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Possible Treatment of *Mycobacterium Lepramatous* with Bovine Milk

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Abstract: Biochemical etiology of *M. lepromatous* suggests binding to a sialyl group on the endothelial cell surface. This binding is known to occur for binding of ICAM 1, VCAM 1 and VLA 4 to TLR-2. Post translational modification on TLR 2, on the cell surface and which initiates cell's immune response, is, here, postulated to contain a sialyl group for binding to *M. lepromatous*. We hope bBinding of M. lepromatous to sialyl (α2->3) galactosyl (β 1->4) linked TLR-2, on human gut endothelial cells is may be prevented with ingestion of bovine milk because of the presence of sialylated lactose 6' phospho di-phospho asparaginyl sulfo tyrosine dipeptide in bovine milk. To establish the structure of this bovine milk component through the use of mass spectrometry and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is sought. And thereby propose the use of bovine milk to treat M. lepromatous. Since no viable way to test bovine milk components for binding to *M. lepromatous* due to the difficulty of culturing the microbe testing in humans is suggested to be done. Here, to we characterize the structure of milk oligosaccharide, which is essential to moving forward with treatment of *M. lepromatous* infection with bovine milk.

Keywords: Mass Spectrometry, HPAEC-PAD, Molecular Characterization, Bovine Milk, Possible *M. Lepramatous* Treatment

1. Introduction

Mycobacterium lepramatous infection causes leprosy and its effects are devastating to those infected. Currently thalidomide which is known to have horrible birth defects to the progeny of those contracting the disease and who cannot afford other treatments has been used for treatment. The US FDA re-approved use of the drug in 1998 after banning it in 1960, with the concomitant use of birth-control. Clearly an inexpensive treatment that addresses the issues of multi-drug resistance and safety by giving an attainable treatment regimen would help those impoverished people with this disease, help to eradicate the disease and allow wanted pregnancies while preventing deformities. TLR-2 on endothelial cells binds to *M. lepramatous* as a cell entry mechanism for the organism via an epitope thought to contain sialyl groups. A sialylated oligosaccharide could be used to mimic TLR-2[1, 2], on endothelial cell surfaces to bind *M. lepromatous*, to prevent infection by this microbe. A possible sialylated oligosaccharide is present in bovine milk. The isolation, [3] and structural characterization, by the techniques noted previously[4] for proposed new structure for the major component of bovine milk is described. This molecule is N acetamido deoxy neuraminyl (α 2->3) 6'phospho galactosyl (β 1->4) glucosyl di-phospho aspariginyl sulfo tyrosine dipeptide. Phosphorylated sialylated oligosaccharide has been found in bovine colostrum. [5] This derivative from bovine milk may act as a TLR 2 mimetic to prevent *M. lepromatous* infection. The dipeptide from milk, readily available even in third world countries, in order to make such a treatment available even to impoverished peoples is described. Here we make the case for the structure noted. Mass spectral evidence is used on the milk component and a novel chemistry on the isolated molecule, and subsequent mass spectral evidence, is used to aid in structural characterization of this molecule. Safety issues from introducing sialylated molecules into the human

body would be alleviated due to the known safety of bovine milk.

2. Methods

2.1. Bovine Skim Milk Isolation and Analysis

Bovine milk component was isolated as noted. [3] Pipet 1.00 mL of skim milk into a 15 mL centrifuge tube and next add 10.0 mL ethanol (95%) and then centrifuge the mixture to pellet the protein. The isolate was subjected to ESI mass spectrometry. The original method for isolating this molecule is as follows: Bovine skim milk (commercial, 100 µL) was placed in a micro-centrifuge tube. To this tube was added H_2O (18 mega Ω resistivity, 1.00 mL). The contents of the tube were pushed through an NH_4^+ form cation exchange cartridge (Thermo Fisher Scientific, Sunnyvale, CA, USA). This cartridge in the H^+ form can be converted to the NH_4^+ form cation exchange cartridge by pushing 40 mL of 1 N NH_4OH through it and then pushing H_2O until the pH of the effluent is pH 10 or below.

