Supporting Information

Engineered Ribosyl-1-Kinase Enables Concise Synthesis of Molnupiravir, an Antiviral for COVID-19

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Abbreviations

AcK – Acetate Kinase
ATP – adenosine 5'-triphosphate
LCAP – Liquid chromatography area percent
MTR kinase – S-methyl-5-thioribose kinase
NNK, NNN, NNS – degenerate codons
PCR – polymerase chain reaction
PO – Pyruvate oxidase
SFP – shake flask powder = lyophilized powder of cell-free extract of enzyme
SSM – site-saturation mutagenesis
SUP – Sucrose Phosphorylase
TEoA – triethanolamine
UP – Uridine Phosphorylase

General experimental details

Reagents and solvents were purchased from commercial suppliers and used as received. All reactions were carried out in a fume hood without special precautions towards air or moisture. NMR chemical shifts are reported in ppm and referenced to residual solvent peaks. Coupling constants are reported in hertz.

Enzyme discovery

A panel of enzyme homologs is screened to discover the most suitable enzyme for evolution. The panel design step is performed computationally for each enzyme family or class via homology search and filtering techniques. To be specific, thousands of homolog sequences are collected and aligned through BLAST search from publicly available protein databases, such as UniProt, GenBank of NCBI or the PDB. This step is followed by a phylogenetic tree construction step to allow systematic selection of the most diverse members in the evolutionary space, using the appropriate thresholds on phylogenetic sub-group size and all-to-all sequence identity. Other filtering criteria such as literature data, structure availability, are additional constraints used to reduce the number of enzymes to be evaluated experimentally. The above computational steps were carried out using 3DM database panel design toolbox[1].

Library design for enzyme evolution

Each round of evolution consisted of multiple libraries, including both site-saturation mutagenesis libraries and combinatorial mutagenesis libraries. Three main strategies were employed to select residues targeted for mutagenesis: (i) sequence-based library design: for an enzyme target, a diverse set of homolog sequences was collected, aligned, and conservation scores of each amino acid at each position were calculated. A conservation threshold was applied to filter amino acids as prospective mutations for experimental evaluation. (ii) Structure-based library design: homology models of each enzyme target were prepared with docked substrates, followed by an *in-silico* mutation analysis to identify the best mutable sites and the best amino acids changes at those positions for evaluation. Structure-based design and visualization tools such as Schrodinger[2] and Pymol[3] were employed for this technique. (iii) Data-driven library design: improved variants from both site-saturation and combinatorial mutagenesis libraries were sequenced in each evolution round to identify specific mutations. Machine learning-based random forest model (using a 3DM deconvolution toolbox)[1] was constructed to triage the beneficial effect of each mutation and guide the combinatorial design in subsequent evolution rounds.

Library construction for directed evolution

For the enzymes reported in the literature, the wild-type variants were codon-optimized for expression in *E. coli* and synthesized and cloned by GenScript into the pET vector system. The uridine phosphorylase (UP) enzyme used as a starting point for evolution was obtained from the *E. coli* genome and cloned into the expression vector pCK900 through restriction enzyme digest. *E. coli* W3110 or BL21(DE3) cells were transformed with the plasmids of interest.

The site-saturation mutagenesis (SSM) libraries[4,5] used degenerate oligos (e.g. NNK, NNS, NNN), introducing mutations through overlap extension PCR. Combinatorial libraries (defined as libraries that were composed of multiple mutations that were recombined to generate variants with several mutations) were produced using standard mutagenesis methods using polymerase chain reaction (PCR). Electrocompetent *E. coli* W3110 or BL21(DE3) cells were transformed with the constructed libraries and plated on LB-agar plates with antibiotic selection, yielding variants as individual colonies.

MTR kinase expression for screening

The 5-S-methylthioribose (MTR) kinase enzyme used as a starting point for evolution was obtained as a codon-optimized gene based on the wild-type protein sequence for the MTR kinase from *Klebsiella spp.* and was cloned into pET30 via NdeI/XhoI restriction sites. The plasmid containing the wild-type MTR kinase was transformed into electrocompetent *Escherichia coli* BL21(DE3) via electroporation. Following a 1 hour outgrowth at 37°C, a portion of the transformation was plated on LB-agar supplemented with 50 micrograms per mL kanamycin and 1 % (w/v) glucose. Colonies were picked and inoculated into a 96-well plate containing 0.2 mL per well of LB Broth (culture media for cells) supplemented with 50 micrograms per mL of kanamycin and 1 % (w/v) glucose. The 96-well plate was shaken at 250 RPM / 30°C overnight. The following day, 0.01 mL per well of culture from each well was used to inoculate the corresponding well of a new 96-well plate containing 0.39 mL per well of ZYM-5052 medium. This culture was grown at 30 °C/250 RPM for 18 hours after which time the cells were pelleted by centrifugation and the supernatants were discarded. After centrifugation, cells were frozen, thawed, and then resuspended in lysis buffer (0.2 mL per well of 50 mM triethanolamine (TEoA)-HCl pH 7.5, 1 mg/mL lysozyme, 0.5 mg/mL polymyxin B sulfate, 1U/mL DnaseI, and 1 mM $MgSO_4$). The plate was then shaken for 2 h at room temperature at 1200 RPM. The lysate was then clarified by centrifugation (4000 x g, 10 minutes). The supernatant following this step was then used in subsequent well-plate reactions.

MTR kinase expression in shake-flasks

A single colony containing the MTR kinase gene was inoculated into a 125 mL flask containing 50 mL of LB broth supplemented with 50 micrograms per mL of kanamycin and 1 % (w/v) glucose. The flask was shaken at 250 RPM / 30°C overnight. The following day, a 2.8 L flask containing 1 L of ZYM-5052 medium was inoculated with a 100-fold dilution of the saturated overnight culture. This culture was grown at 30°C/250 RPM for 18 hours after which time the cells were harvested by centrifugation. After centrifugation, cells were resuspended to a concentration of 0.33 grams of 1 mL of aqueous 50 mM TEoA-HCl buffer pH 7.5. This suspension was shaken at 20°C for 30 minutes, after which time the cells were placed on ice to chill, and then disrupted by high-pressure homogenization (16,000 PSI). The resulting lysate was then clarified by centrifuging at 10,000 x g for 30 minutes. Following centrifugation, the supernatant was frozen and lyophilized.

MTR kinase screening reactions with wild-type enzymes

Reactions were assembled containing tris buffer (100 mM, pH 8.0), potassium chloride (100 mM), 5'-isobutyrylribose (10 mM), ATP (0.4 eq.), magnesium sulfate (1 eq.), proprionyl phosphate (4.5 eq.), uracil (5.3 eq.), sucrose (6.7 eq.), acetate kinase (1 g/L, ACK-101, Codexis, Inc.), wild-type *E. coli* uridine phosphorylase (1 g/L), and sucrose phosphorylase (1 g/L, SUP-101, Codexis, Inc.) and initiated via addition of clarified lysate containing overexpressed MTR kinase (10 vol%). Reactions were sampled at 1h and 3h and analyzed by injection onto an Xbridge Glycan BEH Amide column (4.6 x 150 mm, Waters, 186007275) and eluted on the following gradient:

Sample diluent: 50/50 water/AcN
A: 0.1% TFA in water
B: 0.1% TFA in acetonitrile
1.25 mL/min, 25 C column temperature
Detection by UV at 254 nm with 4 nm bandwidth
Gradient:

Time (min)	% B
0.0	95
5.5	65
5.6	20
7.6	20
7.7	95

Re-equilibrate at initial conditions for 2.0 min

MTR kinase directed evolution screening

Reactions were assembled as indicated in Table S1. Following reaction, samples were quenched via dilution into 50/50 water/acetonitrile and then spin filtered. Samples were then analyzed by injection onto an Acquity BEH Amide (2.1 x 50 mm, Waters, 186004800) and eluted on the following gradient:
A: 2 mM ammonium formate pH 8.5 in 90:10 acetonitrile:water
B: none (isocratic)
0.6 mL/min, 45 C column temperature
Detection by UV at 210 nm with 4 nm bandwidth
Gradient: 0.6 mL/min

Time (min)	% A
0.0	100
1.0	100

Enzyme Preparation for vial and larger-scale reactions

MTR kinase clones were inoculated into a 125 mL flask containing 50 mL of LB Broth supplemented with 50 micrograms per mL of kanamycin and 1 % (w/v) glucose. The flask was shaken at 250 RPM / 30°C overnight. The following day, a 2.8 L flask containing 1 L of ZYM-5052 medium was inoculated with a 100-fold dilution of the saturated overnight culture. This culture was grown at 30 °C/250 RPM for 18 hours after which time the cells were harvested by centrifugation. After centrifugation, cells were resuspended to a concentration of 0.33 grams of 1 mL of aqueous 50 mM TEoA-HCl buffer pH 7.5. This suspension was shaken at 20°C for 30 minutes, after which time the cells were placed on ice to chill, and then disrupted by high-pressure homogenization (16,000 PSI). The resulting lysate was then clarified by centrifuging at 10,000 x g for 30 minutes. Following centrifugation, the supernatant was frozen and lyophilized.

