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Synthesis and Evaluation of C-9 Modified *N*-Acetylneuraminic Acid Derivatives as Substrates for *N*-Acetylneuraminic Acid Aldolase

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Abstract—Several C-9 modified *N*-acetylneuraminic acid derivatives have been synthesised and evaluated as substrates of *N*-acetylneuraminic acid aldolase. Simple C-9 acyl or ether modified derivatives of *N*-acetylneuraminic acid were found to be accepted as substrates by the enzyme, albeit being transformed more slowly than Neu5Ac itself. ^{1}H NMR spectroscopy was used to evaluate the extent of the enzyme catalysed transformation of these compounds. Interestingly, the chain-extended Neu5Ac derivative **16** is not a substrate for *N*-acetylneuraminate lyase and behaves as an inhibitor of the enzyme.

Introduction

In the forward direction, *N*-acetylneuraminic acid aldolase (Neu5Ac aldolase, EC 4.1.3.3) catalyses a retro-aldol condensation of *N*-acetylneuraminic acid (Neu5Ac (**1**)) to produce pyruvate and *N*-acetyl-D-mannosamine (ManNAc) (Scheme 1).[1,2] The synthetically more useful reverse aldol condensation provides **1** from ManNAc, with the equilibrium of the two reactions being modulated by the concentration of pyruvate.[1–3] Neu5Ac aldolase is found in several species of bacteria[1,2,4,5] as well as in mammals,[1,6] and plays an important role in the recycling of Neu5Ac (**1**) in nature.[1,2] The X-ray crystal structure of the active site of Neu5Ac aldolase from *Escherichia coli* has been resolved to 2.2 Å.[7] From the crystallographic data and related studies it has been elucidated for the forward reaction that the enzyme forms a Schiff base between an active site lysine residue and the acetal carbonyl of the open chain form of the substrate (Scheme 1), followed by proton abstraction ultimately resulting in cleavage of the C_3–C_4 bond of Neu5Ac.[5,8,9] Unfortunately, little more is known regarding the precise mechanism of this transformation, although recently it has been suggested that it is the carboxylate group of the substrate which acts as the base in the proton abstaction step.[10]

Several workers have attempted to elucidate the catalytic mechanism of Neu5Ac aldolase by exposing the enzyme to various structurally modified ManNAc[11–16] and Neu5Ac[17–22] derivatives. A vast number of substrate specificity studies for the aldol condensation (i.e. ManNAc + pyruvate to give Neu5Ac) have shown that only pyruvate is accepted as the donor,[23] whilst a wide variety of hexoses, pentoses, D- and L-sugars, are accepted as substrates.[11–15] In contrast, less attention has been paid to the retro-aldol condensation with structurally modified sialic acids. From the few investigations conducted to date it is evident, and perhaps not surprising, that C-4 modified Neu5Ac derivatives are not cleaved by the enzyme and in some instances act as inhibitors.[18,20,21] Studies into the retro-aldol condensation catalysed by Neu5Ac aldolase have shown that 9-deoxy-Neu5Ac is a relatively good substrate.[20,21] Similarly, C-5 modifications are accepted by Neu5Ac aldolase,[21] with the exception of 5-*epi*-derivatives.[21] Glycerol sidechain modifications other than at C-9 of Neu5Ac, involving either stereochemical changes or deoxygenation at C-7 and C-8, typically result in compounds which are cleaved more slowly by the enzyme than the naturally occuring substrate Neu5Ac.[19–21]

As part of our continued interest in the chemistry and biochemistry of structurally modified sialic acid derivatives,[24–26] we thought it of value to prepare a range of C-9 modified Neu5Ac derivatives as probes for *N*-acetylneuraminic acid aldolase. Importantly, our target compounds were designed to explore the steric and structural constraints of the active site of Neu5Ac

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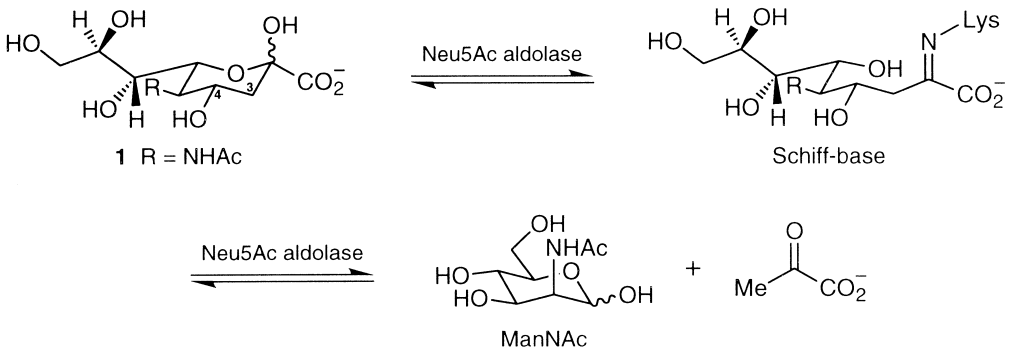


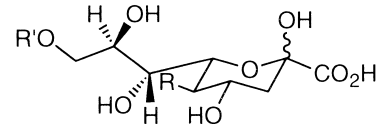
Scheme 1. Neu5Ac aldolase catalysed transformation of Neu5Ac to ManNAc via a Schiff-base.

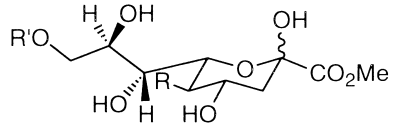
aldolase, as well as examining the effects of different functionalities such as acyl, ether and chain-extended derivatives on the reaction of the modified sialic acid with the enzyme. In addition, we envisaged that many of our simple target compounds would be accessible from Neu5Ac with a minimum of protecting group chemistry.

