Pergamon

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Tetrahedron Letters 44 (2003) 3513–3516

TETRAHEDRON LETTERS

Chemoenzymatic synthesis of [3,9-^{13}C]-labeled NeuAc and KDN

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Received 22 January 2003; revised 5 March 2003; accepted 7 March 2003

Abstract—The chemoenzymatic synthesis of ^{13}C-labeled sialic acid (NeuAc) and 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (KDN) as useful molecular probes for studying the conformation of sialyl or KDN oligosaccharides attached to proteins was performed by using [6-^{13}C]-ManNAc, [6-^{13}C]-Man and [3-^{13}C]-pyruvic acid sodium salt. In the synthesis of the compounds, 5,6-anhydro intermediates were found to easily provide not only 6-^{13}C-labeled but also 5-, and 6-modified NeuAc and KDN analogs. Furthermore, it was demonstrated that identical results are obtained by NMR for both [3,9-^{13}C]-NeuAc and 1:1 mixtures of [3-^{13}C]- and [9-^{13}C]-NeuAc.

Sialyl and KDN[1] glycoconjugates are important cell surface components active in a variety of intercellular recognition events. Therefore, a study of the conformation and dynamics of these sialyl and KDN oligosacharides and their analogs should provide useful insights into how these cell surface oligosaccharides interact with the corresponding receptor molecules. However, although the conformational properties[2,3,4a–c] of sialyloligosaccharide analogs of low molecular weight have been reported by many research groups, the conformation and dynamics of sialyl-oligosaccharides and their analogs attached to glycoproteins have not yet been fully analyzed. To address this problem, ^{13}C-labeled NeuAc has been utilized for the conformational analysis of sialyloligosaccharides on artificial membrane surfaces[5a,b] and for TRNOE experiments.[6] On the other hand, Kajihara et al. developed a concise [3-^{13}C]-labeling method for NeuAc analogs, and reported their preliminary results[2] for the ^{13}C-labeled sialyloligosaccharide, 9-deoxy-9-fluoro-[3-^{13}C]-NeuAc-α-(2-6)-[U-^{13}C]-Gal-β-(1-4)-GalNAc-β-sequence of an intact glycoprotein using a CMP-[3-^{13}C]-NeuAc analog and UDP-[U-^{13}C]-Glc, and the corresponding NMR analysis. In this work, they were not able to observe the H-7 proton of 9-deoxy-9-fluoro-[3-^{13}C]-NeuAc because the HMQC–TOCSY development stopped at H-6. In 2000, they reported novel NMR techniques,[3] HSQC–TOCSY–NOESY–TOCSY, for observation of all protons of NeuAc, H-3 to H-9, even with only a single ^{13}C-atom, at the 3-position of NeuAc, bound to a glycoprotein. However, since combined multi-pulse techniques generally suffer from low sensitivity, we have synthesized [3,9-^{13}C]-labeled NeuAc for convenient observation of all protons of NeuAc from H-3 to H-9 by the HMQC–HOHAHA technique. Further, ^{13}C-labeled KDN was also synthesized by the same strategy.

In this paper, we describe a novel [3,9-^{13}C]-labeling method for NeuAc and KDN via 5,6-anhydro-ManNAc and -Mannose. Another goal of the research described herein is to demonstrate the synthetic merits of mixed samples of [3-^{13}C]- and [9-^{13}C]-NeuAc as opposed to [3,9-^{13}C]-NeuAc. This is advantageous because the mixed samples result in higher yields due to the use of excess amounts of the non-labeled substrate. Equal measurements for di-labeled NeuAc and mixed mono-labeled NeuAc by NMR are also demonstrated.

Synthesis of [3,9-^{13}C]-sialic acid (NeuAc)

The synthesis of [3,9-^{13}C]-NeuAc was achieved as follows. 2-Acetamido-2-deoxy-D-mannose (D-ManNAc) was treated with acetone dimethyl acetal and a catalytic amount of *p*-toluenesulfonic acid in dry acetone at rt. to give the corresponding syrupy di-isopropylydene derivative **1** in 71% yield (Scheme 1). The partial hydrolysis of **1** in 70% aq. AcOH at rt. gave the

Keywords: carbohydrates; labeling; NMR.

