Structures of N-Glycans of Bothrops Venoms Revealed as Molecular Signatures that Contribute to Venom Phenotype in Viperid Snakes

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The complexity of snake venoms has long been investigated to explore a myriad of biologically active proteins and peptides that are used for immobilizing or killing prey, and are responsible for the pathological effects observed on envenomation. Glycosylation is the main post-translational modification (PTM) of viperid venoms but currently there is little understanding of how protein glycosylation impacts the variation of venom proteomes. We have previously reported that Bothrops venom glycoproteomes contain a core of components that markedly define their composition and parallel their phylogenetic classification. Here we extend those observations to eight Bothrops species evaluating the N-glycomes by LC-MS as assigned cartoon structures and detailing those structures separately as methylated analogs using ion-trap mass spectrometry (MS^n). Following ion disassembly through multiple steps provided sequence and linkage isomeric details that characterized 52 unique compositions in Bothrops venoms. These occurred as 60 structures, of which 26 were identified in the venoms of the Jararaca Complex (B. alcatraz, B. insularis, and B. jararaca), 20 in B. erythromelas, B. jararacussu, B. moojeni and B. neuwiedi venoms, and 22 in B. cotiara venom. Further, quantitative analysis of these N-glycans showed variable relative abundances in the venoms. For the first time a comprehensive set of N-glycan structures present in snake venoms are defined. Despite the fact that glycosylation is not template-defined, the N-glycomes of these venoms mirror the phylogeny cladograms of South American bothropoid snakes reported in studies on morphological, molecular data and feeding habits, exhibiting distinct molecular signatures for each venom. Considering the complexity of N-glycan moieties generally found in glycoproteins, characterized by different degrees of branching, isomer structures, and variable abundances, our findings point to these factors as another level of complexity in Bothrops venoms, features that could dramatically contribute to their distinct biological activities.

Snakebites are responsible for many deaths and permanent injuries around the world, making snake envenomation a global public health problem (1, 2). An essential step to getting better treatments for human snakebite victims must be to fully understand the molecular basis of snake envenomation. Snake venoms are composed of proteins and peptides that induce a wide variety of pathophysiological effects. These cocktails of biomolecules are important traits of polygenetic origin that play a fundamental role in prey capture and defense. The challenges involved in the complete characterization of snake venoms are related to their complexity. In the case of viperid venoms, this feature is not only because of the number of different toxin classes, but also to various toxin forms with a high degree of variability in primary structure, resulting from gene duplication and non-synonymous mutations (3–11). On evolution venomous snakes generated different repertoires of toxins to deal with distinct prey types, hence the venom proteome and peptidome may vary at different taxonomic levels, and intraspecifically, because of factors like gender, diet, age, and habitat (12–19). Another source of snake venom complexity is the increase in the number of toxin forms generated by post-translational
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modifications (PTM)\(^1\), however, the extent of this mechanism of variability and its implication for the venom as a trait are still unknown. PTMs found in snake venoms include proteolytic processing, oligomerization, and glycosylation (7, 20–22).

Glycosylation is an important PTM found in many proteins, and database analysis has shown that most proteins have putative sites for glycosylation (23, 24). Protein glycosylation is an energetically costly process for the cell and its occurrence is essential for a myriad of biological processes ranging from structural and functional roles, such as folding, stability, solubility, and protection against proteases, to cell adhesion, cell migration, and signal transduction (24–27). The perspective of glycosylation importance is clearly revealed in multicellular organisms because the elimination of glycoconjugates at an early stage of development precludes survival (28).

The N-glycosylation process occurs at the sequon Asn-X-Ser/Thr, where X is any amino acid, except Pro, in extracellular or secreted proteins (29–31). N-glycans are covalently attached to protein at the first residue (Asn) of the sequon by an N-glycosidic bond. There are three general types of N-glycans in a mature glycoprotein: high-mannose, complex, and hybrid. Five different N-glycan linkages occur, of which N-acetylglucosamine to Asn (GlcNAcβ1-Asn) is the most common (32).

Despite the fact that glycosylation of proteins is a major PTM in viperid and crotalid snake venoms, we still poorly understand its impact regarding variation and stability of venom proteomes. Nevertheless, N-glycan composition studies of isolated toxins and venoms from Elapidae and Viperidae families indicate significant variability (33–46). On the other hand, we have shown that the Bothrops jararaca N-glycan compositions from newborn and adult venoms are similar suggesting that differences in utilization might explain the variable glycosylation levels that were indicated by differential electrophoretic profiles previously reported for B. jararaca newborn and adult venoms (47).

One of the theories on the origin of genes encoding snake venom proteins considers that some genes involved in physiological processes were recruited for expression in the venom glands and then underwent an accelerated evolution process (48, 49). The changed site of action, and substrates, in the case of enzymes, may have led to differences not only in the primary structure but also in PTMs.

Glycosylation is an important feature of the snake venom arsenal as it may provide a significant advantage for the maintenance of homeostasis within the gland lumen by improving protein solubility, enabling the snake to produce an extremely high concentrated toxic fluid. Moreover, N-glycosylation may confer stability to proteins, play a general role in quality control of protein folding on synthesis, and increase protein half-life in the blood stream (26, 43, 50, 51).

The “omics” era brought many advances in the knowledge on the primary structure of snake venom components, but not N- and O-glycosylation. This understanding provides a more comprehensive view that considers additional roles of glycosylation in toxins. The high content of glycosylated proteins in snake venoms and their actions within diverse biological systems in multicellular organisms strongly suggests that essential information is missing in the current databases for an effective understanding of these active molecules in science and medicine. Thus, considering the well-documented variability of viperid venom proteomes, the aim of this study was to analyze the N-linked glycan structures present in the venom of eight species from Bothrops genus (B. jararaca, B. insularis, B. alcatraz, B. cotiara, B. moojeni, B. neuwiedi, B. jararacussu, and B. erythromelas). Based on our qualitative and quantitative analyses, we now report for the first time that the venom glycomics of these species contain a core of N-glycans as signatures that define their composition, which is conserved with evolution in parallel with their proteomes, and other molecular markers that define their phylogenetic classification.

EXPERIMENTAL PROCEDURES

Snake Venoms—Lyophilized venom of B. cotiara, B. insularis, B. jararaca, B. moojeni, B. neuwiedi, B. jararacussu, and B. erythromelas was provided by the Laboratory of Herpetology, Instituto Butantan (São Paulo, Brazil), where the snakes maintenance and milking process are approved by the Ethical Committee for Animal Research (Protocol no. 1296/14). A sample of B. alcatraz venom was provided by Dr. Silvia Regina Travaglia-Cardoso from Museu Biológico, Instituto Butantan (São Paulo, Brazil).

N-Glycan Release—For enzymatic N-deglycosylation of snake venom glycoproteins, 1 mg of lyophilized venom was dissolved in 300 \(\mu\)l of 50 mM sodium bicarbonate pH 8.0, containing 0.09% of sodium dodecyl sulfate and 0.05 M of \(\beta\)-mercaptoethanol, and incubated at 100 °C for 10 min followed by a cooling step at room temperature for 15 min. After cooling, 15 \(\mu\)l of 15% Nonidet P-40 and 15 \(\mu\)l of enzyme and submitted to incubation again for 40 h. In total, 12.5 mU of enzyme were used per sample and 48 h for the enzymatic reaction. After N-glycans releasing, samples were submitted to C18 Solid Phase Extraction (SEP-PAK, Waters Corporation, Milford, MA) to remove proteins and detergent, and dried using a vacuum centrifuge.