Results and Discussion

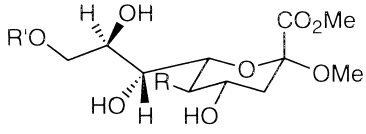
Synthesis of C-9 modified Neu5Ac derivatives

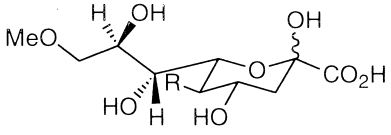
It was anticipated that C-9 acylated derivatives of Neu5Ac would be directly available from Neu5Ac itself, without the need for any laborious protecting group chemistry. Accordingly, the known 9-*O*-acetyl Neu5Ac derivative **2** was prepared by treatment of Neu5Ac with trimethylorthoacetate in DMSO containing *p*-TsOH.[27] In this way, Neu5,9Ac$_2$ (**2**) was obtained in 78% yield after purification, which is comparable to the results obtained by Ogura et al.[27] Unfortunately, initial attempts at forming the sterically more demanding 9-*O*-benzoyl derivative **3** by treatment of Neu5Ac under typical benzoylation conditions (BzCl in pyridine)[28] failed to furnish any of the desired product. After some experimentation it was established that the 9-*O*-benzoyl group could be successfully introduced if the methyl ester **4** was used as substrate instead of Neu5Ac. In this way the 9-*O*-benzoyl derivative **5** of Neu5Ac was obtained in high yield (71% after chromatography).


2 R = NHAc, R' = Ac
3 R = NHAc, R' = Bz

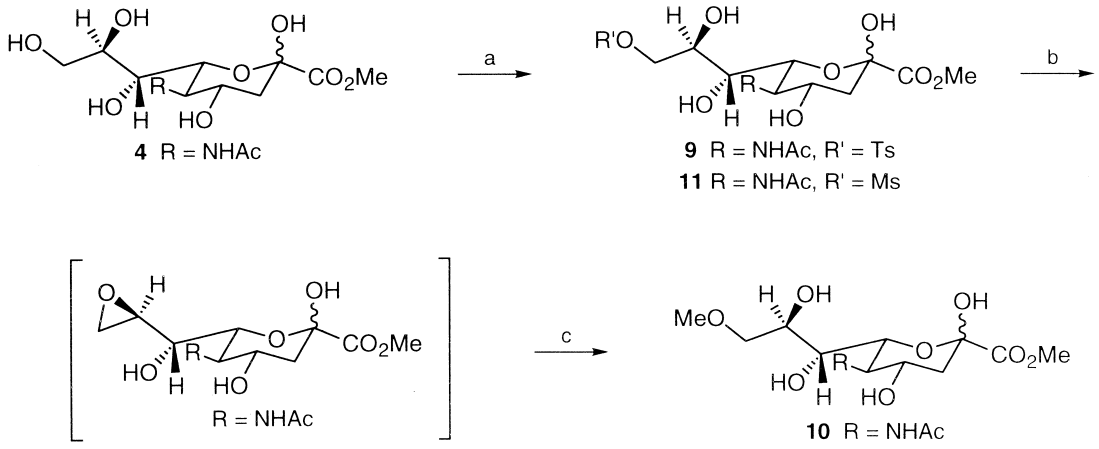

4 R = NHAc, R' = H
5 R = NHAc, R' = Bz

Our preliminary efforts towards the preparation of C-9 ether substituted Neu5Ac derivatives utilised the 9-*O*-methanesulfonyl derivative **6** of Neu5Ac1,α2Me$_2$.[29] Sequential exposure of the mesylate **6** to NaOMe in MeOH followed by acidification afforded the 9-*O*-methyl ethyl **7** in 38% yield after purification. Despite the successful introduction of the 9-*O*-methyl substituent, the moderate chemical yield and the fact that the ether **7** still required both deglycosidation and deesterification before use as a probe for Neu5Ac aldolase prompted further investigation of this transformation. In an attempt to overcome the need for deprotection steps, we aimed to prepare the 9-*O*-methyl ether **8** directly from Neu5Ac itself. Disappointingly, the direct transformation of Neu5Ac to Neu5Ac9Me (**8**) proved fruitless, with several attempts using standard methylation conditions (such as NaH and MeI using various solvents and temperatures)[28] failing to furnish any of the desired compound, instead resulting in complex reaction mixtures.


6 R = NHAc, R' = Ms
7 R = NHAc, R' = Me


8 R = NHAc

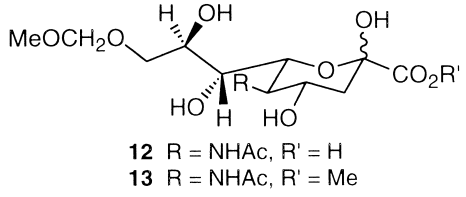
In an alternative approach towards limiting the need for deprotecting steps, we attempted to employ Neu5Ac1Me (**4**) in an analogous sequence to that successfully used in the preparation of **7**. Accordingly, treatment of **4** under tosylation conditions (TsCl/pyridine) gave the desired 9-*O*-tosyl derivative **9** in 55% yield (Scheme 2). Exposure of the tosylate **9** to NaOMe in MeOH followed by acidification (Amberlite IR-120H$^+$ resin) furnished the requisite 9-*O*-methyl ether **10** in moderate yield (Scheme 2). Interestingly, reaction of the 9-*O*-mesylate **11** with NaOMe in MeOH followed by acidification (as described above) resulted in an inse-



Scheme 2. Conditions: (a) TsCl, pyridine, 5 °C, 16 h, 55%; (b) NaOMe, MeOH, rt, 0.5 h; (c) Amberlite IR-120H$^+$ resin (pH = 2), MeOH, rt, 2 h, 53%.

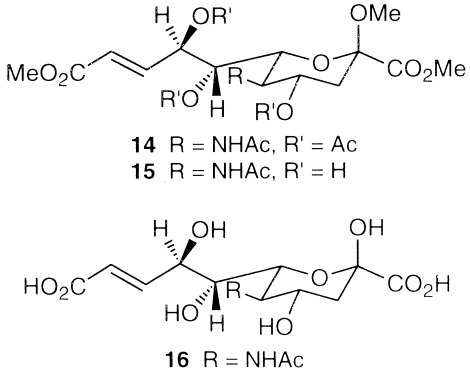
parable mixture of Neu5Ac1,9Me$_2$ (**10**) and unreacted mesylate **11**.

An alternative C-9 ether derivative of Neu5Ac that could be useful for exploring the substrate specificity of Neu5Ac aldolase was the 9-*O*-methoxymethyl ether **12**, since it was envisaged that the extra oxygen atom may participate in a range of additional interactions including hydrogen-bonding interactions. Accordingly, exposure of a solution of **4** in DMF containing Hünigs base to chloromethyl methylether furnished the 9-*O*-methoxymethyl ether **13** in 81% yield after chromatography.



