COMMUNICATION

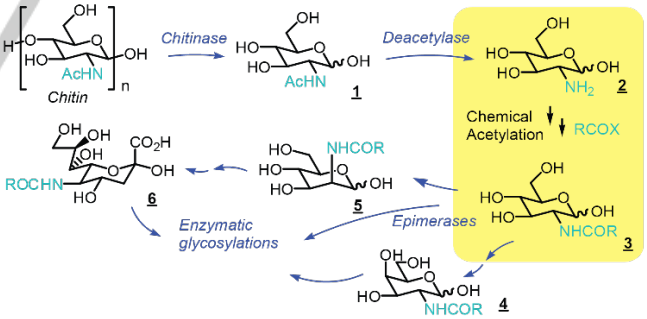
Enzymatic *N*-Acylation Step Enables the Biocatalytic Synthesis of Unnatural Sialosides

Pedro Laborda[+], Yong-Mei Lyu[+], Fabio Parmeggiani, Ai-Min Lu, Wen-Jiao Wang, Ying-Ying Huang, Kun Huang, Juan Guo, Li Liu*, Sabine L. Flitsch*, and Josef Voglmeir*

Abstract: *Selective N-acylation:* Chitin is one of the most abundant and cheaply available biopolymers in Nature. Chitin has become a valuable starting material for many biotechnological products through manipulation of its *N*-acetyl functionality, which can be cleaved under mild conditions using the enzyme family of de-*N*-acetylases. However, the chemo-selective enzymatic re-acylation of glucosamine derivatives, which can introduce new stable functionalities into chitin derivatives, is much less explored. Here we describe an acylase (CmCDA from *Cyclobacterium marinum*) that catalyzes the *N*-acylation of glycosamine with a range of carboxylic acids under physiological reaction conditions. This biocatalyst closes an important gap in allowing for the conversion of chitin to complex glycosides, such as C5-modified sialosides, through the use of highly selective enzyme cascades.

Carbohydrate active enzymes (CAZy) have become valuable catalysts for the highly selective production of complex natural and unnatural glycans and their use in one-pot enzyme cascades is attractive [1]. In particular, glucosamine derivatives are fundamental building blocks for the synthesis of a range of hexosamines and sialylated glycoconjugates [2] and as bioorthogonal chemical reporters for metabolic glycan labelling and engineering [3] as illustrated in Scheme 1. Glucosamine (**2**) itself is a valuable food additive which can be generated from chitin through chemical or enzymatic depolymerization to **1** and de-acetylation [4]. Subsequent epimerases (to generate the galactose and mannose derivatives **4** and **5** respectively) and further elaboration to sialic acids (**6**) and glycoconjugates can also be achieved enzymatically. However, there remains a gap in the enzyme toolkit in that the acylation of glucosamine **2** needs to be performed chemically, requiring activation of carboxylic acids or stoichiometric coupling agents and protecting strategies [5].

Hence, the enzymatic synthesis of *N*-acylated glucosamine derivatives directly from glucosamine and unprotected carboxylic acids would allow for the generation of target structures **3-6** and their derivatives using enzyme cascades directly from chitin (Scheme 1).
In biosynthesis, *N*-acetylation is often mediated through the generation of acetyl precursors such as acetyl coenzyme A or acetyl phosphate, or acetyl adenine [6] and cannot directly utilize glucosamine **2**, but require activated glucosamine-6-phosphate as the acceptor substrate [7]. A more attractive strategy is the reversible enzymatic amine acyl exchange of amines and free carboxylic acids (Scheme 1, compound **2** to **3** using free acid as the acylating agent), thus directly replacing the chemical step. Such enzymatic reaction has been previously described for protease-catalyzed *N*-acylation reactions of peptides or urea [8], but to our knowledge, there is no example of the *N*-acylation of glucosamine. An enzyme-catalyzed approach would not require activation of the carboxylate and in addition has the potential to be highly selective for glucosamine in the presence of other biogenic amines.



Scheme 1. Current enzyme cascades for the conversion of chitin from biomass to complex glycoconjugates, which still require chemical acylation strategies for amino sugars. Here we address the challenge of achieving selective acylation by enzyme catalysis.

