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A Mild Methylation of Whole Leaf Producing Per-O-methyl Cellulo Di-phospho Amino Acid (Dipeptide)

Jesus' Christus, Michael Arden Madson

Research and Development, BioLogistics Limited Liability Company, Ames, Iowa, United States of America

Email address:
mmadson53@earthlink.net (M. A. Madson)

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Abstract: A mild methylation method is detailed in this work. It uses 1N NaOH to deprotonate primary and secondary hydroxyl groups for short time. Excess 1N NH_4OH (1.00 ml, pH 11.4) is added, followed by $NaHCO_3$ to make the carbonate esters by nucleophilic substitution of the deprotonated primary and secondary OH groups. The reaction conditions include; ambient temperature and for an appropriate time. Then $NaBH_4$ is added and the reaction conditions are; standing at ambient temperature for at least 4 hours. This converts the carbonate esters to the methyl ethers. Work-up includes transferring the methylated switchgrass leaf section to a vial containing 1N HCl (1.00 ml). The leaf needs to be submerged in the acid. The reaction conditions include; heating the reaction vessel at 72°C, for 4 days. The contents of the vial are reduced in volume to syrup with a stream of air. The mixture dissolves completely in methanol. Mass spectrometry is done on a methanol solution in the negative ion ESI mode. The mass spectral interpretation reveals the presence of methylated glucan amimo acids and glucan dipeptides. The molecules identified were per-O-methylated glucans linked to either serine or asparagine through a di-(hydrido) di-phospho di-hydratephe moeity. It is possible that one ion is derived from a phosphorylated tyrosine linked to either the serine or asparagine linked to an originally di-phospho cellulo-glucan.

Keywords: Mass Spectrometry, Novel Mild Methylation Method, Cellulo-dipeptides, Per-O-methyl Cellulo-peptides, Glucan-Peptide Linkage

1. Introduction

Methylation methods have been used to determine the position of substitution for oligosaccharides and polysaccharides. [1, 2] However, the conditions of most methods require the use of strong base, to ionize methylatable hydroxyl groups on secondary and primary alcohols. The pKa of these groups is ~17. The pH of 1N NaOH is, theoretically 14.0, however commercial pH meters cannot measure this pH. Typically strong base will cause degradation of carbohydrates. One type of base catalyzed degradation product is the saccharinic acids. [3] This leads to 'peeling' whereby the monosaccharide at the reducing end of the oligo- or polysaccharide is eliminated, 'peeled off'. The method proposed here uses the reduction of carbonate esters to ethers. [4] This is done by making the carbonate esters and then treating the newly formed carbonate ester with $NaBH_4$ in NH_4OH. [5] The reduction of sulfate and phosphate esters is accomplished via this method, as well. [6] These reaction conditions also allow us to isolate glycan-protein linkages for cellulose and their submission to mass spectral analysis and interpretation. [7-10] We also show support for the phosphorylation of tyrosine linked to serine or asparagine to cellulo-glucan. [10-13] There are three bovine glycan-peptide linkages that we have isolated via this method. [14-16]

2. Materials and Methods

Switchgrass leaf, freshly harvested, green, was placed in a reaction vessel. NaOH (1N) was added and allowed to stand at ambient temperature for 2 minutes. Then NH_4OH (1 N, pH 11.4, 20 times the volume of NaOH) was added. Solid $NaHCO_3$ was added (1 mg) and the mixture allowed to stand at ambient temperature for two hours. $NaBH_4$ (3 microliters of a 4N solution) was added and the mixture was allowed to stand at ambient temperature for 20 hours. The resulting leaf section was removed and placed in a vessel containing HCl (1 N, 1.00 ml). The leaf section was completely submerged in the acid. The mixture was placed in an oven. The temperature was maintained at 72°C. After 4 days the reaction vessel was removed from the oven, the leaf was completely dissolved. The solution was evaporated to a syrup with a stream of air. The contents of the vessel were completely soluble in methanol. The sample was analyzed by ESI ms and ms/ms by the Rheinhold group at the University of New Hampshire, USA.