Reduction—Dried samples were dissolved in 400 \(\mu\)l of a borane-ammonia complex solution (10 mg/ml in 28% ammonium hydroxide) and incubated at 50 °C for 2 h with periodical shaking every 30 min. Borane-ammonia was removed using a vacuum centrifuge and residuals were removed by washing 3 times with 2 ml of methanol and dried again.

Desalting—Reduced N-glycans were desalted using Porous Graphitized Carbon (PGC) (Agilent, Santa Clara, CA). One empty SPE cartridge was filled with 250 \(\mu\)l of pre-cleaned PGC and washed with 3 ml water. Reduced N-glycans were dissolved in 1 ml of water and loaded into the cartridge. Sample tubes containing residuals were added with additional 1 ml of water and these portions were also loaded into the SPE cartridge. Following, the cartridges were washed with 8 ml of water and reduced N-glycans were eluted with 6 ml of

\(^{1}\) The abbreviations used are: PTM, post-translational modifications; MS\(^0\), ion trap MS.
40% acetonitrile in 0.1% trifluoroacetic acid and dried using a vacuum centrifuge.

Permethylation—Reduced N-glycans were permethylated using sodium hydroxide beads (Sigma-Aldrich, Saint Louis, MO) packed in spin columns, dimethyl sulfoxide (DMSO) and iodomethane (Sigma-Aldrich). After sodium hydroxide beads preparation by 2 washes with DMSO, samples were dissolved in 100 μl of DMSO and 100 μl of iodomethane. Samples were loaded into the spin column and left to incubation for 30 min at room temperature and collected by centrifugation at 538 × g for 2 min. Additional 100 μl of iodomethane were added to the samples and these were loaded again into the column and incubated for more 30 min at room temperature. Finally, all volumes were collected by centrifugation and sodium hydroxide beads were washed with 300 μl of DMSO and 400 μl of acetonitrile and these portions were collected together and placed on ice. Permethylated N-glycans were cleaned up by liquid-liquid extraction using chloroform and cold water. Dried samples were dissolved in 400 μl of 50% methanol in water.

2-Amino benzoic Acid (2AA) Labeling—Before the labeling procedure, 10% of released N-glycans were cleaned up using PGC cartridges as previously described in the desalting procedure for quality assurance. Briefly, samples were dissolved in 20 μl of 2AA labeling solution (47 mg/ml 2AA, 63 mg cyanoborohydride in 30%/70% acetic acid/DMSO) and incubated at 65 °C for 3 h and cooled to room temperature. Cleaning procedure was carried out using acetone precipitation (adapted from S3). In this step, 1 ml acetone was added to the sample, homogenized by vortex for 30 s and centrifuged at 19,000 × g for 5 min. Then, the top layer (950 μl) was removed by pipetting and discarded. This step was repeated twice, and the sample was dried in a vacuum centrifuge. N-glycans were dissolved in 200 μl of 67% acetonitrile in 33% ammonium formate in water.

LC-MS, Composition and Cartoons. MSn and Structural Detail—Two different approaches to understand structures were applied; an LC-MS, semi-automatic technique provided molecular weight and cartoon structures (Glycomod), and MSn, a precise accounting of structural detail by multiple steps of disassembly using an ion trap mass spectrometer. The former technique provided glycan group classification (high mannose, hybrid or complex), whereas the latter technique extended this understanding to linkage assignments, branching and isomer details. Importantly, this spatial disassembly provides an opportunity to spectrally document results with library standards and advance collaborative research. In this application, activated carbohydrate ions (CID) in a structurally dependent manner providing products that retain some aspects of sequence information with each step of disassembly. In this iterative physiochemical process, oligomers can be spatially sequenced and compiled for library spectral documentation. Two practical factors underpin the importance of this approach; native background ions are lost during the most labile fragments (MS2) at high abundance usually at or adjacent to the polar linkages. Linkage fragment ions of high abundance represent the most facile and proceeding through a disassembly pathway in a structurally dependent manner providing products that retain sequence information with each step of disassembly. In this iterative physiochemical process, oligomers can be spatially sequenced and compiled for library spectral documentation.

Direct spray infusion was performed using a Thermo Nanomate (Advinion, Lithaca, NY) coupled to an LTQ or LTQ Velos Pro mass spectrometer (Thermo Scientific, San Jose, CA). Spray parameters on the Nanomate were set to 1.4–1.8 kV and 0.4 psi nitrogen gas pressure and were adjusted to achieve spray currents between 10–150 nA. The scan rate at positive mode was set to “Enhanced,” AGC target value and maximum injection times were varied according to the signal intensity. For N-glycan mass profile of each sample, a full MS scan was acquired with at least 50 scans and the number of microscans varied between 3 and 10. Activation Q and activation time for CID were left at default values, 0.25 and 30 ms, respectively. Normalized collision energy was set to 35%. Precursor ion selection for MS2 experiments was performed manually and isolation windows were set to capture an entire isotopic envelope. Typically, at least one isolation scan, with collision energy set to 0, was acquired in each CID data file to document the isolated isotopic envelope. The methodology used for spectra acquisition using both spectrometers were the same, except for the activation time for CID (10 ms) and normalized collision energy (40%) used for the LTQ Velos Pro.

Liquid Chromatography Mass Spectrometry—Profiles of N-glycans released from snake venom proteins were obtained by LC-MS using size and charge as properties to carry out the separation. High-performance liquid chromatography was carried out on a Surveyor HPLC system (Thermo Finnigan, San Jose, CA) equipped with fluorescence detector using as stationary phase TSKgel Amide-80 column (2.0 mm [internal diameter] x 150 mm [length], 3 μm [particle size]) (TOSOH, King of Prussia, PA). For separation, the mobile phases were A (acetonitrile), and B (50 mM ammonium formate in water, pH 4.4). The column temperature was set to 40 °C and a flow rate of 189 μl/min was used. The gradient established was: 33–48% B in 32 min; 48–100% B in 0.5 min; 100% B for 2 min. Fluorescence of 2AA–labeled glycans was detected by excitation at 360 nm and emission at 420 nm. Mass spectrometry was performed using a LTQ Velos Pro (Thermo Scientific, San Jose, CA). MS detection was performed using negative mode, scan rate in “Enhanced” mode and the scan range used was m/z 650–2000.

Data Analysis—The raw files were analyzed using Xcalibur Qual Browser (Thermo Scientific, San Jose, CA). The mass values of each peak identified by MSn were previously calculated and these values were used to find the structures in the profile. The final profiles of N-glycans are the extracted ion chromatograms from the isotopic pattern of each structure. These profiles were used for calculation of the peak area of each N-glycan structure for relative quantification. The ratio of each peak area was divided by the sum of peak areas of all glycans from the extracted ion chromatogram of each venom.

