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, (4R, 5R, 6R)- and (4S, 5R, 6R)-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 o-bromomethyl acrylate to functionalised aldehydes. With an α-hydroxy aldehyde, (2R, 3R)-2,3-dihydroxy-4-oxo butanoic acid dipropylamide, the addition was chelation controlled, and the syn product, (6R, 5R, 4S)-6-dipropylcarbamoyl-2-methylidine-4,5,6-trihydroxy-hexanoic acid ethyl ester, was obtained. In contrast, the stereochemical outcome of the addition to (2R, 3R)-N,N-dipropyl-2,3-O-isopropylidene-4-oxobutyramide was consistent with Felkin–Anh control, and the anti adduct, (4R, 5R, 6R)-6-dipropylcarbamoyl-2-methylidine-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. 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.3,4

Evolved enzymes may have broader or altered substrate specificity,3,5 may catalyse reactions with modified levels of stereoselectivity6,7 and may display altered physical characteristics.8,9,13 For example, an enantioselective lipase for the kinetic resolution of chiral esters,10 and a hydantoinase with reversed enantioselectivity,11 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.12 To date, we have concentrated on the evolution of aldolases which catalyse aldol reactions with a modified stereocentre.13 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 IC50 ≈ 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 (IC50 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 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 and/or compatibility with non-aqueous solvents have been evolved.18,21

Scheme 1
Sialic acid aldolase has reasonably broad substrate specificity: although only pyruvate is a competent donor, many six- and five-carbon aldehydes are substrates. 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-acetylmannosamine, 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 aldol condensation as screening substrates. Mutant enzymes in synthetic chemistry, we chose to use the required products of the condensation as screening substrates. In this paper, we describe the preparation of screening substrates for the evolution of enzymes able to accept the aldol condensation as screening substrates. Although the aim was to generate enzymes for use in a protected version of an aldehyde would involve the diastereoselective addition of a pyruvate carbon aldehydes are substrates. 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)

### Table 1 Synthetic transformations of the diester 9

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>Product</th>
<th>Yield (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>1. KOH (3.6 eq.), 85 : 15 MeOH : H2O; 2. EDC, HOBT, Pr2NH, EtoAc</td>
<td>15</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>1. LiOOH (10 eq.), 75 : 25 THF : H2O; 2. EDC, HOBT, Pr2NH, EtoAc</td>
<td>15</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>1. DBU (2.1 eq.), H2O; 2. EDC, HOBT, Pr2NH, EtoAc</td>
<td>11</td>
<td>49</td>
</tr>
</tbody>
</table>

* Yield of purified product. Analysis of the crude reaction mixture by 500 MHz 1H NMR spectroscopy revealed a mixture of the amide 11 and the diamide 15. Analysis of the crude reaction mixture by 500 MHz 1H 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 HOBr gave the required amide 11 (72%) and its diequatorial epimer 16 (4%). The relative configurations of the amides 11 and 16 were deduced by careful analysis of their 500 MHz 1H 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\(_4\), Bu\(_2\)AlH 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\(_4\), Bu\(_2\)AlH 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), was converted into the corresponding acetonide 22 (Scheme 5). The \( \gamma \)-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 \( \gamma \)-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.

*CCDC reference numbers 262317 and 262318. See http://www.ccdc.org/suppdata/ob/b5/r501503k/ for crystallographic data in CIF or other electronic format.

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 \( \alpha \)-bromomethyl acrylate in THF–water was treated with indium powder. The required
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† on the intermediate aldehyde 29 (Fig. 5).

An alternative approach would involve the chelation-controlled 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).†† Acetonide deprotection gave the corresponding 1,2-diol 36.

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.

†† 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 (J2,3 = 6.1 Hz) and 26 (J2,3 = 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 6

Scheme 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 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 J3,4 values for the pyranose forms of the ketones 42 and 43 are consistent with an axial orientated C-4 substituent. The J3,4 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pyranose forms : furanose forms*</th>
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<tbody>
<tr>
<td>41</td>
<td>20 : 0 : 40 : 40</td>
</tr>
<tr>
<td>42</td>
<td>30 : 0 : 40 : 30</td>
</tr>
<tr>
<td>43</td>
<td>15 : 10 : 45 : 30</td>
</tr>
<tr>
<td>45</td>
<td>55 : 0 : 30 : 15</td>
</tr>
<tr>
<td>46</td>
<td>85 : 5 : 10 : 0</td>
</tr>
<tr>
<td>47</td>
<td>80 : 10 : 5 : 5</td>
</tr>
</tbody>
</table>

* Determined (± 5%) by 500 MHz 1H NMR spectroscopy. Initially, a 72 : 14 : 14 mixture of one pyranose and two furanose forms was obtained, which equilibrated to the mixture shown in the Table.
steps and 10% overall yield from d-ribonolactone. Furthermore, both screening methods may also be prepared in 5 steps from a common precursor, 35, derived from d-ribonolactone. The complementarity of the stereo-selectivity of the substrates of 43 and 47 stems from alternative anti- and syn-selective indium-mediated additions 28-29 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 siialidase inhibitors will be described elsewhere. 31

Crystal structure determination of the dipropylamide 11

Crystal data. C9H12N4O4, M = 361.43, monoclinic, a = 8.7774(4) Å, α = 90°, b = 8.3066(4) Å, β = 106.2110(17)°, c = 13.4961(8) Å, γ = 90°, U = 978.239(3) Å³, V = 1509(2) K, space group P2₁, Z = 2, μ(Mo-Kα) = 0.094 mm⁻¹, 10308 reflections, measured 3748 unique (Rint = 0.0739) which were used in all calculations. The final wR(F²) was 0.1247 (all data).

Crystal structure determination of the dipropylamide 25

Crystal data. C9H12N4O4, M = 371.46, orthorhombic, a = 5.73880(10) Å, α = 90°, b = 9.49090(10) Å, β = 90°, c = 38.2430(8) Å, γ = 90°, U = 2082.966(6) Å³, V = 1000(2) K, space group P2₁2₁2₁, Z = 4, μ(Mo-Kα) = 0.087 mm⁻¹, 16140 reflections measured, 4085 unique (Rint = 0.0973) which were used in all calculations. The final wR(F²) was 0.1138 (all data).

Acknowledgements

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References