Of the C-9 modified Neu5Ac derivatives prepared, all except Neu5,9Ac$_2$ (**2**) required deprotection of the methyl ester before exposure to Neu5Ac aldolase. For the 9-*O*-methyl derivative **10** and the 9-*O*-methoxymethyl derivative **13**, saponification was achieved by exposure to dilute NaOH in MeOH, to afford Neu5Ac9Me (**8**) and Neu5Ac9MOM (**12**), respectively, in high yield. The base sensitive nature of the 9-*O*-benzoyl group in **5** precluded the use of NaOH in the saponification of the methyl ester group, as exemplified by the attempted careful saponification (at pH 10.5) of **5** which showed (by TLC analysis) that the 9-*O*-benzoyl group was being hydrolysed more rapidly than the methyl ester. Fortunately, treatment of the 9-*O*-benzoyl derivative **5** with 25% aqueous Et$_3$N[30] furnished Neu5Ac9Bz (**3**) in 72% yield after chromatography. Only a small amount (~10%) of Neu5Ac was isolated from this reaction. Unfortunately, all attempts to hydrolyse the methyl ester group in the 9-*O*-tosyl derivative **9**, using either dilute NaOH in MeOH or aqueous Et$_3$N, failed to yield any of the desired product.

In addition to the compounds described above, we have previously described[31] the synthesis of the C$_{11}$ chain-extended analogue **14** of Neu5Ac. The additional structural features of **14** may provide valuable information regarding the steric and electronic specificity of Neu5Ac aldolase. Interestingly however, attempted NaOMe catalysed deacetylation of **14** resulted in the instantaneous deep red colouration of the reaction mixture (even at −10 °C) and partial decomposition (to a highly polar and unidentifiable yellow material) of the chain-extended Neu5Ac derivative. Whilst yields of up to 60% of the deacetylated material **15** could be obtained under carefully controlled conditions,[32] it was felt that an alternative deprotection strategy was appropriate, particularly since methyl glycoside and methyl ester functionalities are still present in **15**. Towards this end, exposure of the chain-extended compound **14** to acidic Dowex® resin (50W×8) in water at 80 °C gave the fully deprotected compound **16** in 68% yield after chromatography.



Evaluation of C-9 modified Neu5Ac derivatives as substrates for Neu5Ac aldolase

Each of the C-9 modified Neu5Ac derivatives (100 mg) were exposed to Neu5Ac aldolase (from *E. coli*, 1.4 units) using membrane-enclosed enzyme catalysis (MEEC)[33]

at pH 7.4 and 37 °C over a period of 4 days (see Experimental for full details). After this time the solution was concentrated and examined by ^{1}H NMR spectroscopy to determine the ratio of Neu5Ac derivative versus ManNAc derivative. The mixture of Neu5Ac and ManNAc derivatives from each reaction were then separated using chromatography. The results in Table 1 show that, with the exception of the C_{11} chain-extended derivative **16**, all other C-9 modified Neu5Ac derivatives were recognised as substrates by Neu5Ac aldolase. There are clear trends in the relative rates of cleavage of these compounds, which correlate, in part, with the steric demand of the functionality at C-9. For example, the sterically more demanding 9-*O*-MOM ether (**12**) is transformed more slowly than the 9-*O*-methyl ether (**6**) by Neu5Ac aldolase. Similarly, the 9-*O*-benzoyl derivative **3** is cleaved significantly more slowly than the 9-*O*-acetyl derivative **2**. Whilst these observations may suggest that a group as large as a benzoyl moiety could be approaching the limit of the steric tolerance of Neu5Ac aldolase it is worthwhile remembering that a number of 9-*O*-acyl Neu5Ac derivatives, including a sterically demanding BOC-glycyl derivative,[34] can be easily prepared by the reverse enzyme reaction.[14,35] As the enzyme substrate is bound in the acyclic form,[5,9,36] at this stage it is difficult to rationalise the apparent differences in steric tolerance between the forward and reverse Neu5Ac aldolase catalysed transformations. Studies directed towards a better understanding of the steric constraints of Neu5Ac aldolase are continuing.

Since the C_{11} chain-extended Neu5Ac derivative **16** was not recognised as a substrate by Neu5Ac aldolase it was investigated for its inhibitory action against this enzyme. ^{1}H NMR spectroscopy was conveniently used to monitor the rate of transformation of Neu5Ac to ManNAc by Neu5Ac aldolase,[37] since the H3e proton of Neu5Ac (δ∼2.3) and the H1 proton of ManNAc (δ∼5.1/5.2 for α/β) are not obscured by other resonances. The progress of the transformation of Neu5Ac to ManNAc could then easily be determined by measuring the relative integrals of Neu5Ac H3e and ManNAc H1 (full details included in Experimental). In an analogous experiment, the C_{11} chain-extended Neu5Ac derivative **16** (6 mg) was added to a mixture containing Neu5Ac (4 mg) and Neu5Ac aldolase (0.1 units) and the mixture monitored by ^{1}H NMR spectroscopy. The results from these studies are shown in Figure 1, and demonstrate quite clearly that the rate of hydrolysis of Neu5Ac is significantly reduced in the presence of **16**.

It is not clear at this stage why the chain-extended compound **16** is an inhibitor of Neu5Ac aldolase. There are a number of plausible explanations of how this compound might inhibit the catalytic activity of Neu5Ac aldolase, including the possibility that the additional carboxylate group may be interacting with the Schiff-base forming lysine. Additional investigations

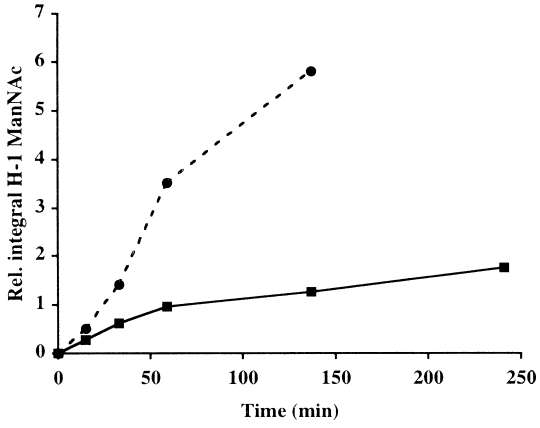


Figure 1. Rate of hydrolysis of **1** by Neu5Ac aldolase in the presence (—■—) and absence (- -●- -) of **16**.

