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Synthesis of screening substrates for the directed evolution of sialic acid aldolase: towards tailored enzymes for the preparation of influenza A sialidase inhibitor analogues†

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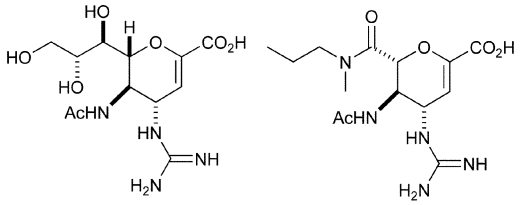
The stereoselective synthesis of two epimeric screening substrates, (4*R*, 5*R*, 6*R*)- and (4*S*, 5*R*, 6*R*)-6-dipropylcarbamoyl-2-oxo-4,5,6-trihydroxy-hexanoic acid, for the directed evolution of sialic acid aldolase is described. The complementary methods relied on stereoselective indium-mediated additions of ethyl α-bromomethyl acrylate to functionalised aldehydes. With an α-hydroxy aldehyde, (2*R*, 3*R*)-2,3-dihydroxy-4-oxo butanoic acid dipropylamide, the addition was chelation controlled, and the *syn* product, (6*R*, 5*R*, 4*S*)-6-dipropylcarbamoyl-2-methylidene-4,5,6-trihydroxy-hexanoic acid ethyl ester, was obtained. In contrast, the stereochemical outcome of the addition to (2*R*, 3*R*)-*N*,*N*-dipropyl-2,3-*O*-isopropylidene-4-oxobutyramide was consistent with Felkin–Anh control, and the *anti* adduct, (4*R*, 5*R*, 6*R*)-6-dipropylcarbamoyl-2-methylidene-4-hydroxy-5,6-*O*-isopropylidene-hexanoic acid ethyl ester, was the major product. Ozonolysis and deprotection gave the screening substrates as mixtures of furanose and pyranose forms, in good yields.

Introduction

High levels of catalytic efficiency, compatibility with aqueous reaction conditions and low levels of side reactions have led to the widespread exploitation of enzymes in organic synthesis.[1] Nonetheless, the narrow substrate specificity of many enzymes limits their potential as general catalysts for synthetic organic chemistry. In addition, Nature rarely provides complementary enzymes for the preparation of all possible stereoisomeric products. Directed evolution offers an opportunity to address these deficiencies, and has huge potential for exploitation in synthetic organic chemistry.[2–4]

Evolved enzymes may have broader or altered substrate specificity,[3,5] may catalyse reactions with modified levels of stereoselectivity[6,7] and may display altered physical characteristics.[8–12]‡ For example, an enantioselective lipase for the kinetic resolution of chiral esters,[13] and a hydantoinase with reversed enantioselectivity,[14] have been generated using directed evolution. In addition, an amine oxidase has been evolved which can catalyse the deracemisation of a wide range of chiral amines.[15] To date, we have concentrated on the evolution of aldolases which catalyse aldol reactions with a modified stereochemical course:[16] this approach enabled the preparation of a diastereoisomeric product from the substrates accepted by the wild-type enzyme.[5,17]

The sialic acid mimetics **1** and **2a** are potent inhibitors of influenza sialidases.[18,19] Indeed, Zanamivir, **1**, inhibits influenza A and B sialidases with $IC_{50} \approx 5$ nM, prevents viral replication *in vitro* and *in vivo* and is marketed as a drug for the treatment of influenza.[19] Its derivative **2a** is a selective inhibitor of influenza A sialidase (IC_{50} for influenza A: 4 nM; and B: 4500 nM), and has been prepared *via* a multi-step reaction sequence involving the oxidative cleavage of the side chain of sialic acid.[20,21] An