Uridine phosphorylase expression for screening

E. coli cells containing recombinant UP-encoding genes from monoclonal colonies were inoculated into 180 µL LB containing 1% glucose and 30 µg/mL chloramphenicol in the wells of 96-well shallow-well microtiter plates. The plates were sealed with O_2-permeable seals and cultures were grown overnight at 30 °C, 200 rpm, and 85% humidity. Then, 10 µL of each of the cell cultures were transferred into the wells of 96-well deep-well plates containing 390 mL TB and 30 µg/mL CAM. The deep-well plates were sealed with O_2-permeable seals and incubated at 30 °C, 250 rpm, and 85% humidity until OD600 0.6-0.8 was reached. The cell cultures were then induced by IPTG to a final concentration of 1 mM and incubated overnight at 30 °C. The cells were then pelleted using centrifugation at 4000 rpm for 10 min. The supernates were discarded and the pellets frozen at -80ºC prior to lysis.

For lysis, 400 µL lysis buffer containing 50 mM TEoA buffer, pH 7.5, 1 g/L lysozyme, and 0.5 g/L polymyxin b sulfate (PMBS) was added to the cell paste in each well. The cells were lysed at room temperature for 2 hours with shaking on a bench top shaker. The plate was then centrifuged for 15 min at 4000 rpm and 4 °C. The clear supernates were then used in biocatalytic reactions to determine their activity levels.

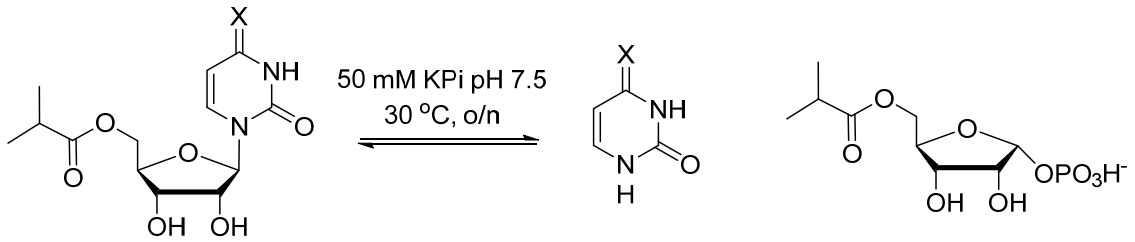
Uridine phosphorylase expression in shake-flasks

Selected HTP cultures grown as described above were plated onto LB agar plates with 1% glucose and 30 µg/mL chloramphenicol, and grown overnight at 37 °C. A single colony from each culture was transferred to 6 mL LB with 1% glucose and 30 µg/ml chloramphenicol. The cultures were grown for 18 h at 30 ºC, 250 rpm, and subcultured approximately 1:50 into 250 mL of TB containing 30 µg/mL chloramphenicol, to a final OD600 of 0.05. The cultures were grown for approximately 195 minutes at 30 °C, 250 rpm, to an OD600 between 0.6-0.8 and induced with 1 mM IPTG. The cultures were then grown for 20 h at 30 °C, 250 rpm. The cultures were centrifuged 4000 rpm x 20 min. The supernate was discarded, and the pellets were resuspended in 30 mL of 20 mM TEoA, pH 7.5, and lysed using a Microfluidizer® processor system (Microfluidics) at 18,000 psi. The lysates were pelleted (10,000 rpm x 60 min) and the supernates were frozen and lyophilized to generate shake-flask enzyme powders (SFP).

Pyruvate oxidase

The pyruvate oxidase genes from *Aerococcus urinaeequi* (PO3), *Streptococcus sanguinis* (PO5), and *Weisella confusa* (PO6) were codon-optimized for *E. coli* and cloned into pET28 in-frame with the N-terminal his-tag. The enzymes were expressed at Prozomix Ltd, UK.

Figure S1. Uridine phosphorylase screening



X = O (**3**) or N-OH (molnupiravir)

	LCAP N-OH cytosine	LCAP uracil
Macaca mulatta MTAP	0.4	3.0
Saccharolobus solfataricus MTAP	0.3	2.8
Mus musculus MTAP	0.2	2.6
Trichomona vaginalis PNP	0.3	3.3
Plasmodium falciparum PNP	0.5	4.0
Escherichia coli TP	0.4	2.5
Rattus norvegicus MTAP	0.5	3.3
Aeropyrum pernix MTAP	0.3	2.9
Pyrococcus furiosis MTAP	0.4	3.6
Saccharomyces solfataricus PNP	0.3	2.7
Plasmodium voelii	0.4	2.7
Homo sapiens TP	0.0	2.5
Escherichia coli UP	**2.1**	**42.6**
Homo sapiens UP	0.4	2.4
Schistosoma mansoni UP	0.5	2.9
Deinococcus geothermalis PNP	0.6	2.8
Bacillaceae sp. PNP	0.4	1.9

MTAP = S-methylthioadenosine phosphorylase
TP = thymidine phosphorylase
UP = uridine phosphorylase
PNP = purine nucleoside phosphorylase
LCAP = liquid chromatography area percent
Note: DNA sequences of nucleoside phosphorylases tested provided at the end of the Supporting Information

Procedure: A panel of uridine phosphorylase enzymes screened for phosphorolytic activity against 5'-isobutyryluridine or molnupiravir (8 mg/mL, final concentration) in the presence of potassium phosphate buffer (50 mM, pH 7.5). Reactions were shaken overnight (30 °C, 1000 RPM). The following day, reactions were sampled and analyzed by injection onto an Xbridge Glycan BEH Amide column (4.6 x 150 mm, Waters, 186007275) and eluted on the following gradient:
A: 0.1% TFA in water
B: 0.1% TFA in acetonitrile
1.25 mL/min, 25 C column temperature
Detection by UV at 254 nm with 4 nm bandwidth
Gradient:

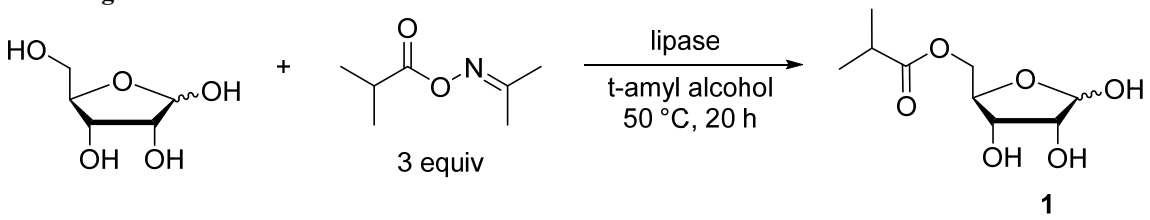
Time (min)	% B

0.0	95
5.5	65
5.6	20
7.6	20
7.7	96

Re-equilibrate at initial conditions for 2.0 min

Fig. S2. Selective 5-acylation of ribose with *O*-isobutyryl acetone oxime

Screening conditions

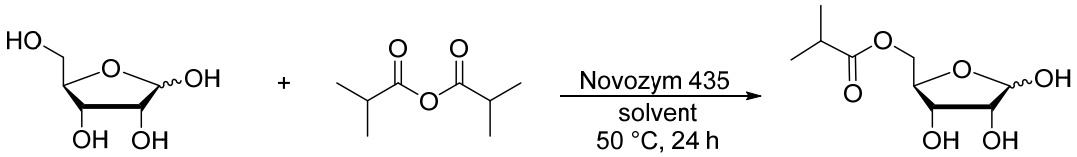


Enzyme Name	5-isobutyryl ribose assay yield%
Novozym 435 (Candida antarctica lipase B)	65
IMMTLL-T2-150 (Thermomyces lanuginosus lipase)	40
IMMRES-T2-150 (Resinase HT lipase)	38
IMMLIPX-T2-150 (Lipex 100 L lipase)	56
IMML51-T2-150 (Novozymes 51032)	61
IMMP6-T2-250 (protease from Bacillus licheniformis)	11
Lipozyme RM IM	10
CDX IMB-103	33

Condition: 10 g/L D-ribose, 10 g/L lipase, 28.6 g/L propane-2-one *O*-isobutyryl oxime, t-amyl alcohol, 50 C, 20 hr

Lipase screening procedure: to a 1-mL glass vial was charged D-ribose (6 mg), lipase (6 mg), propane-2-one *O*-isobutyryl oxime (17.2 mg), and t-amyl alcohol (0.6 mL). The plate was sealed and shaken at 50 °C, 600 RPM for 20 hr. 20 µL of the reaction mixture was taken and diluted with 180 µL 50/50 (v/v) acetonitrile: water for UPLC-CAD analysis. The assay yield was determined using a calibration curve for 5-isobutyryl ribose.

