Organic & Biomolecular Chemistry

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
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Cite this: *Org. Biomol. Chem.*, 2021, **19**, 6644

Facile anomer-oriented syntheses of 4-methylumbelliferyl sialic acid glycosides†

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Received 5th May 2021,
Accepted 23rd June 2021

DOI: 10.1039/d1ob00877c

rsc.li/obc

As part of a program to find new sialidases and determine their enzymatic specificity and catalytic activity, a library of 4-methylumbelliferyl sialic acid glycosides derivatised at the C-5 position were prepared from *N*-acetylneuraminic acid. Both α- and β-4-methylumbelliferyl sialic acid glycosides were prepared in high yields and stereoselectivity. α-Anomers were accessed *via* reagent control by utilising additive CH_3CN and TBAI, whereas the β-anomers were synthesised through a diastereoselective addition reaction of iodine and the aglycone to the corresponding glycal followed by reduction of the resulting 3-iodo compounds. Both anomer-oriented synthetic pathways allow for gram-scale stereoselective syntheses of the desired C-5 modified neuraminic acid derivatives for use as tools to quantify the enzymatic activity and substrate specificity of known sialidases, and potential detection and investigation of novel sialidases.

Introduction

Sialic acids are a family of nonulosonic acids that are found as terminal residues in a wide variety of mammalian and prokaryotic cell lines. As a result of their terminal position in glycoproteins and glycolipids, they have been shown to be implicated in numerous biologically important processes such as cellular recognition, signalling and adhesion.[1] Their presence on cell surfaces are critical to normal cellular function in mammals.[2] Similarly, in bacterial and viral species, sialic acids are vital components for pathogenesis and bacterial nutrition.[3,4] In nature, there are currently over 80 differently modified members of the sialic acid family. As a starting point, we have selected to work on the most common C-5 modified sialic acid derivatives found in mammalian and bacterial cells (Fig. 1). The exact biological consequence of these modifications is still an ongoing endeavour in glycobiology.[5]

The amount of sialic residues incorporated into glycoconjugates within a given cell is typically regulated by the expression levels and activity of sialyltransferase and sialidase enzymes.[6] While sialyltransferases catalyse the addition of sialic acid monomers to their corresponding glycoconjugate moieties, sialidases (also referred to as neuraminidases, EC 3.2.1.18) catalyse the cleavage of sialic acid residues from their component glycan and peptide chains.[7,8] Consequently, sialidases have been shown to be critical components for a myriad of cellular phenomena such as modulation of protein recruitment,[9] cell differentiation,[10] cell signalling[11] and apoptosis.[12] Recently, Bertozzi and co-workers designed an αHER2 antibody-sialidase complex that selectively cleaves α-linked sialoglycans from breast cancer cells in order to induce an immune response against breast cancer in mice.[13]

As the amount of sialic acid molecules incorporated into the cell is regulated by sialidase enzymes, methods to identify and quantify sialidase activity is of paramount interest.[7] Generally, the hydrolytic activity of sialidases can be quantified by using synthetic fluorogenic substrates, such as 4-methylumbelliferylneuraminic acid, or other chromogenic or radiolabelled substrates.[14–17] While the α-4-methylumbelliferyl *N*-acetylneuraminic acid (**17α**) is commercially available, the corresponding β-anomers (to probe the potential existence of β-sialidases) of common C-5 derivatives of sialic acid are not easily accessible. Additionally, the chemistry of 4-methylumbelliferyl sialic glycosides has been left unexplored for

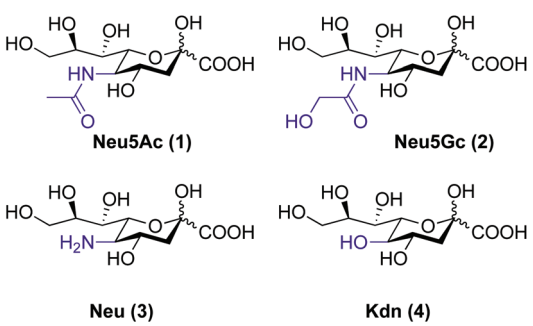

Fig. 1 Selected C-5 derivatised neuraminic acid derivatives found in nature.