Glycomod Search—Full MS of N-glycans from venoms were analyzed manually and single charge mass was used for putative composition search using the Glycomod tool (http://web.expasy.org/glycomod/). All ions are sodium adducts and parameters used in searches were: single charge monoisotopic, with a mass tolerance of 2.0 Da, reduced and permethylated N-glycans, and all monosaccharide classes were considered as possible candidates, with the exceptions of pentoses, glucuronic and deaminohexuronic acids, phosphorylated and sulfated glycans. The discrimination between the monomer stereochemistry was not carried out in this study, and therefore tables contain the result obtained from the Glycomod, describing the monomers without any details. However, the stereochemistry was suggested in the figures according to the entries present in the UniCarbKBB database, the available literature on toxins.
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N-glycans (33–45, 47, 55) and position of each monomer inside the structure.

Computational Methods—The identification of the N-glycan structures ultimately generated a list of N-glycan structure codes, grouped by venom. That list was processed through an in-house Perl program, which computed the total number of unique N-glycan structures, that is, the size of the union of all N-glycan structure codes that appeared at least once in all eight venoms. Using this program, we generated an occurrence matrix, in which the columns are the eight different venoms and the rows are all the unique N-glycan structures. Hence, for each pair <venom, glycan structure>, its respective value is either zero or one, which means absence or presence of that pair, respectively. Clustering analysis was carried out with MATLAB (MathWorks, Natick, MA), using the “clustergram” function. We performed two hierarchical clusterings on the occurrence matrix, one for the venoms and another for the N-glycan structure codes. These clusterings were achieved using an agglomerative method with single linkage and the Jaccard index as the distance metric.

Experimental Design and Statistical Rationale—The experimental design and rationale have been described above for the individual experiments performed in this study.

RESULTS

The main objective of this study was to profile the venom N-glycomes of eight Bothrops species (B. alcatraz, B. cotiara, B. erythromelas, B. insularis, B. jararaca, B. jararacussu, B. moojeni, and B. neuwiedi) in order to explore the relationship between their venom composition and phylogeny. For this purpose, we applied an analytical workflow (Fig. 1) to identify N-glycan structures present in these venoms and to compare their relative abundance between the venoms.

Bothrops Venom N-Glycan Qualitative Analysis—The first steps included the analysis of N-glycans released from venom glycoproteins by PNGase F, followed by reduction and methylation. Identification of putative compositions were established using the monoisotopic mass values acquired by MS, as a query in the Glycomod tool (56).

For identification of composition, the Glycomod results were filtered according to the following specifications: N-glycans belonging to the paucimannose class or containing more than three N-glycolylneuraminic acid/deoxyhexoses residues or deoxyhexoses were excluded. The paucimannose class of glycans were excluded considering that this type of N-glycan occurs only in invertebrates; and previous studies showed that snake glycoproteins usually do not contain a deoxyhexose unit linked to one structure (A1) was detected in all venoms and only 12 compositions do not contain a deoxyhexose unit linked to the N-glycan core (supplemental Fig. S1; Table I). These structures are those identified here by MSn analysis; however, the presence of other structural isomers of these defined compositions in Bothrops venoms cannot be excluded.

A general view of all N-glycans identified in this study, including eight isomers, shows 60 different proposed structures, of which 26 were identified in the venom glycoproteins from species of the Jararaca Complex (B. alcatraz, B. insularis, and B. jararaca), 20 in the species B. erythromelas, B. jararacussu, B. moojeni, and B. neuwiedi, and 22 structures in B. cotiara venom. It was interesting to note that only one structure (A1) was detected in all venoms and only 12 compositions do not contain a deoxyhexose unit linked to the N-glycan core (supplemental Fig. S1; Table I). These structures are those identified here by MSn analysis; however, the presence of other structural isomers of these defined compositions in Bothrops venoms cannot be excluded.

Another general observation is that 58 identified structures were of the hybrid/complex type whereas only two were classified as high mannose type, although these were identified in almost all venoms. Additionally, in the hybrid/complex type structures, two different types of antennae were identified. The venoms of the Jararaca Complex (B. alcatraz, B. insularis,
TABLE I

List of N-glycan compositions/structures released from proteins of *B. alcatraz*, *B. cotiara*, *B. erythromelas*, *B. insularis*, *B. jararaca*, *B. jararacussu*, *B. moojeni*, and *B. neuwiedi* venoms

| Compositions* | Proposed structures | B. alcatraz | B. cotiara | B. erythromelas | B. insularis | B. jararaca | B. jararacussu | B. moojeni | B. neuwiedi |
|---------------|---------------------|--------------|------------|----------------|--------------|------------|---------------|-------------|------------|
| A1            |                     | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| A2            | ![3x](image)        | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B1            | ![2x](image)        | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B2            |                     | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B3            | ![x](image)         | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B4            | ![2x](image)        | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B5            | ![2x](image)        | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B6            | ![2x](image)        | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B7            | ![2x](image)        | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B8            | ![x](image)         | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B9            | ![x](image)         | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B10           | ![x](image)         | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B11           | ![x](image)         | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B12           | ![2x](image)        | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B13           | ![3x](image)        | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B14           | ![x](image)         | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B15           | ![x](image)         | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B16           | ![x](image)         | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B17           | ![2x](image)        | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
| B18           | ![x](image)         | ✓            | ✓          | ✓              | ✓            | ✓          | ✓             | ✓           | ✓          |
### Table I—continued

| Compositions* | Proposed structures | B. altadens | B. coluber | B. erythromelas | B. insularis | B. jararaca | B. jararacussu | B. maurus | B. neuwied |
|---------------|---------------------|-------------|------------|-----------------|-------------|-----------|---------------|----------|----------|
| B19           |                     | ✔           |            |                 |             |           |               |          |          |
| B20           |                     | ✔           |            |                 |             |           |               |          |          |
| B21           |                     | ✔           |            |                 |             |           |               |          |          |
| B22           |                     | ✔           |            |                 |             |           |               |          |          |
| B23           |                     | ✔           |            |                 |             |           |               |          |          |
| B24           |                     | ✔           |            |                 |             |           |               |          |          |
| B25           |                     | ✔           | ✔          |                 |             |           |               | ✔        | ✔        |
| B26           |                     | ✔           |          |                 |             |           |               |          |          |
| B27           |                     | ✔           | ✔          |                 |             |           |               | ✔        | ✔        |
| B28           |                     | ✔           |          |                 |             |           |               | ✔        | ✔        |
| B29           |                     | ✔           |          |                 |             |           |               | ✔        | ✔        |
| B30           |                     | ✔           |          |                 |             |           |               | ✔        | ✔        |
| B31           |                     | ✔           |          |                 |             |           |               | ✔        | ✔        |
| B32           |                     | ✔           |          |                 |             |           |               | ✔        | ✔        |
| B33           |                     | ✔           |          |                 |             |           |               | ✔        | ✔        |
| B34           |                     | ✔           |          |                 |             |           |               | ✔        | ✔        |
| B35           |                     | ✔           |          |                 |             |           |               | ✔        | ✔        |
| B36           |                     | ✔           |          |                 |             |           |               | ✔        | ✔        |
| B37           |                     | ✔           |          |                 |             |           |               | ✔        | ✔        |
| B38           |                     | ✔           |          |                 |             |           |               | ✔        | ✔        |
and B. jararaca) and of B. cotiara showed some hybrid/complex type structures containing disialylated lactosamine (NeuAc-2,8-NeuAc-2,3-Gal-1,4-GlcNAc) as an antenna, whereas in the other venoms an antenna composed by NeuAc-2,3-GalNAc-1,4-GlcNAc was detected (Table I).

