ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

Biocatalytic transamination with near-stoichiometric inexpensive amine donors mediated by bifunctional mono- and di-amine transaminases

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Table of contents

I. General methods and materials 3
1.1 Materials 3
II. Molecular biology protocols 4
2.1 Cloning of spuC genes from Pseudomonas strains: 4
III. Expression and purification of HisTag recombinant proteins 5
3.1 Protein expression protocols 5
3.2 Protein purification 5
3.3 Protein determination and SDS-PAGE analysis 6
Table S3. Purification of recombinant *Pp_spuc*, *Pf_spuC* and *Pc_spuC* expressed in *E.coli* 7
IV. Spectrophotometric activity analysis and determination of kinetic constants 8
4.1 L-Alanine dehydrogenase (L-AlaDh) activity assay screen 8
4.2 Determination of kinetic parameters for substrates 1a-k 9
V. Biotransformations 11
5.1 Analytical scale 11
5.2 Preparative scale 11
5.3 GC-assay conditions 13
5.4 HPLC assay conditions 13

I. General methods and materials

1.1 Materials

Commercially available reagents were used throughout without further purification. Putrescine and all other reagents were purchased from Sigma Aldrich (St Louis, MO, USA) or Acros including anhydrous solvents. Restriction enzymes, T4 ligase, *taq* polymerase, dNTPs and broad protein marker (2-212 kDa) were purchased from New England Biolabs (Ipswich, MA, USA). *Escherichia coli* DH5α and BL21 (DE3) cells were purchased from New England Biolabs (Ipswich, MA, USA). Expression vector pET-28b was purchased from Novagen (Darmstadt, Germany) and was used for gene expression. *Chromobacterium violaceum* (Cv-TA) was purchased from LG standards (ATCC 12472, NCIMB 9178). *Pseudomonas florescens (Asc no. 13500), Pseudomonas chlororaphis subsp. aureofaciens 30-84*, (Asc. no 9392), *Pseudomonas putida* NBRC 14161 (Asc no. 9494), and *Bacillus megaterium* (Asc. No 9376) were purchased from the NCIMB culture collection (Aberdeen, UK).

II. Molecular biology protocols

2.1 Cloning of spuC genes from Pseudomonas strains:

The coding region of the *spuC* gene was amplified by colony PCR from *Pseudomonas putida* NBRC 14161, P*seudomonas chlororaphis subsp. aureofaciens 30-84*, and *Pseudomonas fluorescens* using the primers detailed in Table S1.

Table S1. Primers for cloning the spuC gene from *Pseudomonas putida* NBRC 14161, *Pseudomonas chlororaphis subsp. aureofaciens 30-84*, and *Pseudomonas fluorescens.*

Pf-spuC	(5'-CAGCCATATGATGACCCGCAATAACCCGCAAACCCGTGAA-3') (5'-CACCTCGAGTTAGCCTTGCAACGCACTGAGCGTCAGGTCC-3')
Pp-spuC	(5'- AGTCGACATATGAGCACCAACAACCCGCAAACCCG-3') (5'- ATTCGGCTCGAGCTACCGAATCGCCTCAAGGGTC-3')
Pc-spuC	FW: (5'- GCAAGCATATGAYCAGCAACAAYCCGCAAACCCGTG-3') RV: (5'- GCAAGCTCGAGTTAGCCCTGYAAYGCACTCARSGTC-3')

The *NdeI* and *XhoI* restriction sites are underlined. The following PCR protocol was used: 5 min denaturation at 95 °C and then 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 58 °C and 90 s elongation at 68 °C with a 5 min final extension time at 68 °C. The PCR product was cloned into ZERO Blunt TOPO PCR cloning vector (K2830-20) following the manufacture's protocol.

The alanine dehydrogenase (Aldh) gene from *Bacillus megaterium* was amplified using forward (5'-GCAGCCATATGATTATTGGCGTACCAAAAGAAATC-3') and reverse (5'-GCAAGCTCGAGTTAGAT-AGAAGCTAATTCTTTTTCAAGAG-3') primers (XhoI/*NdeI* restriction sites underlined) and was amplified and cloned into TOPO PCR cloning vector as above.