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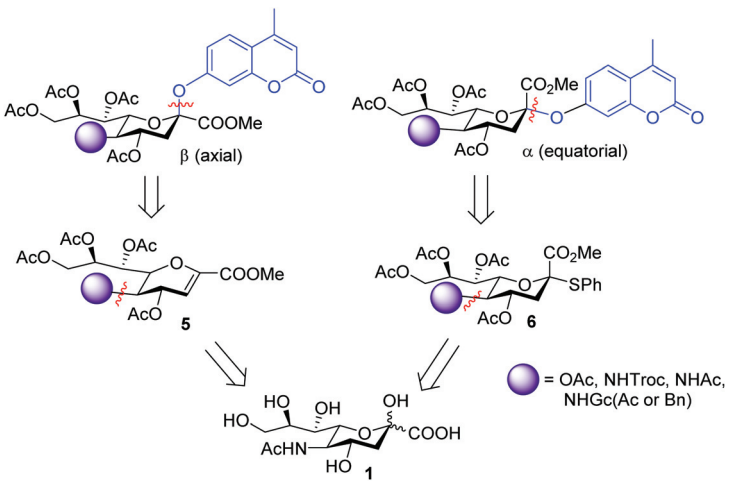
†Electronic supplementary information (ESI) available. See DOI: 10.1039/d1ob00877c

many years despite existing synthetic routes suffering from poor yields, low stereoselectivity and formation of the undesired glycal side product.[18–21] To this end, we report herein synthetic strategies to access C-5 derivatised neuraminic acid substrates with a fluorogenic 4-methylumbelliferyl (4-Mu) aglycone functionality in both anomeric configurations as potential tools to probe sialidase activity and specificity.

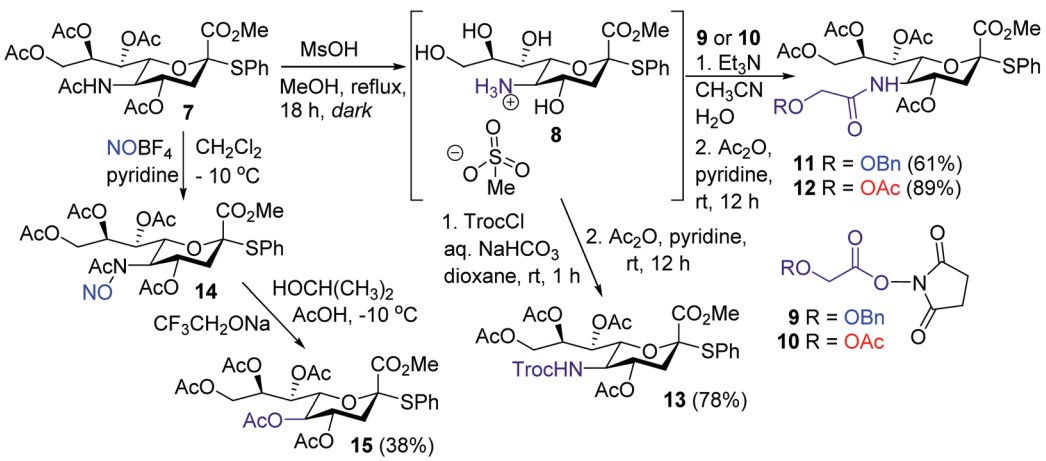
Results and discussion

Our syntheses starts from commercially available *N*-acetylneuraminic acid (**1**) (Scheme 1). The synthetic pathway is divided into two modular anomer-oriented approaches utilising glycal **5** and thiophenol **6** to stereoselectively install the fluorogenic aglycone at the anomeric position. Our previously developed $PhI(OAc)_2/I_2$ mediated diastereoselective olefin addition reaction would give rise to the β-anomers,[22] while we envisioned that the α-anomers could be obtained by utilising solvent control methodology previously investigated by others.[23]