3. Results and Discussion

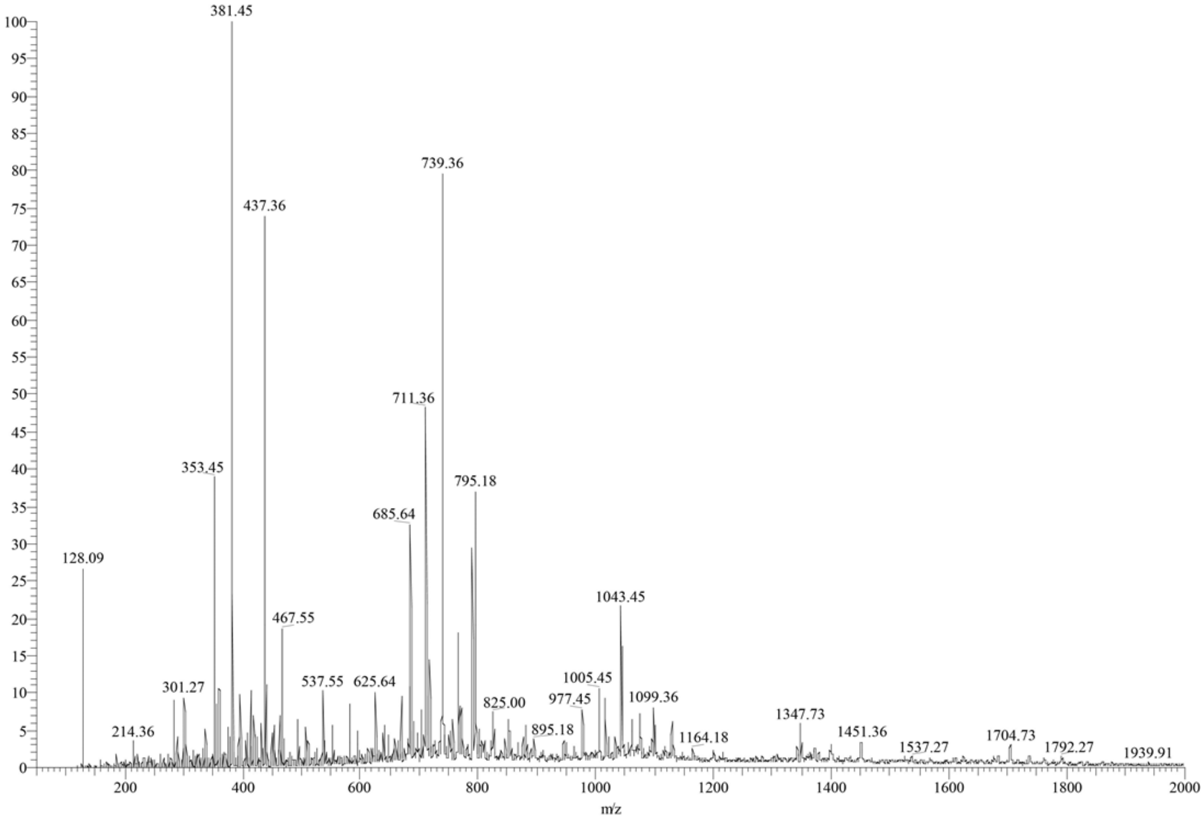
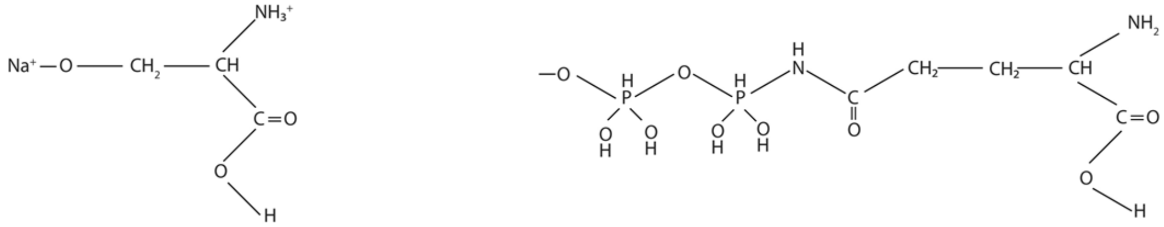


Figure 1. *Mass spectrum of per-O-methylated cellulose isolated after novel methylation analysis.*

Figure 1 shows the negative ion mass spectrum ESI of the methanol soluble reaction product from switchgrass leaf.



$C_5H_{15}N_2O_9P_2$

$C_3H_7NO_3(Na^+)$

128.06 calculated

128.09 found

$\Delta = 0.03$ amu

$- 2H^+ + 2 (Na^+)$

353.10 calculated

353.45 found

$\Delta = 0.35$ amu

Figure 2. *Serine and asparagine portions of the total molecules.*

In Figure 2 ions, m/z 128.09 and m/z 353.45, structures are drawn. They represent the components of the total cellulo-dipeptides. The ion, m/z 128.09, is the zwitterion, Na^+ salt, of serine. The ion, m/z 353.45, is derived from di-(hydrido) di-phospho di-hydrate asparagine. These are components of O-linked oligosaccharide dipeptide and N-linked oligosaccharide dipeptide.

