

**Amino acid mapping–guided scaffold selection and hit identification of
histidine kinase inhibitors from a virtual library of over 12 million compounds**

Supplementary Information

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1. Supplementary Figures

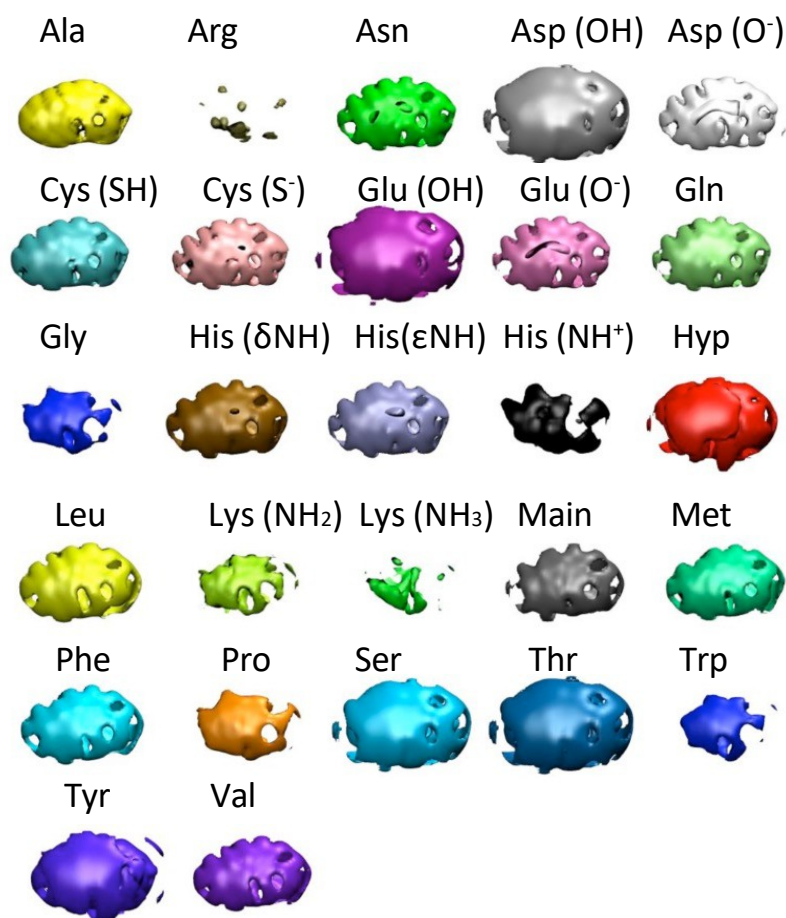


Figure S1 AAM descriptors for all types of amino acids around NQ calculated by AI-AAM.

To calculate the AAM descriptors for all amino acids, we also included variants with different charge states: two types of Asp, two types of Cys, two types of Glu, three types of His, two types of Lys, and one type for the main chain.