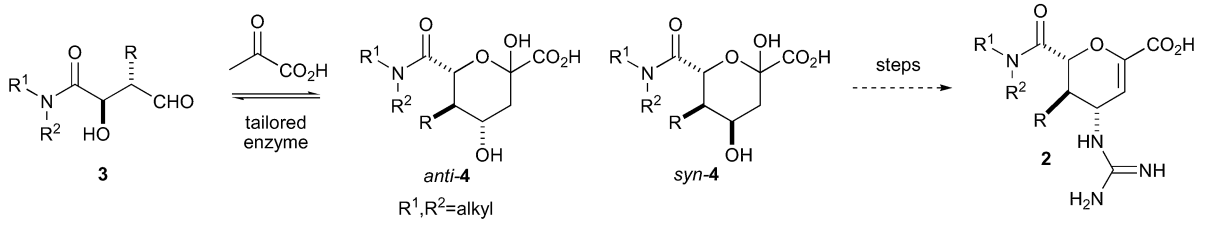


1 **2a**

alternative approach for the preparation of sialic mimetics of general structure **2** could involve an enzyme-catalysed aldol condensation of an aldehyde **3** and pyruvate (→ **4**), followed by functional group manipulation (Scheme 1). Sialic acid aldolase catalyses the reversible aldol condensation between pyruvate and *N*-acetyl mannosamine,[1] and is an ideal starting point for the directed evolution of a suitable tailored enzyme. There are two possible stereochemical outcomes from the aldol condensation of pyruvate and the aldehyde **3** (→ *anti*- or *syn*-**4**), and, ideally, complementary enzymes would be available for the synthesis of either diastereoisomer.

† Electronic supplementary information (ESI) available: experimental details. See http://www.rsc.org/suppdata/ob/b5/b501503k/

‡ Enzymes with enhanced thermostability,[8,9] and/or compatibility with non-aqueous solvents have been evolved.[10–12]



Scheme 1

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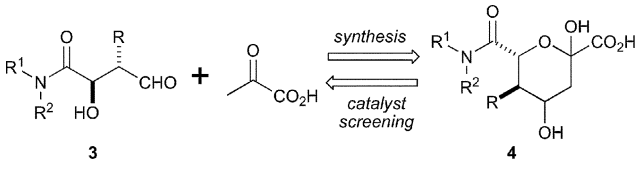
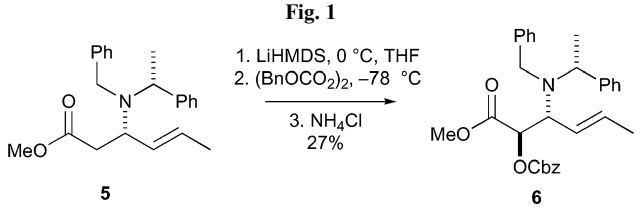


Fig. 1



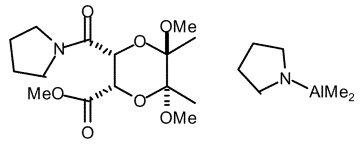
Scheme 2

Sialic acid aldolase has reasonably broad substrate specificity: although only pyruvate is a competent donor, many six- and five-carbon aldehydes are substrates.[1,22] Condensations involving shorter aldehydes are less promising: L- and D-erythrose and threose react at between 0.3% and 5% of the rate of *N*-acetyl mannosamine, and two- and three-carbon aldehydes are not substrates. In this paper, we describe the preparation of screening substrates for the evolution of enzymes able to accept the aldehydes **3** efficiently. A possible screening strategy is shown in Fig. 1. Although the aim was to generate enzymes for use in synthetic chemistry, we chose to use the required products **4** of the condensation as screening substrates. Mutant enzymes able to catalyse the required aldol condensation would also, of course, be able to cleave the screening substrates **4**: one of the cleavage products, pyruvate, may be detected using a coupled enzyme assay. This approach is technically much simpler than detecting the required product of the synthetic reaction, and might enable the evolution of complementary enzymes for the preparation of either of the possible diastereomeric products *anti*- or *syn*-**4**.

Synthesis of protected versions of the aldehydes 3

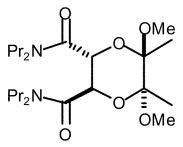
An obvious strategy for the synthesis of the screening substrates **4** would involve the diastereoselective addition of a pyruvate equivalent to a protected version of an aldehyde **3**. With this in mind, the β-amino esters **5, 6** and **7a–b**, in which an aldehyde could be unmasked by ozonolysis of the remaining alkene, were prepared using a Michael addition of the lithium amide **8** as the key step[23] (for example, see Scheme 2). Unfortunately, attempted conversion of the β-amino esters **6** and **7** into the corresponding β-amino dipropylamides was unsuccessful.

