JOC Article

# Glycolipids from Sponges. 20.[1] *J*-Coupling Analysis for Stereochemical Assignments in Furanosides: Structure Elucidation of Vesparioside B, a Glycosphingolipid from the Marine Sponge *Spheciospongia vesparia*

Valeria Costantino, Ernesto Fattorusso, Concetta Imperatore, and Alfonso Mangoni*

*Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli "Federico II", via D. Montesano 49, 80131 Napoli, Italy*

*alfonso.mangoni@unina.it*

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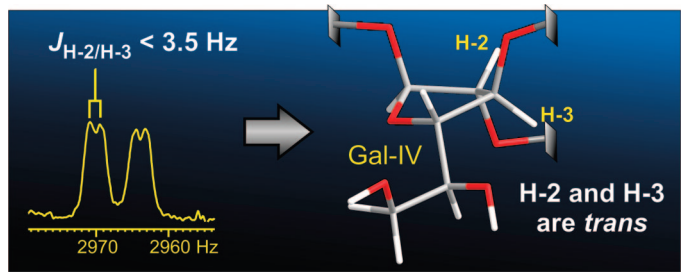


Reinvestigation of the glycosphingolipid composition of the marine sponge *Spheciospongia vesparia* revealed the presence of vesparioside B (**2a**), a new furanose-rich hexaglycosylated glycosphingolipid that is the most complex glycosphingolipid isolated from a marine sponge to date. The structure of the new compound was elucidated using extensive 2D NMR studies and chemical degradation. Particularly useful for structure elucidation of vesparioside B was a quantum mechanical computational study, showing that in furanosides a vicinal coupling constant <2.0 Hz (for H-1/H-2 or H-3/H-4) or <3.5 Hz (for H-2/H-3) is a proof of the *trans* orientation of the relevant protons. This general rule, combined with ROE data, allowed us to elucidate the relative stereochemistry (including anomeric configuration) of the three furanose five-membered rings.

Historically, structure elucidation of oligosaccharides (isolated as such or as part of glycolipids, glycopeptides, or glycoproteins) has been based on chemical means long after two-dimensional NMR methods for structure elucidation had become dominant, if not exclusive, for other classes of compounds. This was probably a consequence of the availability of a simple and effective degradation scheme for oligosaccharides, the Hakomori method,[2] and by the difficulties in dealing with the many overlapping signals that characterize the proton NMR spectra of even simple oligosaccharides. Only in the early 1990s, papers appeared in which NMR methods played a major role in the structure elucidation of carbohydrate chains. A decisive contribution to the development of these methods came from our research group, which since 1994 has been publishing a series of papers on structural elucidations of new glycolipids based on the extensive use of 2D NMR spectroscopy.[1]

Today, the structure of a pyranose sugar is routinely determined by 2D NMR methods, because *J*-coupling analysis provides an easy means for addressing stereochemical issues in a six-membered ring. However, when the sugar is in the furanose form, proton−proton coupling constants are considered to be of little use,[3] and chemical degradation (i.e., sugar analysis) and NOE measurements are generally used for stereochemical assignments.

In this paper we show that, under certain conditions, *J*-coupling values can provide unequivocal information about relative configurations in furanosides. This is demonstrated in the structure elucidation of vesparioside B (**2a**), a unique furanose-rich glycosphingolipid from the marine sponge *Spheciospongia vesparia*, which has previously been shown to contain the simpler diglycosylceramide vesparioside A (**1**).[4]

(1) Part 19: Costantino, V.; Fattorusso, E.; Imperatore, C.; Mangoni, A.; Teta, R. *Eur. J. Org. Chem.* **2008**, 2130–2134.
(2) (a) Hakomori, S. *J. Biochem.* **1964**, *55*, 205–208. (b) Sanford, P. A.; Conrad, H. E. *Biochemistry* **1966**, *5*, 1508–1517.
(3) Claridge, T. D. W. *High resolution NMR Techniques in Organic Chemistry*; Pergamon: Oxford, 1999.
(4) Costantino, V.; Fattorusso, E.; Imperatore, C.; Mangoni, A. *Eur. J. Org. Chem.* **2005**, 368–373.



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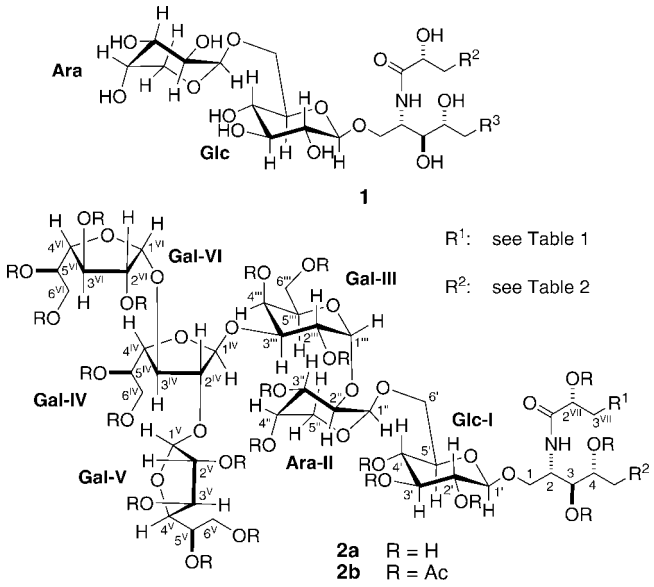
## Results and Discussion

*Spheciospongia vesparia* was collected in the Caribbean Sea (Bahamas Islands) and extracted in sequence with methanol and chloroform. The combined extracts were partitioned between BuOH and water, and the organic layer was subjected to reversed-phase and then normal-phase column chromatography to obtain a glycolipid fraction, which was acetylated with $Ac_2O$ in pyridine. After repeated HPLC separation on $SiO_2$ columns, pure vesparioside B peracetate (**2b**) was obtained. Finally, compound **2b** was deacetylated with MeONa/MeOH to give the natural glycosphingolipids vesparioside B (**2a**).




