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Note

Synthesis of bromoindolyl 4,7-di-*O*-methyl-Neu5Ac: specificity toward influenza A and B viruses

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Abstract

N-Acetylneuraminic acid (Neu5Ac) was converted into the methyl ester methyl ketoside-8,9-epoxy derivative (**8**). Methylation of **8** followed by deprotection gave 4,7-di-*O*-methyl-Neu5Ac (**10**). Compound **10** was converted into the corresponding methyl ester–chloroacetate derivative, which was subsequently coupled to 5-bromo-indol-3-ol to give the chromogenic product (**13**). Deprotection of **13** gave 5-bromo-indol-3-yl 4,7-di-*O*-methyl-Neu5Ac (**5**). The product **5** was specifically cleaved by sialidase from either influenza A or influenza B virus to give an indigo-blue precipitate, but was not cleaved by several bacterial or viral sialidases tested. The properties of product **5** relative to a fluorescent substrate for sialidase were also documented.

Keywords: Chromogenic substrate; Sialidase; Influenza; di-*O*-Methyl-Neu5Ac; Bromoindolyl

For a number of years we have been involved in the development of influenza diagnostic agents [1]. Our approach is based on the enzymatic cleavage of chromogenic ketosides of *N*-acetylneuraminic acid (Neu5Ac) by influenza virus sialidase, and as such, it requires the utilization of a substrate that will exhibit specificity towards the viral enzyme.

The specificity of various sialidase substrates has been studied in the past, and it has already been reported [2] that 4-*O*-methyl-Neu5Ac bound to fetuin showed resistance toward *Vibrio cholerae* sialidase, but was cleaved rapidly by fowl plague virus sialidase. It has also been shown [3] that a 4-*O*-acetyl substitution in sialic acids blocks the action of bacterial sialidases for substrates containing these derivatives, while viral enzymes show significant activity. In another study it was found that the sialidase of *Streptococcus sanguis*, from the oral cavity, did not cleave 4-*O*-acetylated sialic acid derivatives [4].

Accordingly, we proceeded to develop a practical synthesis of 4-*O*-methyl-*N*-acetylneuraminic acid (**1**) [5] from the acetal **2**, and when this was accomplished, the product was coupled to 1-acetyl-5-bromoindo-3-ol (**3c**). Deprotection of the coupled product gave the substrate 5-bromo-indol-3-yl 4-*O*-methyl-*N*-acetylneuraminic acid (**4**) which was purified by HPLC (Liav, unpublished results). In the process of purifying **4**, a byproduct was iso-

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lated. Examination of the structure of this product by mass spectrometry as well as ^{1}H and ^{13}C NMR showed it to be 5-bromoindol-3-yl 4,7-di-*O*-methyl-*N*-acetylneuraminic acid (**5**) (the presence of this product is most likely the result of some dimethylation of the acetal **2**).

Incubation of the two bromoindolyl glycosides, **4** and **5**, with sialidase from influenza A or influenza B virus (see Section 1) resulted in the cleavage of the aglycon, as indicated by the development of the typical indigo-blue color [7]. However, when sialidases from other viruses, such as mumps and parainfluenza, were employed, only the monomethoxy derivative, **4**, was cleaved, and no color was observed in the case of the dimethoxy product **5**. In addition, non-sialidase-producing viruses which could potentially be isolated from respiratory specimens were also unreactive toward **5**. The specificity of **5** as a substrate for influenza virus sialidase was also confirmed when sialidases from several different bacterial sources did not cleave **5**, even when these enzymes were tested at activity levels that were 5–50-fold higher than influenza virus sialidase activity (Table 1).

The remarkable specificity of **5** toward influenza A and B viruses prompted us to devise a synthetic scheme for this product. The most logical approach appeared to be the dimethylation of the readily available acetal **2**, by employing larger amounts of the catalyst and methylating agent than those used in the synthesis of 4-*O*-methyl-Neu5Ac, and following the same sequence of reactions which had led to the substrate 5-bromoindo-3-yl 4-*O*-methyl-Neu5Ac (**4**).

Treatment of **2** with sodium hydride and dimethyl sulfate under the conditions described before [5] failed to give satisfactory results. When slightly more than two molar equivalents of sodium hydride and dimethyl sulfate were used, the major product obtained was the 4-methoxy derivative **6**, and the separation of the monomethoxy and dimethoxy derivatives at this stage was tedious. With larger amounts of the catalyst and dimethyl sulfate, employing DMF as the solvent, hydrolysis of the methyl ester took place, and the formation of the undesired *N*-methyl-*N*-acetyl product could also be observed.

