Development of Fructose-1,6-bisphosphate aldolase enzyme peptide mimics as biocatalysts in direct asymmetric aldol reactions

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1. General information and materials

All solvents and reagents were obtained from commercial sources. Solvents for column chromatography (ethyl acetate and hexanes) were purchased from Protea Chemicals (South Africa) and distilled before use to remove non-volatile components. Aldol reactions were monitored using thin layer chromatography (TLC) plates (0.2 mm silica gel 60 with fluorescent indicator UV254) were obtained from Sigma-Aldrich and further visualization was done by staining with potassium permanganate (KMnO4) solution followed by heating. Purification of aldol products was conducted on Merk normal silica gel (particle size 0.063-0.200 mm) and flash silica gel (particle size 0.040-0.063).

\[^1\]H NMR and \[^13\]C NMR spectra were recorded on either a Bruker AVANCE 300 MHz, Bruker AVANCE 400 MHz or on a Bruker AVANCE III 500 MHz spectrometer. 2D NMR spectra which include COSY, 13C-HSQC, TOCSY, NOESY and ROESY experiments, were recorded at 293, 300, and 308 K on a 500 MHz NMR Bruker III 500 MHz spectrometer. All 2D spectra were recorded at the phase sensitive mode using time proportional phase increment (TPPI). The residual water peak of DMSO-d6 was suppressed by a presaturation pulse of 2 s duration. The first NOESY experiments were recorded with mixing time of 150, 200 and 250 ms; ROESY spectrum were recorded with mixing times of 100, 150, 200, 250 and 300 ms; while TOCSY was recorded with mixing times of 48, 64, 70, 80 and 100 ms; each increment was the sum of 32 scans with a relaxation delay of 2.0 s; 2048 data point were collected per experiment.

The enantiomeric excess (ee) was determined by chiral high performance liquid chromatography (HPLC) analysis on a Dionex HPLC Ultimate 3000 instrument (CHROMELEON version 6.80 software); coupled to a pump and photodiode array detector. A Lux 5µ cellulose-2 column was used for the analysis with hexane and isopropyl alcohol (IPA) as the mobile phase. CD spectra were recorded on a JASCO J-18 spectropolarimeter between the range of 190 to 250 nm in the specified solvents (water and phosphate buffer), with 10 scans at 20°C. Analytical LC-MS analysis was carried out on an Ultra High Performance Liquid Chromatography (Thermo Scientific Ultimate 3000, RS diode array detectors)-High Resolution Mass Spectrometer (Bruker Compact quadruple time of-flight) coupled to Diode Array (215 and 254 nm).

2. Synthesis of peptides catalysts.

Peptide were synthesized on an automated Protein Technologies, Inc PS-3TM peptide synthesizer and manually following the general Fmoc solid phase synthesis.

2.1 General procedure for the automated and manual SPPS synthesis

2.1.1-Swelling, activation and coupling of the first amino acid

The Fmoc-rink amide resin, 600 mg, (0, 160 mmol/g) was swelled in DMF (10.0 mL) for 20 minutes in a 70 mL glass reaction vessel with fritted filters. The DMF was removed by suction and a 20% piperidine solution in DMF (5 mL) was added and mixed by bubbling for 5 minutes with inert nitrogen gas (N2). The piperidine solution was then filtered out and the
resin was washed with DMF (5×5 mL) for 30 sec second per each wash. The first amino acid cysteine (1.20 mmol, 0.2 M), and coupling reagent HBTU (1.14 mmol, 0.19 M) were dissolved in a solution of 1.0 M DIPEA in DMF (6 mL) and added into the reaction vessel. An additional 6 mL of DMF was also added. The reaction mixture was allowed to react for 45 minutes while a gentle flow of N₂ was bubbled into the reaction. For double coupling, the resin was washed with (3×5 mL) DMF, and the coupling was repeated using the same mixture for 60 minutes. The resin was then washed with (3×5 mL) DMF, and (2×5 mL) DCM.

After the first coupling, Fmoc protecting group was removed using (2×10 mL) 20% piperidine solution and the resin was washed with DMF (5×5 mL) before the second amino acid was coupled. A solution containing 6 mL of 1.0 M DIPEA, (1.14 mmol, 0.19 M) HBTU, and (1.02 mmol, 0.2 M) arginine was added to the resin. The reaction mixture was mixed gently by bubbling nitrogen gas for 45 minutes. The remaining amino acids in the sequence were also coupled using the same procedure until the full peptide was synthesized.

2.1.2 General procedure for cleaving peptides from the resin

The resin bound peptide was washed with (3 ×5 mL) DMF and then (3×5 mL) DCM and dried by suction. A cleavage cocktail (10 mL) containing 94% TFA: 2.5% EDT: 2.5% H₂O: 1% TIS was then reacted with the resin bound peptide for 3 hrs. The cleavage solution was filtered off, and the resin was washed with 5 mL of TFA and the filtrated was divided into portions in and poured into 50 ml centrifuged tubes. Cold diethyl ether was added to the filtered solution upon which a white precipitate of the crude peptide was formed. The two samples were centrifuged at 5000 rpm for 10 minutes. The step was repeated 3 times with cold diethyl ether. The obtained white precipitate was then dissolved in 10 mL (60/40; H₂O/MeCN) for further analysis and purification.