Table 1. Survey of C-9 modified Neu5Ac derivatives as substrates for Neu5Ac aldolase

Compound	R′	Ratio from ^{1}H NMR analysis[a]		Ratio from isolated material[b]		Relative cleavage rates (%)
		8-R′-Neu5Ac	5-R′-ManNac	8-R′-Neu5Ac	5-R′-ManNAc	
1	CH_2OH	10.0	20.5	nd	nd	100
8	CH_2OMe	10.0	11.0	10.0	9.5	54
12	CH_2OMOM	10.0	7.4	10.0	7.1	36
2	CH_2OAc	10.0	8.1	10.0	7.5	39
3	CH_2OBz	10.0	2.8	10.0	2.5	14
16	$CH{=}CHCO_2H$	10.0	0.0[c]	10.0	nd[d]	nd[d]

[a]Ratio refers to the average result from at least two concordant experiments and is determined by the analysis of the integral from the ^{1}H NMR spectrum recorded on the entire enzyme reaction product.
[b]Ratio refers to the yield of material after chromatography.
[c]In addition to no transformation, this compound repeatedly caused precipitation of Neu5Ac aldolase.
[d]nd = not determined.

are required in order to understand more fully the nature of the interactions of compounds like **16** within the catalytic site of Neu5Ac aldolase.

In conclusion, this investigation has demonstrated that the retro-aldol condensation catalysed by Neu5Ac aldolase is susceptible to structural modification at C-9 of Neu5Ac. In particular, we have shown by a direct NMR spectroscopic method that large ester (e.g. **3**) or large ether (e.g. **12**) groups at C-9 result in a significant reduction in the rate of Neu5Ac aldolase catalysed transformation to the corresponding ManNAc derivative. In addition, we have demonstrated three different methods for the efficient deprotection of the methyl ester group at C-1 in derivatives of Neu5Ac, which adds an additional degree of flexibility to synthetic manipulations with sialic acid derivatives.

Experimental

General procedures

^{1}H and ^{13}C spectra were recorded using a Brüker DRX-300 spectrometer unless indicated otherwise. Chemical shifts are given in ppm relative to the solvent used ($CDCl_3$: 7.26 for ^{1}H; 77.0 for ^{13}C; CD_3OD: 3.31 for ^{1}H; 49.0 for ^{13}C) or relative to external Me_4Si for D_2O spectra. Two-dimensional DQF-COSY and HMQC experiments were recorded in order to assist with spectral assignment. Typically, the following parameters were used: DQF-COSY—16 scans, 512 slices, relaxation delay 2.0 s; ^{1}H–^{13}C HMQC—48 scans, 256 slices, relaxation delay 2.5 s. FAB mass spectra were obtained using a Jeol JMS-DX 300 mass spectrometer. Infrared spectra were recorded as KBr discs using a Hitachi 270-30 spectrophotometer. Optical rotations were measured using a Jasco DIP-370 digital polarimeter. Reactions were monitored by TLC (Merck silica gel plates GF_{254}, cat. No. 1.05554) and products were generally purified by flash chromatography using Merck silica gel 60 (0.040–0.063 mm, cat. No. 1.09385) or by HPLC using reverse phase C_{18} Waters μBondapak™ columns (analytical: 8×100 mm, part No. WAT085721; preparative: 25×100 mm, part No. WAT038505), detecting at 220 nM with a Waters 484 tunable absorbance detector. All solvents were distilled prior to use or were of analytical grade. Microanalyses were performed at the Department of Chemistry, University of Queensland, Australia. *N*-Acetylneuraminic acid aldolase (EC 4.1.3.3) from *E. coli* was generously provided by Dr. Yoji Tsukada (Kyoto Research Laboratories, Japan).

General procedure for performing reactions with Neu5Ac aldolase

The Neu5Ac derivative (~100 mg) to be treated with Neu5Ac aldolase was weighed accurately into a quickfit test-tube (12×1 cm) and then dissolved in 2.5 mL milli-Q H_2O. The pH of the solution was then adjusted to pH ca. 7.4 with dilute NaOH solution (0.5%, typically ca. 2 mL) and then the total volume of the solution made up to ca. 6 mL with milli-Q H_2O. Neu5Ac aldolase (1.4 units, 5.6 mg per unit) and BSA (3.0 mg) were then dissolved in ca. 200 μL of milli-Q H_2O and added to a dialysis bag.[33] The dialysis bag was then placed in the test-tube and the reaction stirred for four days at 37 °C. The water was removed from the reaction vessel, and H_2O (2×3 mL) stood with the dialysis bag for 30 min. The combined aqueous fractions were concentrated and the total product examined by ^{1}H NMR spectroscopy to establish the ratio of Neu5Ac compound versus ManNAc compound present (see Table 1). Column chromatography then provided the pure components.

General procedure for performing NMR tube reactions with Neu5Ac aldolase

Neu5Ac (**1**) (4 mg) was dissolved in 0.6 mL of 50 mM deuterated PBS (pH 7.4) and a ^{1}H NMR spectrum recorded (600 MHz) at 37 °C representing time = 0. To the NMR tube was added Neu5Ac aldolase (0.4 mg, 0.1 unit), and ^{1}H NMR spectra recorded every ~15–20 min over 4 h. The relative integrals of H3e of Neu5Ac (δ~2.3) and H1 of ManNAc (δ~5.1/5.2 for α/β) were then measured for each time interval.

The identical experiment was then carried out, with the exception that the C_{11} chain-extended derivative **16** (6 mg) was added with the Neu5Ac at the beginning of the experiment (time = 0). In each experiment, ^{1}H NMR spectra were recorded using a Brüker 600MHz DRX spectrometer operating at 37 °C. Spectra were acquired with 32 scans and 16 k data points, over a spectral width of 7800 Hz.