Taken together, the results of this analysis suggest that the N-glycomes of B. alcatraz, B. cotiara, B. erythromelas, B. insularis, B. jararaca, B. jararacussu, B. moojeni, and B. neuwiedi venoms can be classified in three different groups according to their shared N-glycan structures. Group 1 is composed of N-glycans of the species belonging to the Jararaca Complex (B. alcatraz, B. insularis, and B. jararaca), group 2 is composed of the N-glycans of glycoproteins present in B. erythromelas, B. jararacussu, B. moojeni, and B. neuwiedi venoms, whereas group 3 contains only the N-glycans of B. cotiara venom.

N-Glycans of Venoms of the Jararaca Complex (group 1)—N-glycome analysis of the structures released from venom glycoproteins of B. alcatraz, B. insularis, and B. jararaca showed these to contain identical structures (Fig. 2). From their analyses, two of the glycan structures were of the high mannose and 24 of mix of hybrid and complex type. The disialylated lactosamine was the most abundant of the N-glycans, which was present in 23 of the 26 structures.

The analysis of N-glycan structures of hybrid/complex type by MS\textsuperscript{n} was affected by the abundance of sialic acid units in this group, as observed in the MS\textsuperscript{2} spectra of these compositions, where the most abundant fragments were products of sialic acid loss with different charge states. This is because linkages containing sialic acid are weaker compared with other linkages in N-glycans. Therefore, the spectra were more complex, impairing structural

\begin{table}[h]
\centering
\begin{tabular}{|l|l|c|c|c|c|c|c|}
\hline
Compositions* & Proposed structures & B. alcatraz & B. cotiara & B. insularis & B. jararaca & B. jararacussu & B. moojeni & B. neuwiedi \\
\hline
B39 & \[ \text{2 x} \] & & & & & & & & \\
B40 & \[ \text{2 x} \] & & & & & & & & \\
B41 & \[ \text{2 x} \] & & & & & & & & \\
B42 & & & & & & & & & \\
B43 & \[ \text{2 x} \] & & & & & & & & \\
B44 & \[ \text{2 x} \] & & & & & & & & \\
B45 & \[ \text{3 x} \] & & & & & & & & \\
B46 & & & & & & & & & \\
B47 & & & & & & & & & \\
B48 & & & & & & & & & \\
B49 & \[ \text{2 x} \] & & & & & & & & \\
B50 & & & & & & & & & \\
\hline
\end{tabular}
\caption{N-Glycome Analysis of the Structures Released from Venom Glycoproteins of B. alcatraz, B. insularis, and B. jararaca.}
\end{table}

* A indicates high mannose type; B indicates hybrid/complex type.
\checkmark Indicates in which venom the composition/structure was identified.
determination, especially in the case of less abundant structures.

As shown in the analysis of structure B11 (Fig. 3) the MS\(^2\) and MS\(^3\) spectra illustrate how the intensity of the many sialic acid units affected the pathway of fragmentation for topology determination. As the fragments without sialic acid show low relative abundance the next steps of the MS\(^n\) analysis are more laborious, mainly for those structures whose precursor ion has low intensity at the MS\(^1\). In the case of structure B11, the MS\(^2\) analysis (Fig. 3) showed that the most abundant ion has a molecular mass of 3330.2 Da and is present in two charge states (\(m/z\) 1676.44 and +3 with \(m/z\) 1125.32), which is generated by the neutral loss of 375 Da corresponding to the b-ion of permethylated NeuAc. Moreover, other intense ions in this spectrum are the \(m/z\) 1495.88 \(^{12}\), corresponding to the loss of a sialic acid dimer (neutral loss of 736 Da), and the \(m/z\) 1630.36 \(^{12}\), which is the result of the loss of a reduced reducing end with a fucose unit. As evidence of these neutral losses, some signature ions were detected in the low \(m/z\) range of the MS\(^2\) spectrum, such as the b-ions of \(m/z\) 398.08 \(^{12}\) (terminal NeuAc), 759.16 \(^{12}\) (terminal sialic acid dimer), and the y-ion 490.20 \(^{12}\) (reducing end with a fucose). As the presence of sialic acid units could be assigned in the MS\(^2\), we selected the ion \(m/z\) 1307.80 \(^{12}\) for fragmentation at the MS\(^3\) step to confirm the structure topology (Fig. 3). This ion indeed matches the structure after neutral loss of a sialic acid unit (375 Da) and an entire sialic acid dimer (736 Da) and its fragmentation spectrum revealed the loss of the last sialic acid unit (neutral loss of 361 Da) and the lactosaminic structure (neutral loss of 449 Da), confirming the identity of the antenna. Low values of \(m/z\) in MS\(^3\) also corroborated the identification guided by neutral loss, as these structures were found as sodium adducts. Further, the ion \(m/z\) 866.20 \(^{12}\) was submitted to fragmentation and proved that the structure B11 has one antenna on each mannose core unit (supplementary Information).

This is the second report of the NeuAc-2,8-NeuAc epitope from snake venom glycoproteins. The first observation was made by Lin et al. (43) in the analysis of Deinagkistrodon acutus venom and of the serine proteinase acutobin, isolated from this same venom by Wang et al. (44). For the linkage assignment of this sialyl acid dimer, the b-ion \(m/z\) 759 \(^{12}\) (putative NeuAc-2,8-NeuAc-1-ene) was selected for MS\(^3\) fragmentation (Fig. 4A). As described by Ashline et al. (57), the distinction between linkages in positions 7, 8, or 9 is difficult by spectra analysis, and a good way to document this fact was by spectral comparison of differing standards (supplementary Information). Different from the NeuAc-2,8-NeuAc structure, some structures (B46a, B46b, B47a, B47b, B48a, and B48b) have a single NeuGc residue, which was detected in both the terminal (NeuGc-NeuAc) and subterminal sialic acid (NeuAc-NeuGc). The presence of NeuGc in the sialic acid dimer was confirmed by the fragmentation of \(m/z\) 789 \(^{12}\) in the MS\(^3\) analysis (Fig. 4B; supplementary Information). In this case, it was not possible to compare the structure with a standard, however, this fragmentation pattern suggests that the linkage position between the sialic acid units might be 2,8. As mentioned, some monoisotopic masses correspond to more than one composition, and for these cases the existence of isomers was confirmed during the MS\(^n\) steps. For example, in the case of structures B2, B46a and B46b (\(m/z\) 1292.84 \(^{12}\), [M + Na\(^+\)] = 2562.44 Da), it was possible to observe, in MS\(^2\) step, the ions indicating the presence of these three structures (Fig. 5). The main differences between the structures B2 and B46 a/b are the presence of a fucosylated reduced reducing end and the sialic acid dimer containing NeuGc. The first suggestion of the occurrence of these three structures was the identification of sialic acid dimer containing one...
NeuGc unit ($m/z$ 789.36) and the y-ion $m/z$ 490.28 (reducing end fucosylated) (Fig. 5). The ion $m/z$ 1105.48 corresponds to the loss of a terminal NeuAc unit, and is observed in the fragmentation of structures B2 and B46a (Fig. 5A). However, the ion $m/z$ 1090.48 corroborates the structure B46b, as it corresponds to the fragment generated by the loss of a terminal NeuGc (neutral loss of 405 Da) (Fig. 5A). Moreover, the fragmentation of $m/z$ 1105.48 was useful to explain the existence of the structure B46b (Fig. 5B, 5C). Interestingly, other isomeric structures (B4 and B47) were identified in this group of N-glycans (supplementary Information).