In the absence of any obvious candidate enzymes from the literature, we looked for promiscuous activity of known biocatalysts. We previously described a recombinant chitin de-*N*-acetylase CmCDA that is able to specifically and efficiently deacylate *N*-acylglucosamine derivatives (including GlcNAc), whereas no activity towards other carbohydrates such as chitobiose, ManNAc or GalNAc was detected [4].
We also noted similarities in active site residues between CmCDA and a previously reported peptidoglycan deacetylase [9]. The relevant catalytic amino acids appear to be conserved between

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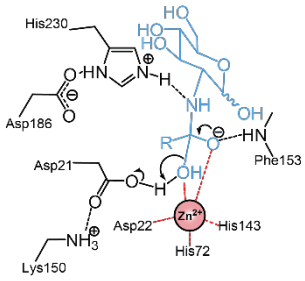
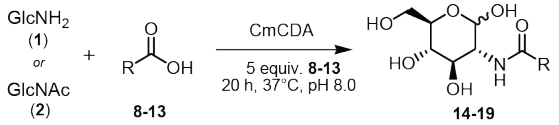
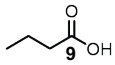
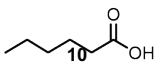
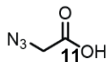
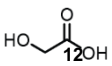
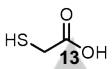


Scheme 2. Proposed *N*-acylation mechanism of glucosamine by CmCDA. The tetrahedral oxyanion intermediate is shown in the catalytic site of CmCDA.

both enzymes, including the His-His-Asp zinc-binding triad coordinating the *N*-acetyl group, as well as the respective histidine and aspartic acid residues acting as the catalytic acid and base. The active site residues suggest acylation *via* the described tetrahedral oxyanion intermediate which would also allow for the reverse the reaction, i.e. the *N*-acylation reaction of glucosamine (**1**) with a carboxylic acid (Scheme 2).

Table 1. CmCDA catalysed *N*-acylation of **1** and transacylation of **2** from carboxylic acids **8-13**.[a]



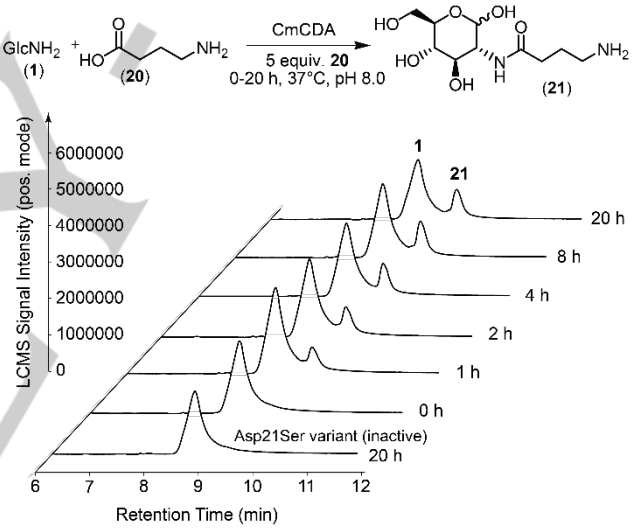
Sugar substrate	Carboxylic acid	Amide product	Conversion[b] [%]
$GlcNH_2$ (**1**)		**2**	69
		**14**	68
		**15**	74
		**16**	48
		**17**	84
		**18**	77
		**19**	77
GlcNAc (**2**)	**8**	**14**	37
	9	**15**	35
	11	**17**	46
	12	**18**	42
	13	**19**	40

[a] 1 mL reactions consisting of 120 mM **1** or **2**, 600 mM **7-13** (in combination with **1,** or **8**,**9**,**11**,**12**,**13** with **2**), 420 mM Na_2HPO_4 buffer (pH 8.0), 0.4 U CmCDA, 37°C, 20 h. [b] Conversion determined by ^1H NMR.

To our delight CmCDA (expressed recombinantly in *E.coli)* could indeed catalyse *N*-acylation reactions with a series of carboxylic acids: in the presence of 5 equivalents of acetic acid (**7**), propionic acid (**8**), butyric acid (**9**), hexanoic acid (**10**), azidoacetic acid (**11**), glycolic acid (**12**) and thioglycolic acid (**13**), reaction with glucosamine **(2**) yielded the corresponding *N*-acylglucosamines **2** and **14**-**19**, respectively (Table 1).