Synthesis of C-9 modified Neu5Ac derivatives

5-Acetamido-9-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosonic acid (2). To a solution of Neu5Ac (0.50 g, 1.6 mmol) in DMSO (5 mL) under N_2 at rt was added trimethylorthoacetate (2.0 mL, 16 mmol) and *p*-TsOH·H_2O (20 mg). After being stirred at rt for 1.5 h, the mixture was applied directly to a column (8×1 cm) of Dowex® 1-×4 (HCO_2^-) ion exchange resin and the column eluted with H_2O (100 mL) and then formic acid (1 N, 200 mL). The formic acid eluant was concentrated under reduced pressure and then purified by HPLC (reverse phase, 3% CH_3CN in H_2O) to afford Neu5,9Ac_2 (**2**) (0.44 g, 78%) as an amorphous white solid: $[\alpha]_D$ −15.3° (*c* 1.03, H_2O); ν_{max} 3440(br), 1730, 1634, 1554, 1376, 1264 and 1036 cm^{-1}; ^{1}H NMR (D_2O) δ 1.80 (1H, dd, $J_{3a,3e}$ 12.9, $J_{3a,4}$ 11.9 Hz, H-3a), 1.98 (3H, s, AcN), 2.04 (3H, s, AcO), 2.23 (1H, dd, $J_{3e,3a}$ 12.9, $J_{3e,4}$ 4.5 Hz, H-3e), 3.53 (1H, d, $J_{7,8}$ 9.2 Hz, H-7), 3.78–3.89 (2H, m, H-5/H-8), 3.94–4.03 (2H, m, H-4/H-6), 4.10 (1H, dd, $J_{9,9'}$ 11.7, $J_{9,8}$ 5.4 Hz, H-9), 4.29 (1H, dd, $J_{9',9}$ 11.7, $J_{9',8}$ 2.1 Hz, H-9′), assignments confirmed by DQF-COSY; ^{13}C NMR (D_2O) δ 22.9 (OC(O)*Me*), 24.7 (NC(O)*Me*), 41.4 (C-3), 54.7 (C-5), 68.9 (C-9), 69.3 (C-4*), 70.3 (C-7*), 70.8 (C-8*), 72.9 (C-6*), 97.8 (C-2), 175.6 (C-1), 177.0, 177.4 (N*C*(O)Me/O*C*(O)Me), *assignments interchangeable; FABMS: 352 [$(M+1)^+$, 64%], 334 (16), 316 (21), 201 (52), 185 (100). Anal. calcd for $C_{13}H_{21}NO_{10}\cdot0.5H_2O$: C, 43.2; H, 6.3; N, 3.6. Found: C, 43.3; H, 6.15; N, 3.9.

Methyl 5-acetamido-9-*O*-benzoyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonate (5). To a solution of Neu5Ac1Me (**4**) (0.50 g, 1.55 mmol) in pyridine (5 mL) under N_2 at 0 °C was added benzoyl chloride (200 μL, 1.7 mmol). The mixture was allowed to warm to rt and stirred for 15 h before MeOH (ca. 0.5 mL) was added and the solution concentrated. Column chromatography (Silica, EtOAc:MeOH, 10:1; R_f 0.3) afforded Neu5Ac9Bz1Me (**5**) (0.47 g, 71%) as an amorphous white solid: $[\alpha]_D$ −2.7° (*c* 1.03, MeOH); ν_{max} 3415(br), 1735, 1720, 1636, 1552, 1450, 1276, 1122 and 1066 cm^{-1}; ^1H NMR (D_2O) δ 1.84 (1H, dd, $J_{3a,3e}$ 12.8, $J_{3a,4}$ 11.8 Hz, H-3a), 1.93 (3H, s, AcN), 2.23 (1H, dd, $J_{3e,3a}$ 12.8, $J_{3e,4}$ 4.7 Hz, H-3e), 3.64 (1H, d, $J_{7,8}$ 9.3 Hz, H-7), 3.74 (3H, s, CO_2Me), 3.85 (1H, dd, $J_{5,6} = J_{5,4} = 10.1$ Hz, H-5), 3.96–4.04 (3H, m, H-4/H-6/H-8), 4.36 (1H, dd, $J_{9,9'}$ 11.8, $J_{9,8}$ 4.9 Hz, H-9), 4.50 (1H, dd, $J_{9',9}$ 11.8, $J_{9',8}$ 1.3 Hz, H-9′), 7.40–7.45 (2H, m, Ph), 7.52–7.58 (1H, m, Ph), 7.86–7.91 (2H, m, Ph); ^{13}C NMR (D_2O) δ 24.6 (NC(O)*Me*), 41.3 (C-3), 54.8 (C-5), 56.1 (CO_2Me), 69.1 (C-8*), 69.3 (C-9), 70.6 (C-4*), 70.8 (C-7*), 72.9 (C-6*), 97.9 (C-2), 131.2, 131.9, 136.3 (all Ph), 131.7 (ipso Ph), 170.9 (Ph*C*(O)O), 173.9 (C-1), 177.3 (N*C*(O)Me), *assignments interchangeable; FABMS: 450 [$(M+Na)^+$, 100%], 428 [$(M+1)^+$, 90]. Anal. calcd for $C_{19}H_{25}NO_{10}$: C, 53.4; H, 5.9; N, 3.3. Found: C, 53.4; H, 6.1; N, 2.9.

Methyl (methyl 5-acetamido-9-*O*-methyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (7). To a stirred solution of the 9-*O*-mesylate **6**[29] (0.78 g, 1.89 mmol) in MeOH (15 mL) under N_2 at rt was added 2 mL of a freshly prepared NaOMe solution (from 0.15 g of sodium dissolved in 10 mL of MeOH). After 60 min the mixture was acidified (to pH 2) with Dowex® 50W×8 H^+ resin and stirred for a further 2 h, before being filtered and concentrated. Chromatography (Silica, EtOAc:MeOH, 10:1; R_f 0.3) gave **7** (0.23 g, 38%) as a white foam: ν_{max} 3410(br), 1735, 1660, 1172, and 1034 cm^{-1}; ^1H NMR (d_4-MeOH) δ 1.76 (1H, dd, $J_{3a,3e}$ 12.7, $J_{3a,4}$ 11.7 Hz, H-3a), 1.92 (3H, s, AcN), 2.57 (1H, dd, $J_{3e,3a}$ 12.7, $J_{3e,4}$ 4.5 Hz, H-3e), 3.26 (3H, s, 9-OMe), 3.30 (3H, s, 2-OMe), 3.34–3.58 (5H, m, H-4/H-6/H-7/H-9/H-9′), 3.64 (3H, s, CO_2Me), 3.66 (1H, ddd, $J_{5,6} = J_{5,4} = J_{5,NH} = 10.1$ Hz, H-5), 3.87 (1H, ddd, $J_{8,7}$ 8.6, $J_{8,9}$ 5.5, $J_{8,9'}$ 2.2 Hz, H-8); ^{13}C NMR (d_4-MeOH) δ 22.7 (NC(O)*Me*), 41.4 (C-3), 51.9 (2-O*Me*), 53.4 (C-5*), 53.9 (CO_2Me*), 59.4 (9-O*Me*), 68.6 (C-9), 70.2 (C-4*), 71.1 (C-6*), 74.7 (C-7*), 75.3 (C-8*), 100.3 (C-2), 170.8 (C-1), 175.1 (N*C*(O)Me), *assignments interchangeable; FABMS: 374 [$(M+Na)^+$, 70%], 352 [$(M+1)^+$, 90].