In an attempt to alleviate the apparent steric hindrance associated with OH-7 in **2** (difficulties in the chemical synthesis of 7-substituted-Neu5Ac derivatives have already been reported [8]), we sought to replace the acetal group with a less restrictive system, such as the 8,9-epoxy moiety (compounds **8** and **9**).

Treatment of methyl (methyl 5-acetamido-3,5-dideoxy-9-*O*-*p*-tolylsulfonyl-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (**7**) [9] with M sodium methoxide solution in methanol gave the 8,9-epoxide **8** in good yield. Methylation of **8** with dimethyl sulfate as described before [5] proceeded smoothly, and the 4,7-dimethoxy product **9** was isolated after column chromatography in 70% yield. The deprotection of **9** was carried out in two steps: First, the ester was hydrolyzed by treatment with M sodium hydroxide solution, and then

Table 1
Bromoindolyl-4,7-di-*O*-methyl-Neu5Ac is an influenza virus sialidase-specific substrate

Sialidase source	Enzyme activity 4-MU-Neu5Ac (mol. min^{-1})	Fold	Reactivity to bromoindolyl-4,7-di-*O*-methyl-Neu5Ac chromogenic substrate
Influenza A virus	$0.20 \pm 0.005 \times 10^{-9}$	1.00	positive
Arthrobacter ureafaciens	$10.50 \pm 0.500 \times 10^{-9}$	52.50	negative
Streptococcus mitis	$1.06 \pm 0.180 \times 10^{-9}$	5.30	negative
Clostridium perfringens	$10.00 \pm 0.950 \times 10^{-9}$	50.00	negative
Salmonella typhimurium	$10.55 \pm 0.350 \times 10^{-9}$	52.75	negative

the methyl ketoside was treated with Dowex 50 (H^+) resin in 0.025 M hydrochloric acid. The product, 4,7-di-*O*-methyl-Neu5Ac (**10**), was characterized by ^1H and ^{13}C NMR spectroscopy. Treatment of **10** with Dowex 50 (H^+) in dry methanol gave the practically pure methyl ester **11**. Acetylation of **11** with acetic anhydride and pyridine gave a mixture of the anomers, which was purified by column chromatography. Conversion of the acetates mixture into the corresponding chloride, followed by treatment with 1-*N*-acetyl-5-bromoindo-3-ol (**3c**; prepared from the 3-acetoxy derivative **3a** by acetylation to give the diacetate **3b** and subsequent treatment with 90% sulfuric acid [10]) gave a mixture of the bromoindolyl di-*O*-methyl-Neu5Ac product **13**, and a byproduct, most likely the 2,3-dehydro derivative **14**. Column chromatography removed the unchanged chromogen **3c** and minor impurities, but the mixture of the two products was inseparable. Partial deprotection of the mixture gave the esters **15** and **16**, which still could not be separated by chromatography. Finally, deesterification of **15** and **16** gave a mixture which could be easily separated by HPLC, yielding the desired bromoindolyl di-*O*-methyl-Neu5Ac derivative **5**.