Under the same conditions, no product formation was observed using benzoic acid as carboxylic acid donor, suggesting a steric or electronic limitation of CmCDA for bulkier aromatic organic acids. As expected, the amide formation requires an excess of the carboxylic acid in the reaction mixture: whereas 1 equivalent of acid gave low yield, the addition of 5 equivalents shifted the equilibrium to the formation of the amide bond.

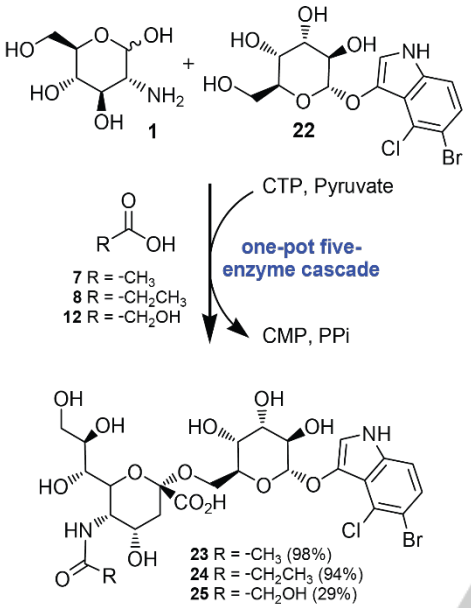
To demonstrate the selectivity of CmCDA for glucosamine in the presence of other amines, **1** was reacted with an excess nonactivated, nonprotected γ-aminobutyric acid (**20**). Whereas no formation of *N*-acyl glucosamine **21** could be observed using the inactive variant CmCDA-Asp21Asn [10], the formation of **21** catalysed by native CmCDA could be observed in a time-course experiment by LC-mass spectrometry (Scheme 3).



Scheme 3. LCMS analysis of CmCDA-catalysed *N*-acylation reaction of **1** and amino acid **20** shows the formation of **21** [11].

Given that CmCDA also catalyses the hydrolysis, we then investigated whether it would be feasible to generate *N*-acyl glucosamine derivatives *via N*-transacylation directly from GlcNAc (**1**). Given the stability of the *N*-acyl bond, this had been achieved chemically before only by using highly fluorinated anhydrides: the exchange of the *N*-acetyl group of unprotected and peracetylated GlcNAc to a *N*-perfluoroacyl group using trifluoroacetic anhydride or heptafluorobutanoic anhydride [12]. Further *N*-transacylation attempts used oleoyl chloride and lauryl chloride as acyl donors of protected GlcNAc in pyridine under reflux [13]. Examples of direct *N*-transacylation are limited, require harsh conditions and commonly the protection of all hydroxyl groups, and are restricted to fluorinated or long-chain acylation reagents. Performing these transacylation reactions with unprotected GlcNAc (**1**) and non-activated carboxylic acids enzymatically would extend the scope to acyl donors which are of interest in the synthesis of sialic acid derivatives, such as azido, hydroxyl or thiol groups [14].

Applying the reaction conditions established for the *N*-acylation reaction of glucosamine (adding 5 equivalents of the acid donor), the overall efficiency of the *N*-transacylation reaction was comparable to the conversion rates of the *N*-acylation reactions (Table 1). Propionic acid (**8**), butyric acid (**9**), azidoacetic acid (**11**), glycolic acid (**12**) and thioglycolic acid (**13**) were explored as acyl donors and showed between 37-46% conversion to the corresponding *N*-acyl glucosamines [15]. ^{1}H NMR analysis of the reaction mixtures showed the disappearance of **2** in all cases whereas **1** could be observed as product of the deacetylation reaction, suggesting that this enzymatic amine acyl exchange consists of two independent (faster) deacetylation and (slower) re-acylation steps.



Scheme 4. One-pot five enzyme cascade for the synthesis of sialosides **23**-**25** from glucosamine and X-gal (**22**).

