Enzyme Engineering

Creation of a Tailored Aldolase for the Parallel Synthesis of Sialic Acid Mimetics

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The exquisite selectivity and efficiency of enzymes has served as an inspiration to chemists for many years.[1] These remarkable properties enable enzymes to guide the assembly of complex products from mixtures of reactants present in low concentrations (in the nM–μM range).[2] Indeed, high levels of substrate specificity, and stereo- and chemoselectivity are hallmarks of enzymatic catalysis.

The most useful catalysts to the synthetic chemist, however, are those that are broadly applicable. Indeed, Sharpless has commented on the synthetic virtues of asymmetric dihydroxylation in terms of its remarkable scope: "It [OsO_4] reacts *only* with olefins and it reacts with *all* olefins (slight poetic license here)".[3] The substrate ranges of many enzymes are rather more restricted which limits their utility in chemical synthesis. The power of directed evolution has been brought to bear on this problem[4] and has, for example, been used to create an amine oxidase with broad substrate specificity and high enantioselectivity[5] and aldolases that modify the stereochemical course of C–C bond formation.[6,7]

Our challenging objective was to broaden the substrate specificity of the carbon–carbon bond-forming enzyme, sialic acid aldolase (*N*-acetylneuraminic acid aldolase), in a manner sufficient for application in the parallel synthesis of sialic acid mimetics. Sialic acid aldolase catalyses the reversible aldol condensation between pyruvate and *N*-acetylmannosamine **3** to give sialic acid **4** (Scheme 1). Although a number of hexoses, pentoses, and their analogues are substrates for this enzyme, condensations that involve shorter aldehydes are less promising: L- and D-erythrose and threose react at between 0.3 and 5% of the rate of *N*-acetylmannosamine, and two- and three-carbon aldehydes are not substrates.[8] The substituted dihydropyran **2** is an influenza A sialidase inhibitor[9] whose activity was optimized from the first potent inhibitor of influenza sialidases, zanamivir (**1**).[10] We decided, therefore, to engineer an aldolase with sufficiently broad substrate specificity to convert four-carbon aldehydes of general structure **5** into the corresponding sialic acid mimetics **6** (Scheme 1).

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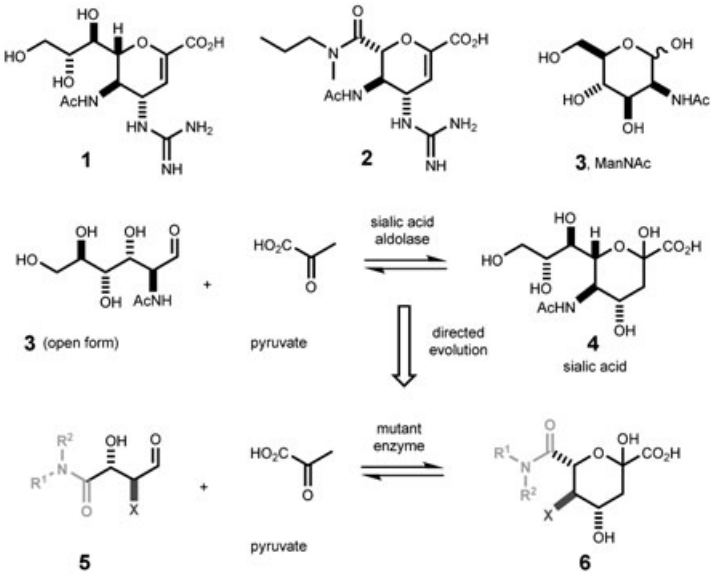


Scheme 1. Directed evolution of an aldolase for application in the parallel synthesis of sialic acid mimetics.