2.2 Purification of the peptides

Peptides were purified via an Agilent 1260 Infinity semi-preparative HPLC system with a UV/VIS detector and an automated fraction collector on a Kinetix® 5 μm B-C18, 100 Å (250 ×230 nm) column. A two-buffer system was employed; Buffer A consisted of 0.1% formic acid in H₂O and buffer B consisted of 0.1% formic acid in CH₃CN. A flow rate of 20 ml/min or 15 ml/min and UV wavelengths of 215 and 254 nm were utilized.

3. Typical procedures for Aldol reaction

3.1 Aldol reactions in acetone in the presence of water

A peptide catalyst (0.0064 mmol, 4 mol%) was added to a vial containing 0.75 mL of 3:1 acetone/water and the mixture was stirred for 15 min. An acceptor aldehyde (0.157 mmol, 24 mg) was then added, and the resulting reaction mixture was stirred vigorously at room temperature for 24-72 hours. The reaction was stopped upon completion as indicated by
TLC and acetone was evaporated under reduced pressure. The crude product was extracted using (3 ×10 mL) ethyl acetate (EtOAc) and 2 mL water. The combined organic phases were dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was then purified by flash silica gel column chromatography using ethyl acetate/hexane (1:3). The pure product was then subjected to chiral-phase HPLC analysis to determine the ee.

3.2 Procedures for the aldol reactions between aromatic aldehydes and cyclohexanone

3.2.1 Aldol reaction between cyclohexanone and aromatic aldehydes (homogenous)

A peptide catalyst (0.0064 mmol, 4 mol%) was added to the ketone (0.45 mL) in water (40µL) and stirred for 15 minutes. An acceptor aldehyde was then added, and the resulting reaction mixture was left to stir for 24-72 hours at room temperature. The reaction was stopped upon completion as indicated by TLC. The reaction was quenched by extraction with (3×10 mL) EtOAc and brine solution (2 mL). The combined organic extract was washed with brine and dried with Na₂SO₄, filtered and concentrated in vacuo. Diastereomeric ratio of the crude product was determined by 1H NMR analysis. The crude aldol product was purified by flash silica-gel column chromatography using EtOAc/Hexane (1:3) and the desired aldol product was subjected to chiral-phase HPLC analysis to determine the ee.

3.2.2 Aldol reaction between cyclohexanone and aromatic aldehydes (heterogenous)

A peptide catalyst (0.0064 mmol, 4 mol%) was added to a solution of a ketone (0.450 mL) and 60 µL of water and stirred for 15 minutes. An acceptor aldehyde (0.157 mmol, 24 mg) was then added to the reaction mixture which was stirred for 24-72 hours. The reaction was monitored by TLC and the reaction was quenched by extraction with (3×10 mL) EtOAc and (1×2 mL) brine solution. The combined organic layers were dried with Na₂SO₄, filtered and concentrated in vacuo. The crude product was purified by flash chromatography on silica gel using EtOAc: hexane (1:3).

3.3 General procedure for aldol reaction between cyclohexanone and aromatic aldehydes in organic solvent

A peptide catalyst (0.0064 mmol, 4 mol%) was added to a vial containing a 0.75 mL solvent mixture (0.5173 mL solvent and 0.237 mL water) and cyclohexanone (0.628 mmol, 70 µL) and the mixture was stirred for 15 minutes. An acceptor aldehyde (0.157 mmol, 24 mg) was then added to the reaction mixture which was stirred for 24 -72 hours. The reaction progress was monitored by TLC and was extracted with EtOAc (3×10 mL) and brine solution
(1×2.0 mL). The combined organic layers were dried over Na$_2$SO$_4$ and concentrated in vacuo. Diastereomeric ratio of the crude product was determined by $^1$H NMR analysis. The crude product was purified by flash chromatography on silica-gel with hexane/ethyl acetate (3:1). The pure products were then analyzed by the chiral HPLC and the ee values were determined.

3.4 Catalyst recyclability for peptide catalyzed aldol reaction

The filtered aqueous layers obtained after extraction of the crude product, were combined and washed with Et$_2$O (3 mL) and C$_2$H$_5$O (4 mL). The peptide was catalyst obtained was after drying and decantation of the resuspended mixture. The recycled peptide was added to a vial containing a 0.75 mL solvent mixture (0.5173 mL solvent and 0.237 mL water) and

4. Analytical data for Aldol products

4-Hydroxy-4-(4'-nitrophenyl)-butan-2-one (TP_A1N4ACE)

![Chemical structure](image)