Our synthesis towards the equatorial α-glycosides of the desired C-5 functionalised Neu5Ac derivatives began with preparation of the common Neu5Ac thiophenol donor **7**, which was prepared in 2 steps from commercially available Neu5Ac.[24] Initial efforts were then focused on derivatising this shared intermediate at the C-5 position to subsequently introduce our requisite fluorogenic glycosides. Accordingly, we converted **7** into its corresponding *N*-glycolyl neuraminic acid by condensation of free amine **8** with activated ester **9** in a mixture of CH_3CN and water affording Neu5Gc thioglycoside **11** in 61% yield over three steps (Scheme 2).[25] Simple replacement of the benzylated activated ester into its acetylated form **10** allowed for the gram scale synthesis of Neu5Gc donor **12** in 89% yield over three steps. Donors **11** and **12** were prepared to determine if the electronic differences in the glycolyl side chain affected the stereochemical outcome of the glycosylation reaction. Similarly, the free amine complex **8** was also treated



Scheme 1 Retrosynthetic analysis of C-5 derivatised sialic acid glycosides.



Scheme 2 Synthesis of acetyl and benzyl *N*-glycolyl, *N*-Troc, and Kdn thioglycoside derivatives.

with 2,2,2-trichloroethoxycarbonyl chloride (TrocCl) to chemoselectively mask the amine functionality. Acetylation of the Troc protected derivative gave Neu5Troc thioglycoside **13** in 78% yield over three steps (Scheme 2). Next, using a modified oxidative deamination methodology originally developed by Zbiral[26] and Ogura,[27] and later applied to thioglycosides by Crich *et al.*,[28] the requisite Kdn thiophenyl sialoside was prepared *via* treatment of **7** with nitrosonium tetrafluoroborate ($NOBF_4$) to furnish the corresponding nitrosyl amide intermediate **14** in an almost quantitative yield (Scheme 2). Subsequent stepwise addition of sodium isopropoxide, trifluoroethanol and acetic acid to the *N*-nitrosoamide species gave the desired oxidatively deaminated Kdn thioglycoside donor **15** in an overall 38% yield.[28]

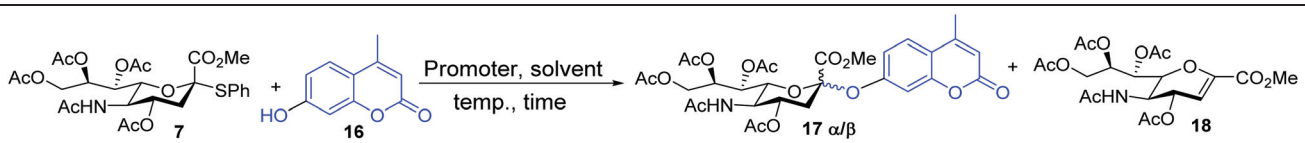
With our C-5 derivatised neuraminic acid thioglycosides in hand, we began glycosylation studies towards the desired 4-methylumbelliferyl α-glycosides. Control glycosylation reactions between donor **7** and our 4-Mu acceptor **16** were carried out, using mainly *N*-iodosuccinimide (NIS) as thiophilic promoter, to optimize the reaction (Table 1).[29–32]

Initially *N*-iodosuccinimide (NIS) and triflic acid (TfOH) in CH_2Cl_2 were tested. Unsurprisingly, at ambient room temperature, large quantities of the competing glycal side product **18** was observed in addition to the hydrolysed donor; 18% of **17α/β** in an anomeric mixture of 1 : 2.2 (α/β) was also recorded (entry 1).[33] Lowering the temperature to either −40 °C (entry 2) or −78 °C (entry 3) gave similar stereoselectivity but higher yield because of less elimination, as expected. The anomers were quite difficult to separate by column chromatography as their retention times on silica gel are very similar. Changing the promotor to dimethyl(methylthio)sulfonium trifluoromethanesulfonate (DMTST, entry 4), resulted in the formation of the glycal side product **18** with only trace quantities of **17** observable by NMR. However, the glycosylation reaction using NIS/AgOTf at −40 °C gave more promising results (entry 5) with a yield of 72% and a slightly better preference for the α-anomer **17α**. Expanding on these conditions, introduction of acetonitrile (CH_3CN), as an additive solvent known to increase α-selectivity in sialidations,[23,29–32] significantly improved the α-selectivity (α/β 8.3 : 1) in addition to giving a higher isolated yield (entry 6). Interestingly, additive tetra-*N*-butylammonium iodide (TBAI, 0.8 eq.) in conjunction with our improved reaction conditions of NIS/AgOTf in the presence of CH_3CN furnished complete α-selectivity (see ESI† for plausible mechanism) with an excellent yield of 91% (entry 8). The addition of TBAI to our reaction mixture was inspired by seminal work on glycosyl iodides in the research group of Gervay-Hague.[34–36] However, to the best of our knowledge, the anomeric selectivity of sialic acids was not investigated in these studies. Compound **17α** was straightforwardly deprotected, as described in the literature,[20] in an 80% yield (see ESI† for details).