Fig. S3. Selective 5-acylation of ribose with isobutyric anhydride



Entry	Solvent	Novozym 435 (wt%)	Conversion (%)	Yield (%)
1	EtOH	50	0	N/A
2	iPrOH	50	8	N/A
3	tBuOH	50	98	N/A
4	THF	50	47	N/A
5	Acetone	50	99	N/A
6	tBuOH	20	86	79
7	Acetone	20	99	94
8	Acetone	10	99	94

Novozym 435 screening procedure:

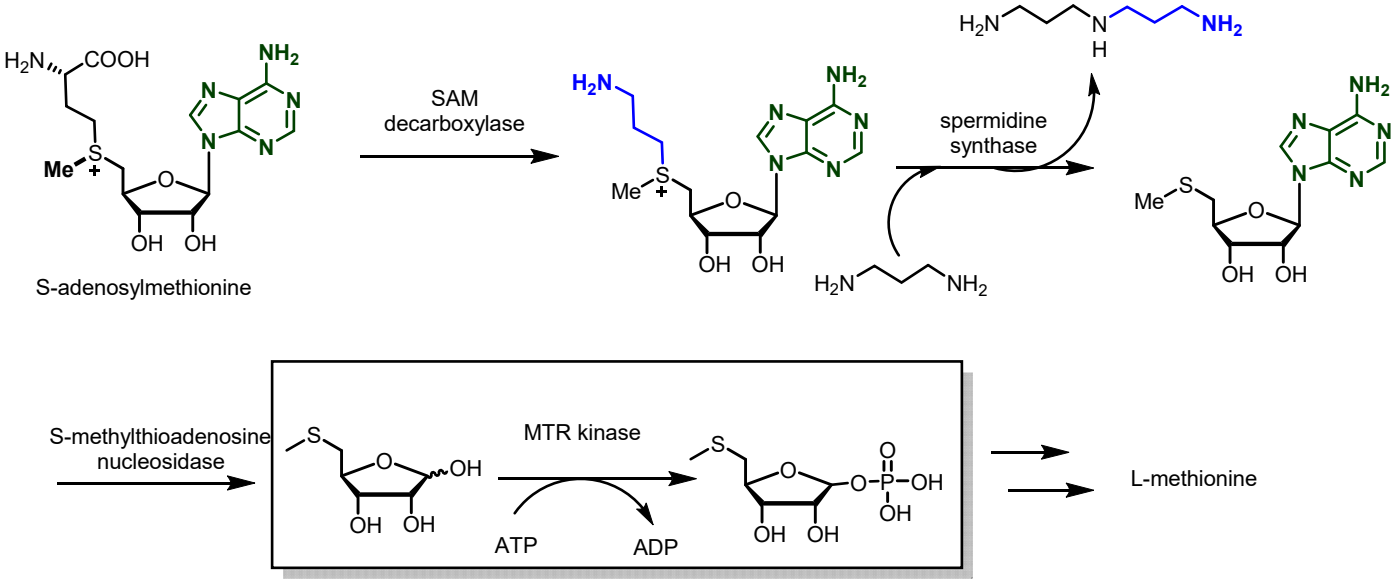
In a vial was added ribose, Novozym 435, solvent, and isobutyric anhydride. The mixture was aged at 50°C for 24 h, and the conversion was determined with a calibrated UPLC-CAD instrument. The assay yield was measured after dissolving the reaction mixture in aqueous acetonitrile and measuring the product absorbance value on a calibrated UPLC-CAD instrument.

UPLC-CAD analysis was performed using Agilent 1290 instrument equipped with a Corona Ultra RS detector by Dionex or Corona Veo R by Thermo Scientific as described below.

Column: Waters XBridge C18 4.6 x 50 mm, 2.5 um particles
Column Temperature: 40 °C
Flow Rate: 2.000 mL/min
Detector: CAD, 35 °C
Diluent: 50:50 v/v Water:Acetonitrile
Mobile phase A: 20 mM TEAA in Water
Mobile phase B: 20 mM TEAA in Acetonitrile
Injection Volume: 5 µL
Gradient: 10%-15% B (0-1.0 min), 15%-90% B (1.0-1.5 min), 90% B (1.5-3.0 min), 90-10% B (3.0-3.1 min), 10% B (3.1-4.0 min).

Figure S4. Natural versus desired reactivity of MTR kinases

Natural pathway for methionine recycling (MTR kinase natural reaction reaction highlighted):



Desired reaction:

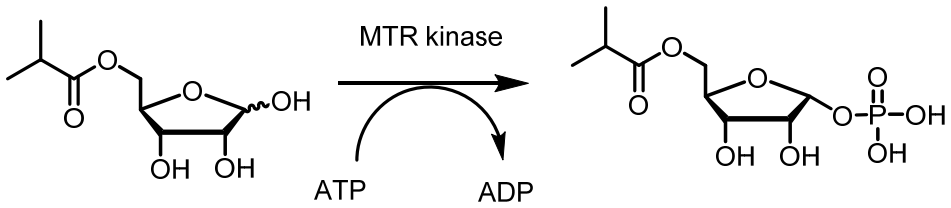


Table S1. Evolution summary of MTR kinase

Round	Backbone mutations (rel. to prev. round)	Assay conditions	Fold improvement in high-throughput assay (rel. to prev. round)	d.r. (α:β)

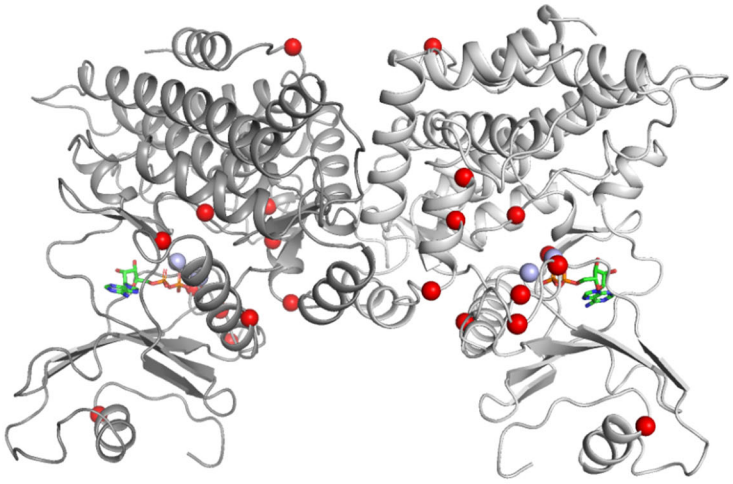
1.1 (natural diversity combinatorial)	none (WT)	100mM TEoA pH 7.5, 1 mM ATP, 10 mM $MgCl_2$, 43 mM proprionyl phosphate, 18 mM uracil, 36 mM 5'-isobutyryl ribose, 72 mM sucrose, 0.05 g/L ACK-101, 0.25 g/L SUP-101, 0.25 g/L UP, 10 vol% lysate	n/a	7:1
1.2 (SSM)	none (WT)	100 mM TEoA pH 7.5, 1 mM ATP, 10 mM $MgCl_2$, 216 mM proprionyl phosphate, 90 mM uracil, 100 mM 5'-isobutyryl ribose, 180 mM sucrose, 2 g/L ACK-101, 2 g/L SUP-101, 2 g/L UP, 5 vol% MTR kinase variant lysate.	n/a	7:1
1.3 (SSM)	None (WT)	100 mM TEoA pH 7.5, 2 mM ATP, 20 mM $MgCl_2$, 120 mM proprionyl phosphate, 90 mM uracil, 100 mM 5'-isobutyryl ribose, 146 mM sucrose, 2 g/L ACK-101, 2 g/L SUP-101, 2 g/L UP, 5 vol% MTR kinase variant lysate.	n/a	7:1
1.4 (SSM)	None (WT)	100 mM TEoA pH 7.5, 2 mM ATP, 20 mM $MgCl_2$, 120 mM proprionyl phosphate, 90 mM uracil, 100 mM 5'-isobutyryl ribose, 146 mM sucrose, 2 g/L ACK-101, 2 g/L SUP-101, 2 g/L UP, 5 vol% MTR kinase variant lysate.	n/a	7:1
2	L79H	100 mM TEoA pH 7.5, 2 mM ATP, 20 mM $MgCl_2$, 120 mM proprionyl phosphate, 90 mM uracil, 100 mM 5'-isobutyryl ribose, 146 mM sucrose, 2 g/L ACK-101, 2 g/L SUP-101, 2 g/L UP, 5 vol% MTR kinase variant lysate.	1.6	>99:1
3	V63P, T173S, F177I, I230L	0.9 mM ATP, 10 mM $MgCl_2$, 0.9 mM TPP, 0.09 mM FAD, 90 mM uracil, 223 mM pyruvic acid, 35 mM K_2HPO_4, 180 mM 5'-isobutyryl ribose, 1 g/L ACK-101, 1 g/L PO6, 1 g/L catalase (Roche), 2 g/L UP, adjust pH to 7.0, 1 vol% MTR kinase variant lysate.	1.5	>99:1
Final variant	H10D, C65A, E68P, A168G, A244V, R384T	-	3.0	>99:1