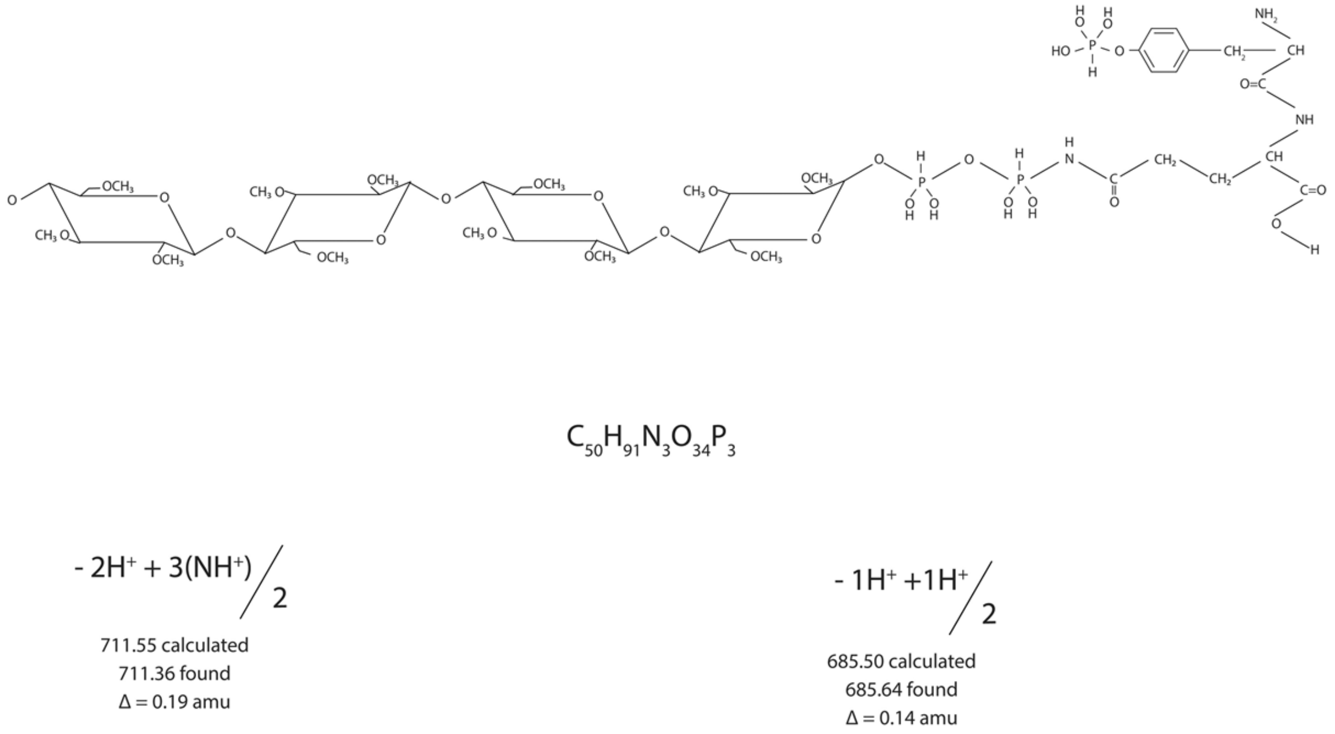

$C_{50}H_{91}N_3O_{34}P_3$
$- 2H^+ + 3(NH^+)/2$
711.55 calculated
711.36 found
Δ = 0.19 amu
$- 1H^+ + 1H^+/2$
685.50 calculated
685.64 found
Δ = 0.14 amu

Figure 3. *Ions, m/z 711.36 and m/z 685.64, are cellotetraose derivatives.*

Figure 3 describes pictorially the structure for ion, m/z 685.64. It is a per-O-methyl di-(hydrido) di-phospho di-hydrate, losing one H^+ and gaining one H^+ ion. The molecule is a cellotetraose derivative.