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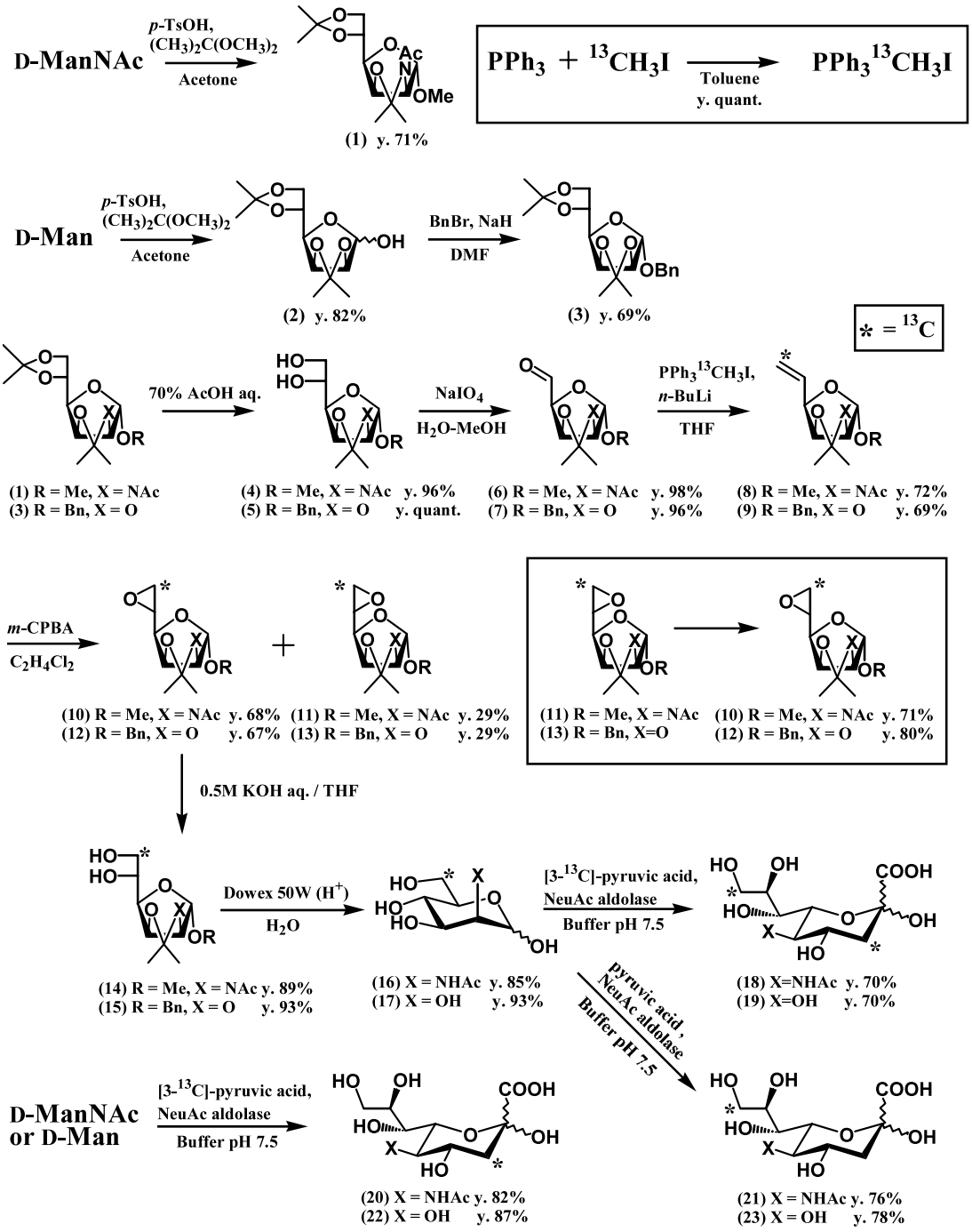
0040-4039/03/$ - see front matter
doi:10.1016/S0040-4039(03)00690-7



Scheme 1.