Yellow solid: $R_f = 0.27$ (30% ethyl acetate/hexane). IR (Vmax/cm$^{-1}$): 3459 (O-H), 3118 (=C-H), 3068 (C-H), 1574 (C=C), 1706(C=O), 1323(C-O). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$: 8.22 (d, 2H, H-7& H-7'), 7.54 (d, 2H, H-6 and H-6'), 5.30 – 5.21 (m, 1H, H-4), 3.55 (brs, J = 3.3 Hz, 1H, OH), 2.87 – 2.77 (m, 2H, H-3), 2.23 (s, 3H, H-1).$^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$: 208.44(C-2), 126.45(C-6), 126.34(C-6'), 123.80(C-7), 123.72(C-7'), 68.62(C-4), 50.83(C-3), 30.77(C-1).HRMS (ESI) m/z: Calculated for C$_{10}$H$_{11}$NO$_4$:209.068, found: 232.0910[M+23]$^+$

4-Hydroxy-4-(2'-nitrophenyl)-butan-2-one (TP_A2N2ACE)

![Chemical structure](image)

Colourless solid: $R_f = 0.32$ (30% ethyl acetate/hexane). IR (Vmax/cm$^{-1}$): 3461 (O-H), 3068(=C-H), 2922 (C-H), 1609 (C=O), 1518 (C=C), 1333(C-O). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$: 8.04 – 7.80 (m, 2H, H-9 and H-8), 7.77 – 7.58 (m, 1H, H-6), 7.50 – 7.38 (m, 1H, H-7), 5.68 (dd, 1H, H-4), 3.79 (brs, 1H, OH), 3.11 (d, 1H, H-3), 2.74 (dd, J = 17.8, 9.4 Hz, 1H, H-3), 2.24 (s, 2H H-1).$^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$: 208.76 (C-2), 147.12(C-10), 138.47(C-5), 133.82(C-7), 128.28(C-6), 128.19(C-9), 124.43(C-8), 65.60(C-4), 51.12(C-3), 30.43(C-1). HRMS (ESI) m/z: Calculated for C$_{10}$H$_{11}$NO$_4$:209.07, found: 232.0583 [M+23]$^+$

4-Hydroxy-4-(4'-chlorophenyl)-butan-2-one (TP_A3CIACE)
Colourless oil: \( R_f = 0.28 \) (30% ethyl acetate/hexane). IR (Vmax/cm\(^{-1}\)): 3421 (O-H), 3117 (C=O), 1701 (C=O), 1519 (C=C), 1315 (C-O). 1H NMR (500 MHz, CDCl\(_3\)) \( \delta \): 7.33 – 7.26 (m, 4H, Ar-H), 5.12 (d, \( J = 9.0, 3.4 \) Hz, 1H, H-4), 3.47 (brs, 1H, OH), 2.89 – 2.72 (m, 2H, H-3), 2.19 (s, 3H, H-1). 13C NMR (126 MHz) \( \delta \): 208.86, 141.25, 133.30, 128.65, 127.03, 69.16, 51.82, 30.76. HRMS (ESI) m/z: Calculated for C\(_{10}\)H\(_{11}\)ClO\(_4\):198.0488, found: 221.0334 [M+23]+.

2-(Hydroxy(4-nitrophenyl)methyl)cyclohexanone (TP_A4N4CY)

Yellowish solid: \( R_f = 0.26 \) (30% ethyl acetate/hexane). IR (Vmax/cm\(^{-1}\)): 3426 (O-H), 3077 (C=O), 2949 (C-H), 1698 (C=O), 1524 (C=C), 1024 (C-O). 1H NMR (500 MHz, CDCl\(_3\)) \( \delta \): 8.21 (d, \( J = 8.9, 2.3 \) Hz, 2H, H-10 and H-10'), 7.50 (d, \( J = 7.5 \) Hz, 2H, H-9 and H-9'), 4.90 (d, \( J = 8.3, 3.0 \) Hz, 1H, H-7), 4.07 (brs, 1H, OH), 2.63 – 2.55 (m, 1H, H-6), 2.54 – 2.45 (m, 1H, H-2a), 2.41 – 2.31 (m, 1H, H-2b), 2.16 – 2.08 (m, 1H, H-3a), 1.72 – 1.64 (m, 1H, H-3b), 1.63 – 1.48 (m, 4H, H-4 and H-3). 13C NMR (126 MHz, CDCl\(_3\)) \( \delta \): 214.73 (C-1), 147.96 (C-11), 127.87 (C-8), 126.59 (C-9 and C-9'), 123.52 (C-10 and C-10'), 74.05 (C-7), 70.14 (C-6), 57.20 (C-5), 42.69 (C-2), 30.77 (C-1, C-2, C-3, C-4). Calculated for C\(_{13}\)H\(_{15}\)NO\(_4\):249.1001, found 272.0890 [M+23]+.