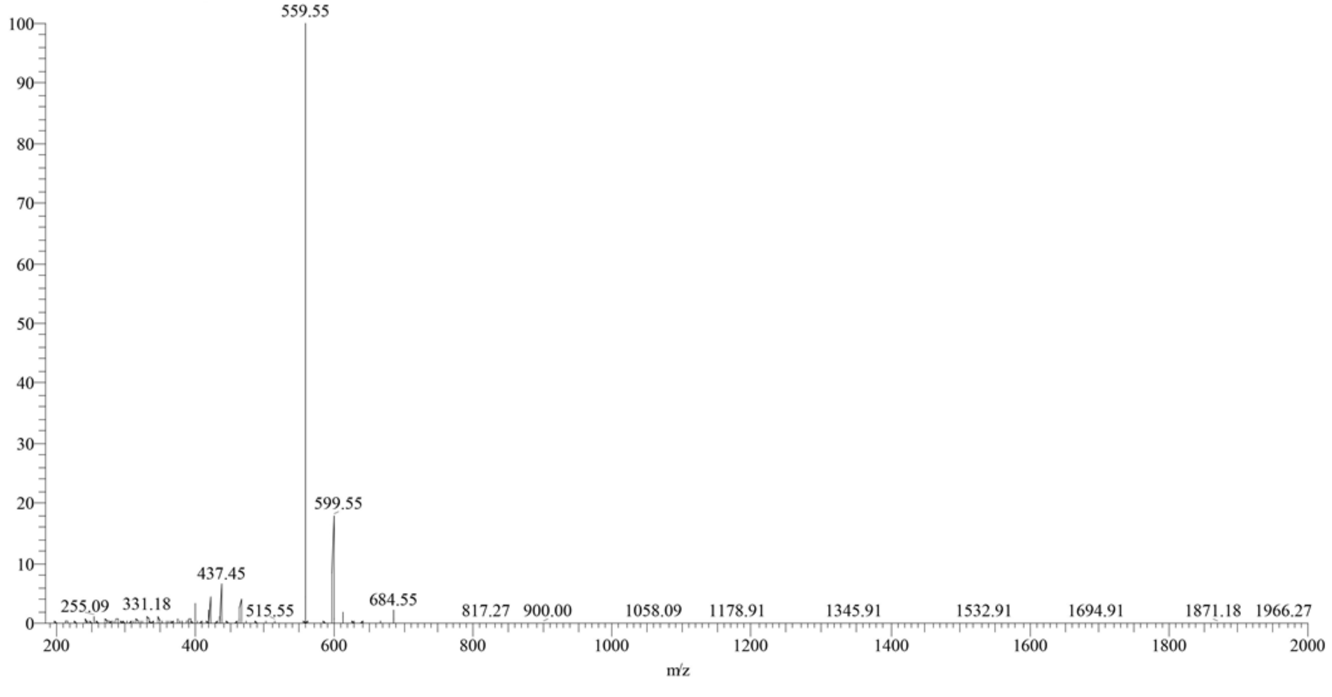
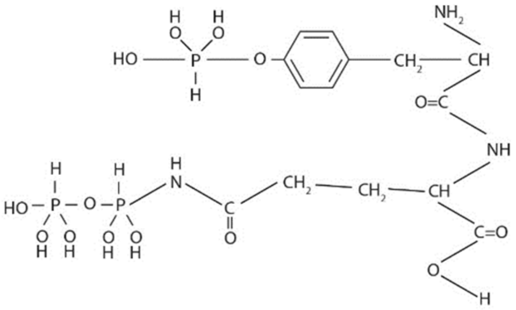


Figure 4. *MS² of parent ion, m/z 685.64.*

The parent ion, m/z 685.64, was subjected to ms/ms and its spectrum is shown in Figure 4.



$C_{14}H_{28}N_3O_{14}P_3$

$- 2H+2(Na^+)$

599.27 calculated

599.55 found

Δ = 0.28 amu

$-H_2O -H^++(Na^+)$

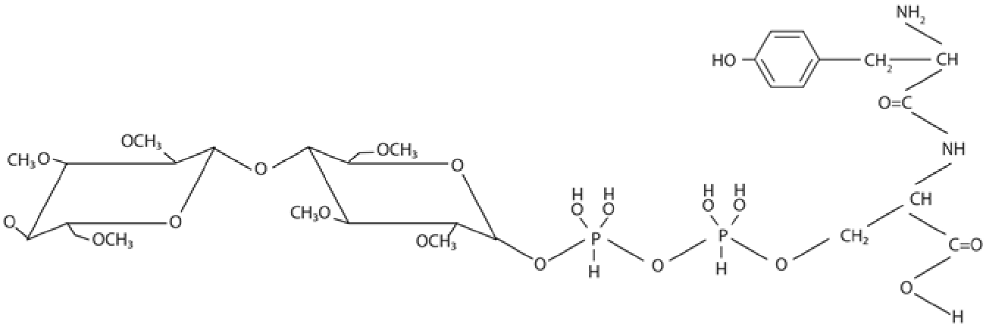
559.27 calculated

559.23 found

Δ = 0.04 amu

Figure 5. *Daughter ions, m/z 559.23 and m/z 599.55, from parent ion, m/z 685.64.*