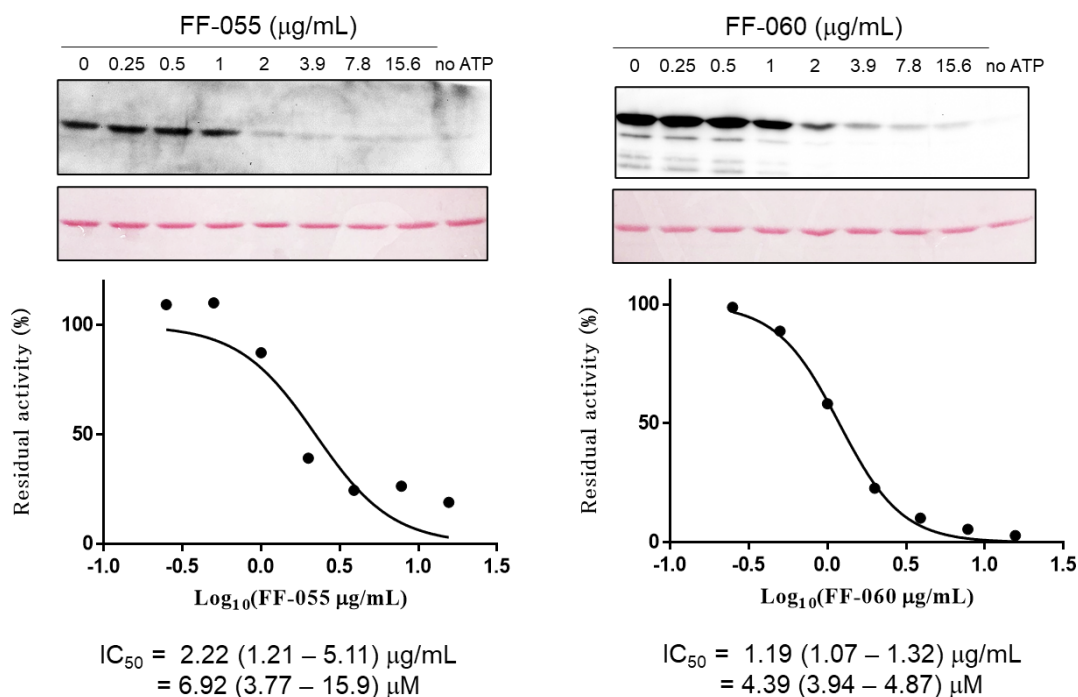


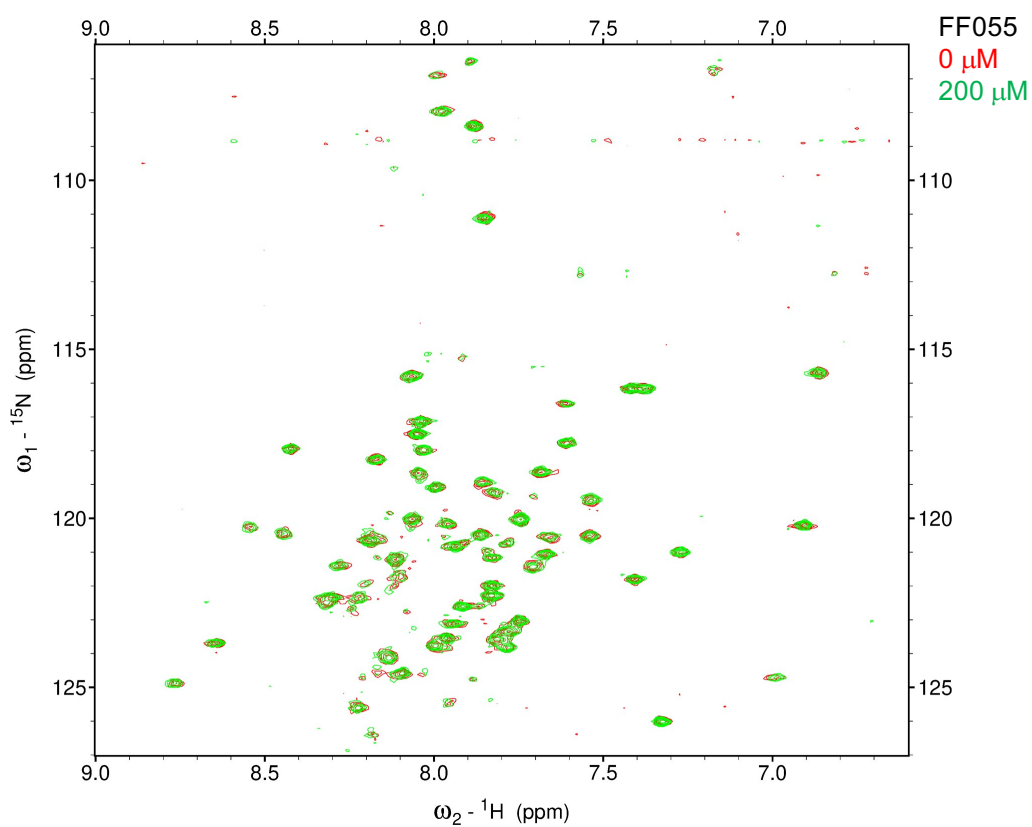
Figure S2 Inhibitory activity of FF-055 and FF-060 against VicK autophosphorylation.

(Upper panels) VicK autophosphorylation activity detected by Western blotting using an anti-N3-phosphohistidine antibody.