2-Hydroxy(2-nitrophenyl)methyl)cyclohexanone (TP_A5N2CY)

Yellow oil: \( R_f = 0.29 \) (30% ethyl acetate/hexane). IR (Vmax/cm\(^{-1}\)): 3454 (O-H), 2922 (C-H), 2853 (C-H), 1693 (C=O), 1512 (C=C), 1199 (C-O). 1H NMR (500 MHz, CDCl\(_3\)) \( \delta \): 7.94 – 7.72 (m, 2H), 7.64 (t, \( J = 7.7 \) Hz, 1H), 7.43 (t, \( J = 7.8 \) Hz, 1H), 5.45 (d, \( J = 7.0 \) Hz, 1H), 4.07 (d, \( J = 37.1 \) Hz, 0H), 2.82 – 2.68 (m, 1H), 2.51 – 2.28 (m, 2H), 1.92 – 1.52 (m, 5H), 1.26 (d, 2H). 13C NMR (126 MHz, CDCl\(_3\)) \( \delta \): 214.96 (C-1), 148.73 (C-13), 136.63 (C-8), 133.07 (C-10), 129.00 (C-9), 128.40 (C-12), 124.10 (C-11), 69.81 (C-7), 57.31 (C-6), 42.85 (C-2), 31.14 (C-5), 27.77 (C-3), 25.00 (C-4). Calculated for C\(_{13}\)H\(_{15}\)NO\(_4\):249.1001, found 272.0890 [M+23]+.
5. LC-MS spectra for TP_Asp and T_ADlys peptide catalysts

Figure S1: LC-MS spectrum of TP_Asp conformation 1 (peak 1)
**Figure S2:** LC-MS spectrum of TP_Asp conformation 2 (peak 2)
Figure S3: LC-MS spectrum of TP_As conformation 3 (peak 3)
Figure S4: LC-MS spectrum of TP_Asp conformation 4 (peak 4)
**Figure S5**: LC-MS spectrum of TP_ADlys conformation 1 (peak 1)
Figure S6: LC-MS spectrum of TP_ADlys conformation 2 (peak 2)
Figure S7: LC-MS spectrum of TP_ADlys conformation 3 (peak 3)
Figure S8: LC-MS spectrum of TP_ADlys conformation 3 (peak 3)
6. NMR spectra for peptide structures

Figure S9: $^1$H NMR spectrum of TP_Asp peptide

Figure S.2-9: $^{13}$C NMR spectrum of TP_Asp peptide
Figure S10: DEPT 135 NMR spectrum of TP_Asp peptide

Figure S11: 2D $^1$H COSY NMR spectrum of TP_Asp peptide
Figure S12: HSQC NMR spectrum of TP_As p peptide

Figure S13: 2D $^1$H TOCSY NMR spectrum of TP_As p peptide
Figure S14: HMBC NMR spectrum of TP_Asp peptide

Figure S15: 2D $^1$H ROESY NMR spectrum of TP_Asp peptide
**Figure S16**: 2D $^1$H ROESY NMR spectrum of TP_ADlys peptide, $^1$HN region

**Figure S17**: $^1$H NMR spectrum of TP_ADlys peptide
Figure S18: $^{13}$C NMR spectrum of TP_ADlys peptide

Figure S19: DEPT 135 NMR spectrum of TP_ADlys peptide
Figure S20: 2D $^1$H COSY NMR spectrum of TP_ADlys peptide

Figure S21: HSQC NMR spectrum of TP_ADlys peptide
Figure S22: 2D $^1$H TOCSY spectrum of TP_ADlys peptide

Figure S23: 2D $^1$H TOCSY spectrum of TP_ADlys peptide, $^1$HN fingerprint region
**Figure S24**: HMBC NMR spectrum of TP_ADLys peptide

**Figure S25**: 2D $^1$H ROESY NMR spectrum of TP_ADLys peptide
Figure S26: 2D $^1$H ROESY NMR spectrum of TP_ADLys peptide, $^1$HN region

Figure S27: 2D $^1$H TOCSY spectrum of TP_ADLys peptide at 308K
Figure S28: 2D $^1$H TOCSY spectrum of TP_ADLys peptide, $^1$HN fingerprint region at 308K

Figure-S29: 2D $^1$H ROESY spectrum of TP_ADLys peptide at 308K
Figure S30: 2D $^1$H ROESY spectrum of TP_ADlys peptide, $^1$HN fingerprint region at 308K.
Selected NMR spectra for aldol products

Figure S31: $^1$H and $^{13}$C NMR spectra of the aldol product TP_A1N4ACE
Figure S32: $^1$H and $^{13}$C NMR spectra of the aldol product TP_A2N2ACE
Figure S33: $^1$H and $^{13}$C NMR spectra of the aldol product TP_A3Cl4ACE
Figure S34: $^1$H and $^{13}$C NMR spectra of the aldol product TP_A4N4CY
Figure S35: $^1\text{H}$ and $^{13}\text{C}$ NMR spectra of the aldol product TP_A5N2CY
Selected chiral HPLC chromatograms for aldol products

Using Acetone as a solvent and substrate (Table 2)

**Figure SR**: Chiral HPLC chromatogram of TP_A1N4ACE –standard

**Figure S36**: Chiral HPLC chromatogram of TP_A1.1N4ACE (entry1)
Figure S35: Chiral HPLC chromatogram of racemate TP_A2N2ACE-S2

