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SHORT COMMUNICATION

# A $^1$H NMR investigation of the hydrolysis of a synthetic substrate by KDN-sialidase from *Crassostrea virginica*

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The mechanism of hydrolysis of 4-methylumbelliferyl 3-deoxy-D-*glycero*-$\alpha$-D-*galacto*-2-nonulopyranosidonic acid (KDN$\alpha$2MeUmb, **4**) by KDN-sialidase isolated from the hepatopancreas of the oyster *Crassostrea virginica* has been monitored by $^1$H NMR spectroscopy. The results of these experiments reveal that KDN-sialidase catalyses the hydrolysis of the synthetic substrate KDN$\alpha$2MeUmb, with initial release of $\alpha$-D-KDN. This is consistent with an overall mechanism for the hydrolysis which proceeds with retention of anomeric configuration. These results agree with earlier NMR studies of other *N*-acetylneuraminic acid-recognising sialidases from both viral and bacterial sources.

*Keywords*: KDN-sialidase, NMR spectroscopy, sialidases, KDN

## Introduction

KDN (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid which is synonymous with 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid and 'deaminated neuraminic acid', **1**) is a novel sialic acid analogue first isolated from the polysialoglycoproteins of rainbow trout (*Salmo gairdneri*) eggs [1]. Since then, KDN-containing glycoconjugates have been found in a variety of organisms [2–7]. It has been shown that the terminal capping of the polysaccharide chains in salmon eggs by KDN protects the saccharides from exosialidase degeneration [8]. The presence of KDN in loach skin mucus was postulated to provide a first line of defence against sialidase-containing organisms [7]. However, the biological functions of KDN remain to be elucidated. Although KDN is structurally very similar to Neu5Ac, KDN-containing glycoconjugates are resistant to bacterial sialidases [9, 10].

Sialidases (E.C. 3.2.1.18) catalyse the release of terminal sialic acids $\alpha$-ketosidically linked to carbohydrate-bearing entities such as glycoproteins and glycolipids [11]. Many of these enzymes are associated with pathogenic microorganisms [12] and are considered excellent targets for drug design. Our recent interest in sialidases from various sources [13–19] and their mechanism of catalysis [20, 21] has provided some valuable insight which has facilitated the design of putative transition state analogues as potential inhibitors of these enzymes. Interestingly, all of the sialidases studied to date, regardless of the source, appear to be retaining glycohydrolases [20, 22, 23].

Based on the substrate specificity, sialidases can be divided into regular sialidases [11] which hydrolyse exclusively Neu5Ac- and Neu5Gc-glycoconjugates but not KDN-glycoconjugates; KDN-sialidases [10, 24, 25] which hydrolyse KDN-glycoconjugates as well as Neu5Ac- and Neu5Gc-glycoconjugates and KDNase [26] which cleaves exclusively KDN-glycoconjugates but not Neu5Ac- and Neu5Gc-glycoconjugates. Given the novelty of this class of enzyme we thought it appropriate to investigate its mechanism of catalysis by

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$^1$H NMR spectroscopy and to compare it with other well-known sialidases.

The hepatopancreas of *C. virginica* provides a convenient source of a highly active KDN-sialidase. This enzyme has been found to cleave 4-methylumbelliferyl α-KDN (KDNα2MeUmb, **4**), KDNα2→3Galβ1→4GlcCer, KDNα2→6Galβ1→4GlcCer and KDNα2→6GalNAc-ol [16]. KDN-sialidase isolated from *C. virginica* preferentially cleaves KDNα2MeUmb (**4**) having a ten-fold greater activity over the corresponding *N*-acetylneuraminic acid-based substrate, Neu5Acα2MeUmb.