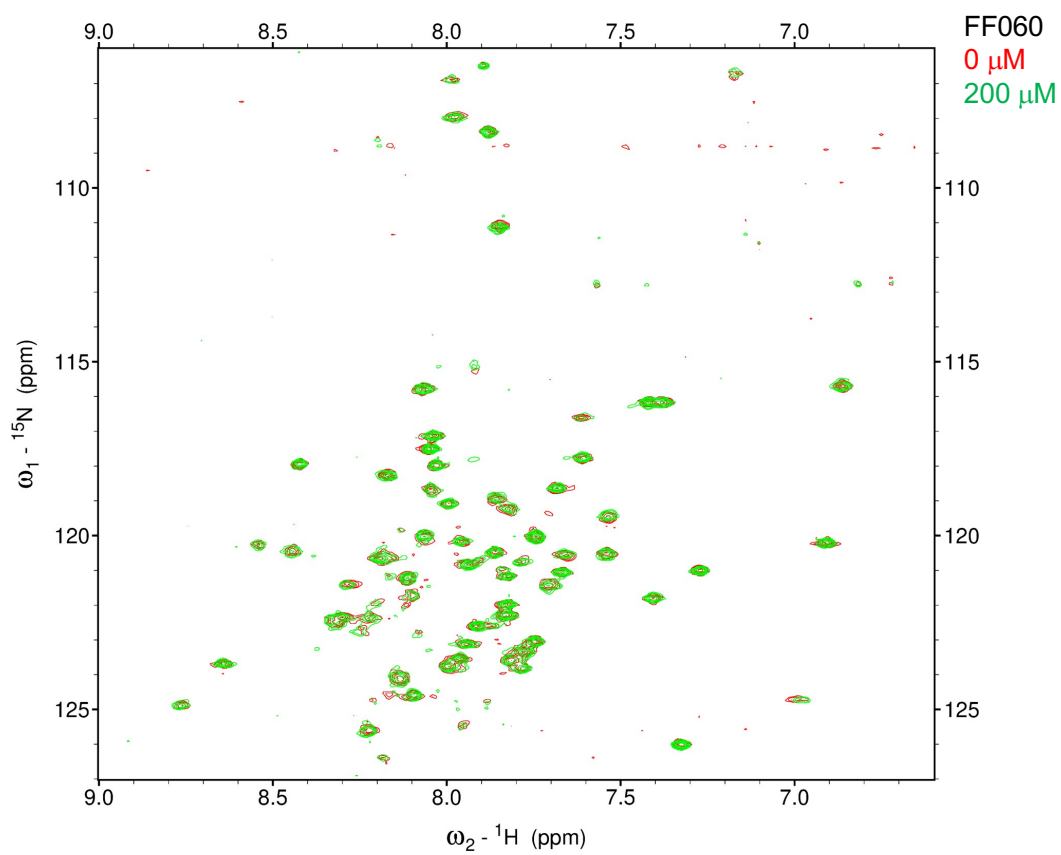
(Lower panels) Ponceau S staining serving as a loading control. Residual activity was plotted against $\log_{10}([\text{inhibitor}])$ and analyzed using GraphPad Prism software (version 7) to determine IC_{50} values. Numbers in parentheses indicate the 95% confidence intervals.