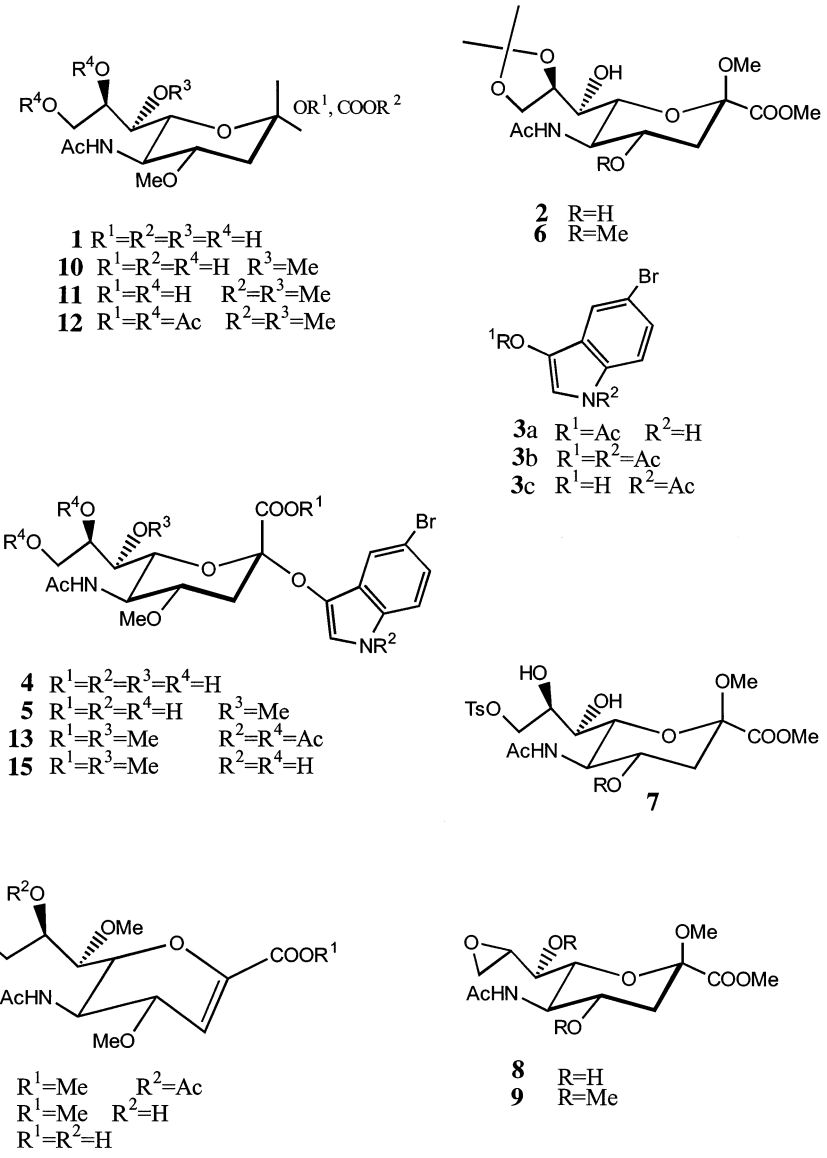
In order to further evaluate **5** as a substrate for sialidase, the catalytic properties of influenza virus sialidase toward **5** were compared to that of the well-known fluorescent substrate 4-methylumbelliferyl Neu5Ac (4-MU Neu5Ac). The optimal pH for influenza virus sialidase toward **5** was 5.4. Nearly 90% of maximal activity was also observed between pH values of 5.2 and 4.8. However, enzymatic activity rapidly declined above pH 5.4 to nearly one-half maximal activity at a pH value of 6.2. In contrast, near maximal (95–100%) influenza virus sialidase activity was observed between pH values of 4.8 and 6.2 when 4-MU Neu5Ac was employed as a substrate. Differences exist in the pH optima of influenza virus sialidase toward different substrates [6]. The activity of influenza virus sialidase upon 4-MU Neu5Ac was higher (13.2×10^{-6} mol min^{-1} L^{-1}) relative to **5** (2.3×10^{-6} mol min^{-1} L^{-1}). Likewise, the affinity of 4-MU Neu5Ac for influenza virus sialidase was also higher ($K_m = 4.67 \pm 0.97 \times 10^{-5}$ M; $n = 3$) compared to **5** ($K_m = 2.63 \pm 0.53 \times 10^{-4}$ M; $n = 4$) (Scheme 1).

1. Experimental

General methods.—Optical rotations were measured with a Jasco P-1020 polarimeter at 24 °C. NMR spectra were measured with a Jeol FX-90QM multinuclear spectrometer unless otherwise stated. Column chromatography was performed with silica gel 70–230 mesh (Aldrich). Thin layer chromatography was performed on Analtech Uniplate silica gel plates. HPLC purification was performed on a Waters system and a C_{18} reverse-phase column (2.2×25 cm) using 1–20% acetonitrile gradient. Microanalyses were performed by Galbraith Laboratories Inc., Knoxville, TN.

Detection of influenza A or influenza B virus.—A throat swab specimen collected from a patient with influenza-like symptoms is placed in a tube containing 1.3 mL of a buffered solution (pH 5.4), and thoroughly squeezed out into the solution to extract the specimen. The swab is then discarded. A filter tip is then placed on the tube and the specimen contents are filtered into a vial containing 1 mg of the bromoindolyl substrate **5**. The vial is then incubated for 30 min at 41 °C, after which time an alkaline stop solution (0.5 mL) is added. The mixture is then transferred into a funnel filtration device [1] and the blue precipitate (indicative of the presence of either influenza A or influenza B virus) is collected on a membrane at the bottom of the device.