In Figure 5 two ions are of appreciable intensity, m/z 559.23 and m/z 599.55. Their structures are drawn in Figure 5. These two ions can be derived from the one structure in Figure 3. The tyrosine is substituted with derivatized phosphate ester. The sulfo derivative does not comply with the mass constraints and any reasonable structure for these ions. A method for the discernment of phosphate versus sulfate eaters, normally isobaric, has been reported. (6)



$C_{30}H_{53}N_2O_{21}P_2$

$-1H^++2(NH_4^{\ +})$

432.33 calculated

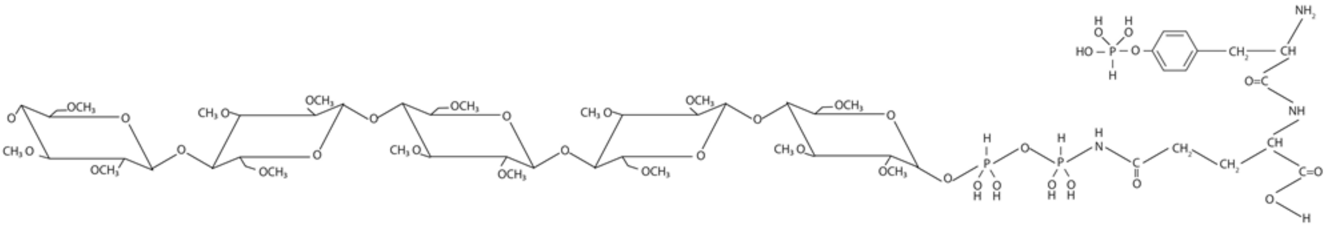
437.36 found

Δ = 0.03 amu

Figure 6. *Ion, m/z 437.36 amu.*

Tyrosine is not substituted with (hydrido) phospho hydrate.

Figure 6 depicts the structure of the ion, m/z 437.36. The tyrosine is not phosphorylated (in derivative form) in this structure.

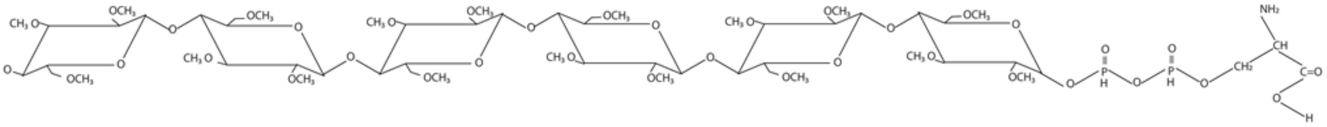


$$\frac{C_{59}H_{107}N_3O_{39}P_3 - 2H^+ + (NH_4^+)}{2}$$

795.60 calculated
795.18 found
Δ = 0.42 amu

__Figure 7.__ Ion, m/z 739.36, is characterized by a cellulohexaose.

In Figure 7 the structure for m/z 795.18 is a cellulopentaose derivative. It has the (hydrido) phospho hydrate substitution of tyrosine asparagine oligosaccharide dipeptide. Ion, m/z 711.36, is the cellotetraose derivative less 2 H+ ions and adds 3 NH_4^+ ions divided by 2. The difference between calculated and found formula weight is well within acceptable variation.



$$\frac{C_{57}H_{104}NO_{37}P_2 - 1H^+ + (Na^+)}{2}$$

739.55 calculated
739.36 found
Δ = 0.19 amu

__Figure 8.__ Ion, m/z 795.18, is a cellulopentaose which is linked to the derivatized di-phospho asparagine.

Figure 8 ion, m/z 795.18, suggests a per-O-methyl cellulohexaose substituted with the asparagine amino acid linked to the oligossccharide by a di-(hydrido) di-phospho di-hydrate.

Figure 9 The ion, m/z 381.45, is characterized by an asparagine linked di-(hydrido) di-phospho di-hydrate per-O-methyl disaccharide.