corresponding 2,3-*N*,*O*-isopropylidene derivative (**4**: mp 101–102°C) in 96% yield. Compound **4** was treated with $NaIO_4$ in MeOH at 0°C to give the carbonyl compound (**6**: mp 74.0–76.0°C) in 98% yield. Wittig reaction of **6** with $PPh_3{}^{13}CH_3I$ and *n*-BuLi in abs. THF gave the corresponding ^{13}C-labeled olefin compound (**8**: mp 66.5–67.0°C) in 72% yield. ^{13}C-Labeled phosphonium salt $PPh_3{}^{13}CH_3I$ was conveniently synthesized by using $^{13}CH_3I$ and triphenylphosphine in toluene in almost quantitative yield. Peracid oxidation of **8** with *m*-CPBA in 1,2-dichloroethane at 70°C gave a mixture of the corresponding 5,6-anhydro derivatives (*manno*-**10**: mp 94.5–95.5°C and *gulo*-**11**: mp 103.5–104.0°C) in 68 and 29% yields, respectively. The required compound **10** was hydrolyzed with 0.5 M aq. KOH in THF (1:1 v/v) at 75°C to give the labeled 5,6-di-ol derivative **14** in 89% yield. The stereochemistry of **10** was confirmed at this stage by comparing the NMR data of **4** and **14**. It is also possible to convert compound **11** into **10** (80% yield) via the 6-*O*-benzoyl-5-*O*-methanesulfonyl intermediate in order to save the labeled compound. Thus, compound **11** was hydrolyzed under reflux conditions (0.5 M aq. KOH/THF) to give a 5,6-di-ol derivative in 85% yield. The di-ol derivative

was selectively acylated (91% yield) and sulfonated (quantitative yield) to give 6-*O*-benzoyl-5-*O*-methylsulfonyl derivative in good yield. It was then treated with Bu_4NOH/THF at rt to give **10** in 92% yield. 6-^{13}C-labeled D-ManNAc (**16**) was obtained in 85% yield by the hydrolysis of **14** with Dowex 50W (H^+) in H_2O. Finally, [3,9-^{13}C]-NeuAc (**18**) was synthesized by the enzymatic reaction[7] of **16** and 2.5 equiv. [3-^{13}C]-pyruvic acid sodium salt in a solution of phosphate buffer (pH 7.5) containing BSA, NaN_3, and NeuAc aldolase for 16 h at 37°C in 70% yield.

Synthesis of [3-^{13}C]-sialic acid (NeuAc)

3-^{13}C-labeled NeuAc compounds were synthesized by condensation of ^{13}C-labeled pyruvic acid with an excess of ManNAc.[5a] Therefore, **20** was synthesized in a similar manner to that described above using [3-^{13}C]-pyruvic acid sodium salt and an excess amount (5.0 equiv.) of non-labeled ManNAc. The yield of this reaction was 82%.

Synthesis of [9-^{13}C]-sialic acid (NeuAc)

[9-^{13}C]-NeuAc (**21**) was synthesized in a similar manner to that described above using [9-^{13}C]-ManNAc and an excess amount (8.5 equiv.) of pyruvic acid sodium salt. The yield of this reaction was 76%.

Synthesis of [3,9-^{13}C]-3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (KDN)

[3,9-^{13}C]-KDN was synthesized as follows. D-Mannose was treated with acetone dimethyl acetal and a catalytic amount of *p*-toluenesulfonic acid in dry acetone at rt to give the corresponding di-isopropylidene derivative **2** in 82% yield. Then **2** was treated with sodium hydride and benzyl bromide to give the corresponding benzyl α-glycoside (**3**: mp 56–57°C) and β-anomer of **3** in 69 and 27% yields, respectively. In a similar manner as mentioned in the synthesis of [3,9-^{13}C]-NeuAc, compounds **5**–**19** were synthesized as follows. The partial hydrolysis of **3** gave the corresponding 2,3-*O*-isopropylidene derivative (**5**: mp 62–63°C) in quantitative yield. Compound **5** was converted into the syrupy carbonyl compound **7** in 96% yield. Wittig reaction of **7** gave the corresponding 6-^{13}C-labeled compound **9** in 69% yield. Peracid oxidation of **9** gave a mixture of the corresponding 5,6-anhydro derivatives (*manno*-**12**: syrup and *gulo*-**13**: mp 85–86°C) in 67 and 29% yields, respectively. The required compound **12** was hydrolyzed under alkaline conditions to give the labeled 5,6-di-ol derivative **15** in 93% yield. The stereochemistry of **12** was confirmed at this stage by comparing the NMR data of **5** and **15**. It is also possible to convert compound **13** into **12** in 80% yield (four steps). 6-^{13}C-labeled D-mannose (**17**) was obtained in 93% yield by the hydrolysis of **15**. Finally, [3,9-^{13}C]-KDN (**19**) was synthesized by enzymatic reaction[7] of **17** under similar conditions as mentioned in the synthesis of the labeled NeuAc using the same NeuAc aldolase, in 70% yield.