Pf-spuC and *Aldh* genes were subcloned into pET-28b expression vector containing an *N*-terminal His_6 tag with a thrombin linker. Pc-spuC and Pp-spuC, was subcloned into pET-22b expression vector containing a *C*-terminal His tag with a thrombin linker. The inserted genes were in-frame downstream from the ribosome binding site as confirmed via DNA sequencing (Eurofins).

Table S2. Sequence-identity matrix heat map of the transaminases used to draw the cladogram in Figure 1.

[%]	ATA-117	ARS-ATA	Ec-YgjG	Bme-YgjG	Bmy-YgjG	AdATA	VfATA	CvATA	PaSpuC
ATA-117	100.0	16.7	17.0	18.1	17.7	16.4	15.0	16.1	15.5
ARS-ATA	16.7	100.0	25.6	27.8	29.2	25.8	22.9	25.7	28.3
Ec-YgjG	17.0	25.6	100.0	61.5	60.0	28.8	25.0	26.7	28.3
Bme-YgjG	18.1	27.8	61.5	100.0	85.8	29.8	26.0	27.9	28.4
Bmy-YgjG	17.7	29.2	60.0	85.8	100.0	30.3	24.6	25.9	28.7
AdATA	16.4	25.8	28.8	29.8	30.3	100.0	28.9	32.8	35.9
VfATA	15.0	22.9	25.0	26.0	24.6	28.9	100.0	37.3	38.3
CvATA	16.1	25.7	26.7	27.9	25.9	32.8	37.3	100.0	59.6
PaSpuC	15.5	28.3	28.3	28.4	28.7	35.9	38.3	59.6	100.0

III. Expression and purification of HisTag recombinant proteins

3.1 Protein expression protocols

The *spuC* genes from *Pseudomonas putida* NBRC 1416 and *Pseudomonas chlororaphis subsp. aureofaciens 30-84*, were transformed into *E. coli* BL21 (DE3) for yielding *E. coli* BL21 (pET-22b(+)4–Pp_spuC) and *E. coli* BL21 (pET-22b(+)4–Pc_spuC). Similarly, the *spuC* gene from *Pseudomonas fluorescens* F113 in pET-28b(+) expression vector and the *ald* gene from *Bacillus megaterium* DSM 319 in pET-22b(+)-4 expression vector were used to yield *E. coli* BL21 (pET-28b(+)–Pf_spuC) and *E. coli* BL21 (pET-22b(+)4–Bm_AlaDh), respectively. The freshly-prepared strains were cultivated in 600 mL of the LB medium supplemented with 50 µg/mL antibiotic (ampicillin or kanamycin) in 1-L Erlenmeyer flasks at a rotary shaking rate of 220 rpm at 37°C. The recombinant protein expression was induced by adding isopropyl b-D-1-thiogalactopyranoside (IPTG) (0.2 mM, final) when A_{600} reached 0.6 - 0.8. The cell cultures were incubated at 18 °C for 16 h. The cells were harvested by centrifugation (4°C, 3,250xg, 20 min).

3.2 Protein purification

For purification purposes, cell pellets were resuspended (1g of wet cell paste/10 mL) in HEPES buffer (100mM, 1mM PLP, 5mM imidazole, pH 8). The cell pellets were lysed in an iced bath by ultra-sonication by Soniprep 150 (20 cycles of 20s on/20s off). After centrifugation (4^0C, 16,000xg, 20 min) the supernatant was used for protein purification manually or using an AKTA Pure system. Crude extract was then loaded onto a 5 mL HisTrap column. Purification was achieved manually or automated using an AKTA Pure system, using de-gassed HEPES buffer 1 (100 mM, 1mM PLP, 30mM imidazole, pH 8.0) and an elution buffer of HEPES buffer 2 (100 mM, 1mM PLP, 300mM imidazole, pH 8.0).