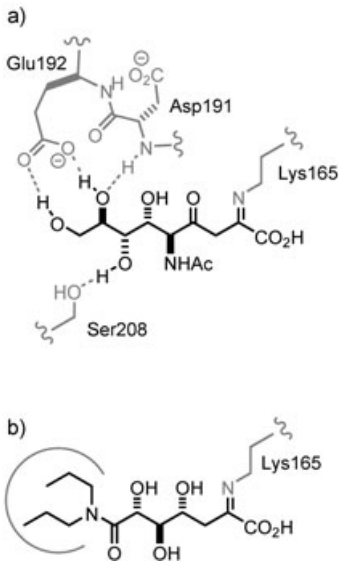
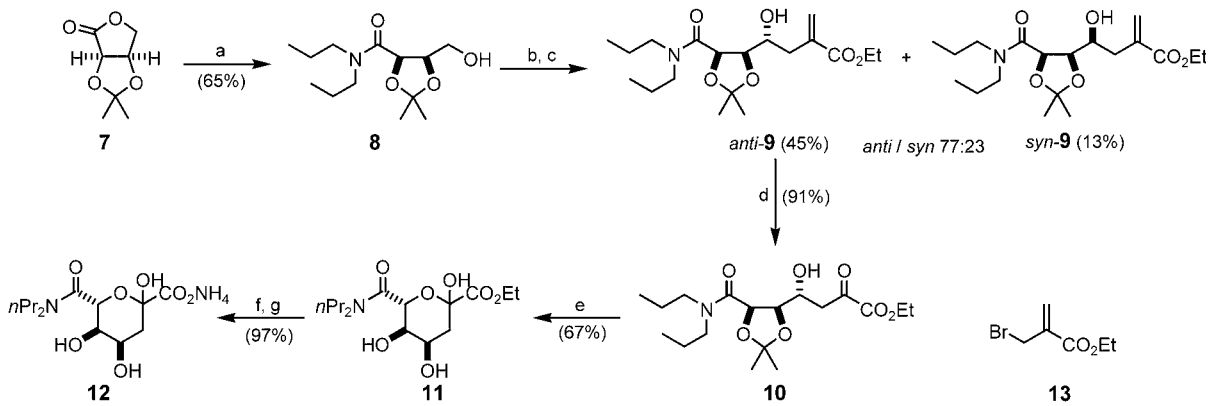


Figure 1. Schematic diagrams of the active sites of sialic acid aldolases: a) residues which have been shown by X-ray crystallography to interact with C7-C9 of 4-oxosialic acid; b) representation of the active site of an evolved enzyme in which a hydrophobic pocket has been sculpted to accommodate the dipropylaminocarbonyl group of the screening substrate.

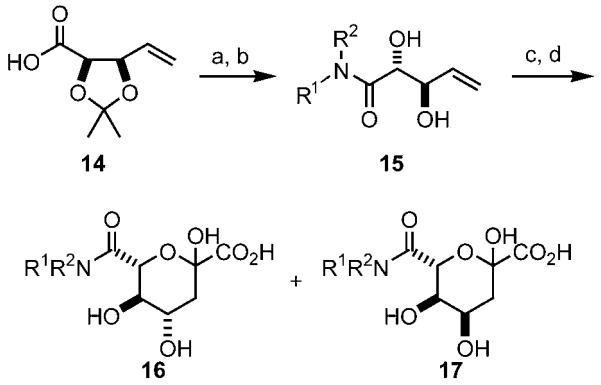
We used a semirational approach, starting with the structure of the sialic acid aldolase from *Haemophilus influenzae*, which has 35% identity and 59% similarity to the corresponding *E. coli* protein. Analysis of the X-ray crystallographic structure[11] of the aldolase in complex with the inhibitor 4-oxosialic acid revealed three residues in contact with the C7–C9 side chain. The corresponding residues in the *E. coli* protein, Asp191, Glu192, and Ser208 were targeted separately by using saturation mutagenesis (Figure 1a). As the aim of this investigation was to evolve an aldolase with broad substrate specificity, we designed a screening substrate **12** with relatively large R^1 and R^2 groups (*n*Pr); it was hypothesized that an active site able to accommodate this rather bulky substrate would also be able to accept a wide range of smaller substrates. Furthermore, the tolerance of the wild-type enzyme toward a wide range of C2-substituted aldehydes[8] was expected to be preserved, as this substituent (NHAc in *N*-acetylmannosamine) is solvent-exposed.[11] A schematic diagram of the designed screening substrate in complex with the mutant enzyme is shown in Figure 1b.