An alternative approach involved the diester **9** in which the enantiotopic carbonyl groups of *meso* tartaric acid have been differentiated. It has previously been shown that the equatorial ester of **9** is more susceptible to attack by the aluminium amide **14**;[24] following this procedure, we were able to isolate the amide **13** but in extremely low yield (6%).The diester **9** was found

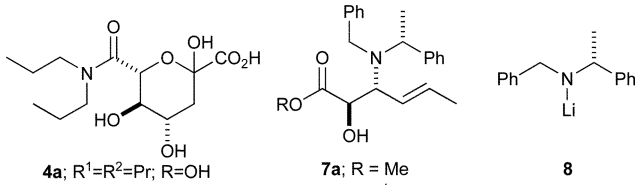


to be highly resistant to aminolysis under a range of reaction conditions: treatment either with neat dipropylamine at 80 °C in a sealed vessel or with an excess of Pr_2NAlMe_2 under a range of reaction conditions§ returned only starting material.

The hydrolysis of the diester **9** (see Table 1 and Scheme 3) was plagued by problems with epimerisation. Treatment of **9** with potassium hydroxide in MeOH–water, and amide formation, gave a mixture of the required amide **11** and the diequatorial diamide **15** (entry 1, Table 1).¶ With lithium hydroperoxide in THF–water, only the diamide **15** was obtained (entry 2). The equatorial ester of **9** is more susceptible to hydrolysis (→ **17**, Scheme 4); however, epimerisation of the axial ester (→ **18**) was competitive with its hydrolysis, and once epimerisation had occurred, hydrolysis to give **19** was rapid. With DBU in water, epimerisation was minimised, and after amide formation, an 80 : 20 mixture of the required amide **11** and the diequatorial diamide **15** was observed (entry 3).

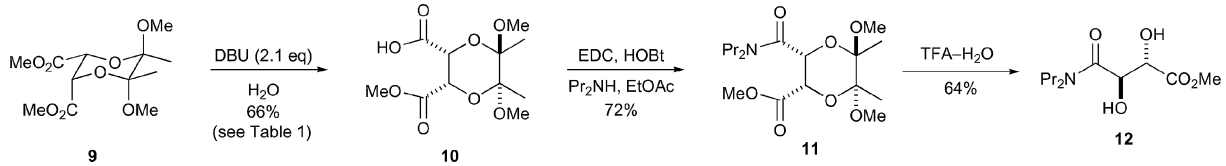


The amide **11** and the diamide **15** were difficult to separate, and so we chose to purify the carboxylic acid **10** (66% yield)



§ The ester **9** (0.1–0.4 M in toluene or dichloromethane) was treated with 4 or 10 equivalents of Pr_2NAlMe_2 at room temperature or 40 °C.

¶ The configuration of the C_2-symmetric diamide **15** was inferred from the simplicity of its 500 MHz ^{1}H NMR spectrum.



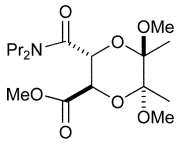
Scheme 3

Table 1 Synthetic transformations of the diester **9**

Entry	Conditions	Product	Yield[a] (%)
1	1. KOH (3.6 eq.), 85 : 15 MeOH–H_2O; 2. EDC, HOBt, Pr_2NH, EtOAc	**15**[b]	23
2	1. LiOOH (10 eq.), 75 : 25 THF–H_2O; 2. EDC, HOBt, Pr_2NH, EtOAc	**15**	90
3	1. DBU (2.1 eq.), H_2O; 2. EDC, HOBt, Pr_2NH, EtOAc	**11**[c]	49

[a] Yield of purified product. [b] Analysis of the crude reaction mixture by 500 MHz ^{1}H NMR spectroscopy revealed a mixture of the amide **11** and the diamide **15**. [c] Analysis of the crude reaction mixture by 500 MHz ^{1}H NMR spectroscopy revealed an 80 : 20 mixture of the amide **11** and the diamide **15**.

after the hydrolysis step (Scheme 3); treatment of the acid **10** with dipropylamine, EDC and HOBt gave the required amide **11** (72%) and its diequatorial epimer **16** (4%). The relative



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configurations of the amides **11** and **16** were deduced by careful analysis of their 500 MHz ^{1}H NMR spectra (for **11**: $J_{2,3}$ = 4.0 Hz; for **16**: $J_{2,3}$ = 10.1 Hz); the relative configuration of **11** was confirmed by X-ray crystallography (Fig. 2)* and the observation of diagnostic NOEs (Fig. 3). Unfortunately, the extremely hindered nature of the axial methoxycarbonyl group of **11** prevented its reduction: treatment with a range of reagents ($LiBH_4$, $^iBu_2AlH$ *etc.*) gave only recovered starting material. Treatment of the amide **11** with TFA–water did, however, give the corresponding diol **12** in 64% yield, whose ester we were unable to reduce using a range of reagents ($LiBH_4$, $^iBu_2AlH$ or $NaBH_4$).