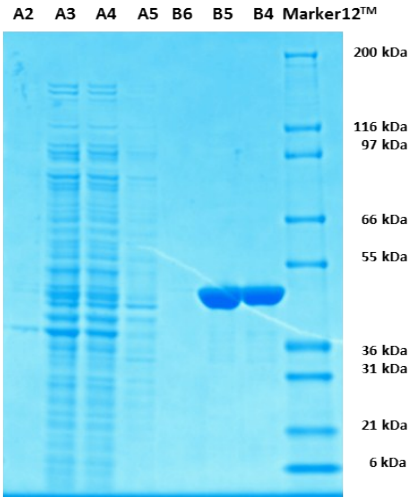


Figure S1. Representative SDS-PAGE analysis for the purification of the recombinant Pp-SpuC protein on AKTA (A2-A5 and B6: flow-through of crude extract during column loading; B5 and B4: fractions of Pp-SpuC protein post HisTag purification; Mark12™ Unstained Standard protein ladder).

The column was initially washed with 5 column volumes HEPES buffer 1. The protein was then eluted with 10 column volumes of 100% elution buffer (50 mM HEPES, 1mM PLP, pH 9.0). 5 mL fractions were collected during the elution phase and a protein gel was run to identify fractions containing the transaminase protein, before these were pooled together. The protein solutions were concentrated in an Amicon® Ultra-15 10K centrifugal filter device and the purified enzyme was flash frozen and stored at –80°C. The purity was analysed by SDS/PAGE and the protein was more than 95% pure.

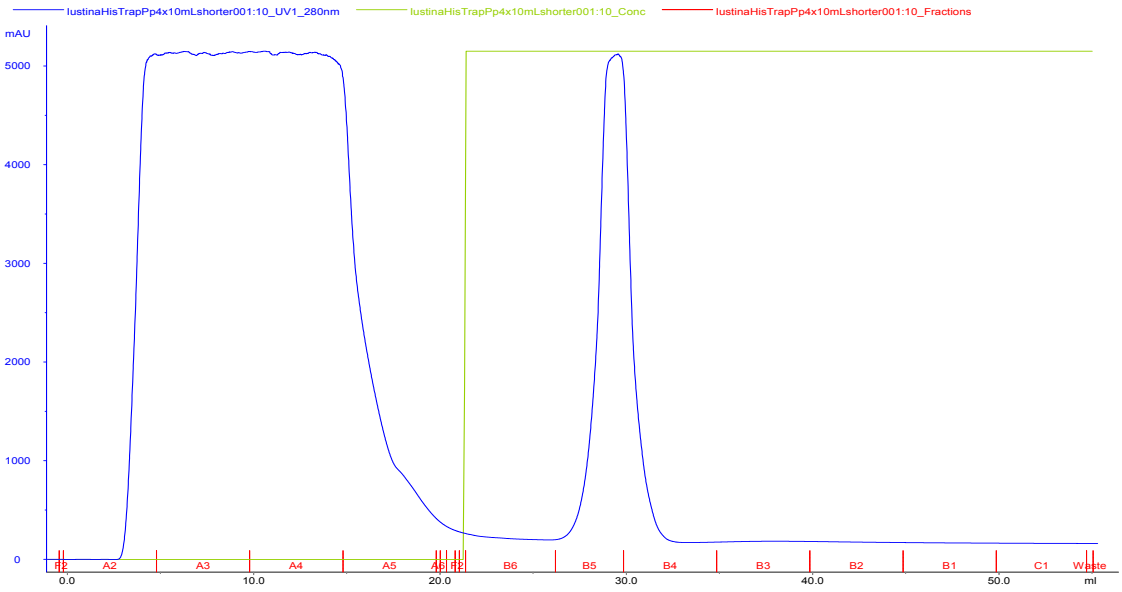


Figure S2. Representative chromatogram for purification of the His_6-tagged Pp-SpuC recombinant protein by metal ion affinity chromatography. Absorbance at 280 nm is shown in blue and the imidazole concentration of the buffer used in green (maximal value: 300 mM). The fractions collected (5mL each) are shown in red.

3.3 Protein determination and SDS-PAGE analysis

Concentrations of soluble protein was analysed by SDS-PAGE with 15% resolving gel and 5% stacking gel in a Tris-glycine buffer system. The gel was stained with Bio-Rad Coomassie Blue 250 kit (Bio-Rad lab, Munich, Germany), with bovine serum albumin (BSA) as a standard protein.