Methyl 5-acetamido-9-*O*-tosyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonate (9). To a stirred solution of Neu5Ac1Me (**4**) (1.05 g, 3.25 mmol) in pyridine (5 mL) at 0 °C under N_2 was added *p*-toluenesulfonyl chloride (0.74 g, 3.90 mmol) and the mixture stirred at 5 °C for 16 h. MeOH (3 mL) was added to the mixture which was then concentrated to a yellow syrup. Chromatography (Silica, EtOAc:MeOH, 4:1; R_f 0.7) gave the tosylate **9** (0.87 g, 55%) as a white foam: $[\alpha]_D$ −16.3° (*c* 1.28, MeOH); ν_{max} 3396(br), 1744, 1640, 1554, 1172, 1132 and 1034 cm^{-1}; ^1H NMR (d_4-MeOH) δ 1.76 (1H, dd, $J_{3a,3e} = J_{3a,4} = 12.0$ Hz, H-3a), 1.90 (3H, s, AcN), 2.10 (1H, dd, $J_{3e,3a}$ 12.0, $J_{3e,4}$ 4.9 Hz, H-3e), 2.32 (3H, s, Ar*Me*), 3.34 (1H, d, $J_{7,8}$ 9.2 Hz, H-7), 3.65 (3H, s, CO_2Me), 3.71–3.98 (5H, m, H-4/H-5/H-7/H-8/H-9), 4.18 (1H, dd, $J_{9',9}$ 9.9, $J_{9',8}$ 1.8 Hz, H-9′), 7.32–7.67 (4H, m, Ar*H*); ^{13}C NMR (d_4-MeOH) δ 24.6 (NC(O)*Me*), 25.7 (Ar*Me*), 43.6 (C-3), 55.2 (C-5), 56.2 (CO_2Me), 69.6 (C-4*), 71.2 (C-6*), 71.9 (C-7*), 73.4 (C-8*), 75.7 (C-9), 98.5 (C-2), 131.0, 133.0, (2×ArH), 136.0 (ArC-4), 148.3 (ArC-1), 173.6 (C-1), 177.1 (N*C*(O)Me), *assignments interchangeable; FABMS: 478 [$(M+1)^+$, 80%; Found: 478.13677 $C_{19}H_{28}NO_{11}S$ requires 478.13831]. Anal. calcd for $C_{19}H_{27}NO_{11}S \cdot H_2O$: C, 46.1; H, 5.9; N, 2.8. Found: C, 46.5; H, 5.8; N, 2.4.

Methyl 5-acetamido-9-*O*-methyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonate (10). To a stirred solution of the tosylate **9** (0.63 g, 1.32 mmol) in MeOH (5 mL) under N_2 at rt was added 2 mL of a freshly prepared NaOMe solution (from 0.15 g of sodium dissolved in 10 mL of MeOH). After 20 min a further 2 mL of the NaOMe solution was added and the mixture stirred for 30 min. The mixture was acidified (to pH 2) with Amberlite IR-120H^+ resin and stirred for a further 2 h, before being filtered and concentrated. Chromatography (Silica, EtOAc:MeOH, 4:1; R_f 0.4) gave **10** contaminated with *p*-TsOH. Ion exchange chromatography (AG-1×4, 100–200 mesh, HCO_2^- form, eluted with H_2O) removed the *p*-TsOH to give Neu5Ac1,9Me_2 (**10**) (0.24 g, 53%) as a colourless solid: $[\alpha]_D$ −10.4° (*c* 0.97, H_2O); ν_{max} 3448(br), 2940, 1744, 1600, 1440, 1376 and 1124 cm^{-1}; ^1H NMR (D_2O) δ 1.89 (1H, dd, $J_{3a,3e}$ 13.1, $J_{3a,4}$ 12.1 Hz, H-3a), 2.03 (3H, s, AcN), 2.29 (1H, dd, $J_{3e,3a}$ 13.1, $J_{3e,4}$ 4.6 Hz, H-3e), 3.36 (3H, s, OMe), 3.49–3.54 (2H, m, H-7/H-9), 3.68 (1H, d, $J_{9',9}$ 10.7 Hz, H-9′), 3.82 (3H, s, CO_2Me), 3.89 (1H, dd, $J_{5,6} = J_{5,4} = 10.2$ Hz, H-5), 4.00–4.10 (2H, m, H-6/H-4); ^{13}C NMR (D_2O) δ 24.8 (NC(O)*Me*), 43.0 (C-3), 56.3 (C-5), 57.8 (CO_2Me), 62.8 (OMe), 71.0 (C-4*), 72.6 (C-6*), 72.8 (C-7*), 74.6 (C-8), 78.1 (C-9), 99.6 (C-2), 173.6 (C-1), 177.8 (N*C*(O)Me), *assignments interchangeable; FABMS: 360 [$(M+Na)^+$, 100%], 338 [$(M+1)^+$, 92; Found: 338.14576 $C_{13}H_{24}NO_9$ requires 338.14511]. Anal. calcd for $C_{13}H_{23}NO_9 \cdot H_2O$: C, 43.9; H, 7.1; N, 3.9. Found: C, 43.7; H, 7.1; N, 4.0%.