## Materials and methods

*Synthesis of Sodium (4-Methylumbelliferyl 3-Deoxy-D-glycero-α-D-galacto-2-nonulopyranosid)onate (KDNα2MeUmb, 4)*

Sodium (4-methylumbelliferyl 3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (KDNα2MeUmb, **4**) was readily synthesised using known chemistry (Fig. 1). Thus, membrane-enclosed enzyme catalysed [27] aldol condensation between D-mannose and sodium pyruvate in the presence of Neu5Ac aldolase (E.C.4.1.3.3) gave an anomeric mixture of 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (KDN, **1**) [28, 29] in good yield. Methyl esterification of **1** using Dowex-50 ($H^+$) resin in methanol [30, 31] afforded methyl 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonate (**2**) in 75% yield. Subsequent exposure of **2** to acetyl chloride [31–33] followed by sodium 4-methylumbelliferate [33] in a one-pot, two step reaction gave methyl (4-methylumbelliferyl 4,5,7,8,9-penta-*O*-acetyl-3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (**3**) in an unoptimized yield of 25%. Base-catalysed deprotection [31–33] of **3** using sodium methoxide and aqueous sodium hydroxide gave the desired KDNα2Me Umb (**4**) in quantitative yield. Spectral data of all compounds were in accordance with those previously reported [28–30, 32, 34].

*KDN-sialidase*

KDN-sialidase was isolated from the hepatopancreas of the oyster *C. virginica* [25]. Using the scheme similar to that described for preparation of starfish KDN-sialidase [24], oyster KDN-sialidase was partially purified using the following chromatographic media: DEAE-Fractogel,

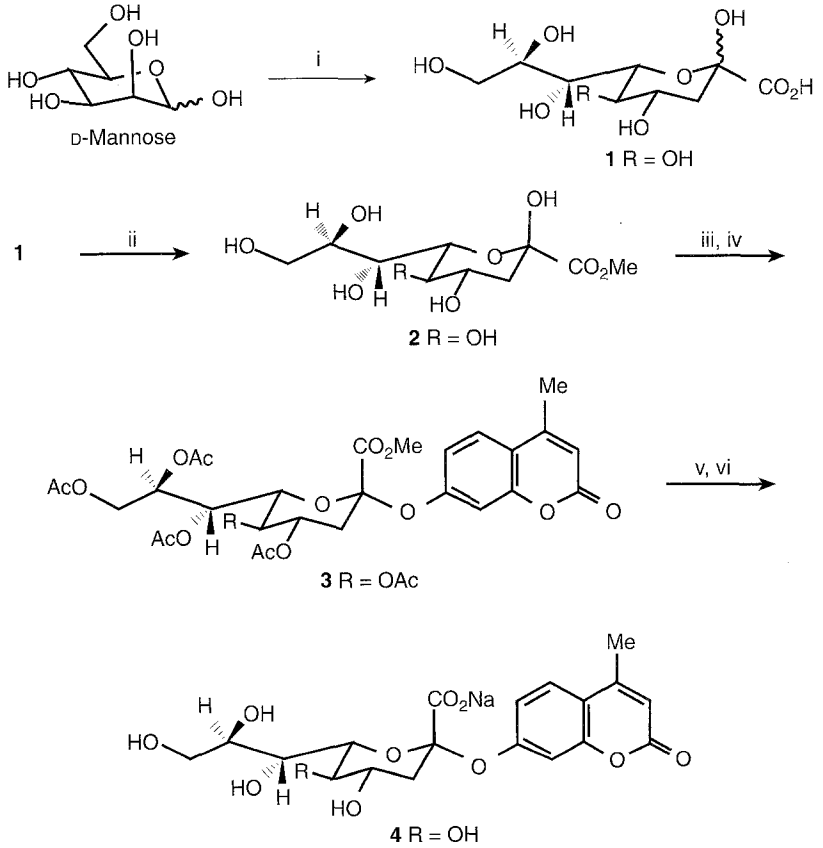


**Figure 1.** Synthesis of sodium (4-methylumbelliferyl 3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (KDNα2MeUmb, **4**).

Sephacryl S200, and $SO_3$-Fractogel. This enzyme was found to be responsible for KDN- and Neu5Ac-cleaving activities [35] and is void of any other sialidase activity. The relative molecular mass of the main KDN-cleaving enzyme was estimated ~ 21 kDa using gel filtration.