Using this rational, the structures B8, B10, B11, B12, B48a, and B48b were submitted to disassembly between the units in the sialic acid dimer and all fragments of the b-ion $m/z$ 759 (NeuAc-2,8-NeuAc-1-ene) following MS$^3$. As a result, the linkage between the sialic acid unit and the lactosamine was determined to be 2,3 in the structures B4a, B4b, B47a, and B47b, in B. jararaca venom, in the structures B6, B10, B11, and B12 in B. insularis venom, and in the structures B8, B9, B11, B48a, and B48b in B. alcatraz venom (supplementary Information). These results also confirmed the presence of the lactosamine unit (Gal-1,4-GlcNAc), based on the similarity between our spectral data and the analysis of carbohydrate standards carried out by Ashline et al. (57).

The structures B6, B11, B12, and B13 were first described by Lin et al. (43) and the linkage analysis by this group indicates that the structures we found have the same linkage type. The structure B43b was also identified by Geyer et al. (39) as the glycan attached to an l-amino acid oxidase found in Calloselasma rhodosoma venom and by Pfeiffer et al. (33) as a carbohydrate chain attached to the serine proteinase ancrod of Agkistrodon rhodostoma venom.

N-Glycans of B. erythromelas, B. jararacussu, B. moojeni, and B. neuwiedi Venoms (group 2)—The analysis of B. eryth-
romelas, B. jararacussu, B. moojeni, and B. neuwiedi venoms resulted in the identification of 20 different structures, of which two belong to the high mannose type and 18 were of the hybrid/complex type. However, different from the N-glycans of the Jararaca Complex venoms, whose structures were identical in all species, only 13 of the 20 structures proved to be identical in this group (Fig. 6). Nevertheless, the seven structures that were not detected in all venoms may however be present in their carbohydrate moieties but in lower abundance. Different from the structures identified in the venoms of the Jararaca Complex, B. erythromelas, B. jararacussu, B. moojeni, and B. neuwiedi venoms antennae containing the composition NeuAc-GalNAc-GlcNAc were identified in 12 out of 13 common N-glycan structures. The linkage chemistry suggestion of these structures is supported by the work of Lochnit and Geyer (38), who analyzed the glycan moiety of batroxobin, a serine proteinase isolated from B. moojeni venom, and identified the structures B26, B27, B28, and B34. The antenna structure was analyzed by the fragmentation of b-ion \( m/z 888^{+1} \) (Fig. 7; supplementary Information) and the linkage between the monosaccharide units was assigned as NeuAc-2,3-GalNAc-1,4-GlcNAc according to the fragmentation of b-ion NeuAc-2,3-GalNAc-1-ene (\( m/z 643^{+1} \)). This finding agreed with the linkages described by Lochnit and Geyer (38). Indeed, glycan analyses of other snake venoms and toxins show a large amount of N-glycan structures containing NeuAc with a 2,3 linkage, instead of 2,6 (33, 34, 36, 38, 39, 43, 44). The 2,6 linkage of NeuAc is likely less common as it was described so far only by Sakai et al. (55) in a serine proteinase from Agkistrodon haly brevicaudus stejneger venom, indicating that on evolution the position 2,3 was preferred for the NeuAc attachment to GalNAc.

The analysis of MS disassembly of the structures from this group was easier than that of group 1, as the presence of sialic acid in low abundance yielded informative fragments. For instance, the MS and MS steps of the analysis of structure B32 (from B. moojeni venom) are shown in Fig. 8. The occurrence of the two losses of terminal sialic acid (each one with 375 Da) excluded the presence of sialic acid dimer as observed in the structures of group 1; besides, the absence of the signature ion of sialic acid dimer (\( m/z 759^{+1} \)) was also a good indication of the absence of this feature in the structures of group 2. Among the low abundant \( m/z \) ions, the ion \( m/z 643.44^{+1} \) also allowed the identification of the terminal portion of the antenna as being NeuAc-GalNAc. At the same time, the ion \( m/z 1234.20^{+2} \) corroborated this deduction as it repres-
sents the parent ion without a terminal NeuAc-GalNAc unit. At the MS³ step of this analysis, after the loss of the first sialic acid unit, the fragmentation elucidated the two antennae compositions as being the same (NeuAc-GalNAc-GlcNAc), and the presence of ion m/z 866.36^+ and its further fragmentation showed that each antenna stayed linked at each core mannose unit (Fig. 8; supplementary Information).

**N-Glycans of B. cotiara Venom (group 3)—** The N-glycans of B. cotiara venom were the most distinct among the eight venoms analyzed in this study, as they share only four structures with those of group 1 (Jararaca Complex venoms), and two structures with group 2 (B. erythromelas, B. jararacussu, B. moojeni, and B. neuwiedi) venoms. The analysis of the N-glycans extracted from B. cotiara venom showed the presence of
22 different structures, which belong to the high mannose type (1 structure) or the hybrid/complex type (21 structures) (Fig. 9). Four of these structures (A1, B11, B43a, and B43b) were also detected in the venoms of the Jararaca Complex. As described for the analysis of the Jararaca Complex venoms, many structures present in B. cotiara venom have the sialic acid dimer and the lactosamine unit. However, as a distinct feature, a bisecting GlcNAc residue was identified in some structures (B14, B15, B18, B19, B20, B21, B22, B24, B42a, B42b, B49a, B50a, and B50b) (Fig. 9). Although in this study bisecting GlcNAc was only detected in B. cotiara venom, this type of structure was previously identified by Gowda et al. (36) in the metalloproteinases RVV-X, from Viperus russeli venom, and VAP2B from Crotaulus atrox venom, by Igarashi et al. (58).

The most abundant structure in this group is B22 (Fig. 10) and its abundance allowed a more detailed structural analysis by MS^n. At the MS^2 spectrum, as occurred in the polysialylated structures of group 1, the loss of the first terminal NeuAc unit was the source of the most abundant peaks. However, in this step it was possible to observe the ion m/z 759.28 and the ion m/z 490.20, indicating the presence of a fucose unit at the reduced reducing end. The MS^n spectrum also suggested the presence of the bisecting GlcNAc in structure B22, as observed by a neutral loss corresponding to a terminal HexNAc (259 Da). To proceed the structural analysis of B22 the ion m/z 1618.12 was selected for fragmentation. The MS^3 fragmentation proved the existence of two units of sialic acid dimer, as after the first loss the ion m/z 759.28 was still present in the spectrum and the ion of this neutral loss was also identified (m/z 1489.08).

For the elucidation of the bisected GlcNAc structure, the MS^2 spectra were analyzed and peaks corresponding to a neutral loss of 259 Da, corresponding to the b-type ion GlcNAc(1-ene), indicated the existence of a terminal GlcNAc residue (supplementary Information). The presence and fragmentation of m/z 1112 and m/z 852 were considered for validation. The analysis of structure B22 revealed the entire topology of the bisected GlcNAc structure (Fig. 11). Except for the presence of the bisected GlcNAc residue of B. cotiara venom, those that contain a sialic acid dimer show the same linkage type identified in the venoms of the Jararaca Complex (supplemental Fig. S1). Another common feature between the
FIG. 9. N-glycan structures released from glycoproteins present in the venom of B. cotiara.