The problems encountered in the preparation of a BDA-protected version of the aldehyde **3** prompted us to prepare the corresponding acetonide instead. The diol **20**, prepared by oxidative degradation of isoascorbic acid (**24**),[25] was converted into the corresponding acetonide **22** (Scheme 5).The γ-lactones **20** and **22** were aminolysed to give the dimethylamides **21a** and **23a** and the dipropylamide **23b**. The effect of the acetonide on the reactivity of the γ-lactones was remarkable: aminolysis with dipropylamide gave a 5% yield of **21b** (from **20**) after 6 days, and a 65% yield of **23b** (from **22**) after 3 days. Deprotection of **23b** (9 : 1 TFA–water) gave the triol **21b**. Unfortunately, attempted selective oxidation of the primary alcohol of **21b** with TEMPO was unsuccessful.

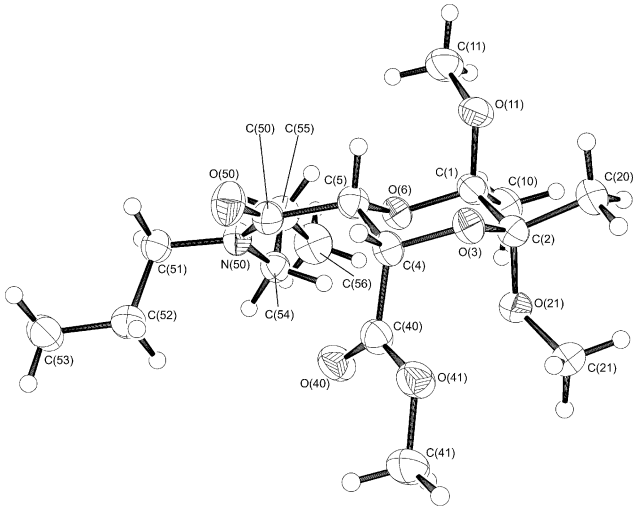


Fig. 2

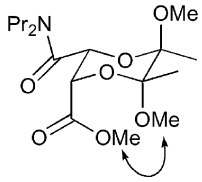
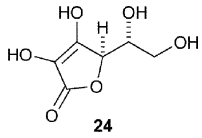


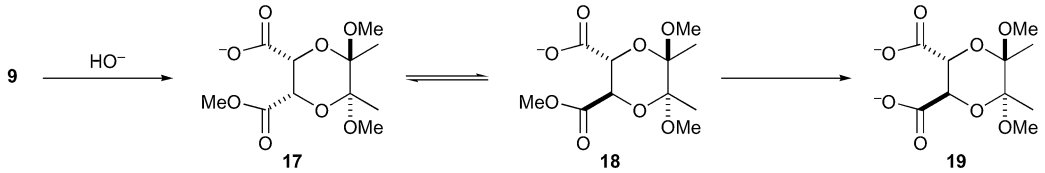
Fig. 3 Diagnostic NOEs for the amide **11**.



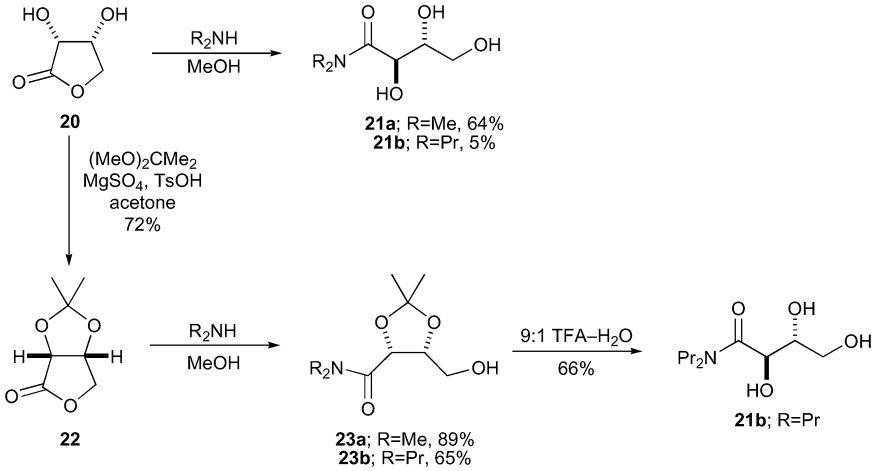
24

Preparation of protected sialic acid mimetics

The alcohol **23b** was converted into the corresponding aldehyde (**29**) using a Swern oxidation, and was used immediately in an indium-mediated allylation without purification.[26–28] A solution of the aldehyde **29** and ethyl α-bromomethyl acrylate in THF–water was treated with indium powder. The required