The effluent from the diluted bovine milk component over the NH_4^+ cation exchange cartridge was immediately frozen for further analysis; by an API 2000 triple quadrapole mass spectrometer, ms and ms^2, by HPAEC-PAD, by monosaccharide component analysis, by acid hydrolysis, HPAEC-PAD analysis, and performance of novel chemistry with a sample of milk oligosaccharide dipeptide. Also performed is ^{13}C NMR on the original of the two sample preparation methods.

2.2. Novel Derivatization Chemistry on Skim Milk Isolate

To a sample (100 µL) from the original method of isolation for this molecule in a micro-centrifuge tube (1.5 mL Eppendorf-like tube, with cap) we added $NaBH_4$ (3 µL from a 4N solution) contained in NH_4OH (1.00 mL, 1N, pH 11.4). This reaction mixture was allowed to stand at ambient temperature for 2 hours, with micro-centrifuge tube capped. The reaction mixture was pushed through an NH_4^+ form cation exchange resin cartridge (Thermo Fisher Scientific, Sunnyvale, CA USA). The effluent was then pushed through a Na^+ form cation exchange cartridge and the contents frozen for storage prior to further analysis by ms on an API 2000 triple quadrapole mass spectrometer.

3. Results and Discussion

Demonstration of structure is performed by techniques [4] for both the un-derivatized and derivatized bovine skim milk component. Analysis via HPAEC-PAD on a sample of the original milk isolate showed the neutral monosaccharides to be galactose and glucose, figure 1.

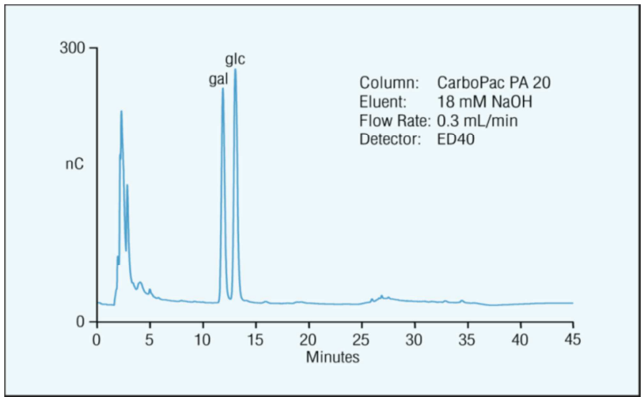

Figure 1. *Hydrolysis of unreduced milk isolate. These are the neutral sugars from sialyl lactosyl-6′-phosphate di-phospho asparaginyl sulfo tyrosine dipeptide. After hydrolysis neuraminic acid, phosphoric acid, sulfuric acid and asparaginyl tyrosine dipeptide would not chromatograph at appreciable retention times in this HPLC program, nor is there any glucosamine. If it were there, it would indicate that the molecule found might be the known milk oligosaccharide, sialyl lactosamine phosphate.*

And the reducing sugar, after reduction by $NaBH_4$ in H_2O, shown to be glucose (data not shown). The mass spectrum of the original isolate is shown in figure 2.

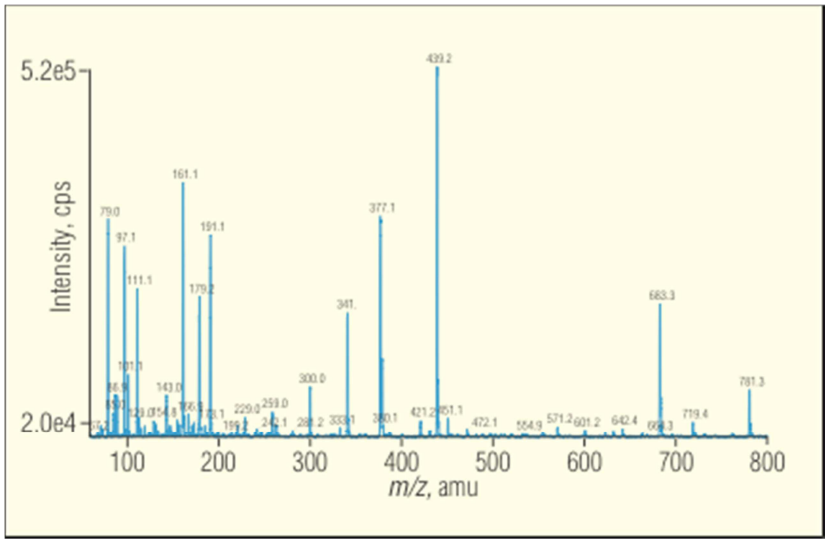

Figure 2. *This is an API 2000 electrospray negative ion mass spectrum of milk trisaccharide isolated by pushing H_2O diluted milk through an ammonium ion cation exchanger. The base peak for this milk fraction is m/z 439.2 with m/z 377.1 nearly equal in size.*