The enzymatic synthesis of C5-substituted sialosides requires *N*-acylglucosamine or *N*-acetylmannosamine derivatives as precursor molecules [16]. The enzymatic synthesis of these compounds would allow the integration of the *N*-acylation reaction in established enzymatic synthesis cascades for unnatural sialosides, thus extending current one-pot enzyme cascades: in a first step, chemically *N*-acylated glucosamine analogues are enzymatically epimerized to the corresponding *N*-acylmannosamines and transformed into the sialic acid derivatives *via* an enzymatic aldol addition with pyruvate. These intermediates can be then used for the synthesis of sialyloligosaccharides *via* enzymatic activation by CMP-sialic acid synthase and sialyltransferases. Previously, we had been successful in using 5-bromo-4-chloro-indolyl-β-D-galacto-pyranoside (X-Gal, **22**) as an acceptor substrate for sialylation reactions, generating valuable labelled oligosaccharides. In the case of incomplete sialylation reactions, residual **22** could simply be removed by treating the reaction mixture with β-galactosidase. The released 5-bromo-4-chloro-3-indole dimerizes to form an insoluble indigo dye. Thus, sialosides containing *N*-acetylneuraminic acid (Neu5Ac **23**), *N*-propanoylneuraminic acid (Neu5Prop **24**) and *N*-glycolylneuraminic acid (Neu5Gc **25**) were synthesized using a five-enzyme one-pot reaction (Scheme 3) by only providing glucosamine (**1**), pyruvate, and acyl donors **7**, **8** and **12** as sialic acid precursors, CTP for the activation of the sialic acid analogues, and **22** as the acceptor substrate for the sialylation reaction (Scheme 3). Sialosides **23** and **24** were obtained in over 94% yield, whereas **25** yielded 29% [17].

In conclusion, we have demonstrated that CmCBA is able to carry out a range of *N*-acylation and *N*-transacylation reactions to generate *N*-acyl glucosamines on preparative scale. This enzyme is key for the development of comprehensive and extended enzymatic cascades, as demonstrated for the first enzymatic total synthesis of sialosides using glucosamine as sialic acid precursor, opening up new avenues in the chemoenzymatic synthesis of glycoconjugates and non-natural analogues.

Acknowledgements

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Keywords: N-acylation • enzymatic synthesis • unnatural sialosides • glycosylation • chitin de-N-acetylases

[1] a) K. M. Koeller, C. H. Wong, *Chem. Rev.* **2000**, 100, 4465-4494. b) R. Sardzik, A. P. Green, N. Laurent, P. Both, C. Fontana, J. Voglmeir, M. J. Weissenborn, R. Haddoub, P. Grassi, S. M. Haslam, G. Widmalm, S. L. Flitsch, *J. Am. Chem. Soc.* **2012**, 134, 4521-4524. c) J. Ye, H. Xia, N. Sun, C. C. Liu, A. Sheng, L. Chi, X. W. Liu, G. Gu, S. Q. Wang, J. Zhao, P. Wang, M. Xiao, F. Wang, H. Cao, *Nat. Catal.* **2019**, 2, 514–522. d) K. Huang, S. L. Flitsch, *Nat. Catal.* **2019**, 2, 479-480. e) H. Yu, X. Chen, *Org. Biomol. Chem.* **2016**, 14, 2809-2818. f) K. W. Moremen, A. Ramiah, M. Stuart, J. Steel, L. Meng, F. Forouhar, H. A. Moniz, G. Gahlay, Z. Gao, D. Chapla, S. Wang, Jeong-Yeh Yang, Pradeep Kumar Prabhakar, Roy Johnson, M. Rosa, C. Geisler, A. V. Nairn, J. Seetharaman, S. C. Wu, L. Tong, H. J. Gilbert, J. LaBaer, D. L. Jarvis, *Nat. Chem. Biol.* **2018**, 14, 156–162.