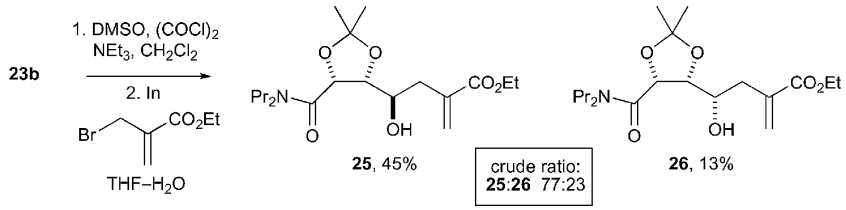


Scheme 4



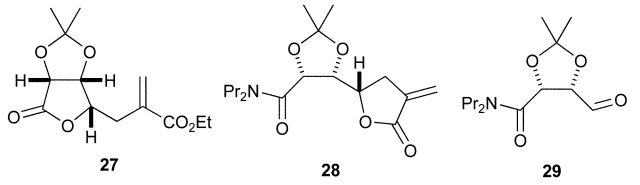
Scheme 5

* CCDC reference numbers 262317 and 262318. See http://www.rsc.org/suppdata/ob/b5/b501503k/ for crystallographic data in CIF or other electronic format.



Scheme 6

addition products **25** and **26** (crude ratio: **25 : 26** 77 : 23) were isolated in 45% and 13% yield respectively (Scheme 6); in addition, the lactones **27** and **28** were each obtained in *ca.* 1% yield.The relative configuration of the major product **25**



was determined by X-ray crystallography (Fig. 4)*, an outcome which is consistent with Felkin–Anh-controlled attack[29] on the intermediate aldehyde **29** (Fig. 5).

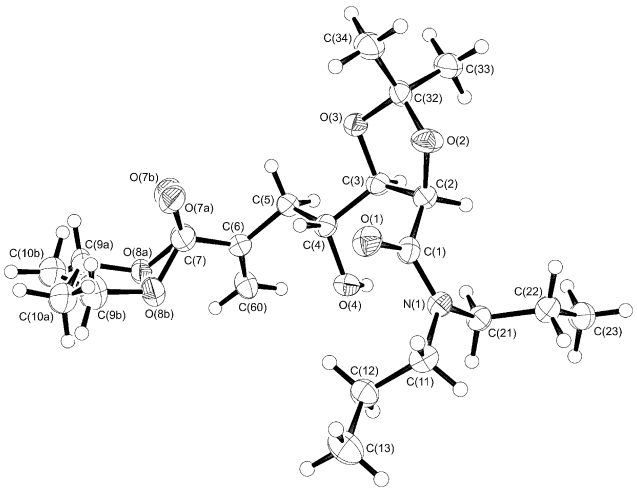
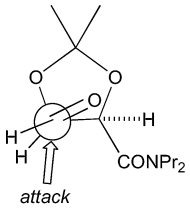


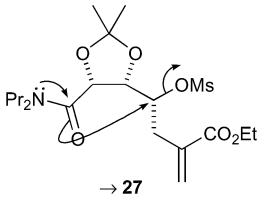
Fig. 4

A strategy for controlling the configuration of the alcohol **26** would involve inversion of its epimer, **25**, either directly or *via* an oxidation–reduction sequence. However, mesylation of the alcohol **25** triggered participation of the amide oxygen to give the lactone **27** (Fig. 6).†† In addition, Swern oxidation of **25** gave a mixture of the regioisomeric α,β-unsaturated esters **30** and **31**. In view of these observations, this strategy was not pursued.