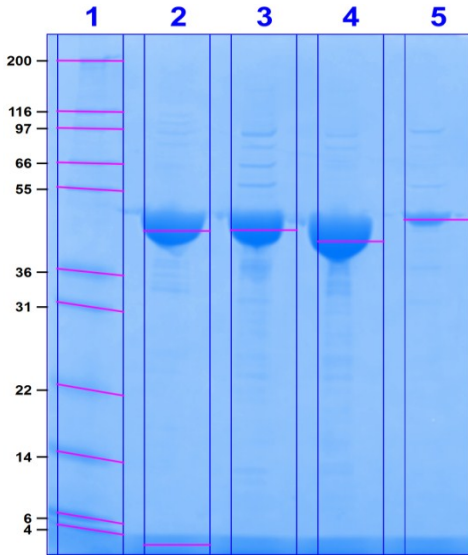


Figure S3. SDS-PAGE analysis of the three *spuC* recombinant proteins and Cv_TA (lane 1: *Marker 12* ; lane 2: *Pp_spuC*; lane 3: *Pf_spuC*; lane 4: *Pc_spuC*; lane 5: *Cv_TA)*

Table S3. Purification of recombinant *Pp_spuc*, *Pf_spuC* and *Pc_spuC* expressed in *E.coli*.

Recombinant protein	Culture medium volume (L)	Cell pellet (g)	Yield pure protein (g)	Purity (%)
Pp_spuC	0.6	5.8	0.200	98
Pf_spuC	0.6	6	0.250	94
Pc_spuC	0.6	5	0.390	95

IV. Spectrophotometric activity analysis and determination of kinetic constants

4.1 L-Alanine dehydrogenase (L-AlaDh) activity assay screen

The transaminase activity was determined on a Tecan Sunrise™ plate reader, using a modified alanine dehydrogenase/ transaminase assay method. The spectrophotometric assay monitors the reduction of the tetrazolium salt INT by NADH, using 1-methoxy PMS as catalyst (λ= 503nm, ε= 5286.2 $M^{-1}cm^{-1}$).

Solution 1 (double-fold concentrated stock) was made up of the following: 2 mM nicotinamide adenine dinucleotide (NAD^+, 100 mM in water), 0.6 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium (INT, 60 mM in DMSO), 0.01 mM 1-methoxy-5-methylphenazinium methyl sulfate (1-methoxy PMS, 1 mM in water), 0.05 mg/mL alanine dehydrogenase from *B. megaterium* (Bm_AlaDh) in 100 mM sodium phosphate buffer pH 8.

The 96-well substrate microplates contained 5 mM amine substrate (double-fold concentrated, in 100 mM sodium phosphate buffer, pH 8) and 0.2 mg/mL transaminase (double-fold concentrated purified enzyme in 100 mM HEPES buffer with 1 mM PLP, pH 8). 100 µL of solution 1 were added to the substrate microplate and the assay was initiated by addition of 2 mM sodium pyruvate (10 mM in water, double-fold concentrated).

Experiments were run on a spectrophotometer at 37 ºC with the measured activity at a wavelength of 503 nm and the absorbance taken every 13 seconds for 60 minutes. One Unit (U) is defined by one µmole of product per minute.

Specific activities against substrates **1a-k** are shown in Table S4.

Table S4. Specific activities for substrates **1a-k** determined using the L-AlaDh-TA assay.

	Specific activity U/mg				
substrate	Pp_spuC	Pf_spuC	Pc_spuC	Ec_yjgj	Cv_TA
1,4-diaminobutane	1.3	0.4	1.4	2.7	nd
1,5-diaminopentane	27.3	16.5	15.1	2.7	7.7
spermidine	15.7	16.5	21.5	2.1	nd
(S)-methylbenzylamine	23.2	25.7	30.4	nd	28.9
isopropylamine	20.4	18.5	18.7	0.05	5.6
butylamine	16.5	19.3	13.5	nd	14.1
1,6-diaminohexane	14.9	18.1	13.5	0.6	10.9
1,7-diaminoheptane	15.2	24.7	14.7	0.45	18.4
1,8-diaminooctane	7.5	2.7	2.3	0.33	4.4
1,9-diaminononane	13	4.4	3.1	nd	4.6
1,10-diaminodecane	2.1	3.8	1.7	nd	2.3

4.2 Determination of kinetic parameters for substrates 1a-k

Kinetic parameters (Tables S5-S7) were deduced by non-linear least-square regression analysis based on Michaelis-Menten kinetics.