The ESI mass spectrum of vesparioside B (**2a**) gave a series of $[M + Na]^+$ pseudomolecular ion peaks at *m*/*z* 1620, 1634, 1648, 1662, and 1676, corresponding to the molecular formulas $C_{75}H_{139}NO_{34} + nCH_2$ ($n = 0-4$). This showed that vesparioside B, like most glycolipids of marine origin, is present in *S. vesparia* as a mixture of homologues. An exact mass measurement confirmed the molecular formula $C_{77}H_{143}NNaO_{34}$ for the ion at *m*/*z* 1648.9364. The region around $\delta$ 0.9 in the $^1$H NMR spectrum of **2a** ($CD_3OD$) contained signals of the terminal methyl groups of *n*-, iso-, and to a lesser extent, anteiso- alkyl chains, showing that the homologues also differed in alkyl chain branching. The general features of the proton spectrum of vesparioside B (**2a**) clearly resembled those of glycolipids, as illustrated by (a) the large band of alkyl chain methylene protons at $\delta$ 1.29, (b) six anomeric protons between $\delta$ 5.38 and 4.67, and (c) many overlapping oxymethine and oxymethylene protons between $\delta$ 4.33 and 3.19.

All subsequent NMR experiments directed to the structure elucidation were performed on the peracetate **2b**, to take advantage of the better signal dispersion in the proton spectrum of a peracetylated sugar chain and the possibility of identifying the glycosylation site of each sugar. The proton NMR spectrum ($C_6D_6$) of compound **2b** showed a $D_2O$ exchangeable doublet at $\delta$ 7.21 (NH-2), indicative of the NH proton of a ceramide. Using this proton as a starting point, analysis of the 2D TOCSY, COSY, ROESY and HSQC showed that the ceramide of vesparioside B is the one most commonly found in glycolipids from sponges and the same as in vesparioside A, i.e., composed of a trihydroxylated, saturated sphinganine and a 2-hydroxy fatty acid (Table S1 in Supporting Information).

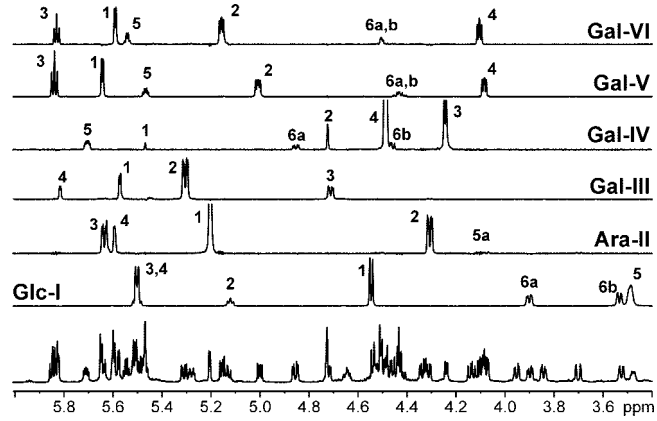



**FIGURE 1.** Sections of the *z*-filtered TOCSY spectra showing the six sugar spin-systems. Rows were extracted at $\delta$ 3.48 (H-5′) for Glc-I, 5.20 (H1″) for Ara-II, 5.31 (H-2‴) for Gal-III, 4.24 (H-3$^{IV}$) for Gal-IV, 5.00 (H-2$^{V}$) for Gal-V, and 5.16 (H-2$^{VI}$) for Gal-VI.

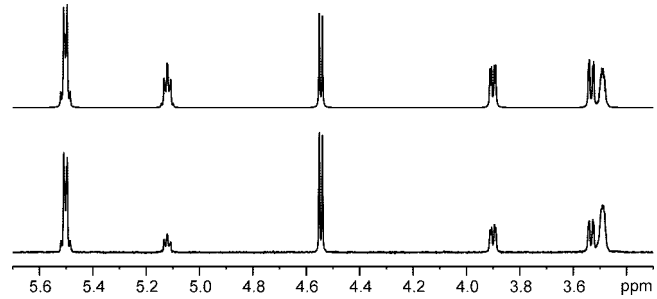



**FIGURE 2.** Simulated spectrum of Glc-I proton spin system (top trace) compared to the experimental spectrum obtained from the relevant section of the *z*-filtered TOCSY spectrum. Simulation parameters are $\delta$(H-1) 4.546, $\delta$(H-2) 5.121, $\delta$(H-3) 5.5037, $\delta$(H-4) 5.5003, $\delta$(H-5) 3.490, $\delta$(H-6a) 3.901, $\delta$(H6b) 3.533, $J$(H-1/H-2) = 8.1 Hz, $J$(H-2/H-3) = 9.7 Hz, $J$(H-3/H-4) = 9.7 Hz, $J$(H-4/H-5) = 9.7 Hz, $J$(H-5/H-6a) = 3.9 Hz, $J$(H-5/H-6b) = 2.6 Hz, $J$(H-6a/H-6b) = −11.0 Hz.

**Carbohydrate Chain: Pyranosides.** Elucidation of the structure of the hexasaccharide chain was more challenging for the severe signal overlapping and the presence of three sugars in the furanose form. Decisive information came from the *z*-filtered version of the TOCSY 2D NMR experiment.[5] In this sequence, the *z*-filter removes any antiphase dispersive component from the spectrum, leading to pure absorption lines. Figure 1 shows sections of the *z*-TOCSY experiment displaying subspectra of the six sugar spin-systems, which allowed us to identify the signals of each sugar.[6] The HSQC spectrum was then used to assign the anomeric proton of each sugar from its correlation peak with the respective anomeric carbon, and the COSY spectrum to determine the sequence of the protons within each sugar. In addition, the in-phase, pure absorption line shape of the multiplets in the slices of the TOCSY spectrum, together with the high digital resolution of the spectrum, allowed us an accurate measurement of all the vicinal proton−proton coupling constants.

The first sugar residue of the saccharide chain (Glc-I) was identified as a $\beta$-glucopyranoside. The shielded chemical shift

(5) Thrippleton, M. J.; Keeler, J. *Angew. Chem., Int. Ed.* **2003**, *42*, 3938–3941.