Enzymatic assays.—Purified or partially purified sialidases from *Arthrobacter ureafaciens*, *Clostridium perfringens*, and *Salmonella typhimurium* were purchased from Sigma. Bacteria cultured from human volunteer throat swab extracts and identified as *Streptococcus mitis* were lysed and then used as a sialidase source. Sialidases were assayed at pH 5.4, except during experiments where the effect of pH on activity was being determined. Enzymatic activity was quantitated using 4-MU Neu5Ac that was synthesized 'in house' (Liav, unpublished data). The fluorescence of cleaved 4-methylumbelliferone was measured using excitation and emission wavelengths λ of 365 and 450 nm, respectively, with an Hitachi F-3010



Scheme 1.

Fluorescence Spectrophotometer. The activity of various sialidases toward **5** was quantitated by measuring the released *N*-acetylneuraminic acid [11]. The kinetic constant K_m was calculated using EnzymeKinetics (Trinity Software, v. 1.2).

In-house evaluation of the substrates for sialidase specificity was conducted with characterized virus strains obtained from the American Type Culture Collection. Influenza A and B virus strains, as well as other sialidase-producing viruses such as parainfluenzas and mumps were tested with the substrate. In addition, non-sialidase-producing viruses which could potentially be isolated from respiratory specimens were also tested. All testing was done from virus cultures grown to maximal titers to obtain the most stringent evaluation of the substrates. Bacterial sialidases were tested with **5** at activity levels of 5–50-fold higher than influenza virus sialidase to demonstrate specificity.

Methyl (methyl 5-acetamido-8,9-anhydro-3,5-dideoxy-D-glycero-α-D-galacto-*2-nonulopyranosid)onate* (**8**).—Methyl (methyl 5-acetamido-3,5-dideoxy-9-*O*-*p*-tolylsulfonyl-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (**7**, 1.66 g) [9] was treated with M NaOMe solution (3.5 mL) in MeOH (20 mL) for 45 min at room temperature (rt). It was neutral-

ized with Dowex 50 (H^+) resin, and the resin was filtered off and washed with methanol. The filtrate was evaporated, and the residue was dried and extracted with acetone. The insoluble solids were filtered off and washed with acetone, and the filtrate was evaporated. The residue was dried and chromatographed on silica gel. Elution with 9:1 CH_2Cl_2–MeOH removed minor impurities. Continued elution with the same solvent system yielded the amorphous product **8** (1.02 g, 95%); $[\alpha]_D$ −62° (*c* 1.0, CH_2Cl_2). Anal. Calcd for $C_{13}H_{21}NO_8$: C, 48.89; H, 6.63; N, 4.38. Found: C, 48.94; H, 6.92; N, 4.02.

Methyl (methyl 5-acetamido-8,9-anhydro-3,5-dideoxy-4,7-di-O-*methyl*-D-glycero-α-D-galacto-*2-nonulopyranosid)onate* (**9**).—A cold (ice-bath) solution of **8** (1.36 g) in MeCN (15 mL) was saturated with nitrogen. Sodium hydride (80% dispersion in oil, 280 mg) was added, and the mixture was stirred under nitrogen for 15 min. Dimethyl sulfate (2.5 mL) was added and stirring in an ice bath, under nitrogen, was continued for an additional 30 min. The mixture was filtered through Celite, and the precipitate washed with dry acetonitrile. The filtrate was evaporated and the residue was dried and extracted with acetone. The insoluble solid material was filtered off and washed with acetone and the filtrate was evaporated. The residue was dried and chromatographed on silica gel. Elution with 15:1 CH_2Cl_2–MeOH removed the oily material. Continued elution with the same solvent system gave the amorphous dimethoxy derivative **9** (1.03g, 69%); $[\alpha]_D$ −27.9° (*c* 1.0, CH_2Cl_2); ^{1}H NMR: δ 3.80 (s, 3 H, CO_2Me), 3.46 (s, 3 H, OMe-4), 3.34 (S, 3 H, OMe-7), 3.28 (s, 3 H, OMe-2), 2.56 (dd, 1 H, $J_{3eq,4}$ 5.3 Hz, $J_{3eq,3ax}$ 13.2 Hz, H-3*eq*), 2.01 (s, 3 H, NAc), 1.77 (dd, 1 H, H-3*ax*). Anal. Calcd for $C_{15}H_{25}NO_8$: C, 51.86; H, 7.25; N, 4.03. Found: C, 51.50; H, 7.20; N, 4.02.