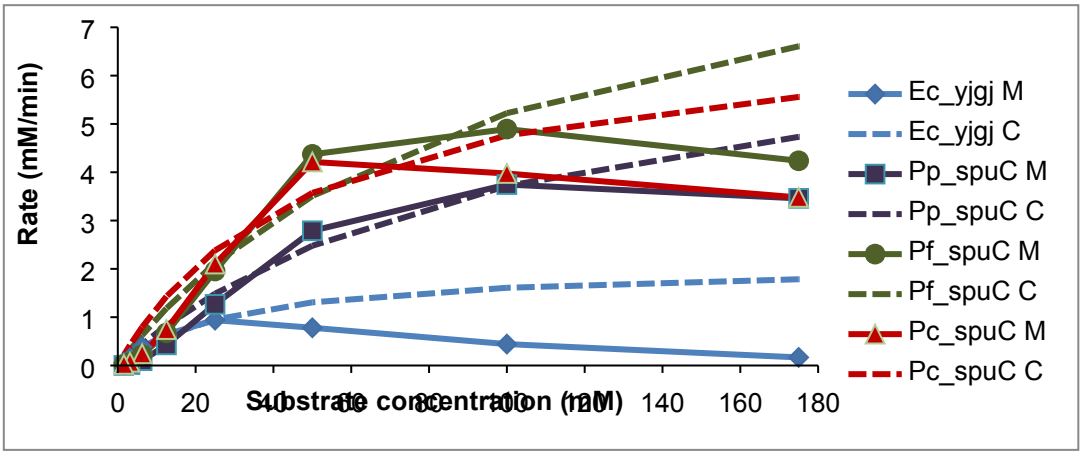


Figure S4. Determination of kinetic constants for Ec_ygjg, Pp_spuC, Pf_spuC and Pc_spuC for spermidine substrate using the L-Aladh/TA liquid phase assay. The measured (M, dotted line) and the calculated (C, solid line) rates of reactions for each protein are plotted against the substrate concentration. The kinetics parameters are determined using non-linear least-squares regression method.

Table S5. Values of Km for substrates **1a-k** determined using the L-AlaDh-TA assay.

	Km (mM)				
substrate	Pp_spuC	Pf_spuC	Pc_spuC	Ec_yjgj	Cv_TA
1,4-diaminobutane	62.34	52.99	59.87	0.47	Nd
1,5-diaminopentane	25	32.38	22.99	0.34	48.04
1,6-diaminohexane	1.41	2.31	4.05	2.54	3.92
1,7-diaminoheptane	1.06	1.02	0.64	0.44	1.09
1,8-diaminooctane	0.59	0.39	0.52	0.53	0.73
1,9-diaminononane	3.48	2.13	1.64	nd	2.11
1,10-diaminodecane	0.91	2.14	0.17	nd	0.34
spermidine	20.82	16.95	25.00	29.9	nd
(S)-methylbenzylamine	1.25	4.64	3.81	nd	1.06
butylamine	5.86	6.05	5.86	nd	10.19

Table S6. Values of Vmax for substrates **1a-k** determined using the L-AlaDh-TA assay.

	Vmax (mM/min)				
substrate	Pp_spuC	Pf_spuC	Pc_spuC	Ec_yjgj	Cv_TA
1,4-diaminobutane	0.13	0.04	0.14	0.27	nd
1,5-diaminopentane	2.73	1.65	1.51	0.27	0.77
spermidine	1.57	1.65	2.15	2.1	nd
(S)-methylbenzylamine	2.32	2.57	3.04	nd	2.89
butylamine	1.65	1.93	1.35	nd	1.41
1,6-diaminohexane	1.49	1.81	1.35	0.06	1.09
1,7-diaminoheptane	1.52	2.47	1.47	0.05	1.84
1,8-diaminooctane	0.75	0.27	0.23	0.03	0.44
1,9-diaminononane	1.3	0.44	0.31	nd	0.46
1,10-diaminodecane	0.21	0.38	0.17	nd	0.23

Table S7. Values of Kcat for substrates **1a-k** determined using the L-AlaDh-TA assay.