(6) All sugar protons could be identified in this way except for the proton at C-5‴ and the two protons at C-6‴ belonging to Gal-I, which did not appear in the TOCSY section shown in Figure 1 because of the small coupling constant between H-4‴ and H-5‴. However, a weak but distinct correlation peak between these two protons was present in the COSY spectrum, which allowed to complete the assignment of the spin system.

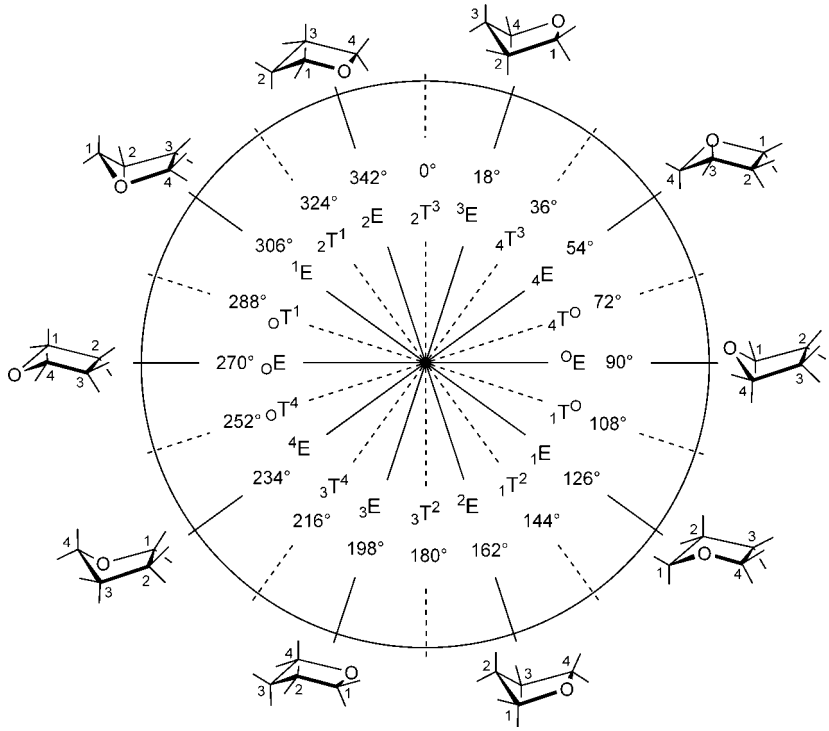



**FIGURE 3.** Pseudorotation wheel showing the 10 envelope conformations of the furanose ring. Each point on the circle represents a specific value of the pseudorotation angle.

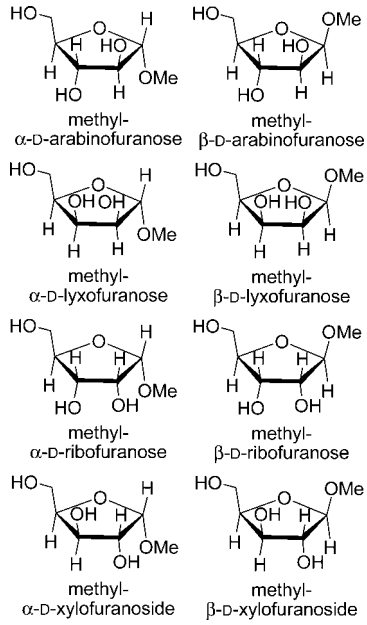



**FIGURE 4.** The eight diasteromeric methyl pentofuranosides.

of H-5′ indicated a pyranose ring, while the large coupling constant (8.1 Hz) of the anomeric proton was indicative of its axial orientation. Direct measurement of the remaining coupling constants of the ring protons was not possible, because H-3′ and H-4′ showed exactly the same chemical shift, leading to a non-first-order spin system. Even so, however, the pure-absorption lineshapes of the signals in the TOCSY subspectrum allowed us to determine NMR parameters of the spin system by spectral simulation (Figure 2). The large coupling constants between each vicinal pair of ring protons clearly indicated that all ring proton are axial. The HMBC spectrum showed that this sugar is linked to the sphinganine O-1 (correlation peak between H-1′ and C-1), and glycosylated at O-6′ (correlation peak between H-1″ and C-6′).

The second sugar residue (Ara-II) contains four methine and one methylene group, suggesting a pentose. The high-field chemical shift of H-5″ and the HMBC correlation peak between this proton and C-1″ indicated a pentopyranose. Coupling constants (see Table S1 in Supporting Information) showed that H-2″ and H-3″ are axial, while H-1″ and H-4″ are equatorial. This sugar residue is therefore a $\beta$-arabinopyranoside. The shielded chemical shift of H-2″ suggested glycosylation at this position, which was confirmed by the HMBC correlation peak of H-1‴ with C-2″.

The third and last pyranose sugar residue (Gal-III) is an α-galactopyranoside. The pattern of coupling constants (Table S1 in Supporting Information) showed the axial orientation of H-2‴ and H-3‴, and the equatorial orientation of H-1‴ and H-4‴. As for H-5‴, it showed a strong ROESY correlation peak with H-3‴, and is therefore axial. Finally, this sugar is glycosylated at position 3, as shown by the chemical shift of H-3‴ ($\delta$ 4.72) and by the HMBC correlations between H-$1^{IV}$ and C-3‴ and between H-3‴ and C-$1^{IV}$.