Table S2. Sites targeted for mutagenesis over the course of MTR kinase evolution

Round #	Screening Goal	Library	Positions
1	Improve activity on 5-isobutyrylribose and selectivity for desired anomer	Combinatorial (1.1)	37, 38, 39, 70, 172, 173, 176, 177, 187, 231, 234, 248, 249, 341, 349, 350
1	Improve activity on 5-isobutyrylribose and selectivity for desired anomer	Single-site mutagenesis (1.2)	35, 36, 37, 38, 39, 40, 41, 42, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 76, 77, 79, 80, 118, 120, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 185, 186, 187, 188, 189, 215, 218, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 244, 245, 246, 247, 248, 249, 250, 251, 257, 258, 259, 260, 261, 262, 264, 265, 337, 338, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353
1	Improve activity on 5-isobutyrylribose and selectivity for desired anomer	Single-site mutagenesis (1.3)	2, 3, 5, 8, 10, 14, 17, 18, 19, 23, 24, 26, 27, 28, 32, 33, 34, 46, 48, 49, 78, 85, 88, 89, 92, 93, 97, 100, 103, 104, 114, 115, 117, 128, 130, 134, 151, 153, 155, 156, 163, 165, 183, 184, 192, 193, 195, 196, 202, 203, 205, 206, 208, 239, 240, 277, 279, 280, 281, 284, 288, 291, 295, 298, 302, 303, 305, 309, 310, 311, 313, 314, 317, 320, 324, 328, 329, 356, 359, 360, 361, 362, 366, 369, 370, 381, 383, 384, 386, 389, 390, 396, 397, 398, 399, 400

1	Improve activity on 5-isobutyrylribose and selectivity for desired anomer	Single-site mutagenesis (1.3)	4, 6, 7, 9, 11, 13, 15, 21, 25, 30, 31, 43, 44, 47, 50, 51, 52, 75, 82, 86, 90, 98, 99, 101, 102, 111, 112, 113, 116, 121, 124, 125, 126, 127, 131, 135, 138, 142, 146, 152, 158, 159, 160, 161, 162, 166, 190, 191, 197, 199, 200, 204, 209, 210, 213, 214, 217, 219, 221, 222, 238, 241, 243, 274, 276, 278, 283, 286, 287, 294, 297, 299, 301, 306, 307, 312, 315, 321, 322, 323, 325, 326, 327, 332, 333, 357, 363, 364, 365, 373, 376, 377, 380, 387, 393, 394
2	Improve activity on 5-isobutyrylribose	Combinatorial	63, 68, 77, 79, 173, 177, 230, 232, 234, 350
3	Improve activity on 5-isobutyrylribose	Combinatorial	10, 65, 68, 77, 85, 168, 171, 177, 232, 244, 349, 350, 370, 384

Fig. S5. Structural model of mutations present in evolved MTR kinase



Locations of mutations in the final evolved variant are shown by red spheres. The MTR kinase dimer homology model was prepared using PDB ID 2PYW[6] as the template. The two chains are shown by different shades of gray. The ADP molecule is illustrated by sticks and the two Mg atoms are shown by light blue spheres in each chain.

MTR kinase evolution round 1.1

MTR kinase evolution screened for improved synthesis of **3** in a cascade reaction from **1**. Reactions were performed in a 96-well format in 2 mL deep-well plates, with 300 µL total volume. Reactions included the components listed in table S1 above. The reactions were set up as follows: (i) reaction components excepting uracil and MTR kinase

lysate were mixed and dispensed as a single solution (240 µL) (ii) 30 µL of a slurry of uracil was dispensed to each well (iii) 30 µL of 2x diluted MTR kinase lysate was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a foil seal, incubated at 30 °C with 1000 rpm shaking for 18-20 h. Reaction aliquots (10 µL per well) were diluted into 190 µL of a 1:1 mixture of acetonitrile/water and then filtered through a 0.2 micron centrifuge filter. The samples were analyzed according to the MTR kinase high-throughput analytical method summarized below.

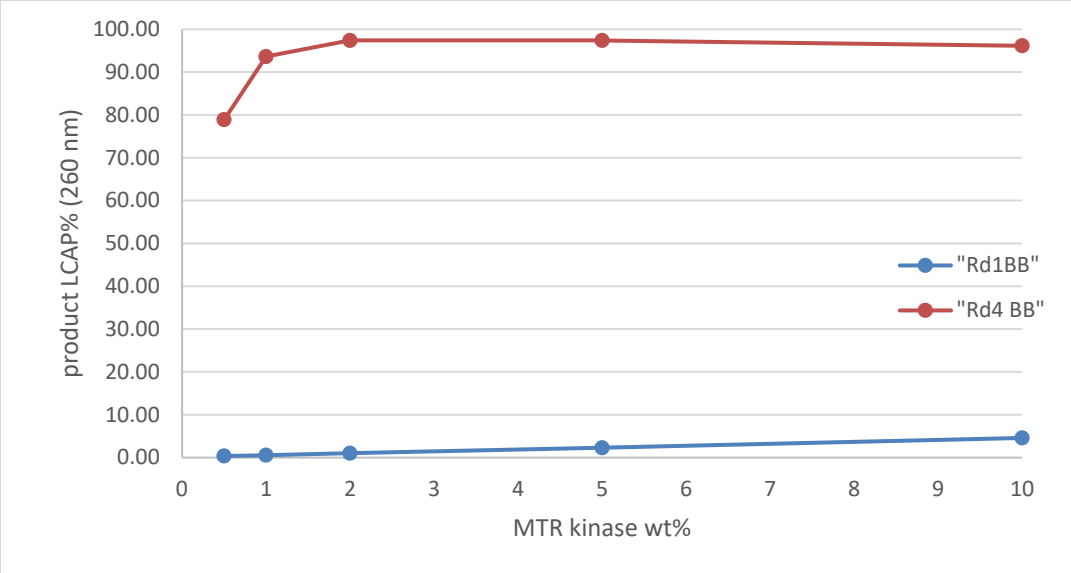
MTR kinase high throughput analytical Method	
Instrument	Agilent 1290 UPLC
Column	Acquity BEH Amide 2.1 x 50 mm
Mobile Phase	Isocratic 10:90 water : acetonitrile with 2 mM ammonium formate pH 6.5
Flow Rate	0.6 mL/min
Run Time	1 min
Substrate and Product Elution order	5'-isobutyryl uridine: 0.28 min Uracil: 0.37 min
Column Temperature	45 ˚C
Injection Volume	1 µL
Detection	UV 254 nm, 360 nm reference, 100 nm reference bandwidth

MTR kinase evolution rounds 1.2, 1.3, 1.4, and 2
Reactions were conducted as described above with the following changes: (i) reaction components excepting uracil and MTR kinase were mixed and dispensed as a single solution (255 µL); (ii) A slurry of uracil was dispensed to each well (30 µL), (iii) MTR kinase lysate was then added to initiate the reaction (15 µL).

MTR kinase evolution round 3
MTR kinase evolution screened for improved synthesis of **3** in a cascade reaction from **1**. Reactions were performed in a 96-well format in 2 mL deep-well plates, with 200 µL total volume. Reactions included the components listed in table S1 above. The reactions were set up as follows: (i) reaction components excepting uracil and MTR kinase lysate were mixed and dispensed as a single solution to each well (160 µL) (ii) 20 µL of a slurry of uracil (100 g/L) was dispensed to each well (iii) 20 µL of 10x diluted MTR kinase lysate was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a foil seal, incubated at 30 °C with 1000 rpm shaking for 18-20 h. Reaction aliquots (10 µL per well) were diluted into 190 µL of a 1:1 mixture of acetonitrile/water and then filtered through a 0.2 micron centrifuge filter.

Fig. S6. Comparison of wild-type MTR kinase Rd1BB and final evolved (Rd4BB) enzymes in the full cascade starting with 5-isobutyrylribose

	Rd1BB	Rd4BB
product LCAP% at 0.5 wt%	0.35	78.90
product LCAP% at 1.0 wt%	0.55	93.62
product LCAP% at 2.0 wt%	1.02	97.41
product LCAP% at 5.0 wt%	2.28	97.39
product LCAP% at 10.0 wt%	4.58	96.16



Procedure: The activity of MTR kinase enzyme powders at 0.5-10 wt% loading, was evaluated using 0.4 M acyl-ribose concentration (90 g/L), 0.833 eq of uracil (38.2 g/L), 1.25 eq of pyruvic acid (36 g/L), KH_2PO_4 (40 mM), 0.0005 eq of FAD, 0.005 eq of ATP, 0.0025 eq of TPP, 0.25 wt% acetate kinase (ACK-101, Codexis), 0.25 wt% catalase (Roche #11650645103), 1.0 wt% PO3, 20 wt% UP Rd2BB (relative to uracil), 25°C, 250 RPM, 85% relative humidity, overnight.