[2] a) C. Meng, A. Sasmal, Y. Zhang, T. Gao, C. C. Liu, N. Khan, A. Varki, F. Wang, H. Cao, *Angew. Chem.* **2018**, *130*, 9141-9145; *Angew. Chem. Int. Ed.* **2018**, *57*, 9003-9007. b) H. Yu, S. Huang, H. Chokhawala, M. Sun, H. Zheng, X. Chen, *Angew. Chem.* **2006**, *118*, 4042-4048; *Angew. Chem. Int. Ed.* **2006**, *45*, 3938-3944. c) S. Wang, Q. Zhang, C. Chen, Y. Guo, M. R. Gadi, J. Yu, U. Westerlind, Y. Liu, X. Cao, P. G. Wang, L. Li, *Angew. Chem.* **2018**, *130*, 9412-9417; *Angew. Chem. Int. Ed.* **2018**, *57*, 9268-9273. d) Z. P. Cai, L. P. Conway, Y. Y. Huang, W. J. Wang, P. Laborda, T. Wang, A. M. Lu, H. L. Yao, K. Huang, S. L. Flitsch, L. Liu, J. Voglmeir, *Molecules* **2019**, *24*, 1368. e) T. Heise, J. F. A. Pijnenborg, C. Bull, N. van Hilten, E. D. Kers-Rebel, N. Balneger, H. Elferink, G. J. Adema, T. J. Boltje, *J. Med. Chem.* **2019**, *62*, 1014-1021.

[3] a) D. J. Vocadlo, H. C. Hang, E. J. Kim, J. A. Hanover, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9116-9121. b) M. Boyce, I. S. Carrico, A. S. Ganguli, S. H. Yu, M. J. Hangauer, S. C. Hubbard, J. J. Kohler, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 3141-3146. c) E. Memmel, A. Homann, T. A. Oelschlaeger, J. Seibel, *Chem. Commun.* **2013**, *49*, 7301-7303. d) Y. Zhu, J. Wu, X. Chen, *Angew. Chem.* **2016**, *128*, 9447–9451; *Angew. Chem. Int. Ed.* **2016**, *55*, 9301–9305. e) W. Qin, K. Qin, X. Fan, L. Peng, W. Hong, Y. Zhu, P. Lv, Y. Du, R. Huang, M. Han, B. Cheng, Y. Liu, W. Zhou, C. Wang, X. Chen, *Angew. Chem.* **2018**, *130*, 1835-1838; *Angew. Chem. Int. Ed.* **2018**, *57*, 1817-1820. f) A. P. Mattey, W. R. Birmingham, P. Both, N. Kress, K. Huang, J. M. van Munster, G. S. Bulmer, F. Parmeggiani, J. Voglmeir, J. E. R. Martinez, N. J. Turner, S. L. Flitsch, *ACS Catal.* **2019**, 9, 8208-8212.[4]

Y. M. Lv, P. Laborda, K. Huang, Z. P. Cai, M. Wang, A. M. Lu, C. Doherthy, L. Liu, S. Flitsch, J. Voglmeir, *Green Chem.* **2017**, *19*, 527-535.

[5] J. Choi, L. J. S. Wagner, S. B. P. E. Timmermans, S. A. Malaker, B. Schumann, M. A. Gray, M. F. Debets, M. Takashima, J. Gehring, C. R. Bertozzi, *J. Am. Chem. Soc.* **2019**, 141, 13442-13453.

[6] a) Petchey, G. Grogan, *Adv. Synth. Catal.* **2019**, *doi*: 10.1002/adsc.201900694. b) A. J. L. Wood, N. J. Weise, J. D. Frampton, M. S. Dunstan, M. A. Hollas, S. R. Derrington, R. C. Lloyd, D. Quaglia, F. Parmeggiani, D. Leys, N. J. Turner, S. L. Flitsch, *Angew. Chem.* **2017**, *129*, 14690-14693; *Angew. Chem. Int. Ed.* **2017**, *56*, 14498-14501.

[7] a) C. P. Selitrennikoff, D. R. Sonneborn, *Biochim. Biophys. Acta.* **1976**, 451, 408-416. b) R. Hurtado-Guerrero, O. Raimi, S. Shepherd, D. M. van Aalten, *FEBS Lett.* **2007**, *581*, 5597-5600.

[8] a) R. Castangia, M. Austeri, S. L. Flitsch, *Angew. Chem.* **2012**, *124*, 13191-13194; *Angew. Chem. Int. Ed.* **2012**, *51*, 13016-13018. b) N. P. Awasthi, R. P. Singh, *J. Oleo. Sci.* **2007**, *56*, 507-509.

[9] D. E. Blair, A. W. Schüttelkopf, J. I. MacRae, D. M. van Aalten, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 15429-15434.

[10] Details of the generation of the CmCDA Asp21Asn enzyme variant are described in the Supporting Information.