**Furanosides: Coupling Constant Computational Study.** The successful use of coupling constant information for stereochemical assignment in pyranosides is based on the conformationally rigid six-membered ring they contain. In addition, for most pyranosides, one of the two possible chair conformations is largely predominant at room temperature, allowing an easy discrimination between the large axial–axial couplings and the small axial−equatorial and equatorial–equatorial couplings. This is not the case for furanosides, where the flexibility of the five-membered ring (leading to the so-called *pseudorotation*) causes large variations in coupling constants between conformers, which prevent such an easy correlation between coupling constant values and relative configuration of adjacent carbon atoms. As a consequence, in our previous work (ref 1 and

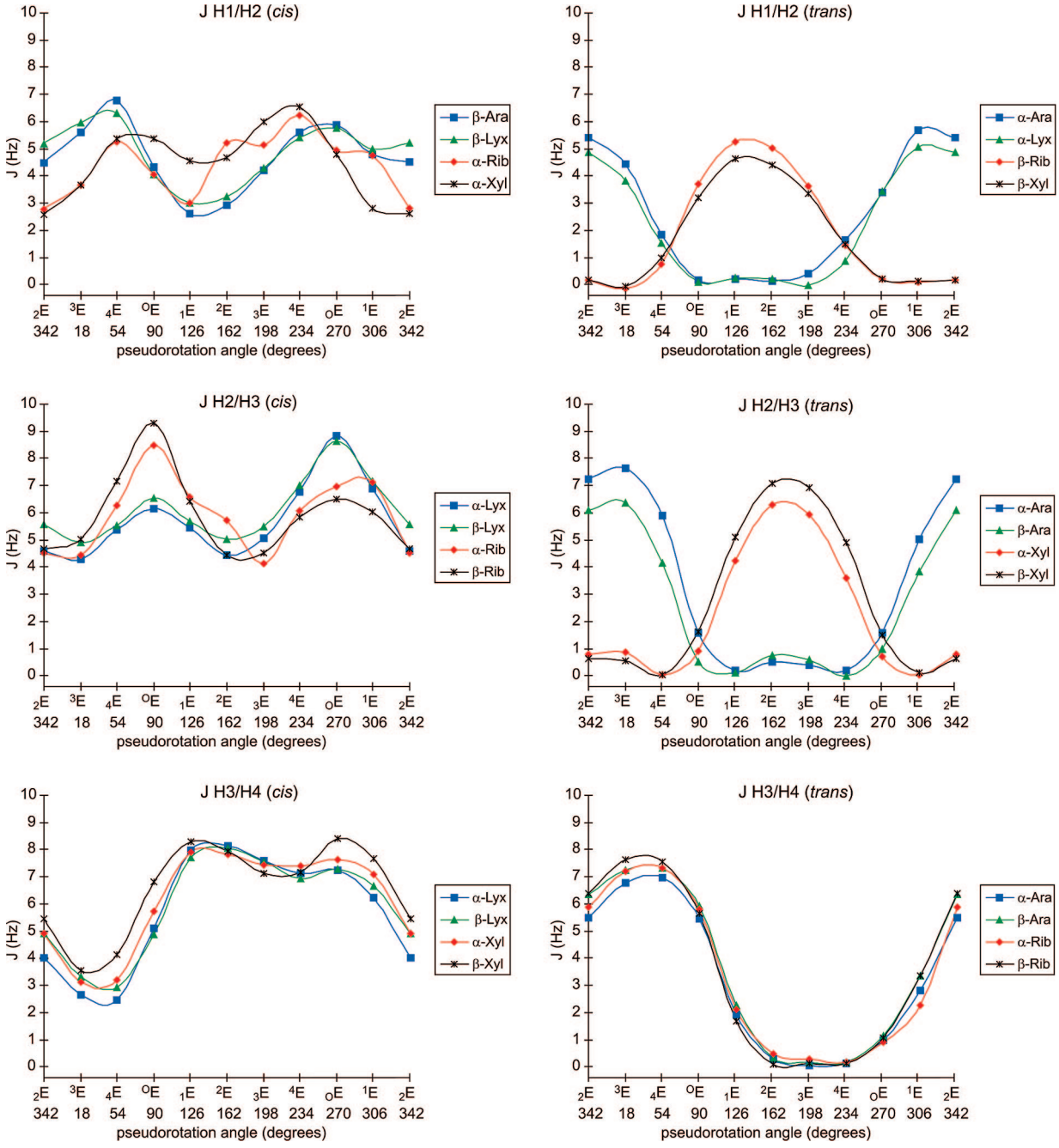



**FIGURE 5.** Vicinal coupling constants versus pseudorotation angle for the eight diastereomeric methyl pentofuranosides calculated at the mPW1PW91/6-311+G(d,p) level. It is apparent that coupling constants between *cis* vicinal protons are never lower than 2.5 Hz for the H-1/H-2 and H-3/H-4 couplings or 4.0 Hz for H-2/H-3 coupling.

previous papers in the series), stereochemical elucidation of furanosides was entirely based on ROESY correlations and/or chemical degradation.

As a matter of fact, some correlation between vicinal couplings and relative configuration of furanosides is present in the literature. As early as in 1963, in a paper reporting the proton NMR spectra of all methyl pentofuranosides,[7] it was clearly recognized that a small vicinal coupling in a furanoside implies that the coupled protons are *trans* oriented. However, this principle has been used in the subsequent structure elucidation work only sporadically and even then in a way that resembles much more the empirical comparison with a model compound than the application of a general rule. The reason for this is probably that the threshold below which a coupling constant can be considered "small" has never been defined, making the practical application of this principle difficult.

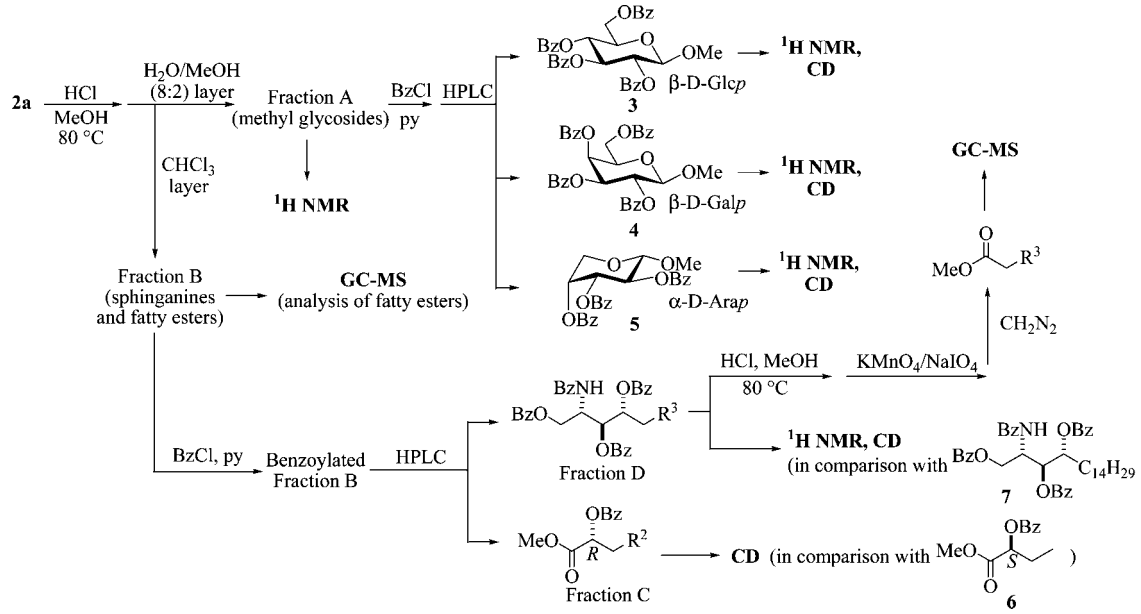
To clarify this point, we undertook an in-depth computational study of coupling constants of furanosides. Coupling constant analysis has been widely used in NMR studies of oligonucleotides[8] to gain information on the conformation of the deoxyribose or ribose rings. Even though these studies were directed