### *$^1H$ NMR experiments*

The hydrolysis of KDN$\alpha$2MeUmb (**4**) by KDN-sialidase was monitored by $^1H$ NMR spectroscopy as a time course reaction on a Bruker AMX 500 spectrometer, at 310 K. The reaction conditions were: KDN$\alpha$2MeUmb (**4**) (4.5 mM), 0.19 mL KDN sialidase (550 $UmL^{-1}$), in 50 mM deuterated sodium acetate buffer (pD 4.6), and 0.1 M $CaCl_2$, total volume 0.6 mL (1U is defined as the amount of enzyme required to catalyse the hydrolysis of 1 nmol substrate $min^{-1}$). Calcium was added to the reaction mixture because a 130% increase in KDN-sialidase activity has been found on addition of a range of divalent metal ions (Li, unpublished data). A spectrum of KDN$\alpha$2MeUmb, without KDN-sialidase, was also acquired under identical spectral conditions for the purpose of providing a 'zero time' spectrum. Spectra were acquired with 16K data points over a spectral width of 6024 Hz, with a relaxation delay of 2 s and 32 scans.

## Results

Figure 2 shows progress of the hydrolysis of KDN$\alpha$2MeUmb (**4**) catalysed by KDN-sialidase as monitored by $^1H$ NMR spectroscopy. The H3 methylene protons of