29 → **25**

Fig. 5

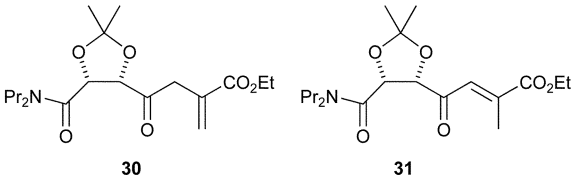


→ **27**

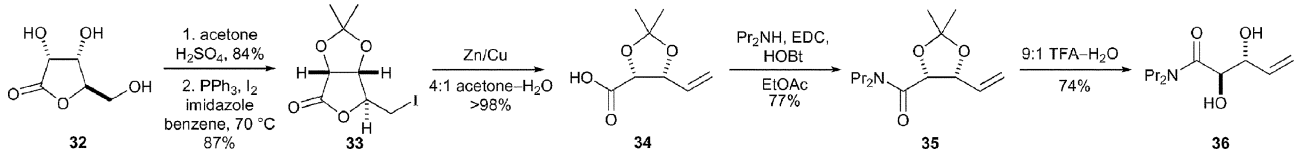
Fig. 6

An alternative approach would involve the chelation-controlled[30] addition of a carbon nucleophile to an analogue of the aldehyde **29**. The γ,δ-unsaturated amide **35** was synthesised from the corresponding acid **34**, prepared by protection of D-ribonolactone, iodination (→ **33**) and reductive fragmentation (Scheme 7).[31] Acetonide deprotection gave the corresponding 1,2-diol **36**.

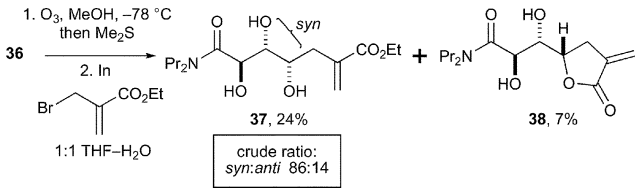
The α,β-dihydroxy aldehyde **39** was prepared by ozonolysis of the corresponding alkene **36**, and was used immediately without purification (Scheme 8).A solution of the aldehyde **39** and ethyl α-bromomethyl acrylate in THF–water was treated with indium powder,[26–28] and the required addition product was obtained in 43% yield (*syn* : *anti* 86 : 14) together with the (*syn*) lactone **38** (7% yield). The high level of *syn* selectivity observed is consistent with chelation-controlled addition[30] to the intermediate aldehyde **39** (Fig. 7). Recrystallisation gave the



†† This result indicated that the lactone **27** had stemmed from lactonisation of the minor diastereomeric adduct (**26**) of the indium-mediated allylation. The conversion of the alcohols **25** ($J_{2,3}$ = 6.1 Hz) and **26** ($J_{2,3}$ = 6.7 Hz) into a common compound demonstrated that **25** and **26** were C-4 epimers (and were, therefore, both *cis* acetonides) and that epimerisation of the aldehyde **29** had not occurred under the conditions of the allylation.



Scheme 7



Scheme 8

addition product **37** in 24% yield as a >98 : < 2 mixture of diastereoisomers.

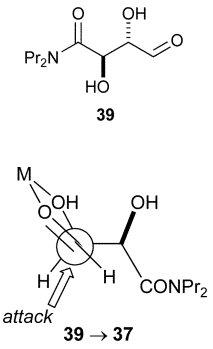


Fig. 7

Preparation of the screening substrates

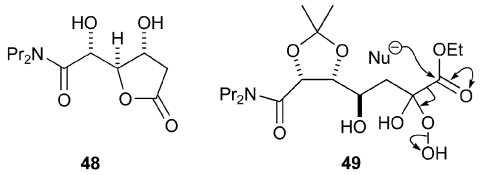
The synthesis of the screening substrates **43** and **47** was completed by deprotection of the diastereomeric esters **25** and **26** (Scheme 9). Ozonolysis of **25**, followed by work-up with aqueous hydrogen peroxide solution, gave the required ketone **40** and the lactone by-product **48** (26%). Presumably, the α-keto ester must have been cleaved under the conditions of the work-up (**49** arrows) to give the by-product, whose formation could be avoided with a reductive work-up with dimethyl sulfide: under these conditions, the required ketone **40** was obtained in 81% yield. Similarly, ozonolysis of **26**, and reductive work-up, gave the corresponding ketone **44**. Acetonide hydrolysis of **40** and **44** gave the diols **41** and **45**. The diol **45** was also prepared more directly by ozonolysis of the α,β-unsaturated ester **37** (>98% yield). Treatment of **41** and **45** with barium hydroxide

Table 2 Ratios of pyranose and furanose forms observed for the compounds **41–43** and **45–47**

Compound	Pyranose forms : furanose forms[a]
41	20 : 0 : 40 : 40
42	30 : 0 : 40 : 30
43	15 : 10 : 45 : 30
45[b]	55 : 0 : 30 : 15
46	85 : 5 : 10 : 0
47	80 : 10 : 5 : 5

[a] Determined (± 5%) by 500 MHz ^{1}H NMR spectroscopy. [b] Initially, a 72 : 14 : 14 mixture of one pyranose and two furanose forms was obtained, which equilibrated to the mixture shown in the Table.