The screening substrate **12** was prepared with the synthetic sequence outlined in Scheme 2. The lactone **7**, readily available by oxidative cleavage of isoascorbic acid,[12] was opened with dipropylamine to give the γ-hydroxyamide **8**. Swern oxidation of **8** and indium-mediated coupling with ethyl α-bromomethylacrylate (**13**)[13] gave the γ-hydroxyamides **9** (*anti*/*syn* 77:33), an outcome consistent with Felkin–Anh-controlled[14] attack on the intermediate aldehyde. Cleavage of the acetonide and hydrolysis of the ester gave the screening substrate **12**.



Scheme 2. Synthesis of the screening substrate **12**. Reagents and conditions: a) $n\mathrm{Pr_2NH}$, MeOH; b) DMSO, $(\mathrm{COCl})_2$, $\mathrm{Et_3N}$, $\mathrm{CH_2Cl_2}$; c) In, **13**, $\mathrm{THF/H_2O}$ (1:1); d) $\mathrm{O_3}$, MeOH, −78 °C then $\mathrm{Me_2S}$; e) TFA/$\mathrm{H_2O}$ (1:1); f) $\mathrm{Ba(OH)_2}$, $\mathrm{EtOH/H_2O}$; g) $(\mathrm{NH_4})_2\mathrm{SO_4}$, $\mathrm{H_2O}$. DMSO = dimethyl sulfoxide; TFA = trifluoroacetic acid.

The library of enzymes produced by saturation mutagenesis of residues 191, 192, and 208, in turn, were screened for useful synthetic activity on the premise that mutant enzymes able to cleave the screening substrate **12** would also be able to catalyze the forward reaction.[6] Libraries of proteins were screened in 96-well plates by analysis of thermally treated crude-cell lysates in which His-tagged mutant proteins had been overexpressed to ≈40% of the total protein content. A coupled enzyme assay was used in which the cleavage of the screening substrate to generate pyruvate was detected spectroscopically at 340 nm by the lactate dehydrogenase catalyzed reduction of pyruvate, with concomitant oxidation of NADH. In this way, a mutant enzyme was identified that contains the Glu192→Asn mutation (E192N), for which substrate specificity [(k_{cat}/$K_{M(12)}$)/(k_{cat}/$K_{M(sialic\ acid)}$)] had been switched 640-fold (Table 1). The k_{cat}/K_M value of the E192N mutant toward the screening substrate **12** is 50-fold higher than that of the wild-type enzyme; indeed, cleavage of substrate **12** by the mutant enzyme was six times more efficient than the wild-type enzyme-catalyzed cleavage of sialic acid!

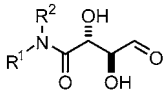


Scheme 3. Parallel synthesis of the sialic acid mimetics **16**/**17** (see Table 2). Reagents and conditions: a) EDC, HOBt, R^1R^2NH, CH_2Cl_2; b) TFA/H_2O (9:1); c) O_3, MeOH, −78 °C then Me_2S; d) E192N (2×10^{-2} mol%), pyruvate, buffer, (pH 7.4). EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt = 1-hydroxybenzotriazole hydrate.

Table 1: Characterization of the kinetics of the wild-type and E192N aldolases.

	Sialic Acid			**12**			
	k_{cat} [min^{-1}]	K_M [mM]	k_{cat}/K_M [min^{-1} mM^{-1}]	k_{cat} [min^{-1}]	K_M [mM]	k_{cat}/K_M [min^{-1} mM^{-1}]	$\frac{k_{cat}/K_{M(12)}}{k_{cat}/K_{M(sialic\ acid)}}$
WT	260 ± 6	4.4 ± 0.3	59 ± 4	74 ± 4	11 ± 1	6.9 ± 1.0	0.12
E192N	170 ± 10	38 ± 5	4.4 ± 0.6	130 ± 3	0.39 ± 0.04	340 ± 30	77

The synthetic utility of the evolved E192N enzyme was investigated with the crude aldehydes **18** generated by ozonolysis of the corresponding γ,δ-unsaturated amides **15**, prepared in parallel from the known γ,δ-unsaturated acid **14**[15] (Scheme 3 and Table 2). With the tertiary amide-containing aldehydes **18a–g** (Table 2, entries 1–7), the enzymatic reactions reached completion well within 3 days, and, after purification by ion-exchange chromatography, the sialic acid mimetics **16**/**17a–g** were obtained in 37–66% yield over the two steps from the corresponding γ,δ-unsaturated amides **15a–g**. In each case, the products **16**/**17** were obtained as ≈80:20 mixtures of epimers (**16**/**17**) which is consistent with thermodynamic stereochemical control.[16]



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The enzymatic reactions of the secondary amide substrates **15h–m** were less efficient (Table 2, entries 8–13). In each case, the required products were observed after 14 days by analysis of crude reaction mixtures by MS and ^{1}H NMR spectroscopy at 500 MHz. The yields of the products **16**/**17h–m**, determined by ^{1}H NMR spectroscopy, were low (19–55%). After purification, the *tert*-butyl amide **16h**/**17h** was obtained in 35% yield over two steps from **15h** as a 45:55 mixture of epimers.