Thereafter, we applied the developed α-selective glycosylation protocol to the other C-5 functionalised sialic acid donors (Table 2). In all cases, high to excellent diastereoselectivity towards the α anomer was observed, proving the optimised glycosylation conditions to be quite general. Complete α-selectivity and excellent yield (89%, **21α**) was observed with the benzylated Neu5Gc(Bn) donor **11**. While good α-selectivity (α/β = 4.3 : 1) was obtained with the acetylated Neu5Gc donor **12**, the isolated yield of the α -anomer product **22α** was slightly lower (72%) than its benzylated counterpart. However, in contrast to the Neu5Ac analogue (**17α/β**), chromatographic separation of the anomers was not an issue. For the Troc-protected donor **13**, an α/β ratio of 19 : 1 of product **23α** was obtained, affording a 75% yield of the α-anomer **23α** after silica gel column chromatography. Complete α-selectivity was again obtained for the acetyl protected Kdn glycosyl donor **15**, giving **24α** in 67% isolated yield. All reactions were performed on gram scale (1–4 g).

Having developed a robust protocol to stereoselectively prepare fluorogenic sialic acid α-glycosides, we began our studies towards the corresponding β-configured 4-methylumbelliferyl glycosides. To determine the feasibility of the diastereoselective glycal addition reaction previously developed

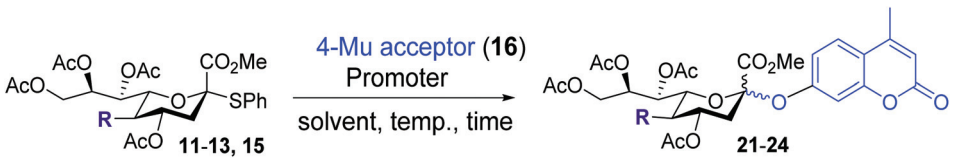
Table 1 Test glycosylation reaction



Entry	Promoter (equivalents)	Solvent	Temp.	Time	Product & anomeric ratio[a]	Yield of **17**[b]
1	NIS/TfOH (1 : 1 eq.)	CH_2Cl_2	rt	12 h	Elimination (**18**), hydrolysis and **17 α/β** = 1 : 2.2	18%
2	NIS/TfOH (1 : 1 eq.)	CH_2Cl_2	−40 °C	12 h	**18** & **17 α/β** = 1 : 1.6	46%
3	NIS/TfOH (1 : 1 eq.)	CH_2Cl_2	−78 °C	24 h	**17α/β** = 1.3 : 1	68%
4	DMTST (1 eq.)	CH_2Cl_2	0 °C	16 h	**18** & trace of **17α/β**	—
5	NIS/AgOTf (1 : 1 eq.)	CH_2Cl_2	−40 °C → rt	12 h	**18**, hydrolysis & **17α/β** = 1.7 : 1	72%
6	NIS/AgOTf (1 : 1 eq.)	CH_2Cl_2 : CH_3CN (1 : 1)	−78 °C	16 h	**17α/β** = 8.3 : 1	87%
7	NIS/AgOTf/TBAI (1 : 1 : 0.4 eq.)	CH_2Cl_2 : CH_3CN (1 : 1)	−78 °C	16 h	**17α**	91%, α only