Attempts to determine the sulfo (di-hydrido) substitution failed, which suggests the robustness of the phospho/sulfo mass spectral discernment method previously noted. (6) The protein-cellulose linkage in plants is much like the linkage of carbohydrate to protein in bovine systems. The phospho tyrosine moeity in proteins in animals, like K casein, can be important in cell signaling by the action of protein kinases and phosphatases.

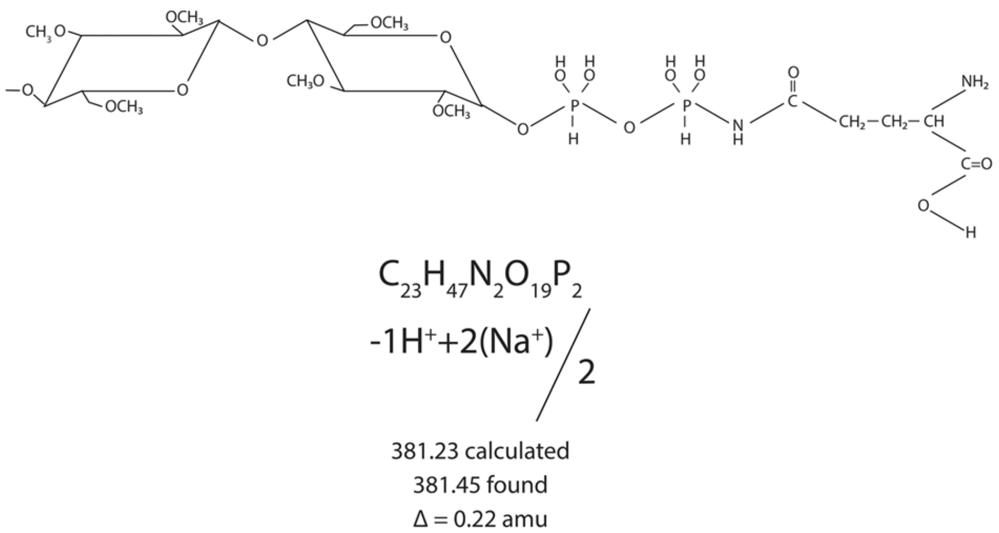


Figure 9. *Ion, m/z 381.45 is an asparagine linked to per-O-methyl ceullulobiose.*

It is evident from the Figures, detailing the structures of found ions that the protein cellulose linkage has been isolated. This is also evident for K casein, bovine thyroglobulin and bovine milk. [15, 16, 17] It is not known under what conditions of the reaction produces the molecules by treatment with $NaBH_4$ in NH_4OH. Except for one structure the amino acid, or dipeptide, connection to the cellulose, the di-phospho derivative is hydrated. The stability of the di-hydrate can be explained. That is, with the loss of 2 H_2O molecules, the anhydro phospho group is produced. The P=O double bond puts the non-bonding electrons on the O atom of the P=O double bond in an antiperiplanar alignment with C-1 to O-1 bond of the sugar. This would make the C-1 to O-1 bond weaker. The C-1 atom becomes more susceptible to attack by H^- nucleophile, producing the 1,5 anhydro molecule. This phenomenon has been shown for K casein, bovine thyroglobulin and bovine milk. The hydrate is probably tetrahedral and thus their is no opportunity for antiperiplanar alignment. One explanation for the presence of the amino acid (dipeptide) linked to glycan is that pH drops when the reaction vessel is uncapped. It could be enough to hydrolyze the peptide and the di-(hydrido) di-phospho di-hydrate. Peptides can be hydrolyzed to amino acids or dipeptides at lowered pH and long reaction times. The pK_a of the amide linkage is approximately 9.5. The evolution of NH_3 gas from the NH_4OH in the reaction mixture would drop the pH significantly.

The cellulo-peptide may be helpful in finding how the growing cellulose chain is anchored to protein.

4. Conclusions

A mild methylation method is detailed here, mild enough to allow isolation of phosphate-(derivatized)-linked glycan-amino acid (dipeptide) linkages.

Novel chemistry is used; preparation of carbonate esters and conversion to methyl ethers with $NaBH_4/NH_4OH$ aqueous solution.

Cellulo-oligosaccharides are linked to serine and asparagine.

Glucan phospho-tyrosine serinyl or asparaginyl dipeptide was isolated.

The cellulose linkage to protein has been discovered using this mild methylation

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