Figure S37: Chiral HPLC chromatogram of TP_A2.1N2ACE (entry 2)
Figure SR: Chiral HPLC chromatogram of TP_A1.2N4ACE (entry 4)

Figure S38: Chiral HPLC chromatogram of TP_A3Cl4ACE (entry 6)
Using 8 mol% for reaction between Acetone and p-nitrobenzaldehyde (Table 5)

**Figure SR:** Chiral HPLC chromatogram of TP_A1.3N4ACE (entry 11)

**Figure S.39:** Chiral HPLC chromatogram of TP-A1.4N4ACE (entry 12)
Effect of substrate: Reaction between cyclohexanone and aromatic aldehydes (Table 3)

Figure SR: Chiral HPLC chromatogram of racemate TP_A4N4CY-R1

Figure S40: Chiral HPLC chromatogram of TP_A4.1N4CY (entry 4)
Figure SR: Chiral HPLC chromatogram of racemate TP_A4N4CY-R4

Figure S41: Chiral HPLC chromatogram of TP_A4.2N4CY (entry 9)
Figure SR: Chiral HPLC chromatogram of racemate TP_A5N2CY-RA5

![Chiral HPLC chromatogram of racemate TP_A5N2CY-RA5](image)

| No. | Ret.Time (min) | Peak Name | Height (mAU) | Area (mAU*min) | Rel.Area (%) | Amount | Type |
|-----|----------------|-----------|--------------|----------------|--------------|--------|------|
| 1   | 28.85          | n.a.      | 0.923        | 0.717          | 0.13         | n.a.   | BMB  |
| 2   | 34.87          | n.a.      | 0.962        | 0.630          | 0.12         | n.a.   | BMB* |
| 3   | 39.28          | n.a.      | 182.083      | 232.316        | 43.02        | n.a.   | BM”  |
| 4   | 44.15          | n.a.      | 194.427      | 306.341        | 58.73        | n.a.   | MB”  |
|     | Total          |           | 378.395      | 540.004        | 100.00       | 0.000  |      |

Figure S42: Chiral HPLC chromatogram of TP_A5.1N2CY (entry 10)
Effect of solvents - Reaction between cyclohexanone and \( p \)-nitrobenzaldehyde (Table 4)

**Figure SR:** Chiral HPLC chromatogram of racemate TP_A4N4CY-R5

**Figure S43:** Chiral HPLC chromatogram of TP_A4.3N4CY (entry 4)
Figure SR: Chiral HPLC chromatogram of racemate TP_A4N4CY-R6

![Chiral HPLC chromatogram of racemate TP_A4N4CY-R6](image)

| No. | Ret.Time (min) | Peak Name | Height (mAU) | Area (mAU*min) | Rel.Area (%) | Amount | Type |
|-----|---------------|-----------|--------------|----------------|--------------|--------|------|
| 1   | 29.93         | n.a.      | 2.726        | 1.960          | 1.32         | n.a.   | BMB* |
| 2   | 34.89         | n.a.      | 2.718        | 2.276          | 1.54         | n.a.   | BMB* |
| 3   | 39.19         | n.a.      | 88.785       | 82.427         | 55.70        | n.a.   | BMB* |
| 4   | 45.06         | n.a.      | 66.283       | 61.318         | 41.44        | n.a.   | BMB* |
| Total|               |           | 160.492      | 147.979        | 100.00       | 0.000  |      |

Figure S44: Chiral HPLC chromatogram of TP_A4N4CY (entry 6)
Figure SR: Chiral HPLC chromatogram of racemate TP_A4N4CY-R7

![Chiral HPLC chromatogram of racemate TP_A4N4CY-R7](image)

| No. | Ret.Time (min) | Peak Name | Height (mAU) | Area (mAU*min) | Rel.Area (%) | Amount | Type |
|-----|----------------|-----------|--------------|----------------|--------------|--------|------|
| 1   | 39.08          | n.a.      | 7.925        | 7.582          | 20.41        | n.a.   | BM*  |
| 2   | 44.17          | n.a.      | 8.280        | 7.602          | 20.40        | n.a.   | BM*  |
| 3   | 45.61          | n.a.      | 18.812       | 21.974         | 59.14        | n.a.   | MB*  |
|     | **Total**      |           | **35.017**   | **37.158**     | **100.00**   | **0.003** |      |

Figure S45: Chiral HPLC chromatogram of TP_A4.5N4CY (entry 9)
Figure SR: Chiral HPLC chromatogram of racemate TP_A4N4CY-R8

Figure S46: Chiral HPLC chromatogram of TP_A4.6N4CY (entry 13)
Figure SR: Chiral HPLC chromatogram of racemate TP_A4N4CY-R10

| No. | Ret. Time min | Peak Name | Height mAU | Area mAU min | Rel. Area % | Amount | Type |
|-----|---------------|-----------|------------|--------------|-------------|--------|------|
| 1   | 18.19         | n.a.      | 49.255     | 25.866       | 3.44        | n.a.   | BM*  |
| 2   | 20.00         | n.a.      | 40.366     | 20.758       | 2.76        | n.a.   | MB*  |
| 3   | 28.14         | n.a.      | 350.081    | 350.136      | 46.53       | n.a.   | M*   |
| 4   | 32.43         | n.a.      | 298.095    | 355.814      | 47.26       | n.a.   | MB*  |
|     |               |           |            |              | Total:      | 727.798 | 752.574 | 100.00 | 0.000 |