(7) Rinehart, K. L.; Chilton, W. S.; Hichens, M.; Von Phillipsborn, W. *J. Am. Chem. Soc.* **1963**, *84*, 3216–3218.

(8) Wuthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.

(9) Altona, C.; Sundaralingam, M. *J. Am. Chem. Soc.* **1972**, *94*, 8205–8212.

**SCHEME 1. Microscale Degradation Analysis of Vesparioside B (2a)**




to *conformational* rather than *configurational* assignment (the configuration of ribose being known a priori), they clearly showed that coupling constants between vicinal ring protons can be reliably predicted as a function of the single parameter, the *pseudorotation angle*, which is used to describe the pucker of the ring (Figure 3).[9]

Therefore, we calculated systematically the dependence of the vicinal proton−proton coupling constants on the pseudorotation angle for each of the eight diasteromeric pentofuranosides (the presence of the additional $CH_2OH$ of hexoses has a negligible influence of the conformation of the five-membered ring) using quantum mechanical DFT methods, which have demonstrated a great efficiency in the calculation of the $^1H-^1H$ spin−spin couplings, with mean absolute errors lower than 0.5 Hz.[10] Among DFT methods, mPW1PW91[11] has been shown to provide good results[10] when used with basis sets of moderate size such as 6-31G(d) and an increased accuracy[12] when used with higher basis sets such as 6-311G(d,p) or 6-311G+(d,p).

Each sugar was modeled as the respective α- or $\beta$-methyl glycofuranosides (Figure 4), and the 10 possible envelope conformations of each sugar (Figure 3) were generated by molecular mechanics by restraining to zero the C1−C2−C3−C4 (for the $^O$E and $_O$E conformation), the C2−C3−C4−O (for $^1$E and $_1$E), the C3−C4−O−C1 (for $^2$E and $_2$E), the C4−O−C1−C2 (for $^3$E and $_3$E), and the O−C1−C2−C3 (for $^4$E and $_4$E) dihedral angles. Each structure generated in this way was optimized by DFT quantum-mechanical calculations using the Gaussian 03 program[13] at the mPW1PW91/6-31G(d) level, and the coupling constants between H-1 and H-2, H-2 and H-3, and H-3 and H-4 were calculated using the same DFT functional and the 6-311G+(d,p) basis set.

When examining the calculated coupling constants (Figure 5), it was immediately apparent that while coupling constants between *trans* vicinal protons vary over a very broad range of values (0−8 Hz), coupling constants between *cis* vicinal protons are never smaller than 2.5 Hz. This trend is related to the fact that, in the furanose rings, dihedral angles that are geometrically accessible to *cis* vicinal protons are in the range between −45° and +45° and therefore cannot reach the values close to 90° that would imply small coupling constants. In contrast, dihedral angles between *trans* vicinal proton are in the range between 85° and 165°, so that both very large and very small couplings can be observed between these protons, depending on the conformation adopted by the sugar.

More in detail, our calculations show that, when H-1 and H-2 are *cis* oriented, the calculated H-1/H-2 coupling is never lower than 2.5 Hz. Therefore, even allowing for some error in the calculation, it is safe to assign H-1 and H-2 as *trans* whenever their coupling constant is lower than 2 Hz. For the H-2/H-3 coupling, the situation is even better, because when they are *cis* oriented the lowest calculated coupling constant is as large as 4.0 Hz, so any coupling smaller than 3.5 Hz means that H-2 and H-3 are *trans*. For the H-3/H-4 coupling, the results are similar to those for H-1/H-2 coupling, and the threshold is again 2 Hz.

In summary, whenever a furanoside vicinal coupling constant is <2 Hz (for H-1/H-2 or H-3/H-4) or <3.5 Hz (for H-2/H-3), the relevant protons are *trans* oriented. Of course, this rule does not work the other way round, and a coupling above the threshold does not provide any information about the stereochemistry of the coupled protons.

It must be noted that this is *not* an empirical rule, because it does not rely on a particular conformation of the furanoside

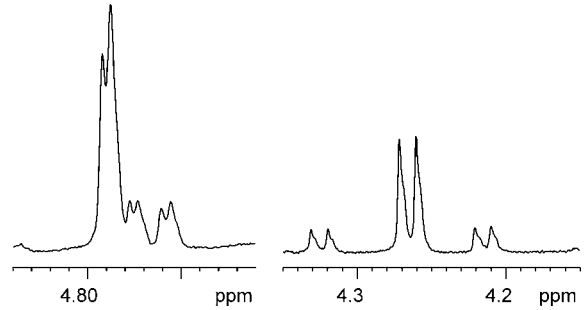



**FIGURE 6.** Anomeric proton regions of the $^1$H NMR spectrum of the methyl glycoside mixture from vesparioside B.

(10) Bassarello, C.; Cimino, P.; Gomez-Paloma, L.; Riccio, R.; Bifulco, G. *Tetrahedron* **2003**, *59*, 9555–9562.
(11) Adamo, C.; Barone, V. *J. Chem. Phys.* **1998**, *108*, 664–675.
(12) Bifulco, G.; Mangoni, A. *Magn. Reson. Chem.* **2008**, *46*, 199–201.