FF054
0 μM
200 μM

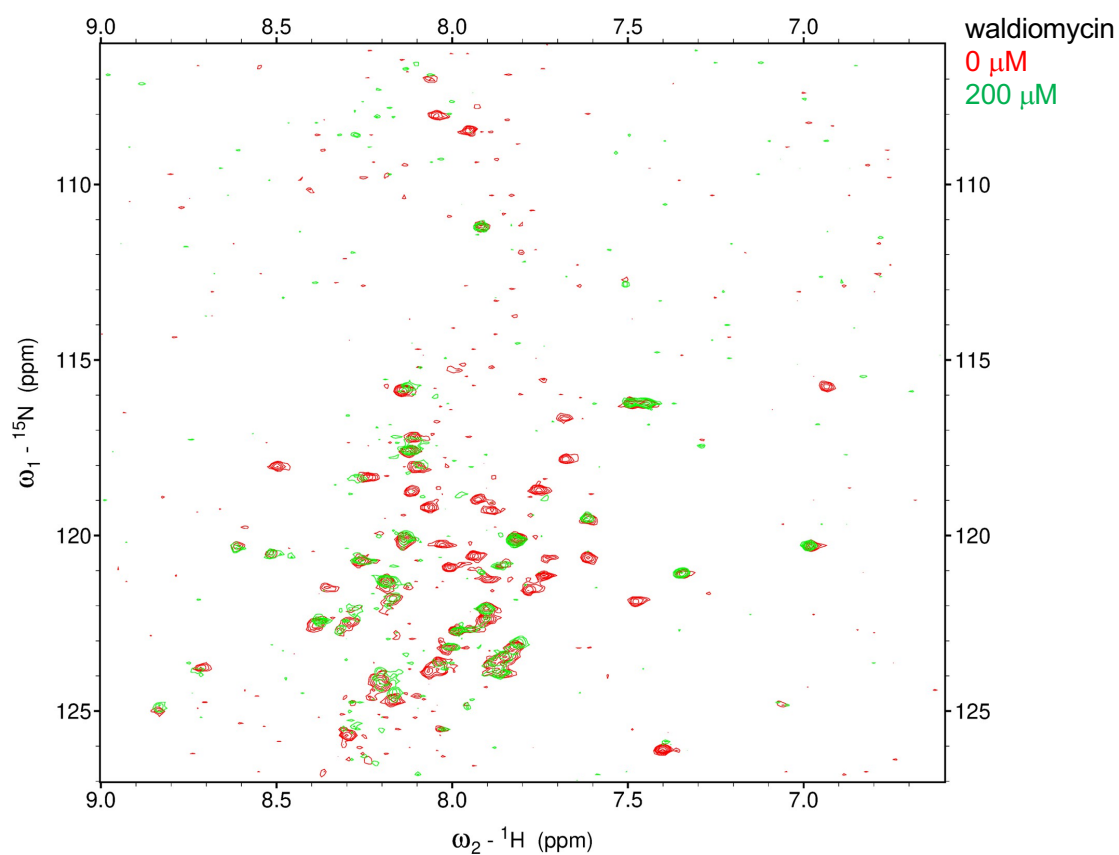
(c) 100 μM EnvZ₂₂₃₋₂₈₉ vs 200 μM FF-055



(d) 100 μM EnvZ₂₂₃₋₂₈₉ vs 200 μM FF-060



(e) 100 μM EnvZ₂₂₃₋₂₈₉ vs 200 μM waldiomycin



(f) 100 μM EnvZ₂₂₃₋₂₈₉ vs 1 mM FF-060

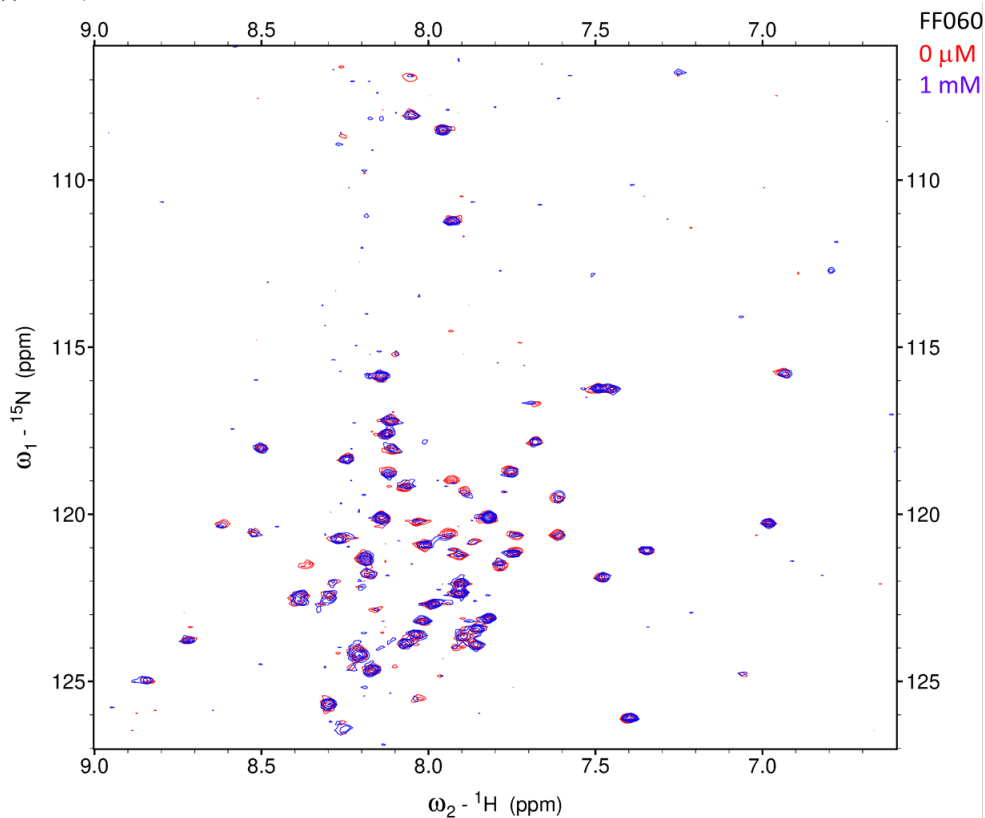
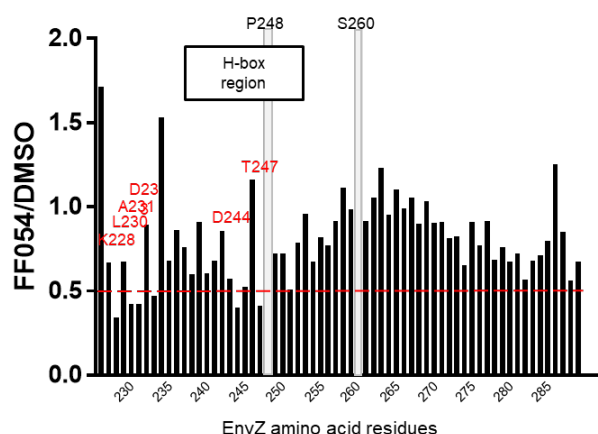