Remarkably, the enantiomeric aldehyde *ent*-**15c**, prepared analogously from D-lyxose,[17] was also a substrate for the evolved aldolase (Table 2, entry 14). After 7 days, the enzymatic reaction was worked up, and the enantiomeric mimetic *ent*-**16c**/*ent*-**17c** was obtained in 32% yield. The catalysis of the aldol reaction was not as efficient in

Table 2: Synthetic utility of the mutant sialic acid aldolase E192N.

Entry	Alkene **15**	R^1	R^2	Yield[a] **15** [%]	Products	*t* [days]	**16**:**17**[b]	Yield **16**/**17**[c,d] [%]
1	**15a**	Et	Et	61	**16**/**17a**	3	82:18	37
2	**15b**	*n*Pr	Me	73[e]	**16**/**17b**	3	82:18	42
3	**15c**	*n*Pr	*n*Pr	78	**16**/**17c**	3	82:18	42
4	**15d**	*n*Bu	*n*Bu	88	**16**/**17d**	3	82:18	66
5	**15e**	$-(CH_2)_4-$		66	**16**/**17e**	3	82:18	48
6	**15f**	$-(CH_2)_5-$		59	**16**/**17f**	3	79:21	55
7	**15g**	$-(CH_2)_2O(CH_2)_2-$		68	**16**/**17g**	3	82:18	47
8	**15h**	*t*Bu	H	79	**16**/**17h**	14	45:55	35 (55)
9	**15i**	*n*Pent	H	81	**16**/**17i**	14	70:30	(13)[f]
10	**15j**	*c*Hex	H	90	**16**/**17j**	14	60:40	(29)[f]
11	**15k**	Ph	H	67	**16**/**17k**	14	60:40	(19)[f]
12	**15l**	(*R*)-CHMeBn	H	74	**16**/**17l**	14	60:40	(30)[f]
13	**15m**	(*S*)-CHMeBn	H	68	**16**/**17m**	14	60:40	(35)[f]
14	*ent*-**15c**	*n*Pr	*n*Pr	78[g]	*ent*-**16c**/*ent*-**17c**	7	64:36	32[h]

[a] Yield of purified product over two steps from the acid **14**. [b] Determined by integration of the ^{1}H NMR spectrum (500 MHz). [c] Yield of purified product over two steps from the corresponding alkene **15**; (yields in parentheses were determined by integration of the ^{1}H NMR spectrum (500 MHz) of the crude reaction mixture). [d] Products were obtained as mixtures of the two pyranose and the two furanose forms: for **16**, ≈75:8:8:7 and for **17**, ≈13:7:44:36. [e] 53:47 mixture of rotomers. [f] The ratio of the two pyranose and two furanose forms was not determined. [g] The enantiomeric acid *ent*-**14** was used. [h] The enantiomeric starting material *ent*-**15c** ($R^1 = R^2 = n$Pr) was used.

this case, however, as thermodynamic equilibration between the epimeric products was not complete (Table 2, compare the 82:18 ratio of epimers, entry 3, with the 64:36 ratio of epimers, entry 14).

In summary, we have engineered an aldolase that was exploited in the parallel synthesis of sialic acid mimetics. The mutant enzyme was most efficient in catalysis of the synthesis of the tertiary amides **16a–g**/**17a–g**, presumably a consequence of the screening assay used; it is well known that "you get what you screen for"[18] and our assay would have selected for enzymes able to accept a range of sterically varied *tertiary* amides. The novel enzyme might be used to catalyze the interconversion of dynamic combinatorial libraries,[19] for example, in the discovery of functional sialic acid mimetics such as paramyxovirus sialidase inhibitors.[20]

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