**TABLE 1. Fatty Acyl Composition of 2a**

| fatty acid methyl ester | % |
|---|---|
| methyl 2-hydroxytetracosane (*n*-$C_{24}$) | 64.5 |
| methyl 2-hydroxypentacosane (*n*-$C_{25}$) | 35.5 |

**TABLE 2. Sphinganine Composition of 2a**

| sphinganine | % |
|---|---|
| (2*S*,3*S*,4*R*)-2-amino-1,3,4-hexadecanetriol (*n*-$C_{16}$) | 14.3 |
| (2*S*,3*S*,4*R*)-2-amino-15-methyl-1,3,4-hexadecanetriol (*iso*-$C_{17}$) | 14.3 |
| (2*S*,3*S*,4*R*)-2-amino-14-methyl-1,3,4-hexadecanetriol (*anteiso*-$C_{17}$) | 8.0 |
| (2*S*,3*S*,4*R*)-2-amino-1,3,4-octadecanetriol (*n*-$C_{18}$) | 44.7 |
| (2*S*,3*S*,4*R*)-2-amino-17-methyl-1,3,4-octadecanetriol (*iso*-$C_{19}$) | 18.6 |

but is based on the geometrical behavior of five-membered rings; for the same reason, the rule can most likely be extended to cover five-membered carbocycles. We are currently evaluating this hypothesis.

**Furanosides: Structure Elucidation.** The sugar glycosylating Gal-III at position 3 (Gal-IV) is a hexofuranoside, as shown by the presence of five methine and two methylene protons in the relevant spin system and by the HMBC correlation peaks of H-$1^{IV}$ with C-3‴ and C-$4^{IV}$. In addition, the high-field chemical shift of H-$2^{IV}$ ($\delta$ 4.73) and H-$3^{IV}$ ($\delta$ 4.24), along with the HMBC correlation peaks of these protons with, respectively, C-$1^{V}$ and C-$1^{VI}$ showed that this sugar is further glycosylated at positions 3 and 4.

Determining the relative configuration of the ring gave us the occasion to test and validate the rule, discussed in the previous section, about coupling constants of furanosides. In fact, coupling constants between H-$1^{IV}$ and H-$2^{IV}$ and between H-$2^{IV}$ and H-$3^{IV}$ were small (<1 and 1.3 Hz, respectively), and according to our rule this indicated the *trans* relationship between the two pairs of protons. In contrast, the coupling constant between H-$3^{IV}$ and H-$4^{IV}$ was 6.1 Hz, therefore providing no information about the relative configuration of C-$3^{IV}$ and C-$4^{IV}$. Fortunately, the ROESY correlation peak between H-$2^{IV}$ and H-$4^{IV}$ showed these protons to be on the same face of the five-membered ring; this was confirmed by the ROE between H-$3^{IV}$ and H-$5^{IV}$. On the basis of these data and considering that the configuration at C-5 (which is not part of the five-membered ring) could not be established spectroscopically, this sugar residue could be either $\beta$-galactofuranoside or its C-5 epimer α-altrofuranoside. Chemical degradation (see below) showed the former to be the case.

The fifth sugar residue (Gal-V), glycosylating Gal-IV at position 2, was also a hexose in the furanose form (HMBC correlation between H-$1^{V}$ and C-$4^{V}$), which was not further glycosylated (chemical shift of H-$2^{V}$, H-$3^{V}$, and H-$5^{V}$ were all above $\delta$ 5). For this sugar, coupling constants between vicinal ring protons were all greater than 4.5 Hz and therefore not informative. Relative configuration at the ring carbons, but not that at C-5, was determined on the basis of the ROESY correlations of H-$4^{V}$ with H-$1^{V}$ and H-$2^{V}$, and of H-$3^{V}$ with H-$5^{V}$, and the sugar was shown to be an α-galactofuranoside and not a $\beta$-altrofuranoside by chemical degradation.

Finally, all NMR data ($^1$H and $^{13}$C chemical shifts, $^1$H multiplicities, and ROESY correlation peaks) of the last sugar (Gal VI) were nearly identical to those of Gal-V (see Table S1 in Supporting Information), thus showing that also Gal-VI is an α-galactofuranoside.

The $^1$H, 13C, COSY, TOCSY, HSQC, and HMBC NMR spectra of the natural (nonacetylated) vesparioside B **2a** were then recorded, and assigned on the basis of the structure so far determined for its peracetyl derivative **2b**.

**Chemical Degradation: Alkyl Chains and Absolute Configuration.** All of the other information needed to complete structure elucidation came from degradation analysis, according to the procedure in use in our laboratory (Scheme 1). Briefly, a small amount (300 $\mu$g) of the glycolipid **2a** was subjected to acidic methanolysis with 1 M HCl in 92% MeOH. The resulting mixture, composed of fatty acid methyl esters, sphinganines, and methyl glycosides, was partitioned between $CHCl_3$ and $H_2O$/MeOH (8:2). The aqueous layer (fraction A) containing methyl glycosides was dried, and the $^1$H NMR spectrum in $D_2O$ of the residue was recorded (Figure 6).

The spectrum contained three doublets in a 4:1:1 ratio at $\delta$ 4.79, 4.78, and 4.76, identified as the anomeric protons of, respectively, methyl α-galactopyranoside, methyl $\beta$-arabinopyranoside, and methyl α-glucopyranoside, and three doublets in a 1:4:1 ratio at $\delta$ 4.32, 4.26, and 4.21, identified as the anomeric protons of, respectively, methyl $\beta$-glucopyranoside, methyl $\beta$-galactopyranoside, and methyl α-arabinopyranoside, by comparison with the proton spectrum of authentic samples. These data showed that the three furanose sugar residues, whose structure could not be completely defined by NMR spectroscopy were, in fact, all galactofuranosides.