Figure S3 ^1H - ^{15}N -HSQC spectra of ^{15}N -labeled EnvZ(233-289) (DHp domain) in the presence of FF-054, FF-055, FF-060 and waldiomycin. (a) 100 μM ^{15}N -labeled EnvZ(233-289) in 5% DMSO. Signals were assigned on the basis of the previous assignment information ^[1]. All the signals except for P248 and S260 were assigned (corresponding to 62 amino acid residues from G226 to R289). The signal intensity of S260 was too weak for assignment. (b) 100 μM ^{15}N -labeled EnvZ(233-289) in 200 μM FF-054 and 5% DMSO. The spectra in the presence of FF-054 (green) were overlaid onto the DMSO control (red). (c) 100 μM ^{15}N -labeled EnvZ(233-289) in 200 μM FF-055 and 5% DMSO. The spectra in the presence of FF-055 (green) were overlaid onto the DMSO control (red). (d) 100 μM ^{15}N -labeled EnvZ(233-289) in 200 μM FF-060 and 5% DMSO. The spectra in the presence of FF-060 (green) were overlaid onto the DMSO control (red). (e) 100 μM ^{15}N -labeled EnvZ(233-289) in 200 μM waldiomycin and 5% DMSO. The spectra in the presence of waldiomycin (green) were overlaid onto the DMSO control (red). (f) 100 μM ^{15}N -labeled EnvZ(233-289) in 1 mM FF-060 and 5% DMSO. The spectra in the presence of FF-060 (blue) were overlaid onto the DMSO control (red).

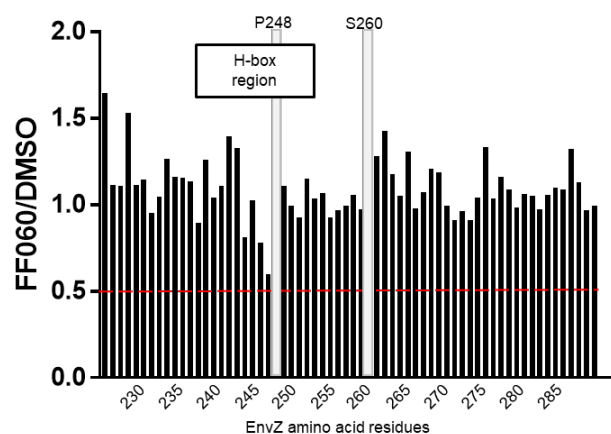
Reference

1. Tomomori, C. et al., Solution structure of the homodimeric core domain of *Escherichia coli* histidine kinase EnvZ. *Nat. Struct. Biol.* **6**, 729-734 (1999)