5-Acetamido-3,5-dideoxy-4,7-di-O-*methyl*-D-glycero-D-galacto-*nonulopyranosidonic acid (4,7-di*-O-*methyl-Neu5Ac*, **10**).—Compound **9** (2.66 g) was treated with M NaOH solution (10 mL) in 50% aq MeOH (30 mL) for 30 min at rt. The mixture was neutralized with Dowex 50 (H^+) and the resin was filtered off and washed with methanol. The filtrate was evaporated and the residue was subsequently treated with Dowex 50 (H^+, 2.5 g) and 0.025 M HCl solution (35 mL) for 3 h at 95–100 °C. The resin was filtered off and washed with MeOH, and the filtrate was evaporated to give the practically pure 4,7-di-*O*-methyl-Neu5Ac product (**10**), isolated as a solid. ^{1}H NMR (D_2O, 300 MHz): δ 3.96 (bd, 1 H, $J_{6,7}$ 1.0, $J_{6,5}$ 10.4 Hz, H-6), 3.83 (t, 1 H, *J* 10.4 Hz, H-5), 3.70–3.63 (m, 2 H, H-9 and H-9′), 3.53 (m, partially overlapped, 1 H, H-4), 3.44 (bdd, 1 H, $J_{8,9}$ 6.3, $J_{8,9'}$ 12.5 Hz, H-8), 3.27 (s, 3 H, OMe), 3.25 (m, 1 H, partially overlapped, H-7), 3.22 (s, 3 H, OMe), 2.30 (dd, 1 H, $J_{3eq,3ax}$ 13.2 Hz, $J_{3eq,4}$ 4.8 Hz, H-3*eq*), 1.90 (s, 3 H, NAc), 1.62 (dd, 1 H, $J_{3ax,4}$ 13.2 Hz, H-3*ax*).

Methyl (5-acetamido-3,5-dideoxy-4,7-di-O-*methyl*-D-glycero-α,β-D-galacto-*nonulopyranosid)onate* (**11**).—The crude **10** (obtained from 2.66 g of **9**) was treated with dry Dowex 50 (H^+, 1.6 g) in MeOH (35 mL). The mixture was boiled under reflux for 90 min, and the resin was filtered off and washed with MeOH. The filtrate was evaporated and the residue was dried to give the crude amorphous ester **11**.

Methyl (5-acetamido-2,8,9-tri-O-*acetyl-3,5-dideoxy-4,7-di*-O-*methyl*-D-glycero-α,β-D-galacto-*nonulopyranosid)onate* (**12**).—The methyl ester **11** was treated with acetic Ac_2O (8 mL) and pyridine (16 mL) in the catalytic presence of 4-dimethylaminopyridine at rt overnight. The mixture was evaporated and the residue was dried and chromatographed on silica gel. Elution with 25:1 CH_2Cl_2–MeOH gave the product **12** as a syrupy anomeric mixture (1.86 g, 51%, based on the anhydro derivative **9**); $[\alpha]_D$ −8.1° (*c* 1.0. CH_2Cl_2); ^{1}H NMR: δ 4.65 (dd, 1 H, $J_{9,9'}$ 12.5, $J_{9,8}$ 2.1 Hz, H-9), 4.24 (dd, 1 H, $J_{9',8}$ 6.5 Hz, H-9′), 4.11 (dd, 1 H, H-8), 3.98–3.68 (m, partially overlapped with CO_2Me), 3.51 (s, 3 H, OMe), 3.35 (s, 3 H, OMe), 2.65 (dd, 1 H, $J_{3eq,3ax}$ 13.4, $J_{3eq,4}$ 4.8 Hz, H-3e*q*), 2.13, 2.06, 2.04 (s, 9 H, OAc), 2.03 (s, 3 H, NAc), 1.75 (dd, 1 H, $J_{3ax,4}$ 11.0 Hz, H-3*ax*) Anal. Calcd For $C_{20}H_{31}NO_{12}$: C, 50.30; H, 6.54; N, 2.93. Found: 49.89; H, 6.54; N, 2.96.