The absolute configuration of all of the sugars was determined by converting the methyl glycosides into the respective perbenzoyl derivatives. CD spectra of perbenzoylated sugars show strong bisignate Cotton effects arising from exciton coupling[14] of the benzoyl chromophores introduced in the molecules, and nice CD spectra can be recorded with only a few micrograms of these derivatives. Therefore, the methyl glycoside fraction was subjected to perbenzoylation, and the methyl glycosides **3**, **4**, and **5** were obtained in the pure form by normal-phase HPLC (Scheme 1).[15] Their CD spectra matched those of the D enantiomer of, respectively, methyl tetra-*O*-benzoyl-$\beta$-glucopyranoside, tetra-*O*-benzoyl-$\beta$- galactopyranoside, and tri-*O*-benzoyl-α-arabinopyranoside.

The $CHCl_3$ layer from the methanolysis (fraction B) was analyzed by GC-MS to identify the fatty acid methyl esters present in the mixture (Table 1) and then perbenzoylated and separated by HPLC, to give a fraction composed of methyl α-benzoyl fatty esters (fraction C) and a fraction composed of perbenzoylated sphinganines (fraction D). The CD spectrum of fraction C was used to determine the absolute configuration of the α-hydroxy fatty acids of vesparioside as *R*, and the $^1$H NMR

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(15) The perbenzoylated α-glycosides of glucose and galactose showed retention times very close to each other under the condition used, so we preferred to analyze the respective $\beta$-glycosides, which were less abundant but easier to separate.

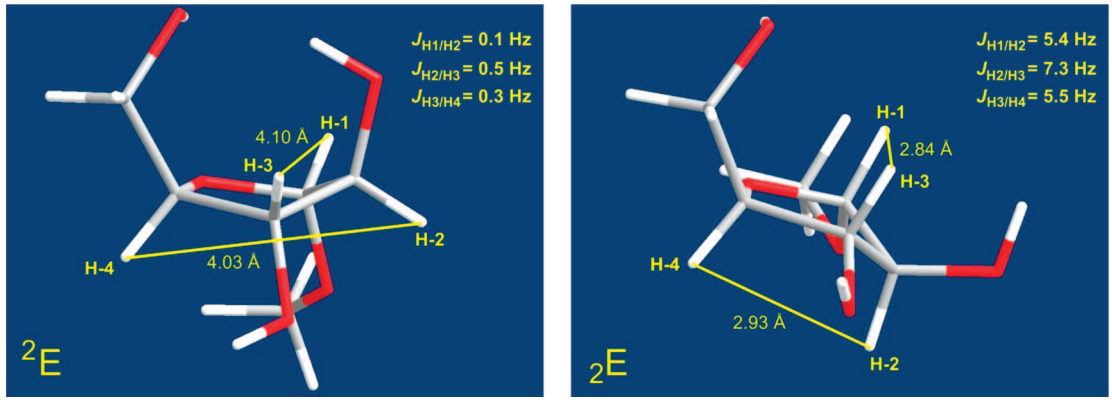


**FIGURE 7.** Selected coupling constants and interatomic distances for the $^2$E (left) and $_2$E (right) conformations of methyl α-D-arabinofuranoside.

and CD spectra of fraction D to determine the relative and absolute configuration of the sphinganines present in vesparioside B as D-*ribo*. Finally, fraction D was again subjected to acidic methanolysis to remove the benzoyl groups and subjected to Lemieux oxidation to convert sphinganines in fatty acids with three less carbon atoms. The fatty acids were converted in methyl esters with $CH_2N_2$ and analyzed by GC-MS, to determine the length and branching of the alkyl chains of the sphinganines of vesparioside B **2a** (Table 2), thus completing its structural elucidation.

## Conclusion

The structure of the furanose-rich glycosphingolipid vesparioside B, the most complex glycosphingolipid isolated from a sponge to date, was successfully determined through combined NMR analysis and chemical degradation. In particular, vicinal proton−proton *J*-couplings were shown to provide useful information on the relative stereochemistry of the furanosides, which combined with NOE data allowed us to fully elucidate the relative stereochemistry (including anomeric configuration) of the three furanose five-membered rings.

Coupling constant analysis (as a proof of 1,2-*trans* relationships) and NOE (as a proof of 1,3-*cis* relationships) appear indeed complementary techniques for structure elucidation of sugar in the furanose form. In fact, in many cases, the conformations in which coupling constants between *trans* protons are diagnostic are those in which NOE is not useful for stereochemical assignments and vice versa. The most striking example of this are the $^2$E and $_2$E conformations of methyl α-D-arabinofuranoside, which are on opposite sides on the pseudorotation wheel. In the $^2$E conformation, the H-1/H-2, H-2/H-3, and H-3/H-4 coupling constants are all close to 0 and are therefore diagnostic; in contrast, protons in the 1,3-*cis* relationship are very far apart (the H-1/H-3 distance is 4.10 Å and the H-2/H-4 distance is 4.03 Å, compared to the 3.98 Å distance between the *trans* H-1 and H-4 protons), and therefore NOE cannot provide any useful information (Figure 7). In contrast, in the $_2$E conformation, vicinal coupling constants are all large and nondiagnostic, while the H-1/H-3 and H-2/H-4 distances are, respectively, only 2.84 and 2.93 Å.

## Experimental Section

**Collection, Extraction, and Isolation.** Specimens of *Sphecio-spongia vesparia* were collected in the summer of 2000 along the coast of Grand Bahamas Island (Bahamas) and identified by Prof. M. Pansini (University of Genoa). They were frozen immediately after collection and kept frozen until extraction. The sponge (220 g of dry weight after extraction) was homogenized and extracted with methanol (3 × 1 L) and then with chloroform (3 × 1 L), and the glycolipid fraction (820 mg) was isolated as described.[4] This was peracetylated with $Ac_2O$ in pyridine at 25 °C for 12 h. The acetylated glycolipids were subjected to HPLC separation on an $SiO_2$ column [eluent, *n*-hexane/EtOAc (2:8)], thus affording a mixture (32 mg) containing **2b** and other glycolipids. Pure vesparioside B peracetate **2b** (5.5 mg) was obtained after further normal-phase HPLC purification [eluent, *n*-hexane/*i*-PrOH (8:2)].