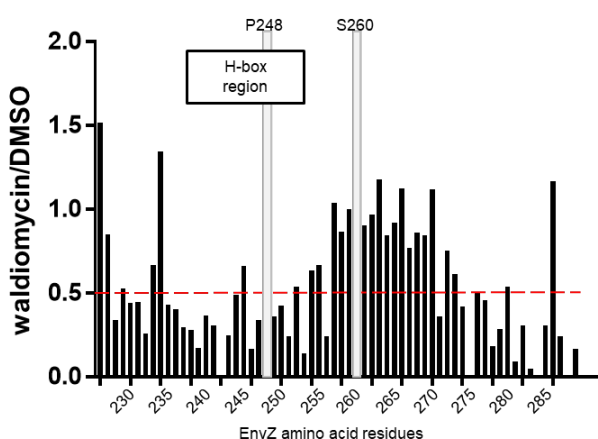
(a) 100 μ M EnvZ₂₂₃₋₂₈₉ vs 200 μ M FF-054



(b) 100 μ M EnvZ₂₂₃₋₂₈₉ vs 200 μ M FF-060



(c) 100 μ M EnvZ₂₂₃₋₂₈₉ vs 200 μ M waldiomycin



(d) 100 μ M EnvZ₂₂₃₋₂₈₉ vs 1 mM FF-060

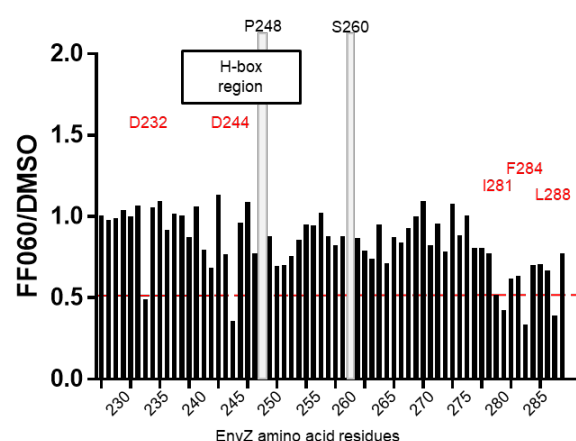


Figure S4 Effect of FF-054, FF-060 and waldiomycin on the signal intensity of amino acid residues within the EnvZ DHp domain in the ^1H - ^{15}N -HSQC spectra.

Ratio of signal intensities of each peak in the ^1H - ^{15}N -HSQC spectra (Fig. S1) between treated and untreated samples is shown. Red dashed line corresponds to 50% reduction in signal intensity. The “H-box region” box indicates the area of the H-box. (a) 100 μ M ^{15}N -labeled EnvZ(233-289) in 200 μ M FF-054; (b) 100 μ M ^{15}N -labeled EnvZ(233-289) in 200 μ M FF-060; (c) 100 μ M ^{15}N -labeled EnvZ(233-289) in 200 μ M waldiomycin; (d) 100 μ M ^{15}N -labeled EnvZ(233-289) in 1 μ M FF-060. Residues showing more than 50% reduction of signal intensities by the treatment are labeled for (a), (b) and (d). As shown in (c), waldiomycin binding reduced the signal intensities of 32 residues to below 50% of the untreated sample.

2. Supplementary Tables

Table S1. Plasmids used in this study.

Plasmids	Description	References
pETBsWalK ₂₀₇₋₆₁₁	Expression plasmid of WalK(207-611) using pET21a (+), His x 6 on N-terminal end	Ishikawa et al. [2]
pETSmVicK ₃₁₋₄₅₀	Expression plasmid of VicK (31-450) using pET22b (+), His x 6 on C-terminal end	Ishikawa et al [2]
pETecEnvZ ₂₂₃₋₄₅₀	Expression plasmid of EnvZ (223-450) using pET22b (+), His x 6 on C-terminal end	Eguchi et al [3]
pETecEnvZ ₂₂₃₋₄₅₀ S242A	EnvZ S242A mutation in pETecEnvZ (223-450)	Eguchi et al [3]
pETecEnvZ ₂₂₃₋₄₅₀ T247A	EnvZ T247A mutation in pETecEnvZ (223-450)	Eguchi et al [3]
pET-His-TEV-EnvZ ₂₂₃₋₂₈₉	Expression plasmid of EnvZ(223-289) using pET22b (+), TEV cleavage site inserted between His x 6 and the N-terminal end of EnvZ*	Eguchi et al [3]

*Gly and Ala remain on the N-terminal end of EnvZ(223-289) after cleavage of His-tag by TEV protease.