Methyl 5-acetamido-9-*O*-methanesulfonyl-3,5-dideoxy-D-*glycero*-β-D-galacto-2-nonulopyranosylonate (11). To a stirred solution of Neu5Ac1Me (**4**) (0.50 g, 1.55 mmol) in pyridine (7 mL) at 0 °C under N_2 was added methanesulphonyl chloride (209 μL, 2.71 mmol) and the mixture stirred for 3 h. MeOH (3 mL) was added and the mixture concentrated to a yellow syrup. Chromatography (Silica, EtOAc:MeOH, 4:1; R_f 0.7) gave the desired 9-*O*-mesylate **11** (0.38 g, 61%): $[\alpha]_D$ −16.6° (*c* 0.9, MeOH); ν_{max} 3388(br), 1736, 1664, 1528, 1356, 1278, 1170, 1132 and 1032 cm^{-1}; ^1H NMR (D_2O) δ 1.85 (1H, dd, $J_{3a,3e} = J_{3a,4} = 12.5$ Hz, H-3a), 1.99 (3H, s, AcN), 2.26 (1H, dd, $J_{3e,3a}$ 12.5, $J_{3e,4}$ 4.6 Hz, H-3e), 3.16 (3H, s, SO_3Me), 3.56 (1H, d, $J_{7,8}$ 9.2 Hz, H-7), 3.77 (3H, s, CO_2Me), 3.81–4.02 (4H, m, H-4/H-5/H-6/H-8), 4.37 (1H, dd, $J_{9,9'}$ 10.6, $J_{9,8}$ 4.9 Hz, H-9), 4.47 (1H, dd, $J_{9',9}$ 10.6, $J_{9',8}$ 1.6 Hz, H-9′); ^{13}C NMR (D_2O) δ 24.7 (NC(O)*Me*), 39.0 (SO_3Me), 41.3 (C-3), 54.7 (C-5), 54.7

(CO_2Me), 69.2 (C-4*), 70.0 (C-6*), 70.3 (C-7*), 70.5 (C-8*), 72.9 (C-6), 75.0 (C-9), 97.9 (C-2), 173.9 (C-1), 175.5 (N*C*(O)Me), *assignments interchangeable; FABMS: 424 [$(M+Na)^+$, 4%], 402 [$(M+1)^+$, 100; Found: 402.10868 $C_{13}H_{24}NO_{11}S$ requires 402.10699].

Methyl 5-acetamido-3,5-dideoxy-9-*O*-methoxymethyl-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonate (13). To a solution of Neu5Ac1Me (**4**) (0.41 g, 1.27 mmol) in DMF (4 mL) under N_2 at 0 °C was added diisopropylethyl amine (775 μL, 4.45 mmol) and then chloromethyl methylether (115 μL, 1.52 mmol). The mixture was allowed to warm to rt and stirred for 48 h before MeOH (ca. 0.4 mL) was added and the solution concentrated. Column chromatography (Silica, EtOAc:MeOH, 4:1) furnished recovered Neu5Ac1Me (0.10 g) together with the title compound **13** [R_f 0.3, 0.29 g, 62% (81% based on recovered Neu5Ac1Me)] as an amorphous white solid: $[\alpha]_D$ −19.5° (*c* 1.05, H_2O); ν_{max} 3380(br), 1746, 1642, 1552, 1440, 1122 and 1032 cm^{-1}; ^1H NMR (D_2O) δ 1.86 (1H, dd, $J_{3a,3e}$ 12.9, $J_{3a,4}$ 11.8 Hz, H-3a), 1.99 (3H, s, AcN), 2.25 (1H, dd, $J_{3e,3a}$ 12.9, $J_{3e,4}$ 4.8 Hz, H-3e), 3.34 (3H, s, OCH_2OMe), 3.54 (1H, d, $J_{7,8}$ 8.7 Hz, H-7), 3.63 (1H, dd, $J_{9,9'}$ 11.0, $J_{9,8}$ 6.1 Hz, H-9), 3.76–3.83 (2H, m, H-8/H-9′), 3.78 (3H, s, CO_2Me), 3.85 (1H, dd, $J_{5,6}=J_{5,4}=10$ Hz, H-5), 3.94–4.05 (2H, m, H-4/H-6), 4.65 (2H, s, OCH_2OMe), assignments confirmed by DQF-COSY; ^{13}C NMR (D_2O) δ 24.6 (NC(O)*Me*), 41.3 (C-3), 54.7 (C-5), 56.1 (CO_2Me), 57.6 (OCH_2OMe), 69.1 (C-4), 70.7 (C-7), 71.4 (C-8), 72.0 (C-9), 72.9 (C-6), 97.9 (C-2), 98.9 (OCH_2OMe), 173.9 (C-1), 177.4 (N*C*(O)Me), *assignments confirmed by HMQC; FABMS: 390 [$(M+Na)^+$, 97%], 368 [$(M+1)^+$, 95]. Anal. calcd for $C_{14}H_{25}NO_{10}$: C, 45.8; H, 6.9; N, 3.8. Found: C, 45.6; H, 7.1; N, 3.1.

5-Acetamido-9-*O*-methyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonic acid (8). Neu5Ac1,9Me_2 (**10**) (0.18 g, 0.54 mmol) was dissolved in MeOH (2 mL) and 2 N NaOH added to pH 13. The mixture was stirred for 4 h at rt and then Amberlite IR 120H^+ resin was added to pH 5. The resin was removed by filtration, washed with aq MeOH (3×10 mL) and the combined filtrate concentrated under reduced pressure. HPLC purification (reverse phase, 5% CH_3CN in H_2O) gave Neu5Ac9Me (**8**) (0.11 g, 62%) as an amorphous mass: ν_{max} 3456(br), 2932, 1742, 1632, 1376, 1120 and 1030 cm^{-1}; ^1H NMR (D_2O) δ 1.82 (1H, dd, $J_{3a,3e}$ 13.0, $J_{3a,4}$ 12.1 Hz, H-3a), 1.98 (3H, s, AcN), 2.24 (1H, dd, $J_{3e,3a}$ 13.0, $J_{3e,4}$ 3.8 Hz, H-3e), 3.32 (3H, s, OMe), 3.48–3.51 (2H, m, H-7/H-9), 3.64, (1H, d, $J_{9',9}$ 10.6 Hz, H-9′), 3.78 (1H, dd, $J_{8,7}$ 8.8, $J_{8,9}$ 6.6 Hz, H-8), 3.88 (1H, dd, $J_{5,6}=J_{5,4}=10.1$ Hz, H-5), 3.97–4.01 (2H, m, H-6/H-4); ^{13}C NMR (D_2O) δ 24.6 (NC(O)*Me*), 41.3 (C-3), 54.6 (C-5), 61.0 (OMe), 69.2 (C-4*), 70.8 (C-6*), 71.1 (C-7*), 72.9 (C-8*), 76.4 (C-9), 98.2 (C-2), 175.6 (C-1), 177.3 (N*C*(O)Me), *assignments interchangeable.