**Vesparioside B Peracetate (2b).** The peracetylated derivative **2b** $[\alpha]^{25}_D = +25$ (*c* 0.3 in $CHCl_3$) was obtained as a colorless oil. $^1$H and $^{13}$C NMR: Table S1 in Supporting Information. Composition in fatty acids: Table 1. Composition in sphinganines: Table 2.

**Deacetylation of 2b.** Compound **2b** (1.9 mg) was dissolved in 950 μL of MeOH, and 50 μL of a 0.4 M solution of MeONa in MeOH was added. The reaction was allowed to proceed for 18 h at 25 °C, the reaction mixture was dried under nitrogen, and the product was dissolved in water (3 mL) and extracted three times with BuOH (3 mL). The organic layer was dried, giving 1.0 mg of the native vesparioside B **2a**.

**Vesparioside B (2a).** White solid, $[\alpha]^{25}_D = +5$ (*c* 0.1 in MeOH); HRESIMS (positive ion mode, MeOH) *m/z* 1648.9364 ($[M + Na]^+$, $C_{77}H_{143}NNaO_{34}$ gives 1648.9389); ESIMS (positive ion mode, MeOH) *m/z* 1676, 1662, 1648, 1634, 1620 ($[M + Na]^+$ series). $^1$H and $^{13}$C NMR: Table S1 in Supporting Information. Composition in fatty acids: Table 1. Composition in sphinganines: Table 2.

**Methanolysis of 2a.** A small amount (300 μg) of **2a** was dissolved in 1 N HCl in 91% MeOH (1 mL), and the obtained solution was kept for about 12 h at 70 °C in a sealed tube. The reaction mixture was dried under nitrogen and partitioned between $CHCl_3$ and $H_2O$/MeOH (8:2). The aqueous layer was concentrated to give mixture of methyl glycosides (fraction A), whereas the organic layer contained a mixture of α-hydroxy acid methyl esters and sphinganines (fraction B).

**Absolute Stereochemistry of Methyl Glycosides from Compound 2a.** Fraction A from methanolysis of compound **2a** was benzoylated with benzoyl chloride (20 μL) in pyridine (200 μL) at 25 °C for 16 h. The reaction was then quenched with MeOH and after 30 min was dried under nitrogen. Methyl benzoate was removed by keeping the residue under vacuum for 24 h with an oil pump. The residue was purified by HPLC (column, Luna $SiO_2$, 5 μm; eluent, *n*-hexane/*i*-PrOH 99.5:0.5, flow 1 mL/min). Three peaks were collected, containing methyl tetra-*O*-benzoyl-β-D-glucopyranoside (**3**, $t_R$ = 28.8 min),[4] tetra-*O*-benzoyl-β-D-galactopyranoside (**4**, $t_R$ = 30.7 min),[16] and tri-*O*-benzoyl-α-D-arabinopyranoside (**5**,

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$t_R$ = 31.8 min).[4] All of the compounds were identified by a comparison of their $^1$H NMR and CD spectra with those reported.

**Analysis of Fraction B.** Fraction B from methanolysis of compounds **2a** was benzoylated as described above, and the crude of reaction was purified by HPLC (column, Luna $SiO_2$, 5 $\mu$m; eluent, *n*-hexane/*i*-PrOH 99.5:0.5, flow 1 mL/min). The chromatogram contained two peaks, which were identified as a mixture of homologues (*R*)-2-benzoyloxy fatty acid methyl esters (fraction C, $t_R$ = 4.2 min) and a mixture of perbenzoylated D-*ribo*-phytosphingosines (fraction D, $t_R$ = 10.4 min) by a comparison of their respective $^1$H NMR and CD spectra with those reported.[4]

**Oxidative Cleavage and GC-MS Analysis of Sphinganines.** Fraction D was debenzoylated by acidic methanolysis as described above and subjected to oxidative cleavage with $KMnO_4$/$NaIO_4$ as described,[17] and the resulting carboxylic acids were methylated with $CH_2N_2$. The obtained esters were analyzed by GC-MS, and the results are compiled in Table 2, expressed in terms of original sphinganines.

**Computational Details.** MM calculations were performed using the CVFF force field[18] and the INSIGHT II/Discover package.[19] Starting coordinates of the methyl pentofuranosides were taken from X-ray structures when available[20,21] or, for methyl α-ribofuranoside, $\beta$-lyxofuranoside, and $\beta$-xylofuranoside, from the respective $\beta$-, α-, and α-anomers by inverting the configuration of C-1.

The $^O$E and $_O$E conformers of each furanoside were generated by minimizing energy of the relevant starting structure while restraining the dihedral angle C1—C2—C3—C4 to zero, the O—C1—C2—C3 angle between −10° and −40° (for $^O$E) or +10° and +40° (for $_O$E), and the C2—C3—C4—O angle between +10° and +40° (for $^O$E) or −10° and −40° (for $_O$E). The remaining conformers were generated similarly.

Structures from molecular mechanics were then optimized at the DFT level using the Gaussian03W package.[13] Optimizations of the envelope conformers were performed at the mPW1PW91/6-31G(d) level, freezing to zero the relevant dihedral angle (C1—C2—C3—C4 for the $^O$E and $_O$E conformers, C2—C3—C4—O for the $^1$E and $_1$E conformers, and so on). Finally, the coupling constant calculations on the optimized geometries were performed using the mPW1PW91 functional and the 6-311G+(d,p) basis set. Cartesian coordinates of the final structures and calculated coupling constants for each structure are reported in Supporting Information.

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**Supporting Information Available:** General experimental procedures. Table S1 with NMR data of compounds **2a** and **2b**. $^1$H NMR spectrum, HMQC NMR spectrum, and ESI MS spectrum of vesparioside B (**2a**). $^1$H and $^{13}$C NMR and COSY, TOCSY, ROESY, HMQC, and HMBC 2D NMR spectra of peracetyated vesparioside B (**2b**). $^1$H NMR and CD spectra of compounds **3**, **4**, and**5.** Cartesian coordinates of the optimized envelope conformers of pentopyranosides. Calculated $^1$H−$^1$H coupling constants of methyl pentofuranosides. This material is available free of charge via the Internet at http://pubs.acs.org.

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