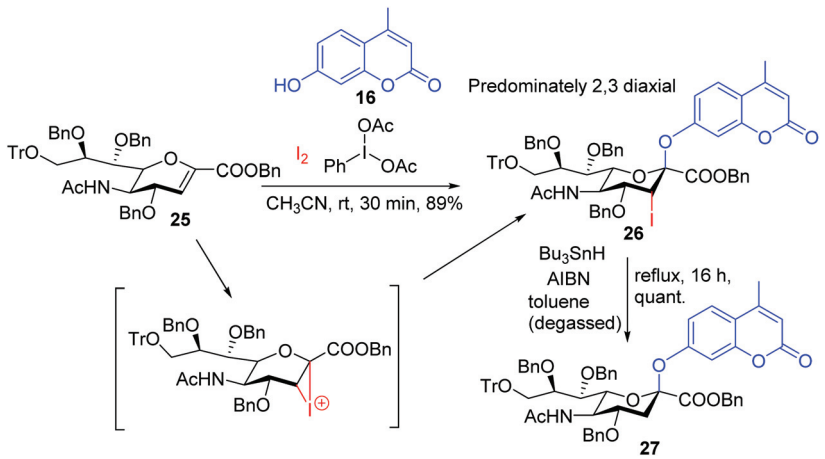
[a] Determined by ^{1}H NMR analysis of the crude reaction mixture. [b] Isolated yields.

Table 2 Optimised conditions for glycosylation of C-5 functionalised sialic acid derivatives



R	Promoter (equivalents)	Solvent	Temp.	Time	Product & anomeric ratio[a]	Yield of α-anomer[b]
NH(BnGc), **11**	NIS/AgOTf/TBAI (1 : 1 : 0.4 eq.)	CH_2Cl_2 : CH_3CN (1 : 1)	−78 °C	16 h	**21α** only	89%
NH(AcGc), **12**	NIS/AgOTf/TBAI (1 : 1 : 0.4 eq.)	CH_2Cl_2 : CH_3CN (1 : 1)	−78 °C	24 h	**22α/β** 4.3 : 1	72%
NHTroc, **13**	NIS/AgOTf/TBAI (1 : 1 : 0.4 eq.)	CH_2Cl_2 : CH_3CN (1 : 1)	−78 °C	12 h	**23α/β** 19 : 1	75%
OAc, **15**	NIS/AgOTf/TBAI (1 : 1 : 0.4 eq.)	CH_2Cl_2 : CH_3CN (1 : 1)	−78 °C	24 h	**24α** only	67%

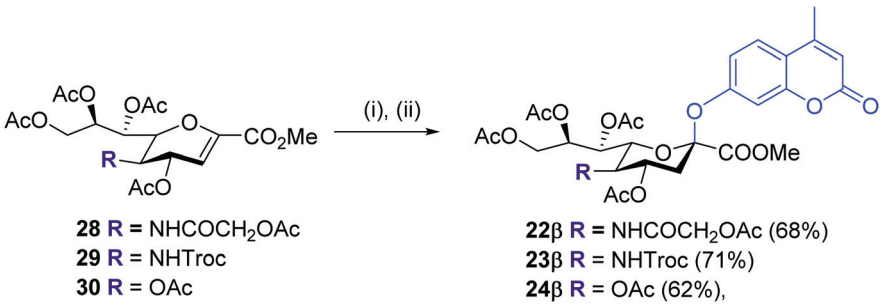
[a] Determined by ^{1}H NMR analysis of the crude reaction mixture. [b] Isolated yields. 4 Å molecular sieves were used in all the reactions.



Scheme 3 Diastereoselective addition of **16** to **25** to furnish **27**.

in our laboratory,[22] we carried out an initial test reaction with 9-*O*-trityl protected per-benzylated Neu5Ac glycal **25** (Scheme 3). Treatment of **25** with $PhI(OAc)_2$, molecular iodine and 4-Mu acceptor **16** gave the desired β anomer **26** in 95% selectivity (α/β = 1 : 19). Importantly, since in enzyme reactions even small contaminations of the wrong anomer can give false positives, it was found that the 3-iodo anomers were easily separated by silica gel column chromatography. Tributyl tin hydride (Bu_3SnH) reduction of the iodo function then afforded compound **27** in 89% overall yield. The anomeric configuration was determined by measuring $^3J_{C\text{-}1,\ H\text{-}3}$ coupling constant of a selectively decoupled coupled ^{13}C NMR experiment. Additionally, the relative anomeric ratio was measured by integrating the H-3eq proton after reduction of the iodo species.[37] Degassing the reaction mixture *via* 'freeze–pump–thaw' method was critical for the successful reduction of the iodide species in this reaction.