In this figure there are several ions. They are: m/z 781.3, in figure 3, m/z 683.3, in figure 4, m/z 439.2 in figure 4, m/z 377 in figures 5 and 6, m/z 341.3 in figure 7, m/z 191.1 in figure 5, m/z 179.2 and m/z 161.1 in figures 8(b) and 8(a), m/z 111.1 in figure 9.

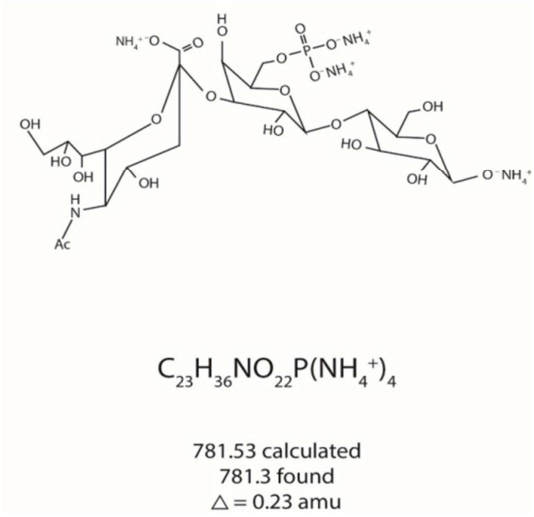

Figure 3. *Diluted skim milk NH_4^+ cartridge ms ion from figure 2.*

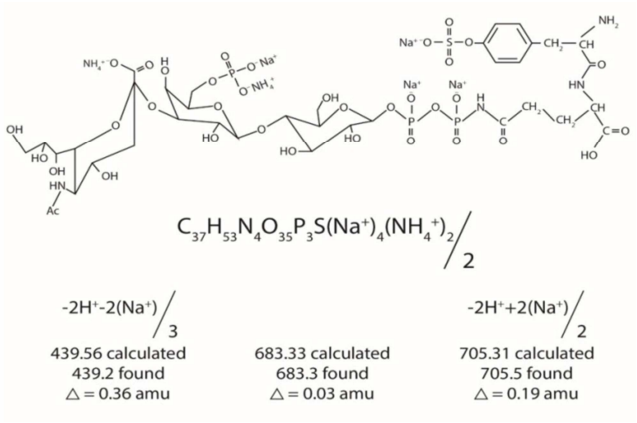

Figure 4. *Three ions, from milk isolate, isolated by NH_4^+ cartridge, found in figure 2.*

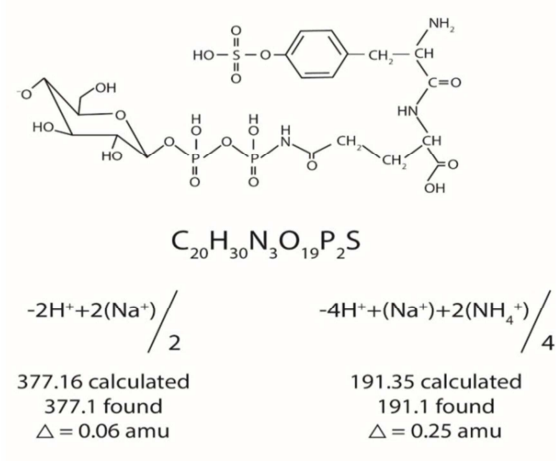

Figure 5. *Two ions from ms, figure 2, isolated by NH_4^+ cartridge with diluted skim milk.*

