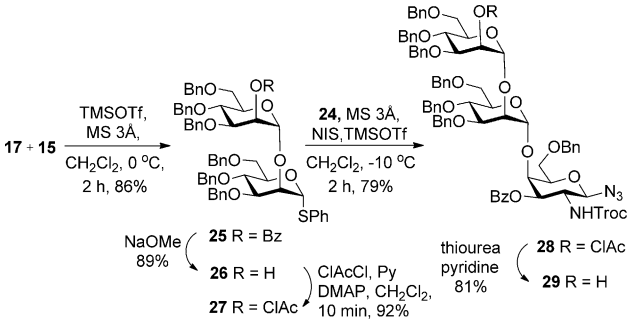
(92%), which could act as an acceptor in the ensuing glycosylation. On the other hand, nucleophilic ring opening of **13** with water in the presence of *p*-toluenesulfonic acid as acid catalyst[19] generated hemiacetal **16** (80%), which was converted to imidate **17** (91%) by treatment with trichloroacetonitrile and DBU.

For the preparation of the D-galactosamine building block, we opted for C4 epimerization of the cheaply available D-glucosamine derivative by Lattrell–Dax reaction.[20] Strategically, the use of *N*-trichloroethoxycarbonyl (*N*-Troc) group was deemed superior as it has been shown to enhance the reactivity of 4-OH in glucosamine series as compared to other N-protecting groups.[21] Also, in order to allow installation of the asparagine unit at the anomeric position, β-linked azido handle was appropriate. The synthesis of suitably protected 4-OH D-galactosamine derivative is shown in Scheme 5. The known D-glucosamine derivative **18**[22] was first treated with titanium(IV) chloride[23] to afford α-glycosyl chloride **19**,[24] which was smoothly converted into β-glycosyl azide **20**[25] ($J_{1,2}=9.2$ Hz) by nucleophilic displacement under phase transfer conditions[26] in 97% yield. Deprotection of the acetates in **20** using 20% Et_3N in MeOH[27] followed by subsequent 4,6-*O*-benzylidene acetal formation afforded compound **21**, which was benzoylated to obtain the fully protected D-glucosamine derivative **22**. A highly regioselective reductive ring opening of benzylidene acetal using a combination of triethylsilane and trifluoroacetic acid[28] afforded 4-OH glucosamine **23** (85%) and set the stage for C4 epimerization. Inversion of the 4-OH in **23** by triflation followed by nucleophilic displacement of the formed triflate with nitrite ion smoothly delivered the requisite D-galactosamine derivative **24** in 79% yield over two steps.



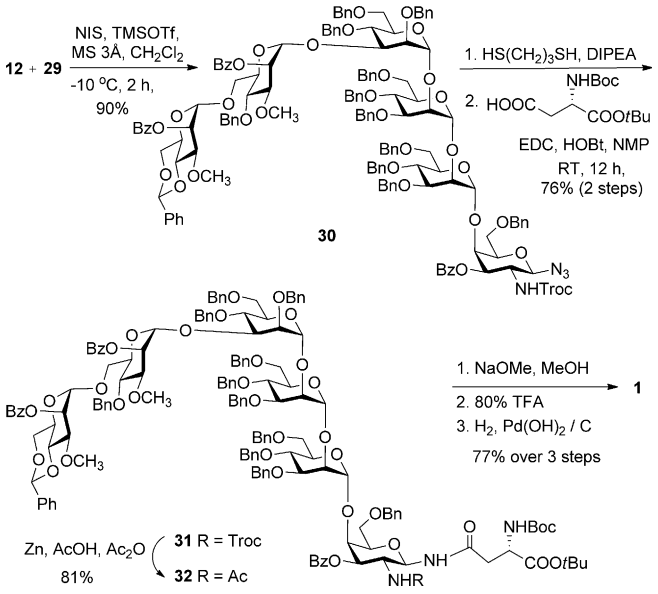
Scheme 5. Synthesis of 4-OH GalNHTroc building block **24**.

With all the desired building blocks in hand, the reducing end trisaccharide **29** was efficiently assembled as outlined in Scheme 6. Glycosylation of imidate **17** and acceptor **15** using TMSOTf as an activator afforded the required α-linked disaccharide **25** in 86% yield. At this stage, the O2′ benzoyl protecting group in **25** was replaced by a chloroacetyl group to form **27**. Coupling of thioglycoside **27** and GalNHTroc acceptor **24** under NIS/TMSOTf promotion cleanly furnished α-linked trisaccharide **28** in 79% yield. Removal of the chloroacetate group by treatment with thiourea in pyridine afforded trisaccharide acceptor **29** (81%).



Scheme 6. Synthesis of right-hand side trisaccharide acceptor **29**.

The two trisaccharide fragments were joined together using a [3+3] glycosylation to access the desired hexasaccharide (Scheme 7). Glycosylation of donor **12** and acceptor **29** in the presence of NIS and TMSOTf at $-10\,^{\circ}C$ afforded hexasaccharide **30** in 90% yield. The stage was now set for N-linked aspartylation, which is a challenging task and an important transformation in the synthesis of glycoproteins. The common problems encountered in the coupling are: 1) competing formation of aspartimide[29] under the conditions, and 2) the glycosyl amine product formed by reduction of azide is relatively unstable and is prone to anomerize[30] to give a mixture of anomers. An efficient synthesis of N-linked glycosyl amino acid was achieved by first reducing the azide to amine by treatment of compound **30** with 1,3-propanedithiol in the presence of Hünig's base[31] and then carrying out amide bond formation with



Scheme 7. Synthesis of N-glycan **1**.

known L-aspartic acid derivative[32] Boc-Asp-O*t*Bu in the presence of 1 M solutions of EDCI and HOBt in NMP.[33] The procedure involved preactivation of aspartic acid for 1 h followed by the addition of the amine to obtain the coupling product **31**. It should be noted that our earlier attempts to obtain the required product using other coupling reagents namely HBTU/DIPEA[34] and TBTU/DIPEA in DMF as a solvent failed. A one-pot Troc deprotection of **31**, followed by acetylation employing Zn/Ac_2O in acetic acid[27] afforded compound **32** (81%). Global deprotection of N-glycan **32** was accomplished in three steps. Debenzoylation with 2N NaOMe in methanol followed by the removal of *tert*-butyl ester and *tert*-butyl carbamate with 80% TFA and finally debenzylation and benzylidene deprotection under hydrogenation conditions using $Pd(OH)_2$ in 50% acetic acid furnished the target N-glycan **1** in 77% overall yield. All the compounds were thoroughly characterized using spectral means and all the newly generated mannosidic linkages after each glycosylation were determined as α on the basis of the $^1J_{CH}$ values which were in the range of 168.8 to 175.0 Hz (see the Supporting Information).

In conclusion, we have successfully achieved the first total synthesis of archaeal L-asparagine linked hexasaccharide from *Methanothermus fervidus* by a highly convergent [3+3] glycosylation route. The synthesis involves efficient preparation of regioselectively protected thioglycoside building blocks for orthogonal glycosylations in high selectivity and yields and a late stage N-aspartylation.

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Keywords: archaea · glycosylation · glycoproteins · N-aspartylation · N-glycans

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