Performing the same reaction but starting from the acetylated Neu5Ac glycal **18** resulted in significantly lower diastereoselectivity (1 : 2.1) and a 60% yield of **17β**. However, since also these stereoisomers were easily separated, it was decided to extend this methodology to the other C-5 functionalized sialic acid derivatives using the acetylated glycals as precursors because of their easier accessibility, especially on a large scale, and facile deprotection (no risk of reducing the 4-methylumbelliferyl moiety). Their respective glycals, **28–30**, were easily accessed from the previously prepared thioglycosides **12**, **13**, and **15**. The sialyl thioglycosides were initially converted into the corresponding bromides and *in situ* elimination of the resulting bromides by addition of Et_3N furnished our desired 2,3-anhydro derivatives **28–30** in yields ranging from 85–96% (see ESI† for synthesis of glycals). Glycal formation was confirmed by high resolution mass spectrometry and by the presence of the diagnostic alkene H-3 proton peaks in ^{1}H NMR spectra at around 5.98 ppm. With the C-5 derivatised glycals in hand, their conversion into the desired β analogues was investigated (Scheme 4). Accordingly, the acetylated form of Neu5Gc (**28**) was treated with our optimised reaction conditions of $PhI(OAc)_2$ and I_2 in a mixture of CH_3CN and CH_2Cl_2 at room temperature. Excess equivalents of 4-Mu (**16**, 5 eq.) were necessary for higher yields. Completion of the reaction (between 20 and 30 min) was monitored by TLC and rapidly worked up. It should be noted that leaving the reaction on for longer than required results in a complex mixture. Again, a relatively low α/β-ratio of 1 : 3 was observed. After anomer separation followed by reduction of the iodide functionality using



Scheme 4 Reagents & conditions: (a) $PhI(OAc)_2$, I_2, **16**, CH_3CN : CH_2Cl_2, rt, 20–30 min; (b) Bu_3SnH, AIBN, degassed toluene, reflux, 18 h.

Bu_3SnH/AIBN, the β-4-Mu-Neu5Gc derivative **22β** was isolated in a 68% yield over the two steps.

The NeuTroc (**29**) and Kdn (**30**) glycals were also subjected to the same addition and reduction conditions and the desired 4-methylumbelliferyl β-glycosides **23** and **24** were isolated in 71% and 62% yield respectively, following tributyl tin hydride mediated reduction (Scheme 4). The observed lower β-selectivity for **18** (1 : 2.1, α : β), **28** (1 : 3, α : β), **29** (1 : 2.7, α : β), and **30** (1 : 3.5, α : β) was attributed to our chosen ester protecting groups, but all the diastereomeric mixtures were easily separated before the tin hydride reduction. Consequently, the decrease in stereoselectivity is offset by the ability to generate large quantities of pure β-4-methylumbelliferyl sialic glycosides following chromatographic separation of the 3-iodo species. It should be noted that no previous syntheses of the β-umbelliferyl sialic glycosides for Kdn, Neu, and Neu5Gc have been reported in literature.

Conclusions

In summary, a library of C-5 derivatised 4-methylumbelliferone sialic acid derivatives (Neu, Neu5Ac, Neu5Gc, Kdn) were synthesised in high yields from *N*-acetylneuraminic acid. The α anomers were stereoselectively accessed by synergistic acetonitrile-mediated solvent control, aided by additive TBAI. Excellent to good yields and α-stereoselectivity was observed for all the C-5 functionalised sialic derivatives. Similarly, the β-anomers were selectively synthesised *via* a preferential diastereoselective addition reaction to our C-5 derivatised neuraminic acid glycal analogues. Both anomer-oriented synthetic pathways allow for the gram scale stereoselective synthesis of the desired C-5 modified neuraminic acid derivatives for further chemoenzymatic studies and should be applicable to other sialic acid analogues. It is our hope that the synthesis of these derivatives will allow for the fluorometric quantification of the enzymatic activity and substrate specificity of known sialidases, and the detection of novel sialidases found in nature.

Experimental section

Full experimental details and procedures are disclosed in the ESI.†

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Financial support from Research Council of Norway (BIOTEK2021, Nr 254780) and Science Foundation Ireland (13/IA/1959) is gratefully acknowledged. Drs Yannick Ortin and Jimmy Muldoon in University College Dublin are also thanked for performing NMR and mass spectrometry experiments.

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