Table S3. Uridine phosphorylase evolution summary

Round	Backbone mutations (rel. to prev. round)	Assay conditions	Fold improvement in high-throughput assay (rel. to prev. round)
1	none (WT)	11 mM 5'-isobutyryluridine, 50 mM sodium phosphate pH 7.4, 10 mM KCl, 1 vol% uridine phosphorylase variant lysate, 35 °C, 600 RPM, 16 h	n/a
2	E91I	67 mM 5-isobutyryl-1-phosphate, 67 mM uracil, 50 mM TEoA-HCl pH 7.5, 0.1 vol% uridine phosphorylase variant lysate, 30 °C, 600 RPM, 16 h	4.9
3	A252M	67 mM 5-isobutyryl-1-phosphate, 67 mM uracil, 50 mM TEoA-HCl pH 7.5, 0.1 vol% uridine phosphorylase variant lysate, 30 °C, 600 RPM, 16 h	1.9
4	K14E, V35L, H58W, A200R, I239L, T244S	67 mM 5-isobutyryl-1-phosphate, 67 mM uracil, 50 mM TEoA-HCl pH 7.5, 0.06 vol% uridine phosphorylase variant lysate, 30 °C, 600 RPM, 16 h	2.1
5	L20M	67 mM 5-isobutyryl-1-phosphate, 67 mM uracil, 50 mM TEoA-HCl pH 7.5, 0.04 vol% uridine phosphorylase variant lysate, 30 °C, 600 RPM, 16 h	1.3
Final variant	V17S, D40A, K51N, P111G, L132G, L137M, H190R, K192Q, A200E, T248V	-	2.4

Table S4. Sites targeted for mutagenesis over the course of uridine phosphorylase evolution

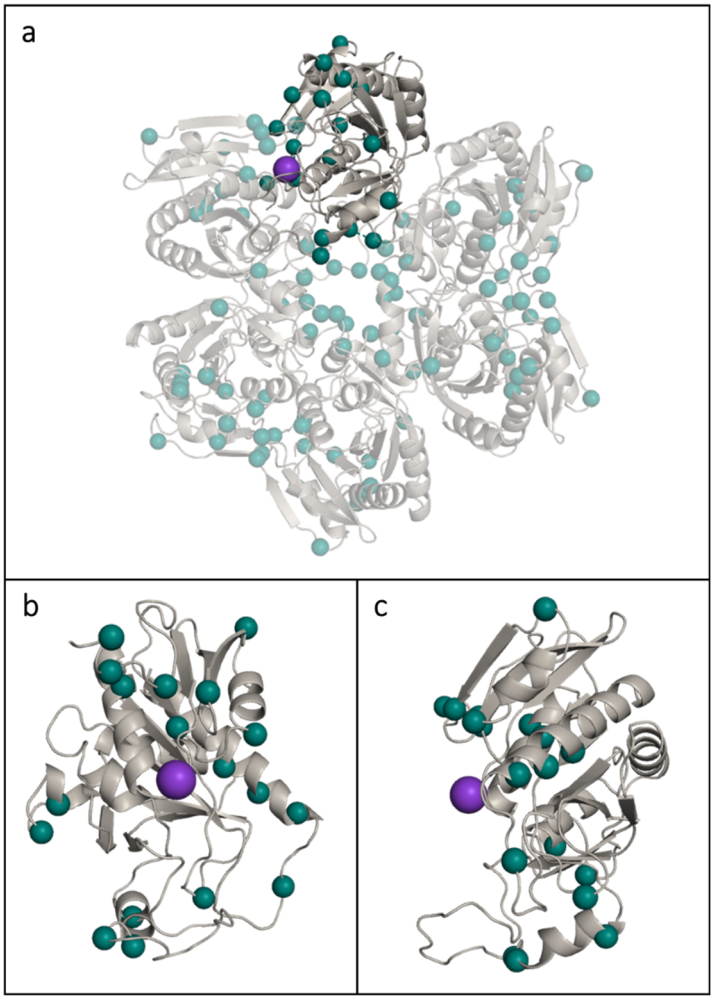
Round #	Screening Goal	Library	Positions
1	Specific activity for phosphorolysis (reverse direction)	Single-site mutagenesis	17, 18, 20, 34, 35, 36, 38, 39, 40, 42, 44, 45, 58, 60, 61, 75, 76, 77, 78, 80, 82, 83, 84, 85, 86, 87, 88, 91, 101, 103, 105, 106, 107, 108, 109, 110, 113, 114, 115, 116, 117, 119, 121, 124, 127, 128, 133, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 195, 204, 205, 206, 207, 210, 211, 212, 213, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 244, 245, 246, 248, 249, 250, 252, 253, 256

2	Specific activity in forward direction with decreased catalyst loading	Single-site mutagenesis	13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 92, 93, 94, 95, 96, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 139, 141, 142, 143, 147, 148, 151, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 188, 191, 194, 195, 196, 197, 198, 199, 200, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 259, 260
		Combinatorial	14, 17, 18, 20, 42, 58, 84, 103, 108, 158, 190, 192, 227, 239, 242, 246, 247, 248, 251
3	Specific activity in forward direction with decreased catalyst loading	Combinatorial	14, 35, 44, 58, 58, 106, 111, 119, 122, 171, 194, 196, 200, 227, 229, 239, 244

4	Specific activity in forward direction with decreased catalyst loading	Single-site mutagenesis	20, 22, 25, 32, 40, 44, 47, 49, 50, 51, 54, 65, 66, 72, 74, 91, 97, 98, 101, 103, 108, 111, 112, 113, 117, 119, 132, 137, 146, 147, 151, 153, 156, 157, 158, 163, 171, 189, 190, 191, 192, 195, 196, 199, 200, 201, 202, 204, 214, 217, 218, 220, 226, 239, 241, 242, 243, 245, 246, 247, 249, 251, 252, 255, 262
5	Specific activity in forward direction with decreased catalyst loading	Combinatorial	17, 20, 40, 51, 57, 63, 74, 108, 111, 117, 132, 137, 146, 153, 190, 191, 192, 200, 212, 218, 242, 248, 253

Fig. S7. Structural visualizations in UP

Structural visualization of amino-acid differences between the evolved and the wild-type *E. coli* UP based on a reported crystal structure (PDB ID 1K3F)[7]. (a) Hexameric structure of UP (trimer of dimers). (b) Front view of UP monomer; mutated residues are marked as teal spheres. (c) Side view of UP monomer; mutated residues are marked as teal spheres.



UP evolution round 1

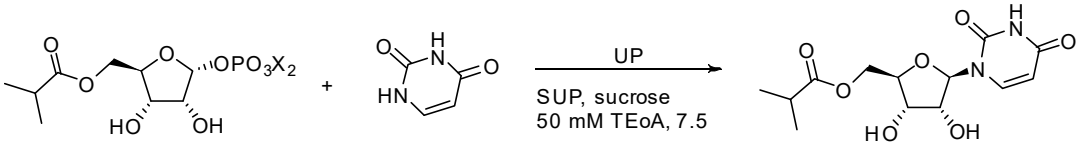
The initial round of evolution was conducted in the phosphorolytic direction given the limited availability of **2** available at that time, owing to lack of a sufficiently active MTR kinase enzyme. Reactions were performed in a 96-well format in 2 mL deep-well plates, with 100 µL total volume. Reactions included 11.1 g/L (3.5 mM) 5'-isobutyryl uridine, 10 mM potassium chloride, 50 mM sodium phosphate, pH 7.4, and 10x diluted UP lysate. The reactions were set up as follows: (i) all the reaction components, except for UP, were pre-mixed in a single solution and 87.5 µL of this solution was then aliquoted into each well of the 96-well plates (ii) 12.5 µL of 10x diluted UP lysate was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a foil seal, incubated at 35 °C with 600 rpm shaking for 18-20 h. The reactions were quenched with 100 µL of a 1:1 mixture of acetonitrile in 20 mM TEoA, pH 7.5 buffer. The quenched reactions were shaken for 3 min on a tabletop shaker followed by centrifugation at 4000 rpm for 10 mins at 4°C to pellet any precipitate. Supernate (50 µL) was then transferred into a 96-well round bottom plate prefilled with 100 µL of 1:1 mixture of acetonitrile in 20 mM TEoA, pH 7.5 buffer. The samples were analyzed according to the HILIC analytical method summarized below.

HILIC Analytical Method	
Instrument	ThermoScientific U3000 UPLC with UV Detection
Column	Xbridge glycan BEH amide, 3.5 µm, 4.6 x 100 mm

Mobile Phase	Isocratic 20:80 water with 0.1% TFA: acetonitrile with 0.1% TFA
Flow Rate	1.0 mL/min
Run Time	2.25 min
Substrate and Product Elution order	5'-isobutyryl uridine: 0.93 min Uracil: 1.25 min
Column Temperature	25 ˚C
Injection Volume	2 µL
Detection	UV 254nm Detector: Thermo VWD-3400; Peak width 0.02min; Collection rate= 200Hz; Time Constant = 0.12s

UP evolution round 2

UP-Rd2BB was selected as the parent enzyme based on the results of screening variants toward the synthesis of 5'-isobutyryl uridine from 5'-isobutyryl ribose-1-phosphate and uracil.