Synthesis of [3-^{13}C]-KDN

[3-^{13}C]-KDN (**22**) was synthesized in a similar way as mentioned above by the use of [3-^{13}C]-pyruvic acid sodium salt and an excess amount (5.0 equiv.) of non-labeled ManNAc. The yield of this reaction was 87%.

Synthesis of [9-^{13}C]-KDN

[9-^{13}C]-KDN (**23**) was synthesized in a manner analogous to that described above using [9-^{13}C]-ManNAc and an excess amount (8.5 equiv.) of pyruvic acid sodium salt. The yield of this reaction was 78%.

NMR data for each ^{13}C-labeled compound **18**–**23** are given in Ref. 8. NMR data demonstrating identical results for the HMQC–HOHAHA spectra of [3,9-^{13}C]-NeuAc and a 1:1 mixture of [3-^{13}C]- and [9-^{13}C]-NeuAc are shown in Figures 1, 2, and 3. It is possible to analyze the chemical shifts and *J* values of all protons by this technique.

As described above, a practical synthesis of minimal ^{13}C-labeled NeuAc and KDN should facilitate studies on the conformational properties and dynamic behavior of oligosaccharides that contain NeuAc and KDN. This minimal and efficient labeling method may also be extended to other important component monosaccharides, such as mannose, mannosamine, galactose, and fucose which are currently difficult to study due to $^3J=0$ coupling constants.

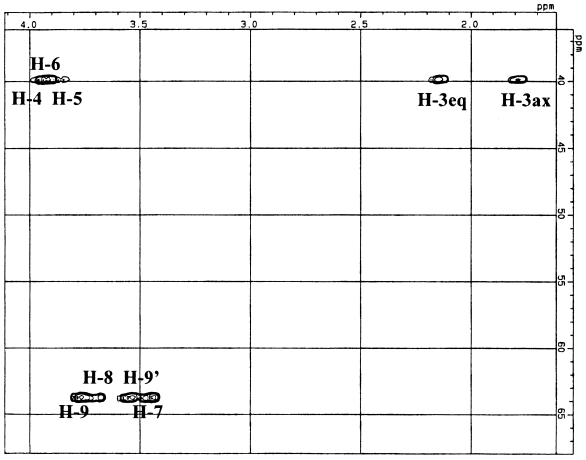


Figure 1. HMQC–HOHAHA spectrum of [3,9-^{13}C]-NeuAc.

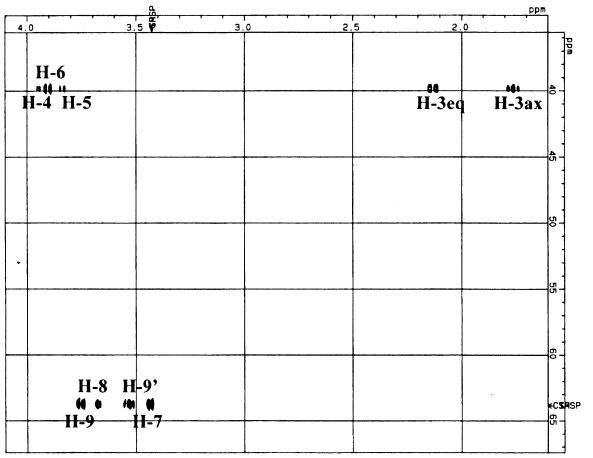


Figure 2. HMQC–HOHAHA spectrum of a 1:1 mixture of [3-^{13}C]- and [9-^{13}C]-NeuAc.