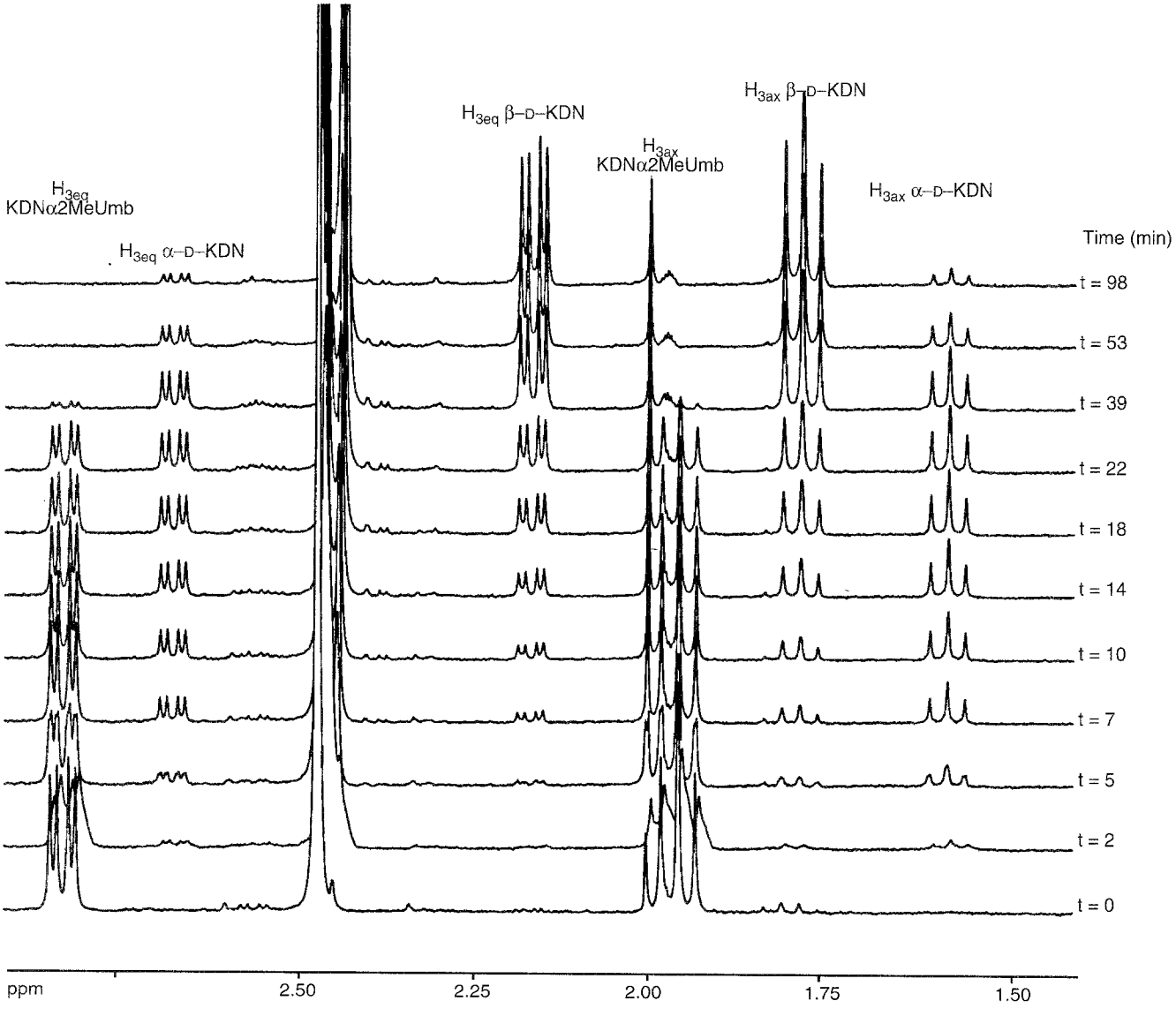


**Figure 2.** Progress of the KDN-sialidase reaction monitored by 500 MHz $^1H$ NMR spectroscopy. Spectral data were acquired at times indicated on the spectra. The reaction was performed with KDN$\alpha$2MeUmb (**4**) (4.5 mM) at 37 °C in 50 mM deuterated sodium acetate buffer (0.6 mL, pD 4.6) in the presence of KDN-sialidase isolated from the hepatopancreas of the oyster, *Crassostrea virginica*.

KDN$\alpha$2MeUmb are a convenient monitor of the progress of the hydrolysis reaction because they resonate in a spectral region which is free of other KDN resonances. Moreover, the chemical shift of the H3 protons easily identify the anomeric configuration of the possible hydrolysis products, $\alpha$-D-KDN (**1**$\alpha$) and $\beta$-D-KDN (**1**$\beta$).

The first spectrum which corresponds to t = 0 min shows the spectral region from 1.7 to 3.0 ppm of KDN$\alpha$2MeUmb (**4**) at 310 K, in 0.6 mL of 50 mM deuterated sodium acetate buffer (pD 4.6) and 0.1 M $CaCl_2$. These conditions were identical to those used for the time course reaction except for the addition of the KDN-sialidase. In this spectrum the $H_{3eq}$ and $H_{3ax}$ protons of KDN$\alpha$2MeUmb (**4**) are clearly seen at 2.82 and 1.95 ppm respectively. The methyl resonance of the 4-methylumbelliferyl aglycone is at 2.46 ppm.

The remaining spectra show the hydrolysis reaction after the addition of the KDN-sialidase. At t = 2 min, additional $H_{3eq}$ and $H_{3ax}$ protons corresponding to the $\alpha$-anomer of KDN (**1**$\alpha$) at 2.66 and 1.58 ppm, respectively, are just visible. These signals increase in intensity as the reaction proceeds with a concomitant decrease in the intensity of the H3 proton signals at 2.82 and 1.95 ppm as KDN$\alpha$2MeUmb (**4**) is hydrolysed. The intensity of these resonances steadily increase until ~ 38 min, but after this time decrease. This is due to mutarotation of the initial reaction product $\alpha$-D-KDN (**1**$\alpha$) to $\beta$-D-KDN (**1**$\beta$) as shown in Fig. 3. Mutarotation of KDN (**1**) occurs quite quickly after the liberation of the $\alpha$-D-KDN (**1**$\alpha$) because by t = 5 min the $H_{3eq}$ and $H_{3ax}$ protons of $\beta$-D-KDN (**1**$\beta$) are visible at 2.16 and 1.78 ppm respectively. The hydrolysis of KDN$\alpha$2MeUmb (**4**) goes to completion as can be seen from spectra after 38 min. Mutarotation of $\alpha$-D-KDN (**1**$\alpha$) continues until final equilibrium values for the anomeric mixture of ~ 94% $\beta$ and 6% $\alpha$-D-KDN (**1**$\beta$ and **1**$\alpha$ respectively, as determined by integration of the H3 signals) are established. This is not unexpected as the $\beta$-anomer of KDN (**1**$\beta$) is the more thermodynamically-stable anomer of KDN and these values are comparable to the final equilibrium anomeric ratio seen for *N*-acetylneuraminic acid [22].