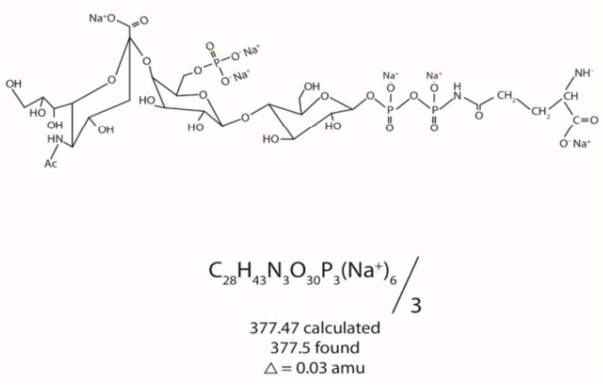

Figure 6. *Structure of m/z 377.5 from ms figure 2 of diluted skim milk on NH_4^+ cartridge.*

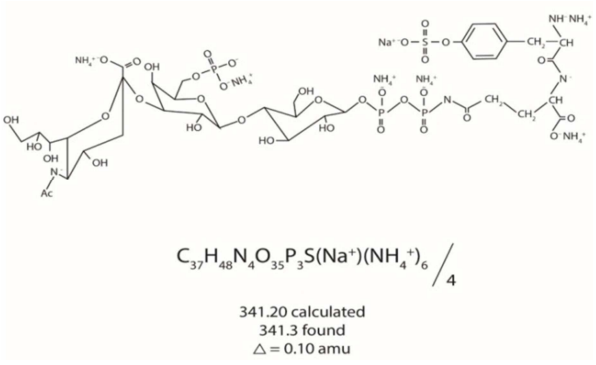

Figure 7. *Ion from ms, shown in figure 2, from NH_4^+ cartridge of diluted skim milk.*

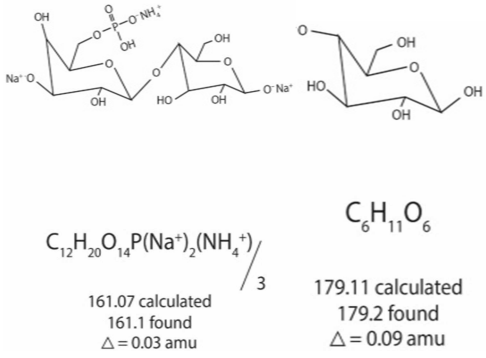

Figure 8. *Ion from ms , in figure 2, from NH_4^+ cartridge's effluent of diluted skim milk.*

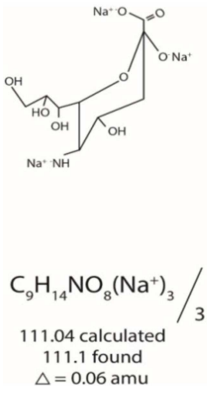

Figure 9. *Skim milk isolate from NH_4^+ cartridge, ion from ms in figure 2.*

All calculated ion masses are within 0.56 amu of found mass to charge ratios for these ions. This difference is well within the precision of the API 2000 triple quadrapole mass spectrometer. From the milk component isolated by ethanol extraction, we obtained the ESI single quadrapole mass spectrum, figure 10.

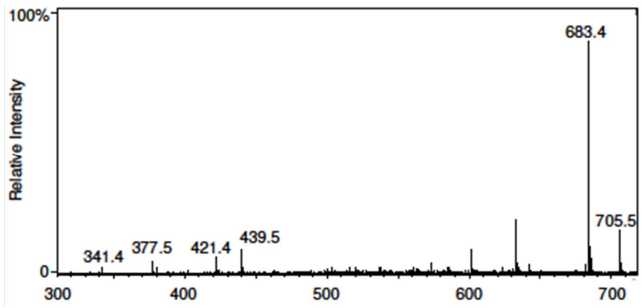

Figure 10. *ESI Mass spectrum of bovine milk oligosaccharide di-phospho aspariginyl sulfo tyrosine dipeptide isolated by extraction with ethanol from bovine milk.*

The ions are; m/z 705.5 and m/z 683.3 and m/z 439.2, the latter two ions in figure 4, and m/z 377 in figures 5 and 6. The small ion from the latter spectrum, m/z 421, can be attributed to lactose 6'-phosphate less one H^+ ion.