FIG. 8. Spectra of MSⁿ analysis of structure B32 found in B. moojeni venom. On the left side is shown the structure of B32 N-glycan and the MS² spectrum of this structure. On right side is shown the same structure after one sialic acid loss (ion with m/z 1356.40⁻²) and its respective MS/MS spectrum. One antenna was chosen for representation of the neutral loss.

MS² of m/z 1036.8⁻³

MS³ of m/z 1036.8⁻³ _1356.40⁻²
Jararaca Complex and *B. cotiara* venom N-glycomes was the presence of NeuGc in some sialic acid dimers, in the two possible positions (NeuAc-NeuGc and NeuGc-NeuAc) (supplementary Information).

**Isomers of Structure A1**—The A1 structure belongs to the high mannose type and occurs in many glycoproteins, from different sources, and its isomers were detailed by Prien et al. (59). In our MSn analysis of A1, fragmentation in the MS2, as previously described by Prien et al. (59) and Zelanis et al. (47), revealed as the most intense ion the \( m/z 1302^{+3} \) (neutral loss of 293 Da from \( m/z 1595^{+1} \)) (supplementary Information). In the MS3 spectrum the most intense ion was \( m/z 1084^{+3} \), which corresponds to the loss of a terminal hexose (neutral loss of 218 Da). The spectra of MS4 and MS5 of structure A1 (supplemental Figs. S3 and S4) showed very similar fragmentation profiles between the eight venom N-glycomes. This finding suggests that the most abundant isomer is the same in these *Bothrops* venoms, although the existence of other less abundant isomers cannot be excluded.

**Cluster of All Identified Structures**—Fig. 12 shows a graphical visualization of the two hierarchical clusterings of the 52 identified N-glycan compositions considering for each pair (venom, structure) the presence or absence of a given unique composition. According to this, the venoms of the Jararaca Complex (*B. alcatraz*, *B. insularis*, and *B. jararaca*) formed a cluster distinct from the other cluster composed by *B. erythromelas*, *B. jararacussu*, *B. moojeni*, and *B. neuwiedi* venoms, whereas *B. cotiara* venom showed the most distinct N-glycomic composition.

**Bothrops Venom N-glycan Quantitative Analysis**—The N-glycan quantitative analysis was based on the comparison of the relative abundance of the structures between the venoms. In the group 1 of N-glycans, identified in the venoms of the Jararaca Complex (*B. alcatraz*, *B. insularis*, and *B. jararaca*), the structure B11 is the most abundant component in the three venom N-glycomes and was considered as the 100% abundance reference (Fig. 13A). The overlaid LC/MS profiles of these three venoms showed a perfect match of retention time of their N-glycan structures, confirming the observation that they have the same glycosylation pattern, as revealed by the qualitative analysis (supplemental Tables S1–S8). Interestingly, in the case of structures B10, B43a, and B43b, the separation of structural isomers was detected by the different retention times for the same mass. For structure B10, the
isomers can be related to the position of the terminal GlcNAc unit, for which three positions are possible: (1) linking to the same mannose of NeuAc-2,8-NeuAc-2,3-Gal-1,4-GlcNAc antenna; (2) linking to the other free core mannose; (3) in a bisecting position of the core. The structures B43a and B43b were described as structural isomers, which differ in the position of the NeuAc: the B43a form contains the sialic acid dimer and the B43b form has two monosialylated antennae. The mass corresponding to these two structures was found in double peaks, confirming the presence of these two isomers in the Jararaca Complex venoms (Fig. 13 A).

The comparison of the normalized areas of LC-MS profiles of the N-glycan structures of venoms of the Jararaca Complex (Table II) shows similar abundances, except for structures A2, B2, B4, B10, B46, and B47. For instance, the relative abundance of A2 structure is 3.5 times higher in B. alcatraz venom than B. insularis venom, whereas structure B10 is 3.3 times more abundant in B. jararaca venom than in B. insularis venom.

The overlaid of LC-MS profiles of N-glycans of group 2 (B. erythromelas, B. jararacussu B. moojeni, and B. neuwiedi venoms) was generated using all identified structures and confirmed the differences observed in the qualitative analysis of these venoms (Fig. 13B). The first observation from Fig. 9B is that these four venoms share the most abundant structures and that the quantitative differences are more prominent in this group of N-glycans than in those of the Jararaca Complex venoms (group 1). The analysis showed variable LC-MS/MS profiles except for the N-glycans of B. moojeni and B. neuwiedi venoms, which were very similar. The comparison of the normalized areas of LC-MS profiles of the N-glycan structures of these four venoms (Table III) showed that B32 is the most abundant structure of this N-glycan group. The data also revealed that structures B25, B26, B27, and B40 are present in more abundance in B. moojeni venom than in B. neuwiedi venom, even though these venoms share all structures identified in this study. Moreover, the structure B37 is present in significantly higher abundance in B. jararacussu venom.

Although the A1 structure was detected in all N-glycomes analyzed in this study, in the venoms of B. erythromelas, B. jararacussu, B. moojeni, and B. neuwiedi the relative abun-

**Fig. 11.** MS^n analysis performed with structure B22 present only in B. cotiara venom glycoproteins (the structure is shown on the top of the right side). The presence of a monocharged ion of m/z 1112^+1 represents the core with the N-bisecting GlcNAc without fucosylated reducing end, which was selected for fragmentation (A). B, Mass spectrum of fragmentation of m/z 852^+1 (ion m/z 1112^+1 after GlcNAc(1-ene) neutral loss (259 Da). C, Mass spectrum of fragmentation of m/z 648^+1 (ion m/z 852^+1 after mannose arm loss).
Fig. 12. *Bothrops* venom clustering according to the composition of N-glycans. Graphical visualization of the two hierarchical clusterings of the venom N-glycome characterization. For each venom, a given structure is either present (red) or absent (black).
Fig. 13. Overlaid LC-MS profiles of the N-glycans present in the venom glycoproteins of the Jararaca Complex (A), *B. erythromelas*, *B. jararacussu*, *B. moojeni*, and *B. neuwiedi* (B) and *B. cotiara* (C) and structures that have been assigned in the extracted ion chromatogram.
dance of A1 is much lower than in those of the Jararaca complex.

Compared with the other Bothrops venoms, the N-glycans of B. cotiara venom (group 3) showed a unique quantitative profile, although it shares four structures with those of the Jararaca Complex (Fig. 13C). Considering the area of the most abundant structure in this venom (B22) as a 100% reference, the abundance of the A1 structure would be around 27% (Table IV). Another interesting observation is the fact that some structures (B14, B15, B16, B19, B21, B42a, and B42b, B43a, and B43b) showed more than one retention time because of the presence of distinct structural isomers in B. cotiara venom, some of which were not identified in this study.

**DISCUSSION**

In this study, we approach glycan structural studies using two different MS related methods. First, an overview of probable structures established primarily based on biological inference that is rapid, automated and summarized in cartoons. Secondly, a more exacting approach of controlled multistage disassembly (MSn) to well known or synthetic fragments confirmed by spectral documentation. All stereo and structural isomers are resolved in this latter method. The stereo and structural isomers that are a common component of glycosylation are numerous and specific. A comprehensive understanding of glycosylation structure remains fundamental.