[11] 1 mL reaction were performed using 25 mM $GlcNH_2$ (**1**), 125 mM aminobutyric acid (**20**), 200 mM Phosphate buffer, and 0.4 U CmCDA at 37 °C. Further details in the Supporting Information.

[12] a) B. Nilsson, S. Svensson, *Carbohyd. Res.* **1978**, 62, 377-380. b) P. Rota, P. Allevi, R. Colombo, M. L. Costa, M. Anastasia, *Angew. Chem.* **2010**, *122*, 1894-1897; *Angew. Chem. Int. Ed.* **2010**, *49*, 1850-1853. c) G. Despras, A. Alix, D. Urban, B. Vauzeilles, J. M. Beau, *Angew. Chem.* **2014**, *126*, 12106-12110; *Angew. Chem. Int. Ed.* **2014**, *53*, 11912-11916.

[13] a) Y. Yang, B. Yu, *Tetrahedron Lett.* **2007**, *48*, 7049-7052. b) I. García Álvarez, G. Corrales, E. Doncel Pérez, A. Muñoz, M. Nieto Sampedro, A. Fernández Mayoralas, *J. Med. Chem.* **2007**, *50*, 364-373.

[14] P. R. Wratil, R. Horstkorte, W. Reutter, *Angew. Chem.* **2016**, *128*, 9632-9665; *Angew. Chem. Int. Ed.* **2016**, *55*, 9482-9512.

[15] CmCDA catalysed conversion of GlcNAc (**2**) in the presence of propionic acid (**8**), butyric acid (**9**), azidoacetic acid (**11**), glycolic acid (**12**) and thioglycolic acid (**13**) into the corresponding *N*-acylglucosamine derivatives **14**, **15**, and **17**-**19** were monitored using ^{1}H NMR (Supporting Information).

[16] a) M. Knorst, W. D. Fessner, *Adv. Synth. Catal.* **2001**, *343*, 698–710. b) J. E. Reyes Martinez, R. Sardzik, J. Voglmeir, S. L. Flitsch, *RSC Adv.* **2013**, *3*, 21335–21338. c) H. Yu, K. Lau, V. Thon, C. A. Autran, E. Jantscher-Krenn, M. Xue, Y. Li, G. Sugiart, J. Qu, S. Mu, L. Ding, L. Bode, X. Chen, *Angew. Chem.* **2014**, *126*, 6805-6809; *Angew. Chem. Int. Ed.* **2014**, *53*, 6687-6691. d) A. Santra, A. Xiao, H. Yu, W. Li, Y. Li, L. Ngo, J. B. McArthur, X. Chen, *Angew. Chem.* **2018**, *130*, 2979-2983; *Angew. Chem. Int. Ed.* **2018**, *57*, 2929-2933.

[17] Enzymatic synthesis of X-gal sialoside using an one-pot five-enzyme cascade consisting of: a) CmCDA-catalysed *N*-acylation to *N*-acylglucosamines; b) PhGn2E-catalysed epimerisation to the corresponding mannosamine derivatives; c) EcAldolase –catalysed aldol reaction to *N*-acylneuraminic acid derivatives; d) NmCTT-catalysed synthesis of cytidine-5-monophospho-*N*-acylneuraminic acid derivatives. e) PdST6-catalysed sialylation reaction of X-gal. Further details in the Supporting Information.

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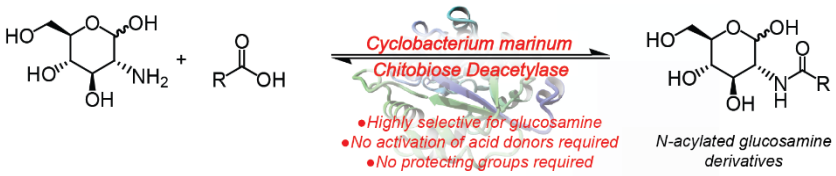

Selective N-acylation: The enzyme Chitobiose Deacetylase *Cyclobacterium marinum* was shown to have promiscuous *N*-acylation activity using a range of free carboxylic acids and glucosamine as substrates. This selective *N*-acylation step provides the last missing link for the synthesis of natural and C5-modified sialosides from chitin and *N*-acetyl glucosamine using a fully enzymatic reaction cascade.

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