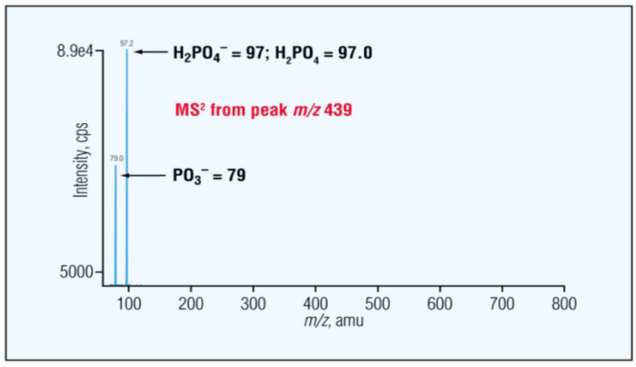

Figure 11. *MS^2 of m/z 439 from unreduced skim milk trisaccharide. Note evidence for phosphate or phosphate/sulfate substitution of this species at m/z 79.0 and m/z 97.0.*

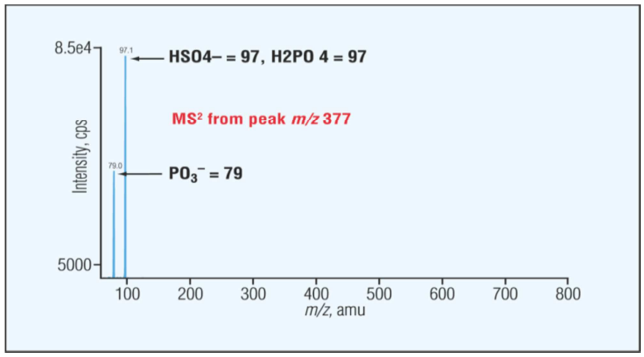

Figure 12. *MS^2 of m/z 377 from unreduced skim milk trisaccharide. Note evidence for phosphate or phosphate/sulfate substitution of the ion m/z 79.0 and m/z 97.0.*

Ions m/z 439.2 and 377 from the former spectrum were subjected to ms^2. Both ms^2 spectra, figures 11 and 12, give the ions m/z 79.0 and m/z 97.0. The former ion of the two ms^2 ions is diagnostic of phosphate ester substitution and the latter ms^2 ion can arise from phosphate or sulfate substitution or both substitutions.

In figures 4 and 7 we draw a structure that represents the whole molecule. This molecule contains the N-acetamido deoxy (α 2->3') 6' phospho galactosyl (β 1->4) glucosyl di-phospho asparaginyl sulfo tyrosine dipeptide found in bovine milk. This is the first report of this molecule in bovine milk. It is the first report of this molecule from any source. Figure 4 and 7 suggests a fully substituted oligosaccharide dipeptide structure for this milk component. In the following figure 8, the complete oligosaccharide dipeptide less the neuraminic acid substituent derived from the original mass spectrum, describing both ions, m/z 179.2 and m/z 161.1, are drawn. The next figure 5 depicts the full dipeptide less the N acetamido deoxy neuraminyl 6' phospho galactosyl portion of the oligosaccharide dipeptide; the ions, m/z 191.1 and m/z 377.1. Figure 9 describes pictorially the ion m/z 111.1. The presence of this ion shows evidence for N-acetamido deoxy neuraminyl presence in this oligosaccharide. From the next figure, figure 6, an alternate structure for the ion m/z 377, (377.5) is drawn. In another slide, figure 3 we have drawn the fragmented oligosaccharide portion of the molecule. This ion, m/z 781.3, has not been identified until this report.

To aid in identification of the bovine milk oligosaccharide a sample of the originally isolated bovine milk trisaccharide phospho di-phospho aspariginyl sulfo tyrosine dipeptide with $NaBH_4$ in NH_4OH (pH 11.4) was reacted as noted in the experimental section of this report.

There are two types of H^- substitution that produce the molecule shown in figure 13. The first chemistry, is described [6] for the method of H^- insertion of sulfate versus phosphate discernment of esters of carbohydrate and, [6] concerning the H^- insertion of carbonate to produce formic acid, formaldehyde and methanol from carbon dioxide in NH_4OH containing $NaBH_4$. [7] It produces hydride substituted phosphate esters and di-hydride substituted sulfate esters in the case of the milk component.