The molecular route of the N-glycosylation process in eukaryotes includes the N-glycan precursor synthesis followed by the transfer in a bloc to the Asn residue belonging to a polypeptide chain inside the ER and the glycan processing steps of the antennae inside ER and Golgi Complex until the secretion or membrane incorporation (28, 60). The process of N-glycosylation of reptile toxins is fully unknown, however a comparative transcriptomic survey on body tissues from B. jararaca indicated that the molecular mechanism probably follows the same rules described for other eukaryotic systems (49). The study showed the analysis of transcriptomes of B. jararaca organs, and transcripts identified in the venom gland encoded proteins involved in N-glycosylation process such as the enzyme STT3a, responsible for the transfer of the glycan precursor from the lipid to the Asn residue of the nascent polypeptide chain, and an alpha-sialyltransferase 8f transcript, suggesting that sialic acid dimers present in venom glycoproteins might have \( \alpha \)-anomeric configuration. This study also showed that the venom gland tissue contained a higher level of transcripts of the protein glycosylation pathway compared with other snake organs.

The diversification of N-glycan compositions revealed by our analyses of venoms from the Bothrops genus probably
B. jararaca characterization of the venom proteomes and glycoproteomes of
and B. alcatraz speciation of terrestrial venoms, their identical glycosylation patterns (group 1), re-
the evolutionary process. In the case of the Jararaca Complex reflects the divergent paths followed by each species during
the evolutionary process. In the case of the Jararaca Complex
venoms, their identical glycosylation patterns (group 1), re-
ferred in this study, can be a consequence of the recent speciation of terrestrial B. jara
caca, and the insular B. insularis and B. alcatraz snakes (61). This is agreement with the char-
acterization of the venom proteomes and glycoproteomes of B. jara
caca and B. insularis venoms, which showed very similar profiles (46). However, these species significantly diverge in their diet habits. Although B. jara
caca is considered a generalist species, for B. insularis birds are the major source of
food, and B. alcatraz apparently evolved paedomorphic feeding habits in that adults maintain the diet of juveniles, which consists of small ectothermic prey such as small lizards and amphibians and centipedes (62). Indeed, the close evolution-
ary relationship between these species and their diet-related differences suggest that the conservation of the venom carbohydrate repertoire of their venoms is likely not related to their feeding habits.

| Composition | Normalized area |
|-------------|-----------------|
|             | B. alcatraz | B. insularis | B. jararaca |
| A1          | 0.221       | 0.182       | 0.130       |
| A2          | 0.015       | 0.004       | 0.009       |
| B1          | -           | -           | -           |
| B3          | 0.015       | 0.024       | 0.020       |
| B5          | 0.005       | 0.004       | 0.003       |
| B6          | 0.011       | 0.015       | 0.014       |
| B7          | 0.011       | 0.017       | 0.013       |
| B8          | 0.018       | 0.014       | 0.026       |
| B9          | 0.087       | 0.099       | 0.089       |
| B10         | 0.029       | 0.015       | 0.050       |
| B11         | 0.372       | 0.395       | 0.402       |
| B12         | 0.028       | 0.034       | 0.042       |
| B13         | 0.034       | 0.022       | 0.027       |
| B43         | 0.068       | 0.084       | 0.085       |
| B44         | 0.006       | 0.004       | 0.005       |
| B45         | 0.013       | 0.011       | 0.013       |
| B2          | 0.003       | 0.003       | 0.001       |
| B46         | 0.063       | 0.068       | 0.068       |
| B47         | 0.002       | 0.004       | 0.001       |
| Total       | 1           | 1           | 1           |

- Indicates composition that was identified in the qualitative analysis but not in the quantitative analysis.

Interestingly, except for B. neuwiedi, which feeds only on mammals, B. moojeni, B. erythromelas and B. jararacussu are generalist species (62), and their N-glycome contents (group 2) showed some conservation, such as the antennae composed of NeuAc-GalNAc-GlcNAc, which were identified in 12 out of 13 common N-glycan structures. However, despite these common features, six nonsialylated structures were particular to some of these venoms, suggesting that they might be species-specific N-glycome features.

The clearly distinct profile of B. cotiara venom glycans (group 3) was characterized by the presence of bisecting GlcNAc residues. Interestingly, the introduction of GlcNAc in a β-(1–4)-linkage to the mannose residue at the base of the trimannosyl core of N-glycans suppresses the action of other glycosyltransferases, preventing elongation of N-glycan chains (63). Roles of the bisecting GlcNAc have been postulated to be related with the biological function of proteins involved in cell adhesion, migration, growth, and differentiation (64, 65). In the case of integrins, the presence of bisecting GlcNAc was related to the inhibition of integrin-mediated cell spreading, migration, and phosphorylation (64). The identification of N-glycans containing bisecting GlcNAc only in B. cotiara venom proteins needs further analysis regarding the functional role of this monosaccharide; however, the recently identified binding specificity of the mouse dendritic cell inhibitory receptor 2 to biantennary complex-type glycans containing bisecting GlcNAc (66) suggests that in this snake venom this sugar residue might participate in the modulation of the response from immune system cells.

In fact, concerning feeding habits, B. cotiara is a strictu sensu specialist, feeding only on mammals during its entire lifespan (62). In this regard, phylogenetic correlates of feeding habits in pitvipers of the genus Bothrops closely resemble our results on hierarchical clustering of N-glycan compositions. Feeding habits have long been associated to the variability in snake venoms and, in this context, toxin microheterogeneities such as N-linked glycans may be regarded as functional signatures of snake venom variability that would allow distinct species to deal with different prey types. This fine-tuning mechanism is both (1) parsimonal, as changing the glycan composition or N-glycosylation site occupancy helps to generate distinct glycoforms and (2) effective, as structural changes may result in new biological targets toward different prey types (47). However, a study on the characterization of the venom gland proteome of B. jararaca showed that glyco-
sylation-related proteins represented a small fraction (17%) of all proteins involved with PTMs that were detected by mass spectrometry in the tissue. Nevertheless, the diversity of glycoproteins in viperid snake venoms is huge, and therefore it is possible to conceive that the use of N-sequons and the extension of N-glycan chains are rather basic forms in the structural diversification of toxins and not influenced by diet. Hence, it is possible to hypothesize that on the venom evolu-
tion process the carbohydrate variability contributes more
in the differentiation between the species than the prey preference.

In general, the proteomic analyses of Bothrops venom proteomes result in the identification of around 80–150 proteins, depending on number of amino acid sequences that are available in the protein databases for each species; however, the number of spots observed on venom 2-D gels is usually over 200. Because each spot may contain more than one protein, the total number of unique proteins on a venom 2-D gel is far greater than that of unique proteins identified by LC-MS/MS analysis of tryptic peptides. This is clearly because of the various levels of glycosylation in snake venom glycoproteins, which affect protein isoelectric point and molecular mass, and act as a mechanism of variability, which has implications for the venom as a trait.