Figure S47: Chiral HPLC chromatogram of TP_A4.7N4CY (entry 15)

| No. | Ret. Time min | Peak Name | Height mAU | Area mAU min | Rel. Area % | Amount | Type |
|-----|---------------|-----------|------------|--------------|-------------|--------|------|
| 1   | 0.06          | n.a.      | 0.005      | 0.000        | 0.00        | n.a.   | BMB  |
| 2   | 26.19         | n.a.      | 54.929     | 38.662       | 29.15       | n.a.   | BMB* |
| 3   | 29.42         | n.a.      | 65.175     | 64.604       | 48.70       | n.a.   | BMB* |
| 4   | 36.15         | n.a.      | 4.826      | 3.142        | 2.37        | n.a.   | BMB* |
| 5   | 40.01         | n.a.      | 25.817     | 26.244       | 19.78       | n.a.   | BMB* |
|     |               |           |            |              | Total:      | 140.752 | 132.052 | 100.00 | 0.000 |
Aldol products using 8 mol% between cyclohexanone and p-nitrobenzaldehyde (Table 5)

**Figure SR**: Chiral HPLC chromatogram of racemate TP_A4N4CY-R13

| No. | Ret.Time | Peak Name | Height | Area | Rel.Area | Amount | Type |
|-----|----------|-----------|--------|------|----------|--------|------|
| 1   | 34.68    | n.a.      | 0.066  | 0.017| 0.00     | n.a.   | BMB* |
| 2   | 40.58    | n.a.      | 0.008  | 0.001| 0.00     | n.a.   | BMB* |
| 3   | 45.26    | n.a.      | 185.230| 193.103| 49.21    | n.a.   | BMB* |
| 4   | 52.62    | n.a.      | 146.419| 199.321| 50.79    | n.a.   | BMB* |
| Total: |        |           | 331.723| 392.442| 100.00  | 0.000  |      |

**Figure S48**: Chiral HPLC chromatogram of TP_A4.10N4CY (entry 5)

| No. | Ret.Time | Peak Name | Height | Area | Rel.Area | Amount | Type |
|-----|----------|-----------|--------|------|----------|--------|------|
| 1   | 33.89    | n.a.      | 2.422  | 1.802| 1.26     | n.a.   | BMB* |
| 2   | 36.40    | n.a.      | 1.243  | 0.033| 0.65     | n.a.   | BMB* |
| 3   | 44.50    | n.a.      | 49.146 | 49.059| 34.41    | n.a.   | BMB* |
| 4   | 51.20    | n.a.      | 47.979 | 58.946| 39.94    | n.a.   | BMB |
| 5   | 63.28    | n.a.      | 27.664 | 33.849| 23.74    | n.a.   | MB*  |
| Total: |        |           | 128.454| 142.589| 100.00  | 0.000  |      |
Figure SR: Chiral HPLC chromatogram of racemate TP_A4N4CY-R12

| No. | Ret.Time (min) | Peak Name | Height (mAU) | Area (mAU*min) | Rel.Area (%) | Amount (%) | Type |
|-----|---------------|-----------|--------------|----------------|--------------|------------|------|
| 1   | 33.15         | n.a.      | 0.855        | 0.598          | 0.18         | n.a.       | BM*  |
| 2   | 38.69         | n.a.      | 0.010        | 0.003          | 0.00         | n.a.       | BMB* |
| 3   | 43.29         | n.a.      | 156.876      | 167.356        | 49.27        | n.a.       | BMB* |
| 4   | 49.64         | n.a.      | 123.206      | 171.693        | 50.55        | n.a.       | BMB  |
|     |               |           |              |                |              |            |      |
| Total|               |           | 286.947      | 339.650        |              | 100        |      |

Figure S49: Chiral HPLC chromatogram of TP_A4.9N4CY (entry 1)

| No. | Ret.Time (min) | Peak Name | Height (mAU) | Area (mAU*min) | Rel.Area (%) | Amount (%)  | Type |
|-----|---------------|-----------|--------------|----------------|--------------|-------------|------|
| 1   | 34.49         | n.a.      | 356.697      | 372.865        | 31.78        | n.a.        | BM*  |
| 2   | 38.65         | n.a.      | 383.506      | 603.075        | 51.40        | n.a.        | MB*  |
| 3   | 40.00         | n.a.      | 66.234       | 53.515         | 4.56         | n.a.        | Rd   |
| 4   | 53.91         | n.a.      | 91.311       | 143.847        | 12.26        | n.a.        | BMB* |
|     |               |           |              |                |              |             |      |
| Total|               |           | 897.748      | 1173.302       |              | 100        |      |

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Figure **SR**: Chiral HPLC chromatogram of **racemate TP_A4N4CY-R15**