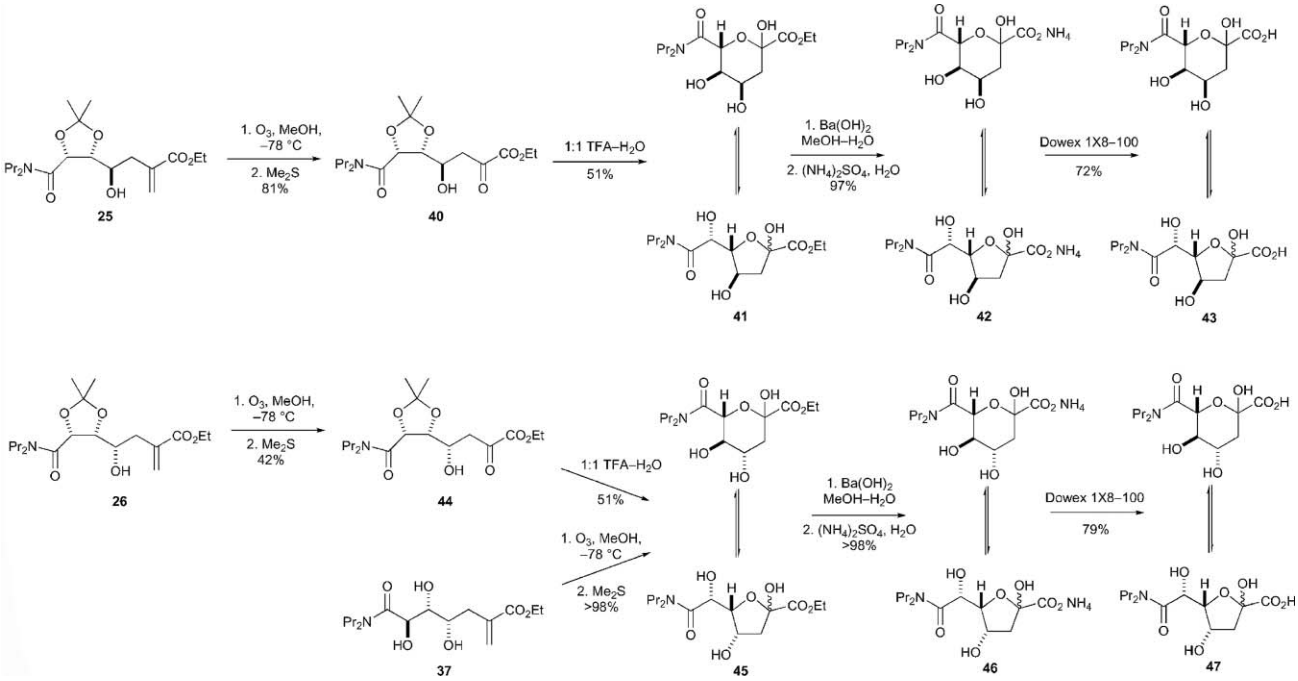
in methanol–water, cation exchange, and purification by ion exchange chromatography, gave the sialic acid mimetics **43** and **47**.



The ketones **41–43**, and **45–47** existed as mixture of pyranose and furanose forms (see Table 2 and Scheme 9); confirmation that all signals in the spectra of these compounds were due to interchanging anomeric forms was provided by 500 MHz exchange spectroscopy (EXSY) NMR experiments. NMR spectroscopic details of each of the forms of **42, 43, 46** and **47** are summarised in Table 3. The $J_{3,4}$ values for the pyranose forms of the ketones **42** and **43** are consistent with an axial orientated C-4 substituent. The $J_{3,4}$ values for the pyranose forms of the ketones **46** and **47** are consistent with an equatorial orientated C-4 substituent.