The second type of H^- chemistry we observe is described below. Because the milk oligosaccharide di-phospho aspariginyl sulfo tyrosine dipeptide has a hydrido-phospho group at the anomeric carbon of the 'reducing monosaccharide,' the anomeric carbon to oxygen bond is weakened through a non-bonding orbital anti-periplanar from the phosphorous to oxygen double bond which weakens the 'reducing monosaccharide' anomeric carbon, C-1, to anomeric oxygen bond, O-1 bond. Also, the O-5 non-bonding orbital contributes double bond character to the O-5 to C-1 bond. This is borne out by the long-postulated transition state for SN_1 nucleophilic substitution mechanisms.

The reaction produces the 1, 5 anhydro glucitol substituted with N acetamido deoxy neuraminyl (hydrido) phospho galactosyl group, as shown in figures 14 and 15, ions m/z 369.2 and m/z 395.5, respectively, from the mass spectrum in figure 13.

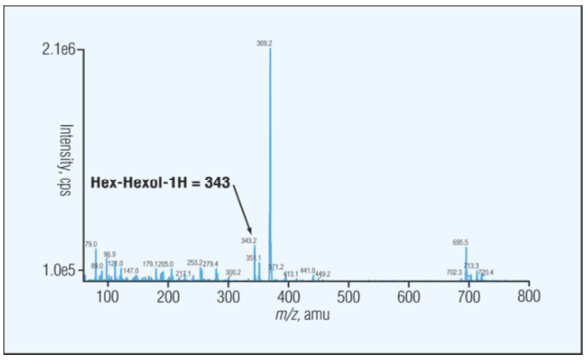

Figure 13. *Mass spectrum of title molecule originating from bovine milk.*

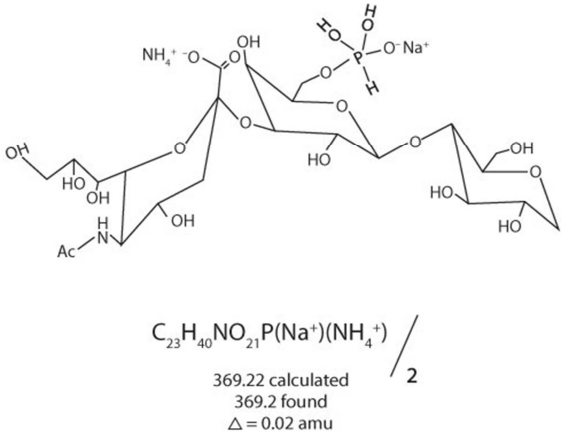

Figure 14. *Ion from mass spectrum of bovine milk treated with $NaBH_4$ in NH_4OH.*

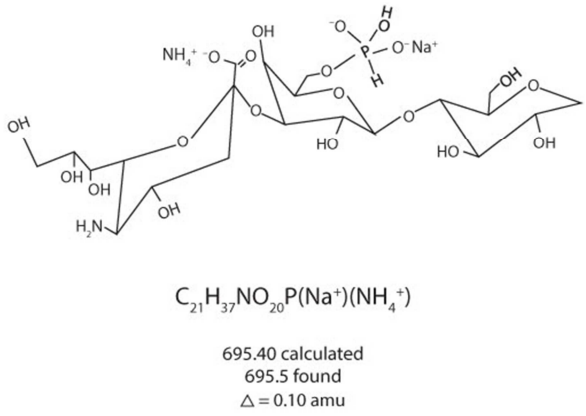

Figure 15. *Ion from mass spectrum of bovine milk treated with $NaBH_4$ in NH_4OH.*

For figure 16 the ion, m/z 79.0, less than 20% of the base peak, is drawn to show the di (hydrido) di-phospho group, an integral portion of the starting underivitized bovine milk oligosaccharide dipeptide. Typically this ion is diagnostic for phosphate substitution. However, to draw an ion with formula weight 79.0 from the starting hydrogen substituted phosphate group could not be done. In figure 17, the ion extended beyond the di (hydrido) phospho group to include the aspariginyl portion of the dipeptide is drawn. Lactose reduced by $NaBH_4$ in H_2O, less one H^+ ion, would produce the isobaric ion mass, 343.2 amu. The problem with its presence, in the reaction mixture, is that the pKa of the lactose alditol hydrogen would be expected to be over 13. But the molecule experiences only pH 11.4, nearly two orders of magnitude less than the required pKa for ionization. Therefore, the drawn structure is more probable, which is still less than 20% of the base peak.