Reactions were performed in a 96-well format in 2 mL deep-well plates, with 100 µL total volume. Reactions included 67 mM 5'-isobutyryl ribose-1-phosphate solution, 7.5 g/L (67 mM) uracil, 91 g/L (267 mM, 4 equiv.) sucrose, 0.019 g/L (0.25 wt% wrt uracil) SUP-101, 50 mM TEoA, pH 7.5, and 100-fold diluted UP lysate. The reactions were set up as follows: (i) all the reaction components, except for UP, were pre-mixed in a single solution and 90 µL of this solution was then aliquoted into each well of the 96-well plates (ii) 10 µL of 100-fold diluted UP lysate was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a foil seal, incubated at 30 °C with 600 rpm shaking for 18-20 hours. The reactions were quenched with 400 µL of a 1:1 mixture of acetonitrile in 20 mM TEoA, pH 7.5 buffer. The quenched reactions were shaken for 5 min on a tabletop shaker followed by centrifugation at 4000 rpm for 10 mins at 4 °C to pellet any precipitate. Supernate (30 µL) was then transferred into a 96-well round bottom plate prefilled with 120 µL of 1:9 mixture of acetonitrile in 20 mM TEoA, pH 7.5 buffer. The samples were analyzed according to the reverse-phase analytical method summarized in the table below.

UP evolution rounds 3-5

Reactions were performed in a 96-well format in 2 mL deep-well plates, with 100 µL total volume. Reactions included 67 mM 5'-isobutyryl ribose-1-phosphate solution, 7.5 g/L (67 mM) uracil, 91 g/L (267 mM, 4 equiv.) sucrose, 0.019 g/L (0.25 wt% wrt uracil) SUP-101, 50 mM TEoA, pH 7.5, and 100-fold diluted UP lysate. The reactions were set up as follows: (i) all the reaction components, except for UP, were pre-mixed in a single solution and 90 µL of this solution was then aliquoted into each well of the 96-well plates (ii) 10 µL of 100x diluted UP lysate was then added into the wells to initiate the reaction. The reaction plate was heat-sealed with a foil seal, incubated at 30 °C with 600 rpm shaking for 18-20 h. The reactions were quenched with 400 µL of a 1:1 mixture of acetonitrile in 20 mM TEoA, pH 7.5 buffer. The quenched reactions were shaken for 5 min on a tabletop shaker followed by centrifugation at 4000 rpm for 10 mins at 4 °C to pellet any precipitate. Supernate (30 µL) was then transferred into a 96-well round bottom plate prefilled with 120 µL of 1:9 mixture of acetonitrile in 20 mM TEoA, pH 7.5 buffer. The samples were analyzed according to the reverse-phase analytical method summarized in the table below.

Reverse-Phase Analytical Method	
Instrument	ThermoFisher Vanquish UPLC with UV Detection

Reverse-Phase Analytical Method	
Column	Atlantis HSS T3, 1.8 µm, 2.1 x 100mm
Mobile Phase	Mobile Phase A: water with 0.1% TFA Mobile Phase B: acetonitrile with 0.1% TFA
Gradient	0-1.0 min 5%B, 1.0 – 1.1 min 50% B, 1.1 – 2.7 50% B, 2.7 – 2.8 min 5% B, 2.8 – 3.25 min 5% B
Flow Rate	0.3 mL/min
Run Time	3.25 min
Substrate and Product Elution order	Uracil : 1.00 min 5'-isobutyryl uridine : 2.52 min
Column Temperature	40 ˚C
Injection Volume	10 µL
Detection	UV 254nm Detector: Vanquish Diode Array Detector HL; Peak width 0.2min; Collection rate= 2Hz; Time Constant = 2s

Fig. S8. Improvement of UP activity over evolution when run as the forward assay (as described in UP Evolution Rounds 3-5).

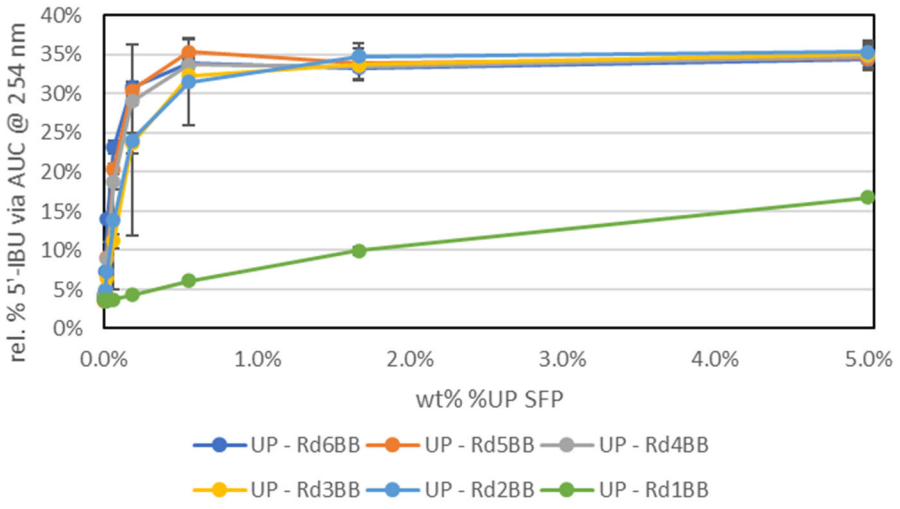


Fig. S9. Improvement of UP activity over evolution in the context of the cascade, varying SFP concentration for each UP variant from 0 – 5 wt%.

Reaction conditions: 37 g/L uracil, 1 equiv. 5'-isobutyryl ribose-1-phosphate, 0.5 mol% TPP, 0.5 mol% ATP, 0.05 mol% FAD, 1.25 equiv. pyruvic acid, 0.1 equiv K_2HPO_4, 0.5 wt% acetate kinase (ACK-101, Codexis) 1.9 wt% evolved MTR-kinase (Rd4), 0.5 wt% catalase (Roche #11650645103), 0.8 wt% pyruvate oxidase 5 (PO5), 10 mM $MgCl_2$. Reactions were run at 25 °C, 250 rpm, 85% humidity for 18 h, followed by quench and analysis (as described in UP Evolution Rounds 3-5).

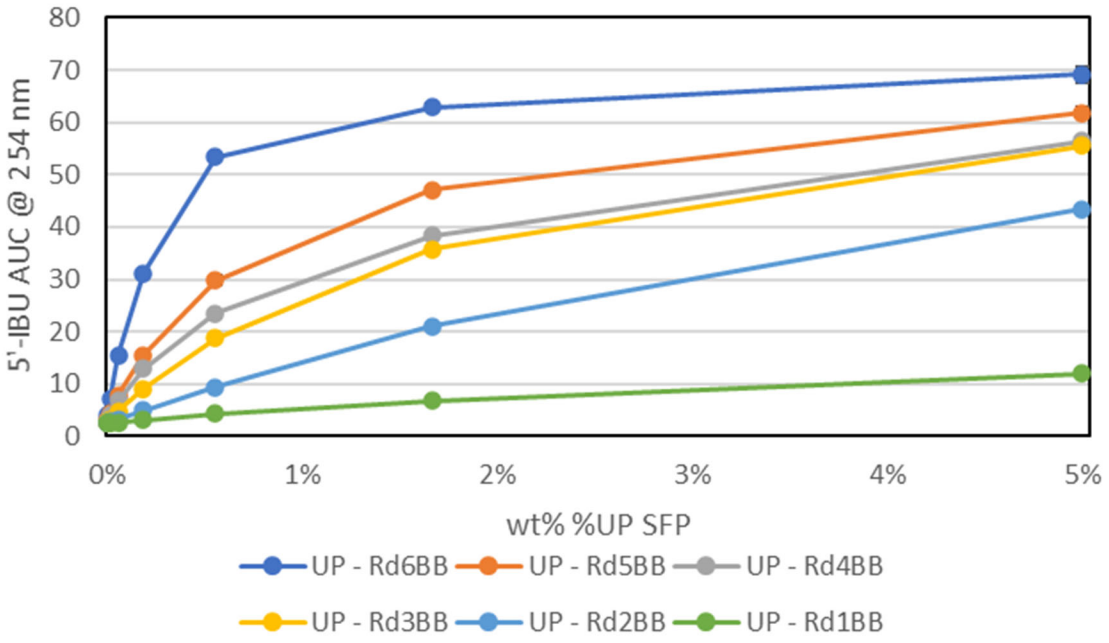
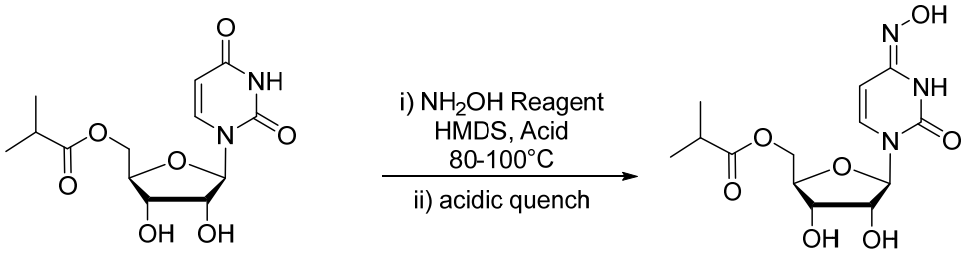


Fig. S10. Testing of selected uridine phosphorylase variants

Enzyme tested	Enzyme loading	
	1.1 wt% UP	**0.42 wt% UP**
Rd4BB	69.3	36.9
Rd5BB	89.9	48
Rd6BB	100	97.3