	Kcat (s^{-1})				
substrate	Pp_spuC	Pf_spuC	Pc_spuC	Ec_yjgj	Cv_TA
1,4-diaminobutane	1.11	0.34	1.19	2.33	nd
1,5-diaminopentane	23.15	14.38	12.86	2.33	6.68
spermidine	13.32	14.38	18.31	18.17	nd
(S)-methylbenzylamine	19.68	22.41	25.89	nd	25.09
butylamine	13.99	16.82	1.50	nd	12.24
1,6-diaminohexane	12.64	15.77	11.49	0.51	9.46
1,7-diaminoheptane	12.89	21.53	12.51	0.38	15.97
1,8-diaminooctane	6.36	2.35	1.95	0.28	3.82
1,9-diaminononane	11.03	3.83	2.64	nd	3.99
1,10-diaminodecane	1.78	3.31	1.44	nd	1.99

V. Biotransformations

5.1 Analytical scale

All biotransformations were carried out in 2 mL Eppendorf tubes, in a volume of 0.5 mL. To a mixture of amine substrate (25 mM from a 500 mM stock in 50 mM HEPES buffer, 1mM PLP, pH 9) and ketone (5 mM from a 500 mM stock solution in DMSO or MeOH), was added the enzyme (2mg/mL, purified or as cell-free lysate, prepared as described). The reactions were placed in a shaking incubator at 30°C and 250 rpm for 12 hours. The reactions were quenched by addition of 10 M NaOH (100µL), followed by extraction with methyl tert-butyl ether (300 µL). The organic phase was dried on $MgSO_4$ and analysed on normal phase chiral HPLC or GC-FID. For the GC-FID analysis, the samples were derivatized to the corresponding acetamides by addition of 30 µL Et_3N and 20 µL Ac_2O.

5.2 Preparative scale

In a 250 mL conical flask, the amine (20 mM, 2 eq) was dissolved in 50 mM HEPES buffer, containing 1mM PLP, pH 9 and the transaminase, Pp_spuC (60 mg from a stock of 20 mg/mL in buffer prepared as above) was added. The pH of the mixture was adjusted to 9 and the ketone (10 mM, 1eq) in DMSO (2.5% v/v) was added. The reaction was placed in a shaking incubator, 200 rpm, 30°C for 24h and the progress was followed by HPLC, with samples prepared as described in the analytical scale procedure. Samples were taken every hour for an initial period of 5 hours, followed by sampling at 19h, 21h and 29 h, respectively. The time course of the reaction is shown in Figure S5.