Summary

The synthesis of the diastereoisomeric screening substrates **43** and **47** was described. The routes were amenable to the synthesis of each substrate on >500 mg scale: the screening substrate **43** was prepared in 9 steps and 7% overall yield from D-isoascorbic acid, and its epimer **47** was prepared in 10



Scheme 9

Table 3 NMR spectroscopic details of **42, 43, 46** and **47** (recorded in D_2O)

Compound	Form	δ/ppm C-2	δ/ppm H-3	δ/ppm H-4	δ/ppm H-5	δ/ppm H-6	J/Hz $H3_a$–$H3_b$	J/Hz H3–H4	J/Hz H4–H5	J/Hz H5–H6
42	fur(1)	96.7	1.88, 1.82	3.95[a]	3.64	4.70	15.0	3.4, 3.4	3.2	9.9
	fur(2)	104.7	2.31, 1.77	4.30[a]	3.95[a]	4.38	14.1	7.3, 2.6	[b]	5.6
	pyr	104.3	2.11, 2.03	4.30[a]	3.84	4.31	14.1	6.8, 5.6	3.9	6.8
43	pyr(1)	95.5	2.15, 2.11	4.20	3.86	4.95	15.0	3.4, 3.4	3.4	9.8
	pyr(2)	[b]	2.54, 1.90	4.16	3.93	4.93	14.1	5.1, 2.6	3.0	9.0
	fur(1)	103.2	2.64, 2.09	4.52	4.25	4.46	14.5	6.9, 2.6	2.6	7.3
	fur(2)	102.9	2.42, 2.39	4.57	4.10	4.54	15.0	5.8, 6.4	3.9	6.8
46	pyr(1)	97.6	2.11, 2.11	3.92	3.62	4.56	13.1	5.1, 11.7	9.4	9.4
	pyr(2)	[b]	2.50, 1.84	3.84	3.60	4.45	12.8	5.1, 12.0	9.4	9.4
	fur	[b]	2.37, 2.27	4.40	4.12	4.78	14.5	0, 4.7	2.8	9.0
47	pyr(1)	96.4	2.23, 1.84	3.95	3.64	4.61	13.3	5.1, 11.5	9.4	9.4
	pyr(2)	[b]	2.60[a], 1.68	3.79	3.64[a]	4.33	12.8	5.1, 12.0	9.4	9.4
	fur(1)	[b]	2.60[a], 2.34	4.55[a]	4.16	4.80	15.0	[b], 5.6	3.4	9.0
	fur(2)	[b]	2.60[a], 2.17	4.55[a]	4.10	4.87	14.5	[b], 0	3.4	9.0

[a] Part of a multiplet centred on this chemical shift. [b] Not determined.

steps and 10% overall yield from D-ribonolactone. Furthermore, both screening substrates may also be prepared in 5 steps from a common precursor, **35**, derived from D-ribonolactone. The complementarity of the stereoselective syntheses of **43** and **47** stems from alternative *anti*- and *syn*-selective indium-mediated additions[26–28] of ethyl α-bromomethyl acrylate to the functionalised aldehydes **29** and **36**. It was possible to switch between Felkin–Anh[29] and chelation control,[30] allowing the synthesis of either diastereomeric series at will. The application of the screening substrates **43** and **47** in the directed evolution of tailored aldolases for the synthesis of analogues of influenza A sialidase inhibitors will be described elsewhere.[32]

Crystal structure determination of the dipropylamide 11

Crystal data. $C_{17}H_{31}NO_7$, $M = 361.43$, monoclinic, $a = 8.7774(4)$ Å, $\alpha = 90°$, $b = 8.3066(4)$ Å, $\beta = 96.2110(17)°$, $c = 13.4961(8)$ Å, $\gamma = 90°$, $U = 978.23(9)$ Å^3, $T = 150(2)$ K, space group $P2_1$, $Z = 2$, μ(Mo–Kα) $= 0.094$ mm^{-1}, 10308 reflections measured, 3748 unique ($R_{int} = 0.0739$) which were used in all calculations. The final wR (F^2) was 0.1247 (all data).*

Crystal structure determination of the dipropylamide 25

Crystal data. $C_{19}H_{33}NO_6$, $M = 371.46$, orthorhombic, $a = 5.73880(10)$ Å, $\alpha = 90°$, $b = 9.49090(10)$ Å, $\beta = 90°$, $c = 38.2430(8)$ Å, $\gamma = 90°$, $U = 2082.96(6)$ Å^3, $T = 100(2)$ K, space group $P2_12_12_1$, $Z = 4$, μ(Mo–Kα) $= 0.087$ mm^{-1}, 16140 reflections measured, 4085 unique ($R_{int} = 0.0973$) which were used in all calculations. The final wR (F^2) was 0.1138 (all data).*

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