Procedure: In a 24 well plate, uracil (83 mg, 0.74 mmol) was added to each of 6 wells. In a 50 mL flask, 19.2 g of 20.6 wt% solution containing 5-isobutyrylribose (3.93 g, 17.9 mmol) was combined with potassium phosphate tribasic (341 mg, 1.61 mmol), pyruvic acid (1.96 g, 22.3 mmol), magnesium chloride hexahydrate (91 mg, 0.45 mmol) and water (8 mL). The pH was adjusted to 7.0 with 8 N KOH. To the solution was added thiamine pyrophosphate disodium salt trihydrate (41.1 mg, 0.089 mmol), adenosine 5'-triphosphate disodium salt trihydrate (54.0 mg, 0.089 mmol), and flavin adenine dinucleotide disodium salt (7.4 mg, 8.9 µmol). The pH was adjusted to 7.0. The volume was 31.4 mL, and 8.9 mL water was added to 40.3 mL total.
To the solution were added the enzyme powders sequentially:
Catalase (Roche #11650645103, 9.9 mg, 0.25 wt%)
Pyruvate oxidase PO3 (39.3 mg, 1.0 wt%)
Acetate kinase (Codexis, ACK-101, 9.9 mg, 0.25 wt%)
Methylthioribose kinase MTRK rd 4 BB (118 mg, 3.0 wt%)
2.02 mL of the solution was added to each of the 16 uracil-containing wells in the 24-well plate (for 196 mg isobutyryl ribose each).
Each well was charged with a uridine phosphorylase solution (11 mg/mL uridine phosphorylase in pH 7.0 25 mM phosphate buffer), either 75 µL for 0.42 wt% (1.0 wt% vs. uracil), or 189 µL for 1.1 wt% (2.5 wt% vs. uracil). The plate sealed with an air-permeable film and was shaken at 250 rpm, 25 °C, 85% humidity.
After 20.5 h, mixtures were allowed to settle, and supernatants sampled for HPLC analysis.

Table S5. Screening for Step 3 Conditions



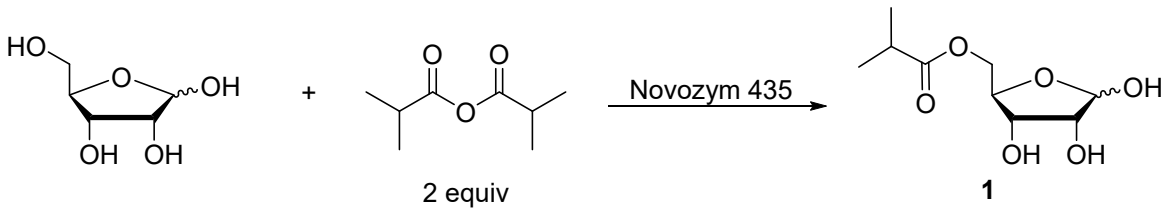
3 **molnupiravir (MK-4482)**

Entry	NH_2OH Reagent	Solvent	Acid	Conversion
1	NH_2OH•HCl	Sulfolane	none	0
2	NH_2OH•HCl	Sulfolane	TfOH	27
3	NH_2OH•HCl	Sulfolane	H_2SO_4	85
4	$(NH_2OH)_2$•H_2SO_4	Sulfolane	H_2SO_4	89
5	$(NH_2OH)_2$•H_2SO_4	2-MeTHF	H_2SO_4	79
6	$(NH_2OH)_2$•H_2SO_4	DME	H_2SO_4	89
7	$(NH_2OH)_2$•H_2SO_4	none	H_2SO_4	89
8	$(NH_2OH)_2$•H_2SO_4	none	$(NH_4)HSO_4$	69
9	$(NH_2OH)_2$•H_2SO_4	none	$(NH_4)HSO_4$ + 0.5 equiv imidazole	99

In a vial was added **2**, hydroxylamine reagent, solvent, HMDS, then acid. The resulting mixture was aged at 80-100°C for 24 h, and the conversion was determined with a calibrated UPLC instrument. The assay yield was measured after dissolving the reaction mixture in aqueous acetonitrile and measuring the product absorbance value on a calibrated UPLC instrument.

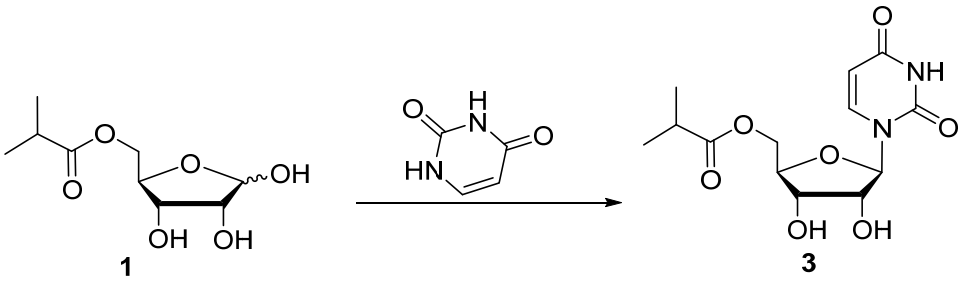
Fig. S11. Conditions for multigram scale reactions

Step 1



To a 2 L vessel is charged ribose (50 g), Novozym435 (5 g, commercially available from Novozymes (catalog # 3925009-810)), acetone (1 L), and isobutyric anhydride (2.0 equiv). The contents were heated and aged at 50°C for 12 h. The mixture was cooled, filtered, and the solid rinsed with acetone. The filtrate was concentrated under vacuum to approximately 150 mL total volume. MTBE (200 mL) was added, and the organic solution was extracted four times each with 100 mL of water. The aqueous mixture was partially concentrated under vacuum to a final weight of 332 g. 92% yield as determined with a calibrated UPLC-CAD instrument. The solution was used directly in the following step.