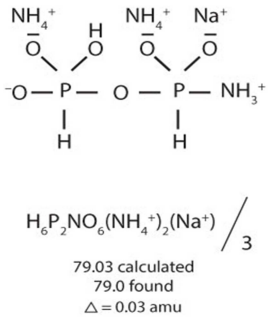

Figure 16. *Ion from mass spectrum of bovine milk treated with $NaBH_4$ in NH_4OH.*

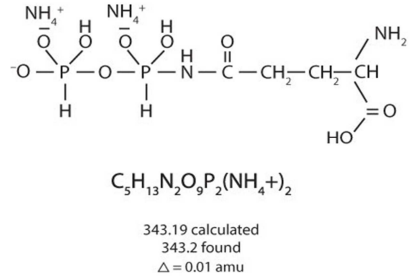

Figure 17. *Ion from mass spectrum of bovine milk treated with $NaBH_4$ in NH_4OH, probably not the Hex-Hexol as noted in the spectrum because of the pH necessary to abstract a proton from the disaccharide alditol.*

In figure 18 a structure for m/z 351.1 is drawn. It is the other portion of the total dipeptide after cleavage into the oligosaccharide portion and this portion, which would be in the reaction mixture, the di (hydrido) phospho asparaginyl (di-hydrido) sulfo tyrosine dipeptide. In figure 19, the ion to be the reacted whole molecule from bovine milk less the oligosaccharide portion of the resulting molecule to include the di (hydrido) phospho aspariginyl (di-hydrido) sulfo tyrosine dipeptide is shown. Still this ion is much less than the base peak in relative intensity.

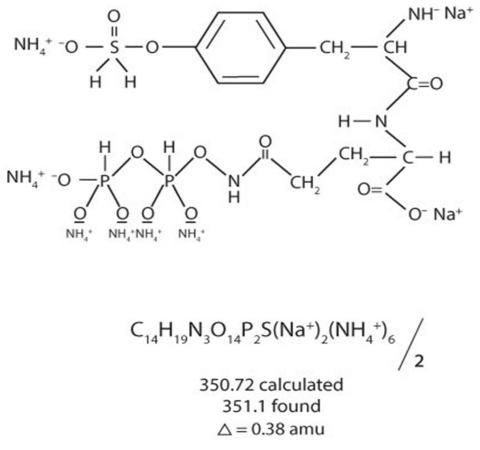

Figure 18. *Ion from mass spectrum of bovine milk treated with $NaBH_4$ in NH_4OH.*

Figure 19 shows the whole molecule, reacted with $NaBH_4$ in NH_4OH, which is first derivatized at the di phospho groups and sulfo group and then the H^- anion attacks the anomeric carbon producing the substituted 1, 5 anhydro glucitol derivative.