5-Acetamido-9-*O*-methoxymethyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonic acid (12). Prepared from the 9-*O*-MOM ether **13** using the saponification procedure detailed above to give **12** in 72% yield after HPLC (reverse phase, 3% CH_3CN in H_2O) as an amorphous mass: ν_{max} 3400(br), 1710, 1632, 1550, 1375, 1120 and 1032 cm^{-1}; ^1H NMR (D_2O) δ 1.82 (1H, dd, $J_{3a,3e}$ 12.7, $J_{3a,4}$ 11.6 Hz, H-3a), 1.98 (3H, s, AcN), 2.19 (1H, dd, $J_{3e,3a}$ 12.7, $J_{3e,4}$ 4.7 Hz, H-3e), 3.34 (3H, s, OCH_2OMe), 3.51 (1H, d, $J_{7,8}$ 9.2 Hz, H-7), 3.64 (1H, dd, $J_{9,9'}$ 12.0, $J_{9,8}$ 6.5 Hz, H-9), 3.76–3.82 (2H, m, H-8/H-9′), 3.84 (1H, dd, $J_{5,6}=J_{5,4}=10.1$ Hz, H-5), 3.91–4.02 (2H, m, H-4/H-6), 4.64 (2H, s, OCH_2OMe), assignments confirmed by DQF-COSY; ^{13}C NMR (D_2O) δ 24.7 (NC(O)*Me*), 41.5 (C-3), 54.7 (C-5), 57.6 (OCH_2OMe), 69.3 (C-4), 70.8 (C-7), 71.5 (C-8), 72.1 (C-9), 73.0 (C-6), 98.0 (C-2), 98.9 (OCH_2OMe), 175.5 (C-1), 177.5 (N*C*(O)Me), assignments confirmed by HMQC; FABMS: 398 [$(M+Na)^+$, 8%], 376 [$(M+1)^+$, 13], 354 (100).

5-Acetamido-9-*O*-benzoyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosonic acid (3). To a solution of Neu5Ac9Bz1Me (**5**) (0.15 g, 0.35 mmol) in H_2O (1 mL) was added Et_3N (0.25 mL)[30] and the mixture stirred at rt for 1.5 h. Concentration of the mixture under reduced pressure and column chromatography (Silica, EtOAc:MeOH:H_2O, 3:2:0.1) afforded Neu5Ac (R_f 0.2, 18 mg, 17%) together with Neu5Ac9Bz (**3**) (R_f 0.3, 104 mg, 72%) as an amorphous white solid: ν_{max} 3380(br), 1708, 1630, 1376, 1314, 1278, 1122 and 1032 cm^{-1}; ^1H NMR (D_2O) δ 1.76 (1H, dd, $J_{3a,3e}$ 13.0, $J_{3a,4}$ 11.6 Hz, H-3a), 1.92 (3H, s, AcN), 2.17 (1H, dd, $J_{3e,3a}$ 13.0, $J_{3e,4}$ 4.5 Hz, H-3e), 3.69 (1H, d, $J_{7,8}$ 8.6 Hz, H-7), 3.87 (1H, d, $J_{6,5}$ 9.6 Hz, H-6), 3.90–4.10 (3H, m, H-4/H-5/H-8), 4.36 (1H, dd, $J_{9,9'}$ 11.7, $J_{9,8}$ 5.0 Hz, H-9), 4.47 (1H, dd, $J_{9',9}$ 11.7, $J_{9',8}$ 2.0 Hz, H-9′), 7.42–7.47 (2H, m, Ph), 7.57–7.62 (1H, m, Ph), 7.91–7.97 (2H, m, Ph); ^{13}C NMR (D_2O) δ 24.7 (NC(O)*Me*), 42.0 (C-3), 54.9 (C-5), 69.1 (C-9), 69.6 (C-8*), 70.6 (C-4*), 70.9 (C-7*), 72.8 (C-6*), 99.4 (C-2), 131.4, 132.0, 136.5 (all Ph), 131.8 (ipso Ph), 171.1 (C-1), 177.3 (N*C*(O)Me), 179.2 (Ph*C*(O)O), *assignments interchangeable; FABMS: 436 [$(M+Na)^+$, 59%], 414 [$(M+1)^+$, 33]. Anal. calcd for $C_{18}H_{23}NO_{10}\cdot 1.5H_2O$: C, 49.1; H, 5.9; N, 3.2. Found: C, 49.1; H, 5.5; N, 3.0.

5-Acetamido-9,10-didehydro-3,5,9,10-tetradeoxy-10-carboxy-D-*glycero*-β-D-*galacto*-2-deculopyranosonic acid (16). A solution of the C_{11} chain-extended Neu5Ac derivative **14**[31] (200 mg, 0.38 mmol) in H_2O (10 mL) containing Dowex® 50W×8 acidic resin (500 mg) was heated at 80 °C for 48 h. After cooling to rt the mixture was filtered, the resin washed with H_2O and the solution concentrated. HPLC chromatography (reverse phase, H_2O) gave **16** (92 mg, 68%) as an amorphous white solid: ν_{max} 3350(br), 1710, 1642, 1554, 1376, 1272, 1124 and 1036 cm^{-1}; ^1H NMR (D_2O) δ 1.83 (1H, dd, $J_{3a,3e}$ 12.5, $J_{3a,4}$ 11.7 Hz, H-3a), 1.99 (3H, s, AcN), 2.26 (1H, dd, $J_{3e,3a}$ 12.5, $J_{3e,4}$ 4.5 Hz, H-3e), 3.51 (1H, d, $J_{7,8}$ 8.3 Hz, H-7), 3.88 (1H, dd, $J_{5,6}=J_{5,4}=10.1$ Hz, H-5), 3.98–4.07 (3H, m, H-4/H-6), 4.35 (1H, m, H-8), 6.05 (1H, d, $J_{10,9}$ 15.7 Hz, H-10), 7.08 (1H, dd, $J_{9,10}$ 15.7, $J_{9,8}$ 4.9 Hz, H-9), assignments confirmed by DQF-COSY; ^{13}C NMR (D_2O) δ 24.7 (NC(O)*Me*), 41.5 (C-3), 54.7 (C-5), 69.3 (C-4), 72.4 (C-8), 72.8 (C-6), 73.1 (C-7), 98.2 (C-2), 124.1 (C-10), 152.1 (C-9), 172.6, 175.8 (2×CO_2H), 177.4 (N*C*(O)Me), assignments confirmed

by HMQC; FABMS: 372 [$(M+Na)^+$, 18%], 350 [$(M+1)^+$, 44].

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