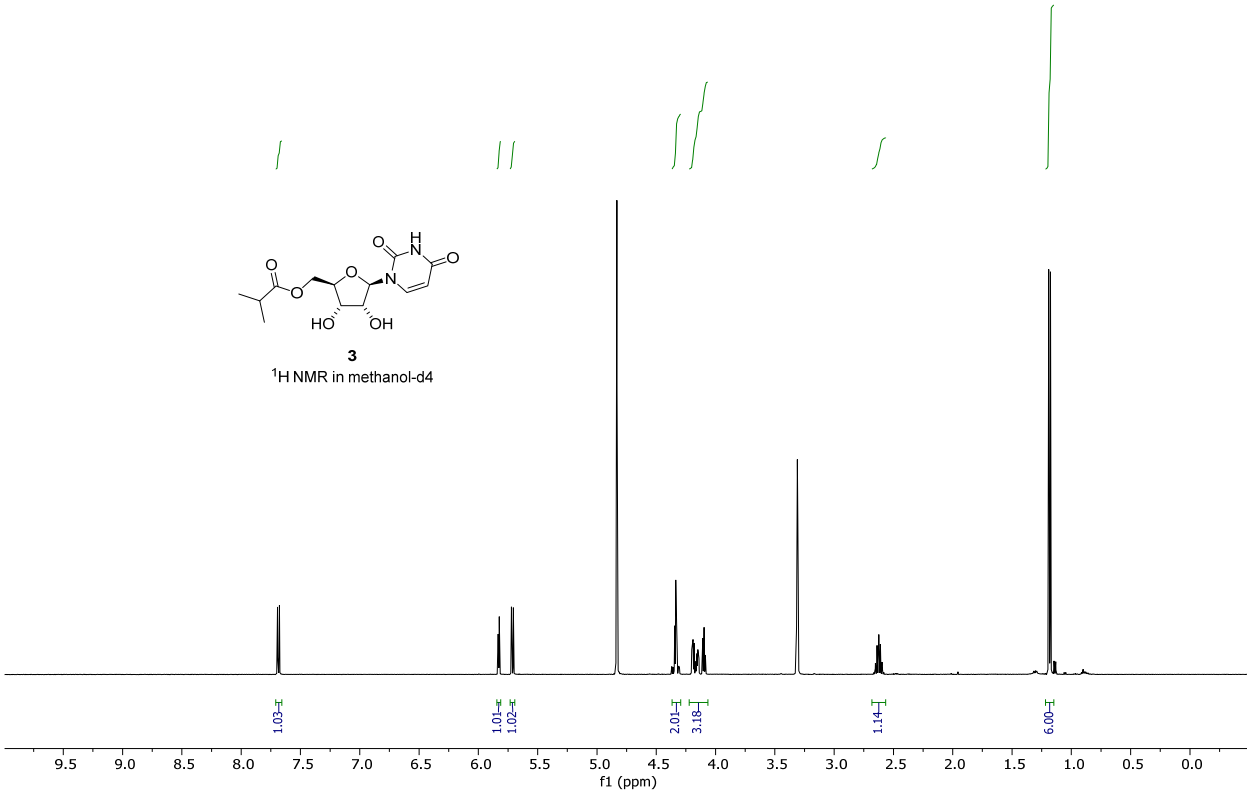
Step 2



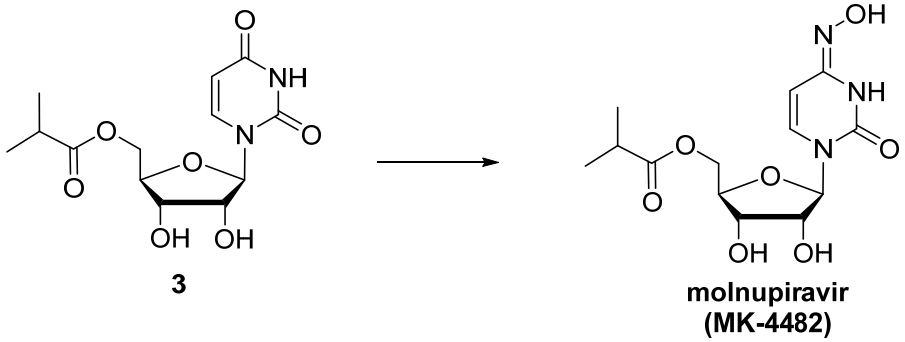
In a 1000 mL flask, charged 5-isobutyryl ribose (72 g, 327 mmol, 349.5 g as 20.6 wt% aqueous solution coming directly from the previous step), followed by pyruvic acid (36.0 g, 409 mmol), potassium phosphate dibasic (5.52 g, 31.7 mmol) and $MgCl_2 \cdot 6H_2O$ (8 mmol 1.63 g). The pH was adjusted to ~7.1 with approximately 67 mL 8 N KOH, then the volume of the solution was adjusted to approximately 800 mL by adding water. To the solution, thiamine pyrophosphate (0.753 g, 1.635 mmol), adenosine triphosphate disodium salt hydrate (0.989 g, 1.635 mmol), and flavin adenine dinucleotide disodium salt (0.136 g, 0.163 mmol), and performed a final pH adjustment to 7.0 with 8 N KOH. In parallel, uracil (30.5 g, 272 mmol), 180 mg acetate kinase (ACK-101, Codexis), 720 mg pyruvate oxidase, 180 mg catalase (Roche #11650645103), 3.6 g of evolved MTR-kinase (Rd4) and 6.1 g of evolved UP varian (Rd2) t was added to a clean and dry Optimax vessel, followed by the 5' isobutyryl ribose solution and Antifoam 204 (500 µl, commercially available form Sigma-Aldrich). Using the overhead stirring available with the OptiMax reactor, the mixture was agitated at 25°C at 1000 rpm while air was sparged through a tubing to supply oxygen at 0.5 slpm in the solution for 16 h. The solution was checked by HPLC to show the formation of 5'-isobutyryl uridine.

The mixture was transferred to a round bottom flask with Celite diatomaceous earth (86 g), MeTHF (850 mL), and ammonium sulfate (258 g), and heated at 75 °C for 30 min. The mixture was cooled to ambient

temperature, filtered, and the aqueous phase was discarded. The organic phase was washed twice with water (86 mL, then 43 mL). The solvent was exchanged to EtOAc and heptane was added to crystallize the product, then aged at 0-5 °C. The crystalline product was collected and washed with a 1:1.5 mixture of EtOAc:heptane to afford ((2R,3S,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl isobutyrate in 87% isolated yield (>99.5 wt% as determined by QNMR and a calibrated UPLC instrument).



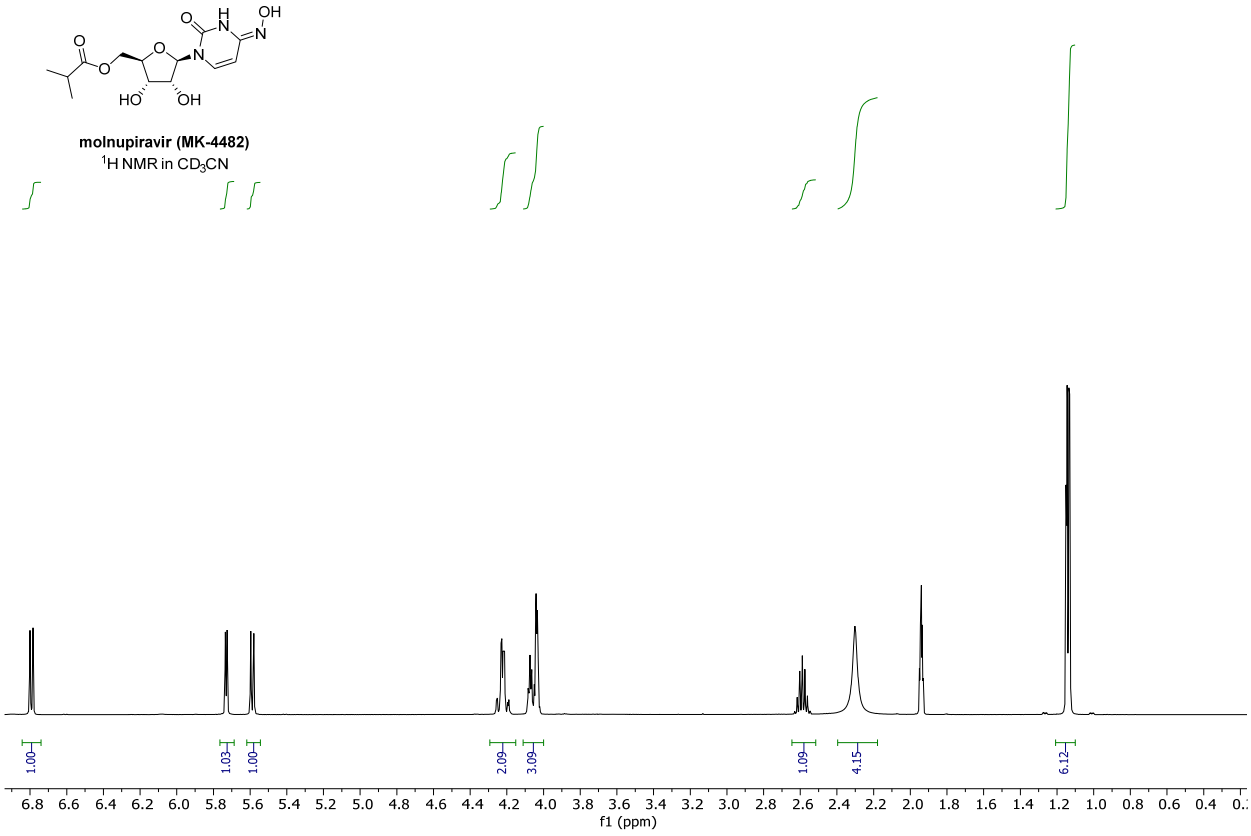
Step 3



A 100 mL vessel with overhead stirrer was set up under nitrogen. The vessel was charged with HMDS (68.0 ml, 320 mmol), and the reaction temperature was raised to 76°C. Imidazole (1.362 g, 20.00 mmol) was added, and the mixture was stirred at 78°C for 30min during which time all the imidazole had dissolved. Ammonium

hydrogen sulfate (11.51 g, 100 mmol) was added, the temperature was adjusted to an external temperature of 75°C, and the mixture was stirred for 30min. Hydroxylamine sulfate (8.21 g, 50.0 mmol) was added. ((2R,3S,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl isobutyrate (12.57 g, 40 mmol) was added, and the reaction mixture was aged with stirring at 75-85°C for 6h, then cooled to ambient temperature.

Water (30.00 ml) was added, and the mixture was transferred to a separatory funnel using 3 mL of water and 2 portions of heptane (5.00 ml) to complete the transfer. The aqueous phase was discarded. The organic phase was washed twice with water (10.00 ml), and the aqueous phases were discarded. Formic acid (6.00 ml, 156 mmol) was added in a single portion, and the mixture was stirred at 50°C for 1h. Water (25.00 ml) was added with stirring, and the mixture was transferred to a separatory funnel using water (10.00 ml) and heptane (5.00 ml). The aqueous phase was separated, and the organic phase was extracted with water (10.00 ml). The organic phase was discarded. The combined aqueous extracts were basified with ammonium hydroxide (1.01 mL, 72.0 mmol). EtOAc (50.0 ml) and ammonium sulfate (40.0 g, 302 mmol) were added, and the mixture was heated to 50°C to give two homogeneous phases. The phases were separated at 50°C, and the aqueous phase was extracted with EtOAc (50.0 ml). The EtOAc extracts were combined and concentrated to approximately 40 mL volume. EtOAc (50.0 ml) was added, and the mixture was concentrated to approximately 40 mL volume. Again EtOAc (50.0 ml) was added, and the mixture was concentrated to approximately 40 mL volume. The resulting slurry was maintained at reflux (75-80°C) for 15min and gradually cooled to 60°C over 30min. MTBE (40.0 ml) was added, then the mixture was cooled to 0°C over 2h. The slurry was filtered, and the filter cake was washed with MTBE (40.0 ml) and dried under nitrogen stream to provide ((2R,3S,4R,5R)-3,4-dihydroxy-5-((Z)-4-(hydroxyimino)-2-oxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methyl isobutyrate in 86% isolated yield (>99.5 wt% purity as determined by a calibrated UPLC instrument).



Sequences

>Rd1_WT_MTR_kinase_klebsiella spp.

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> Rd2_MTR_kinase

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