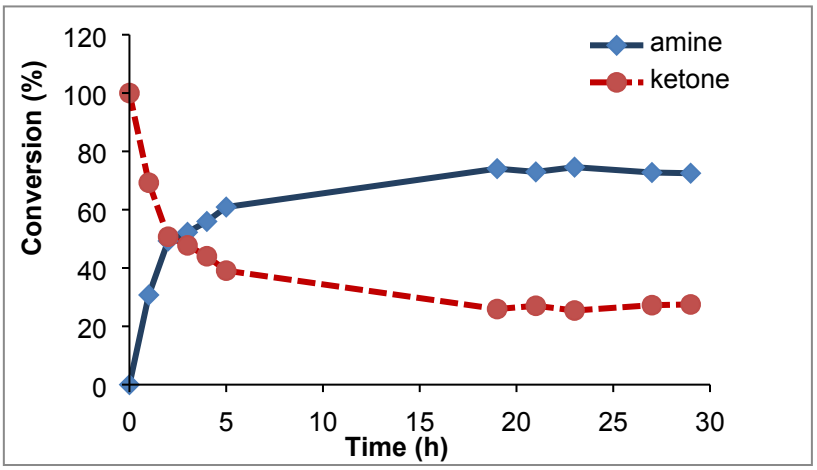
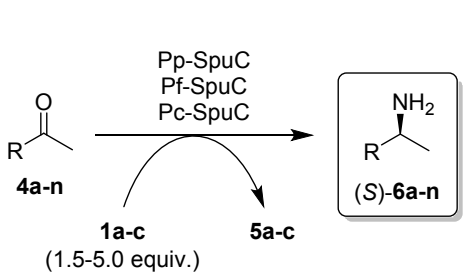
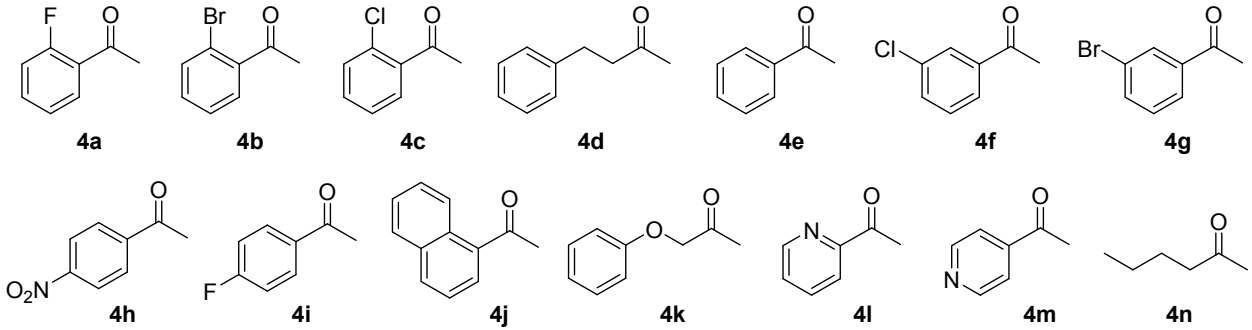


Figure S5. Kinetics of the transamination of 2-fluoroacetophenone (10 mM) by Pp_spuC, using cadaverine **1b** as amino donor (20 mM, 2 eq). The formation of the (*S*)-2-fluoromethylbenzylamine is shown in solid blue line, whilst the concentration of the 2-fluoroacetophenone is shown in red dotted line.

Table S8. Amination of ketones 4a-m with diamine donors **1a-c**.

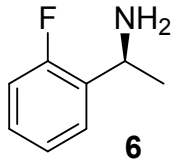




	Diamine **1a**						Diamine **1b**						Diamine **1c**					
	Pp-SpuC		Pf-SpuC		Pc-SpuC		Pp-SpuC		Pf-SpuC		Pc-SpuC		Pp-SpuC		Pf-SpuC		Pc-SpuC	
Subs.	c (%)[a]	ee (%)[a]	c (%)[a]	ee (%)[a]	c (%)[a]	ee (%)[a]	c (%)[a]	ee (%)[a]	c (%)[a]	ee (%)[a]	c (%)[a]	ee (%)[a]	c (%)[a]	ee (%)[a]	c (%)[a]	ee (%)[a]	c (%)[a]	ee (%)[a]
4a	84	>99	92	>99	92[b]	>99	95	>99	95	>99	97	>99	83	>99	92	>99	97	>99
4b	80	>99	68	>99	89[b]	>99	68	>99	65	>99	93	>99	67	>99	65	>99	93	>99
4c	67	>99	75	>99	88[b]	96	88	>99	83	>99	91	>99	79	>99	81	>99	96	>99
4d	37[b]	69	34[b]	75	51[b]	9	64[b]	>99	59[b]	90	72	74	47	>99	41	50	90	36
4e	12[b]	>99	12[b]	>99	13[b]	82	42[b]	>99	25[b]	>99	34	>99	13	>99	13	>99	30	>99
4f	5[b]	>99	33[b]	>99	35[b]	>99	16[b]	>99	44[b]	>99	62	>99	8	>99	27	>99	74	>99
4g	63[b]	>99	5[b]	>99	85[b]	85	80[b]	>99	46[b]	>99	56	>99	62	>99	13	>99	86	>99
4h	41	>99	57	>99	99[b]	>99	59	>99	63	>99	93	>99	55	>99	63	>99	99	>99
4i	12	>99	11	>99	13[b]	94	28	80	13	>99	35	>99	13	>99	14	>99	37	93
4j	32[b]	>99	27[b]	>99	37[b]	>99	59[b]	>99	10[b]	>99	80	>99	28	>99	12	>99	38	>99
4k	92	>99	91	>99	35[b]	>99	99	96	98	>99	87	>99	88	56	81	>99	60	>99
4l	79	>99	77	>99	29[b]	83	96	>99	58	>99	5	>99	65	>99	91	>99	71	>99
4m	58	>99	49	>99	62[b]	>99	95	>99	73	>99	57	>99	55	>99	11	>99	44	>99