![Chiral HPLC Chromatogram of TP_A4N4CY-R15](image)

| No. | Ret.Time (min) | Peak Name | Height (mAU) | Area (mAU*min) | Rel.Area (%) | Amount Type |
|-----|----------------|-----------|--------------|----------------|--------------|-------------|
| 1   | 35.00          | n.a.      | 2.391        | 1.759          | 1.73         | n.a. BMB^*   |
| 2   | 40.78          | n.a.      | 2.615        | 1.975          | 1.95         | n.a. BMB^*   |
| 3   | 45.79          | n.a.      | 20.731       | 20.635         | 20.34        | n.a. BMB^*   |
| 4   | 52.77          | n.a.      | 17.529       | 19.034         | 18.77        | n.a. BM^*    |
| 5   | 54.67          | n.a.      | 47.354       | 58.025         | 57.21        | n.a. MB^*    |
| **Total** |                |            | 90.620       | 101.428        | 100.00       | 0.000        |

Figure **S50**: Chiral HPLC chromatogram of **TP_A4.13N4CY** (entry 4)
Figure SR: Chiral HPLC chromatogram of racemate TP_A4N4CY-R14

Figure S51: Chiral HPLC chromatogram of TP_A4.14N4CY (entry 6)
**Figure SR**: Chiral HPLC chromatogram of racemate TP_A4N4CY-15

![Chiral HPLC chromatogram of racemate TP_A4N4CY-15](image)

| No. | Ret. Time min | Peak Name | Height mAU | Area mAU*min | Rel. Area % | Amount | Type |
|-----|---------------|-----------|------------|--------------|-------------|--------|------|
| 1   | 25.95         | n.a.      | 27.625     | 16.077       | 5.92        | n.a.   | BMB* |
| 2   | 29.94         | n.a.      | 24.588     | 17.622       | 6.48        | n.a.   | BMB* |
| 3   | 37.05         | n.a.      | 89.999     | 77.484       | 28.51       | n.a.   | BMB* |
| 4   | 42.70         | n.a.      | 33.210     | 0.000        | 0.00        | n.a.   | BMB* |
| 5   | 44.23         | n.a.      | 127.454    | 160.564      | 59.09       | n.a.   | bMB* |
| Total|               |           | 302.857    | 271.747      | 100.00      | 0.00   |      |

**Figure S52**: Chiral HPLC chromatogram of TP_A4.15N4CY (entry 7)
Figure SR: Chiral HPLC chromatogram of racemate TP_A4N4CY-R16

Figure S53: Chiral HPLC chromatogram of TP_A4.16N4CY (entry 8)
Figure SR: Chiral HPLC chromatogram of racemate TP_A4N4CY-R17

Figure S54: Chiral HPLC chromatogram of TP_A4.17N4CY (entry 9)
Catalyst Recycling: Reaction between cyclohexanone and p-nitrobenzaldehyde (Table 6)

Figure SR: Chiral HPLC chromatogram of racemate TP_A4N4CY-R18

Figure S55: Chiral HPLC chromatogram of TP_A4.18N4CY (entry 5)
Table A: Analytical data and Chiral-phase HPLC spectra analysis for selected aldol products using Lux 5µm Cellulose-1 chiral column

| Structure | Aldol product | Eluent; %ratio | Flow rate; Wavelength (nm) | Retention time (min) |
|-----------|---------------|----------------|----------------------------|----------------------|
|           | **TP_A1.1N4ACE** | Hexane/IPA; 85/15 | 0.6 mL; 254 | $t_{R\text{major}} = 23.38$; $t_{R\text{minor}} = 30.42$ |
| ![Structure](image1) |               |                |                            |                      |
|           | **TP_A1.2N4ACE** | Hexane/IPA; 85/15 | 0.6 mL; 254 | $t_{R\text{major}} = 24.52$; $t_{R\text{minor}} = 32.07$ |
| ![Structure](image2) |               |                |                            |                      |
|           | **TP_A1.3N4ACE** | Hexane/IPA; 85/15 | 0.6 mL; 254 | $t_{R\text{major}} = 22.37$; $t_{R\text{minor}} = 27.88$ |
| ![Structure](image3) |               |                |                            |                      |
|           | **TP_A1.4N4ACE** | Hexane/IPA; 85/15 | 0.6 mL; 254 | $t_{R\text{major}} = 23.13$; $t_{R\text{minor}} = 28.80$ |
| ![Structure](image4) |               |                |                            |                      |
|           | **TP_A2.1N2ACE** | Hexane/IPA; 90/10 | 0.6 mL; 254 | $t_{R\text{major}} = 22.75$; $t_{R\text{minor}} = 26.31$ |
| ![Structure](image5) |               |                |                            |                      |
|           | **TP_A2.2N2ACE** | Hexane/IPA; 90/10 | 0.6 mL; 254 | $t_{R\text{major}} = 19.31$; $t_{R\text{minor}} = 22.29$ |
| ![Structure](image6) |               |                |                            |                      |
|           | **TP_A3Cl4ACE** | Hexane/IPA; 85/15 | 0.7 mL; 254 | $t_{R\text{major}} = 14.77$; $t_{R\text{minor}} = 16.79$ |
| ![Structure](image7) |               |                |                            |                      |
|           | **TP_A4.1N4CY** | Hexane/IPA; 94/6 | 0.6 mL; 254 | $t_{R\text{minor}} = 39.09$; $t_{R\text{major}} = 44.17$ |
| ![Structure](image8) |               |                |                            |                      |
|           | **TP_A4.2N4CY** | Hexane/IPA; 94/6 | 0.6 mL; 254 | $t_{R\text{major}} = 38.63$; $t_{R\text{minor}} = 43.60$ |
| ![Structure](image9) |               |                |                            |                      |
|           | **TP_A5.1N4CY** | Hexane/IPA; 95/5 | 0.5 mL; 254 | $t_{R\text{major}} = 39.28$; $t_{R\text{minor}} = 44.15$ |
| ![Structure](image10) |               |                |                            |                      |
|           | **TP_A5.2N4CY** | Hexane/IPA; 95/5 | 0.5 mL; 254 | $t_{R\text{major}} = 38.63$; $t_{R\text{minor}} = 43.60$ |
| ![Structure](image11) |               |                |                            |                      |