The presence of the same isomer of high mannose type (A1 structure) in such diverse sources of N-glycans suggests that this structure might play a fundamental role in protein folding and quality control inside the endoplasmic reticulum, or other general function not directly related to the protein biological activity. As such, a possible function for the high mannose type N-glycans was suggested to be to act as chaperone-like molecules and aid in the protein correct folding (67). The fact that the relative abundance of the A1 structure is higher in the venoms of the Jararaca complex (B. jararaca, B. insularis, and B. alcatraz) suggests that it may be important for acquisition and retention of functional conformation of proteins in these specific venom proteomes whereas in the other venoms the protein folding process may be less dependent on high-mannose type N-glycans.

In a previous work, a pattern of clustering was obtained by the analysis of the proteomes and of the subproteomes of lectin-binding proteins of the same Bothrops venoms (46), that is like the clustering of N-glycan compositions obtained in this study. Both clusters resemble the phylogeny cladograms of South American bothropoid snakes reported in studies on morphological, molecular data and feeding habits (62, 68). These data suggest that N-glycans may also play a role in shaping the signature of venom proteins in the Bothrops species sampled in this study and, despite the variable abundance of components of toxin families in these venoms, their contents of N-glycans seem to have evolved as traits that surpass venom variation and reflect the phylogenetic relationships among these species. Further, the results showing vari-

### Table III

Comparison of the normalized areas of LC-MS profiles of venom N-glycan compositions identified by the quantitative approach in the venoms of B. erythromelas, B. jararacussu, B. moojeni and B. neuwiedi

| Composition | B. erythromelas | B. jararacussu | B. moojeni | B. neuwiedi |
|-------------|-----------------|---------------|------------|-------------|
| A1          | 0.018           | 0.032         | 0.033      | 0.038       |
| A2          | x               | -             | -          | -           |
| B1          | x               | -             | -          | -           |
| B25         | x               | x             | 0.018      | 0.002       |
| B26         | 0.001           | x             | 0.028      | 0.001       |
| B27         | x               | x             | 0.018      | 0.003       |
| B28         | x               | -             | -          | -           |
| B29         | 0.016           | 0.009         | 0.014      | 0.024       |
| B30         | 0.133           | 0.103         | 0.147      | 0.116       |
| B31         | 0.013           | 0.016         | 0.017      | 0.038       |
| B32         | 0.453           | 0.501         | 0.359      | 0.488       |
| B33         | 0.013           | 0.017         | 0.021      | 0.014       |
| B34         | 0.174           | 0.170         | 0.165      | 0.162       |
| B35         | 0.046           | 0.018         | 0.017      | 0.017       |
| B36         | 0.056           | 0.043         | 0.016      | 0.028       |
| B37         | x               | 0.021         | 0.004      | -           |
| B38         | 0.045           | 0.051         | 0.039      | 0.050       |
| B39         |                 |               |            |             |
| B40         | 0.026           | 0.015         | 0.101      | 0.015       |
| B41         | 0.006           | 0.004         | -          | 0.003       |
| Total       | 1               | 1             | 1          | 1           |

- Indicates compositions that were identified in the qualitative analysis but not in the quantitative analysis.

x Indicates compositions not detected in venom in both qualitative and quantitative analyses.
lytic activity on casein was not affected by this treatment. These authors hypothesized that sialic acid units of bilitoxin-1 would play a role in positioning the toxin on the basement membrane of capillaries by interacting with selectins on endothelial cells (77).

As for the recognition of carbohydrates by lectins, it has been suggested that the existence of sialic acid dimers in toxins could be related to their targeting to specific sialic acid-binding lectins, the so-called Siglecs (sialic acid-binding Ig superfamily lectins) that have wide expression in immune cells, including natural killer cells (39, 43). The proximity of one disialylated oligosaccharide to the active site in an L-amino acid oxidase from Calloselasma rhodostoma venom was suggested to be important for its binding to Siglecs and the expression of its apoptotic activity (39). Moreover, the carbohydrate moiety of the snake venom serine proteinase autobin was suggested to play a role in the substrate accommodation inside its active site because a recombinant form of the protein, lacking disialylated N-glycans, generated different hydrolysis products than those of the native enzyme (44).

The presence of sialic acid units at the terminal end of glycan chains of proteins can however have a broader impact in the snake venom proteomes, as it may affect the net charge and, thus, the isoelectric point (pl) of proteins, as previously shown by 2-D electrophoresis that on removal of N-glycans many B. jararaca venom protein spots showed a shift toward the basic region of the 2-D gel (46). The same study provided evidence for the presence of glycan chains containing sialic acid in viperid venoms by the identification of many glycoproteins of seven Bothrops venoms that showed affinity to wheat germ agglutinin, whose carbohydrate recognition regions includes sialic acid and N-acetyl-glucosylamine (46). Previously, Nawarak et al. (41) had shown that proteins from ten venoms from the Viperidae and Elapidae families showed affinity for lectins with specificity for sialic acid. In the present study, the fact that 50 out of 60 different structures identified in Bothrops venom proteins contain at least one sialic acid at their non-reducing ends suggests that this monosaccharide likely play a role in the toxin-induced immune-response in mammalian envenomation involving Siglecs.

**CONCLUSIONS**

In this work we have assessed the qualitative and quantitative N-glycan compositions of eight Bothrops venoms, using LC-MS and MS^n. From the point of view of post translational modifications, the variability in Bothrops venoms as well as in their envenomation outcomes, may be considered an emerging feature derived from the puzzling pattern of N-glycan arrangements (compositions, structures and abundances) in glycoproteins. In this context, the combinatorial set of N-glycans that decorate venom toxins has a remarkable role in shaping protein microheterogeneities, which in turn

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**Table IV**

| Composition | Normalized area |
|-------------|----------------|
| A1          | 0.083          |
| B1          | -              |
| B11         | 0.040          |
| B15         | 0.030          |
| B16         | 0.010          |
| B17         | 0.020          |
| B18         | 0.046          |
| B19         | 0.146          |
| B20         | 0.143          |
| B21         | 0.081          |
| B22         | 0.306          |
| B23         | 0.005          |
| B24         | 0.017          |
| B49         | 0.034          |
| B50         |                |
| B14         | 0.014          |
| B42         |                |
| B43         | 0.024          |
| Total       | 1              |

- Indicates composition that was identified in the qualitative analysis but not in the quantitative analysis.
may influence several fundamental properties of snake venom toxins such as the interaction with biological targets in the prey. Because the most abundant toxin classes of Bothrops venoms are composed of glycosylated proteins (i.e. metalloproteinases and serine proteinases), the study of glycan composition in addition to the characterization of whole proteomes and glycoproteomes are important means for understanding the process of evolution of the complexity and variability of these venoms.

Because post-translational modifications such as glycosylation are key to ensure molecular diversity for protein scaffolds, we suggest that the subproteomes of glycoproteins mirror the phenotypic plasticity found in congeneric species evidencing distinct molecular signatures for each venom. On evolution, the use of the arsenal of N-glycan structural features including different degrees of branching, isomer structures, and variable abundances, snake venoms were endowed with another level of complexity that could dramatically impact their distinct biological activities. Hence, this proper fine-tuning mechanism (i.e. the presence of distinct glycan structures or different levels of protein glycosylation) would enable the toxins present in the venom of a given species to deal with different types of molecular targets, a paramount feature related to the survival of any venomous species.

DATA AVAILABILITY

Raw MS data are available at the MassIVE website (ftp://massive.ucsd.edu/MSV000081579).

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