Expt. Cond.: 5 mM **4a-n**, 1.5 equiv. **1a-c**, 1mM PLP, 1% v/v DMSO, 2 mg mL^{-1} purified SpuC, 50 mM HEPES buffer, pH 9.0, 40°C, 250 rpm, 24 h.
a: determined by HPLC on a chiral stationary phase. b: in these cases 5. equiv. of amine donor were used to increase the conversion values.

After 30 h, the reaction was stopped by addition of 10 M NaOH until the pH reached 12. The reaction was filtered through a short pad of Celite and extracted with MTBE (3 x 10 mL); the combined organics were washed with brine (1 x 20 mL) and dried on Na_2SO_4 and concentrated in vacuo to afford the amine as a pale yellow oil (55 mg).



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^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (td, *J* = 7.7, 2.0 Hz, 1H), 7.25 – 7.17 (m, 1H), 7.12 (td, *J* =7.5, 1.3 Hz, 1H), 7.01 (ddd, *J* = 10.9, 8.1, 1.3 Hz, 1H), 4.39 (q, *J* = 6.7 Hz, 1H), 1.42 (d, *J* = 6.7 Hz, 3H)

5.3 GC-assay conditions

GC-MS method: Hewlett Packard HP 6890 equipped with a HP-1MS column, a HP 5973 Mass Selective Detector and an ATLAS GL FOCUS sampling robot; method: 90^{0}C for 0 min, then 10^{0}C min^1 to 200C, hold for 10 min. GC FID method: Agilent 6850 equipped with a Gerstel Multipurposesampler MPS2L and an Agilent CHIRASIL-DEX CB 25 m x 0.25 mm DF=0.25 column; method: 120^{0}C isothermal, 15 min. The amines were analyzed by GC-FID as their corresponding acetamides, following derivatization as described previously.

5.4 HPLC assay conditions

Chiral normal phase HPLC was performed on an Agilent system (Santa Clara, CA, USA) equipped with a G1379A degasser, G1312A binary pump, a G1367A well plate autosampler unit, a G1316A temperature controlled column compartment and a G1315C diode array detector. CHIRALCEL® OD-H Analytical (Daicel, Osaka, Japan), 250 mm length, 4.6 mm diameter, 5 µm particle size) column was used. The typical injection volume was 15 µl and chromatograms were monitored at 265 nm. Solvent mixtures are given in *n*-hexane/isopropanol ratios (+0.1% diethylamine v/v).

Table S9. Retention times for substrates and products

Ketone	Product	Amine	*n*-Hex/*i*-PrOH ratio
4a (4.59 min)	**6a**	(*R*)- 7.3 min, (*S*)- 7.81 min	95:5
4b (6.93 min)	**6b**	(*S*)- 9.83 min	95:5
4c (5.82 min)	**6c**	(*R*)- 7.3 min, (S)-9.07 min	95:5
4d (8.44 min)	**6d**	(*R*)- 13.01 min, (S)-18.28 min	95:5
4e (5.93 min)	**6e**	(*S*)- 11.09 min	95:5
4f (5.57 min)	**6f**	(*S*)- 9.41 mn	95:5
4g (5.81 min)	**6g**	(*S*)- 10.07 min	95:5
4h (11.37 min)	**6h**	(*S*)- 14.89 min	90:10
4i (5.53 min)	**6i**	(*S*)- 8.92 min	95:5
4j (9.05 min)	**6j**	(*S*)- 15.57 min	95:5
4k (10.5 min)	**6k**	(*R*)- 11.41 min, (S)- 17.87 min	90:10
4l (4.92 min)	**6l**	(*S*)- 8.3 min	90:10
4m (4.96 min)	**6m**	(*S*)- 11.45 min	90:10