Using cyclohexanone as solvent for reaction between cyclohexanone and aromatic aldehydes (Table 4-2)
### Selected Aldol products using organic solvents (Table 4-3)

| Compound       | Solvent     | Volume | λ (nm) | t<sub>major</sub> | t<sub>minor</sub> |
|----------------|-------------|--------|--------|-------------------|------------------|
| TP_A4.3N4CY    | Hexane/IPA; 95/5 | 0.7mL  | 254    | 37.91             | 42.57            |
| TP_A4.4N4CY    | Hexane/IPA; 95/5 | 0.6mL  | 254    | 39.19             | 45.06            |
| TP_A4.5N4CY    | Hexane/IPA; 94/6 | 0.7mL  | 254    | 39.08             | 45.61            |
| TP_A4.6N4CY    | Hexane/IPA; 95/5 | 0.6mL  | 254    | 43.19             | 48.48            |
| TP_A4.7N4CY    | Hexane/IPA; 96/4 | 0.8mL  | 254    | 29.42             | 26.19            |

### Using 8 mol% catalyst loading (Table 4-5)

| Compound       | Solvent     | Volume | λ (nm) | t<sub>major</sub> | t<sub>minor</sub> |
|----------------|-------------|--------|--------|-------------------|------------------|
| TP_A4.10N4CY   | Hexane/IPA; 94/6 | 0.6mL  | 254    | 44.50             | 51.20            |
| TP_A4.9N4CY    | Hexane/IPA; 94/6 | 0.7mL  | 254    | 34.49             | 38.65            |
| TP_A4.13N4CY   | Hexane/IPA; 94/6 | 0.6mL  | 254    | 45.79             | 54.67            |
| TP_A4.14N4CY   | Hexane/IPA; 95/5 | 0.6mL  | 254    | 43.66             | 50.82            |
| TP_A4.15N4CY   | Hexane/IPA; 94/6 | 0.6mL  | 254    | 37.05             | 44.23            |
| TP_A4.16N4CY   | Hexane/IPA; 95/5 | 0.7mL  | 254    | 48.57             | 55.54            |
| TP_A4.17N4CY   | Hexane/IPA; 95/5 | 0.8mL  | 254    | 42.08             | 50.40            |
| TP_A4.18N4CY   | Hexane/IPA; 95/5 | 0.8mL  | 254    | 40.92             | 48.72            |
Selected NMR spectra for determination of the syn/anti (dr) for aldol products by $^1$H NMR

**Figure S56:** $^1$H NMR spectrum of the crude product of TP_A4.2N4CY

**Figure S57:** $^1$H NMR spectrum of the crude product of TP_A4.3N4CY
Figure S58: $^1$H NMR spectrum of the crude product of TP_A4.4N4CY

Figure S59: $^1$H NMR spectrum of the crude product TP_A4.5N4CY
Figure S60: $^1$H NMR spectrum of the crude product of TP_A4.6N4CY

Figure S61: $^1$H NMR spectrum of the crude product of TP_A4.8N4CY
Figure S62: $^1$H NMR spectrum of the crude product of TP_A5.1N2CY

Figure S63: $^1$H NMR spectrum of the crude product of TP_A5.2N2CY
Figure S64: $^1$H NMR spectrum of the crude product TP_A4.9N4CY

Figure S65: $^1$H NMR spectrum of the crude product TP_A4.10N4CY
Figure S66: $^1$H NMR spectrum of the crude product TP_A4.12N4CY

Figure S67: $^1$H NMR spectrum of the crude product of TP_A4.14N4CY
**Figure S68:** $^1$H NMR spectrum of the crude product of TP_A4.16N4CY

**Figure S69:** $^1$H NMR spectrum of the crude product TP_A4.17N4CY