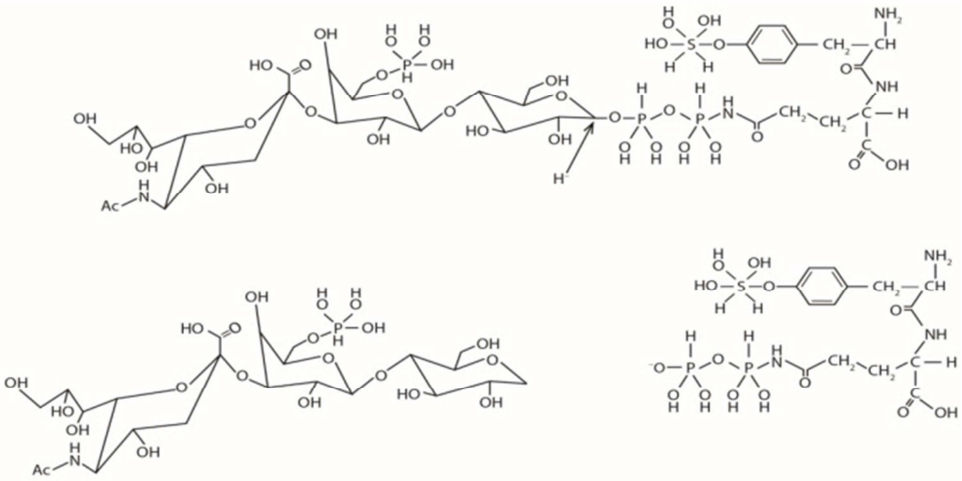

Figure 19. *Molecules from bovine milk treated with $NaBH_4$ in NH_4OH. Hydride attacks the activated and hydrated hydrido phosphoryl group.*

The phosphorylated trisaccharide di-phospho aspariginyl sulfo tyrosine dipeptide structure is attested to, by the cumulative body of mass spectral evidence shown and the body of mass spectral evidence for the molecule after reaction with $NaBH_4$ in NH_4OH.

The ^{13}C NMR of the isolated molecule from bovine milk gives the tabulated spectral peaks, figure 20. This is nearly identical to the standard lactose ^{13}C NMR spectral peaks as published. [8] When preparing a sample for both ^1H NMR and ^{13}C NMR the solution needs to be repeatedly evaporated in ^2H_2O to exchange ^1H atoms for ^2H atoms. In the case of bovine milk isolate, the concentration of the sample would produce an acidic mixture, even as the sodium salt, an appreciable amount of acid would be present. This molecule includes highly acid sensitive acidic components; N-acetamido deoxy neuraminic acid, phosphate ester and sulfate ester which have low pKas. Lactose would be the probable product of repeated evaporations for sample preparation treatment. Therefore neither ^1H NMR nor ^{13}C NMR would produce evidence for the molecule from bovine milk. [9]

Sugar	C-1	C-2	C-3	C-4	C-5	C-6
α-glc	91.8*	71.2	71.4	78.4	70.1	59.9
β-gal	102.9	70.9	72.5	68.5	75.4	61.0

*In ppm

Figure 20. *Table of ^{13}C NMR of Chemical Shifts of Isolated Carbohydrate from Skim Milk.*

In figure 21 the preparation difficulties with the originally isolated milk component is supported. On standing at ambient temperature the isolate decomposes to lactose in less than, or equal, to 4 minutes, which is shown in this chromatogram.

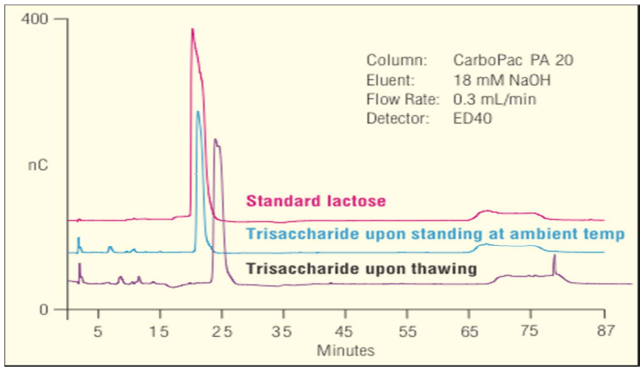

Figure 21. *Unknown component from milk upon thawing, then standing at ambient temperature, compared to lactose. This indicates that this fraction contains mainly one component and that it decomposes to lactose under the stated conditions.*

The structure of the molecule discovered in bovine milk and, the structure for the molecule after its reaction with, $NaBH_4$ in NH_4OH, have not been previously reported. This is the first report for both molecules' existence and attests to their possible efficacy in various diseases, 3'sialyl lactose is known to have efficacy in; malaria, [10] Parkinson's disease and cancer metastasis, [11] and other disease states. [12-16]

4. Conclusion

Therefore, bovine milk, itself, could act as a treatment for leprosy. The safety of bovine milk is unequivocal throughout the world to include the Western hemisphere and the third world. With an inexpensive and relatively widely available treatment for *M. lepramotous* infection and its following disease etiology, treatment regimens encompassing the whole lifetime of the disease, using bovine milk, would be achievable and multi-drug resistance would be minimized.

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