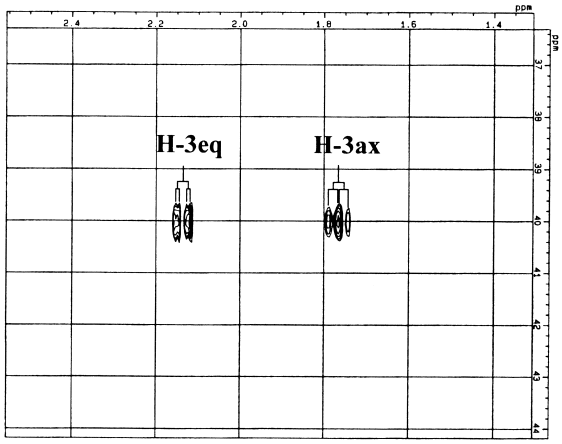


Figure 3. HMQC–HOHAHA spectrum of a 1:1 mixture of [3-^{13}C]- and [9-^{13}C]-NeuAc (expansion).

Acknowledgements

This work was partly supported by 'High-Tech Research Center Project' of Ministry of Education, Science, Sports, and Culture, Japan. The authors thank Professor Y. Kajihara of Yokohama City University for NMR measurements.

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8. ^{1}H and ^{13}C NMR data for ^{13}C-labeled compounds **18**–**23**: **18**: ^{1}H NMR (D_2O, 500 MHz) 3.95 (1H, dddd, $J_{4,3ax}=11.9$ H, $J_{4,3eq}=5.0$ Hz, $J_{4,5}=10.4$ Hz, $J_{4,C}=1.6$ Hz, H-4), 3.93 (1H, dd, $J_{6,5}=10.4$ Hz, $J_{6,7}=1.0$ Hz, H-6), 3.80 (1H, dd, H-5), 3.71 (1H, ddd, $J_{9,8}=2.8$ Hz, $J_{9,9'}=11.9$ Hz, $J_{9,C}=144.8$ Hz, H-9), 3.62 (1H, dddd, $J_{9',8}=6.4$ Hz, H-8), 3.50 (1H, ddd, $J_{9',C}=143.8$ Hz, H-9′), 3.43 (1H, dd, $J_{7,8}=9.2$ Hz, H-7), 2.19 (1H, ddd, $J_{3eq,3ax}=12.4$ Hz, $J_{3eq,C}=132.4$ Hz, H-3eq), 1.92 (3H, s, -NHAc), 1.76 (1H, dddz, $J_{3ax,C}=130.0$ Hz, H-3ax); ^{13}C NMR (D_2O, 125 MHz) 64.12 (C-9), 40.20 (C-3); **19**: ^{1}H NMR (D_2O, 500 MHz) 3.88 (1H, dddd, $J_{4,3ax}=11.6$ Hz, $J_{4,3eq}=4.9$ Hz, $J_{4,5}=9.5$ Hz, $J_{4,C}=2.1$ Hz, H-4), 3.84 (1H, dd, $J_{6,5}=9.5$ Hz, $J_{6,7}=1.2$ Hz, H-6), 3.78 (1H, ddd, $J_{9,C}=143.1$ Hz, $J_{9,8}=2.8$ Hz, $J_{9,9'}=11.6$ Hz, H-9), 3.75 (1H, dd, $J_{7,8}=8.9$ Hz, H-7), 3.67 (1H, ddd, $J_{8,9'}=6.4$ Hz, H-8), 3.57 (1H, ddd, $J_{9',C}=142.8$ Hz, H-9′), 3.49 (1H, dd, H-5), 2.10 (1H, ddd, $J_{3eq,3ax}=13.0$ Hz, $J_{3eq,C}=132.4$ Hz, H-3eq), 1.66 (1H, ddd, $J_{3ax,C}=128.8$ Hz, H-3ax); ^{13}C NMR (D_2O, 125 MHz) 63.5 (C-9), 39.25 (C-3); **20**: ^{1}H NMR (D_2O, 500 MHz) 3.91 (1H, dddd, $J_{4,3ax}=11.9$ Hz, $J_{4,3eq}=4.6$ Hz, $J_{4,5}=10.4$ Hz, $J_{4,C}=1.6$ Hz, H-4), 3.89 (1H, dd, $J_{6,5}=10.4$ Hz, $J_{6,7}=1.0$ Hz, H-6), 3.80 (1H, dd, H-5), 3.73 (1H, dd, $J_{9,8}=2.4$ Hz, $J_{9,9'}=11.9$ Hz, H-9), 3.65 (1H, ddd, $J_{8,7}=9.2$ Hz, $J_{8,9'}=6.4$ Hz, H-8), 3.51 (1H, dd, H-9′), 3.42 (1H, br dd, H-7), 2.12 (1H, ddd, $J_{3eq,3ax}=12.8$ Hz, $J_{3eq,C}=132.8$ Hz, H-3eq), 1.95 (3H, s, -NHAc), 1.73 (1H, ddd, $J_{3ax,C}=129.4$ Hz, H-3ax); ^{13}C NMR (D_2O, 125 MHz) 39.67 (C-3); **21**: ^{1}H NMR (D_2O, 500 MHz) 3.92 (1H, ddd, $J_{4,3ax}=10.0$ Hz, $J_{4,3eq}=4.9$ Hz, $J_{4,5}=10.1$ Hz, H-4), 3.88 (1H, dd, $J_{6,5}=10.4$ Hz, $J_{6,7}=1.0$ Hz, H-6), 3.81 (1H, dd, H-5), 3.73 (1H, ddd, $J_{9,8}=2.8$ Hz, $J_{9,9'}=11.9$ Hz, $J_{9,C}=144.0$ Hz, H-9), 3.66 (1H, ddd, H-8), 3.50 (1H, ddd, $J_{9',8}=6.4$ Hz, $J_{9',C}=138.9$ Hz, H-9′), 3.41 (1H, br dd, $J_{7,8}=9.2$ Hz, H-7), 2.11 (1H, dd, $J_{3eq,3ax}=13.1$ Hz, H-3eq), 1.95 (3H, s, -NHAc), 1.73 (1H, dd, H-3ax); ^{13}C NMR (D_2O, 125 MHz) 64.11 (C-9); **22**: ^{1}H NMR (D_2O, 500 MHz) 3.83 (1H, dddd, $J_{4,3ax}=12.0$ Hz, $J_{4,3eq}=5.2$ Hz, $J_{4,5}=9.7$ Hz, $J_{4,C}=2.0$ Hz, H-4), 3.79 (1H, dd, $J_{6,5}=9.7$ Hz, $J_{6,7}=1.2$ Hz, H-6), 3.68 (1H, dd, $J_{9,8}=2.8$ Hz, $J_{9,9'}=11.5$ Hz, H-9), 3.70 (1H, dd, $J_{7,8}=8.9$ Hz, H-7), 3.65 (1H, ddd, $J_{8,9'}=6.3$ Hz, H-8), 3.50 (1H, dd, H-9′), 3.43 (1H, dd, H-5), 2.05 (1H, ddd, $J_{3eq,3ax}=13.2$ Hz, $J_{3eq,C}=132.8$ Hz, H-3eq), 1.61 (1H, ddd, $J_{3ax,C}=129.4$ Hz, H-3ax); ^{13}C NMR (D_2O, 125 MHz) 39.67 (C-3); **23**: ^{1}H NMR (D_2O, 500 MHz) 3.82 (1H, ddd, $J_{4,3ax}=12.0$ Hz, $J_{4,3eq}=5.2$ Hz, $J_{4,5}=9.7$ Hz, H-4), 3.78 (1H, dd, $J_{6,5}=9.7$ Hz, $J_{6,7}=1.1$ Hz, H-6), 3.64 (1H, ddd, $J_{9,C}=146.1$ Hz, $J_{9,8}=2.5$ Hz, $J_{9,9'}=12.0$ Hz, H-9), 3.71 (1H, dd, $J_{7,8}=8.5$ Hz, H-7), 3.62 (1H, ddd, $J_{8,9'}=6.3$ Hz, H-8), 3.50 (1H, ddd, $J_{9',C}=140.7$ Hz, H-9′), 3.43 (1H, dd, H-5), 2.02 (1H, dd, $J_{3eq,3ax}=13.2$ Hz, H-3eq), 1.62 (1H, dd, H-3ax); ^{13}C NMR (D_2O, 125 MHz) 63.8 (C-9).