Methyl (5-bromoindol-3-yl 5-acetamido-8,9-di-O-*acetyl-4,7-di*-O-*methyl-3,5-dideoxy*-D-glycero-α-D-galacto-*nonulopyranosid)onate*

(**13**).—A solution of **12** (380 mg) in CH_2Cl_2 (5 mL) and AcCl (0.5 mL) was saturated with dry HCl. The mixture was then stirred for 5h at rt, and evaporated. The residue was dried under vacuum. A solution of the crude chloride in acetone (5 mL) was saturated with nitrogen at rt. 1-Acetyl-5-bromo-3-indol-ol (**3c**, 208 mg) was added. Stirring under nitrogen was continued, and 5 min later M NaOH solution was added. After an additional 5 min, more NaOH solution (0.2 mL) was added, and at this point the reaction mixture turned green. The mixture was stirred for 30 min more, and evaporated. The residue was dried and chromatographed on silica gel. Elution with 25:1 CH_2Cl_2–MeOH gave the unchanged indole derivative **3c**. Continued elution with the same solvent system gave a syrupy mixture of the coupled product **13** and 2,3-dehydro derivative **14**, as indicated by NMR. Attempts to separate the two products by using different conditions were unsuccessful.

5-Bromoindol-3-yl 5-acetamido-3,5-dideoxy-4,7-di-O-*methyl*-D-glycero-D-galacto-*nonulopyranosidonic acid* (**5**).—The foregoing mixture of **13** and **14** was treated with M NaOMe solution (0.2 mL) in MeOH (5 mL) for 40 min at rt. It was neutralized with Dowex 50 (H^+) and the resin was filtered off and washed with MeOH. The filtrate was evaporated and the residue was dried and chromatographed on silica gel. Elution with 5:1 CH_2Cl_2–MeOH removed residual yellow color. Continued elution with the same solvent system yielded a mixture of the methyl esters **15** and **16** which was still inseparable. Finally, this mixture was treated with M NaOH solution (0.5 mL) in 50% aq MeOH (5 mL) for 1 h at rt. The mixture was evaporated and the residue was purified by reverse-phase HPLC. The 2,3-dehydro derivative **17** was eluted first (retention time 10 min); $[\alpha]_D + 22°$ (*c* 1.0, H_2O); ^1H NMR (D_2O): δ 6.04 (d, *J* 1.8 Hz, H-3), 3.51 (s, 3 H, OMe), 3.49 (s, 3 H, OMe), 2.15 (s, 3 H, NAc). The desired product **5** (61 mg, 14.5%, based on the dimethoxy ester **11**) had a retention time of 60 min, and was isolated as a solid, $[\alpha]_D - 41°$ (*c* 1.0, H_2O); ^1H NMR data (in D_2O, 300 MHz): δ 7.85 (d) and 7.07–7.02 (m) (4 H, indolyl), 4.03 (t, $J_{5,4}$ 9.5, $J_{5,6}$ 9.5 Hz, H-5), 3,90 (dd, $J_{6,7}$ 1.9 Hz, H-6), 3.85 ($J_{8,7}$ 8.3, $J_{8,9}$ 2.3, $J_{8,9'}$ 4.6 Hz, H-8), 3.78 (dd, $J_{9,9'}$ 12.4 Hz, H-9), 3.63 (dd, H-9′), 3.43 (s, 3 H, OMe), 3.38 (dd, H-7), 3.34 (s, 3 H, OMe), 3.29 (m, $J_{4,3eq}$ 4.7, $J_{4,3ax}$ 11.8 Hz, H-4), 3.03 (dd, $J_{3eq,3ax}$ 11.8 Hz, H-3*eq*), 1.95 (3, 3 H, NHAc), 1.68 (t, H-3 *ax*); ^{13}C NMR (in D_2O with 1,4-dioxane as the internal standard): δ 175.0 (CO_2H); 173.4 (CO–NAc); 132.9, 132.1, 125.4, 124.2, 121.7, 118.3; 114.4; 112.7 (indolyl); 104.9 (C-2); 79.5 (C-4); 78.8 (C-8); 74.2 (C-6); 72.1 (C-7); 63.1 (C-9); 61.1 (OMe-7); 57.6 (OMe-4); 50.8 (C-5); 37.7 (C-3); 23.1 (*Me*–NAc).

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