## Conclusion

KDN-sialidase from *C. virginica* hydrolyses the synthetic substrate KDN$\alpha$2MeUmb (**4**) with the initial product being the $\alpha$-anomer of KDN (**1**$\alpha$). Release of $\alpha$-KDN (**1**$\alpha$) as the first product of substrate cleavage is consistent with KDN-sialidase being a retaining enzyme i.e. KDN-sialidase catalyses the hydrolysis of $\alpha$-ketosides with overall retention of anomeric configuration, as detailed in Fig. 3.

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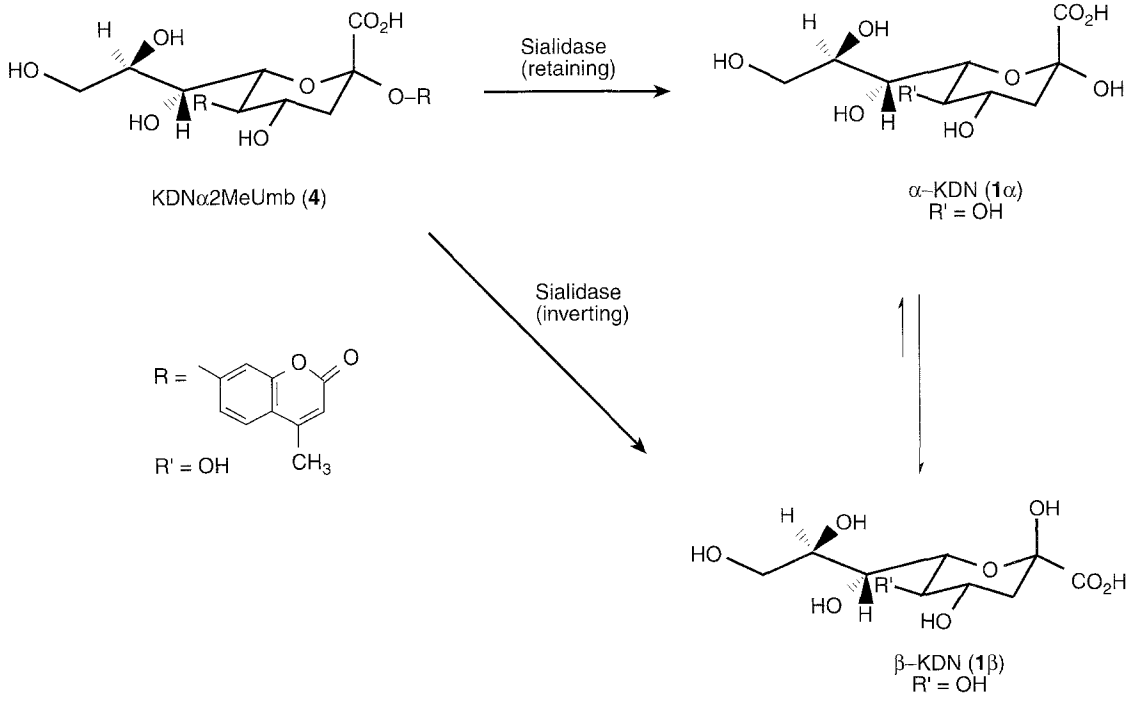


**Figure 3.** Mutarotation of the initial reaction product $\alpha$-D-KDN (**1**$\alpha$) to $\beta$-D-KDN (**1**$\beta$).

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