- Ishikawa T, Eguchi Y, Igarashi M, Okajima T, Mita K, Yamasaki Y, et al. Synthesis and biochemical characterization of naphthoquinone derivatives targeting bacterial histidine kinases. *J Antibiot.* 2024;77:522–32.
- Eguchi Y, Okajima T, Tochio N, Inukai Y, Shimizu R, Ueda S, et al. Angucycline antibiotic waldiomycin recognizes common structural motif conserved in bacterial histidine kinase. *J Antibiot.* 2017;70:251–8.

Table S2 Chemical structures of 714 selected library compounds

The complete list of chemical structures is provided as a separate Excel file:

Table S2_Chemical_structures_of_714_selected_library_compounds.xlsx.

Table S3 227 major scaffolds of the selected 714 library compounds

The complete list of chemical structures is provided as a separate Excel file:

Table S3_227_major_scaffolds_of_the_selected_714_library_compounds.xlsx

Table S4 MIC data of synthesized *4H*-pyrido[1,2-*a*]pyrimidin-4-ones

Test organisms		MIC (µg/ml)															
		FF-008	FF-047	FF-048	FF-053	FF-054	FF-055	FF-058	FF-059	FF-060	FF-061	FF068	FF074	FF081	FF085	FF086	Waldiomycin
<i>Bacillus subtilis</i>	168	4	8	128	2	128	>128	8	128	64	16	>128	32	128	32	>128	8
<i>Staphylococcus aureus</i>	FDA209P	16	>128	>128	8	128	128	16	128	>128	16	>128	32	128	64	>128	8
<i>Enterococcus faecalis</i>	JCM5803	16	>128	>128	16	>128	>128	32	>128	>128	64	>128	64	128	128	>128	128
<i>Escherichia coli</i>	MG1601	>128	>128	>128	>128	>128	>128	32	32	>128	64	>128	>128	>128	>128	>128	>128
<i>Escherichia coli</i>	MG1601 imp4213	8	>128	>128	8	>128	>128	4	4	>128	16	>128	64	128	>128	>128	8

Table S5 AAM similarity of 15 synthesized compounds

Compounds	AAM similarity
FF-008	0.836085
FF-047	0.836207
FF-048	0.821917
FF-053	0.849455
FF-054	0.808139
FF-055	0.860944
FF-058	0.810657
FF-059	0.780804
FF-060	0.812432
FF-061	0.800411
FF-068	0.803538
FF-074	0.783201
FF-081	0.812235
FF-085	0.76335
FF-086	0.820438

3. Supplementary method S1: Calculation of AAM descriptors

AAM (Amino Acid Mapping) descriptors are defined for each conformation of a target compound; thus, different conformations of the same compound yield different descriptors. In this section, we describe the method for calculating AAM descriptors for a given specific conformation. This method represents a partial modification of previously reported procedures [9, 10]. The calculation of AAM descriptors is performed in two steps.

Step 1: Molecular dynamics (MD) simulations are used to calculate the three-dimensional (3D) spatial distribution of each atom in a single unit of each amino acid residue surrounding the compound in aqueous solution. Here, a single amino acid residue unit is defined as a virtual molecular unit that differs from a standard amino acid (general formula: $\text{H}_2\text{N}-\text{CHR}-\text{COOH}$) in that it lacks N-terminal (amino group: $-\text{NH}_2$) and C-terminal (carboxyl group: $-\text{COOH}$) moieties. This corresponds to the structure of an amino acid incorporated as part of a polypeptide chain in peptides or proteins ($-\text{NH}-\text{CHR}-\text{CO}-$). MD simulations were performed using Gromacs (gromacs-5.1.5 package) [11], applying the GAFF2 force field (The Generalized AMBER Force Field 2) [12] and RESP charges (Restrained Electrostatic Potential method) [13] for the compound, the TIP3P model [14] for water molecules, and the ff14SB force field [15] for the single amino acid residue unit.

A simulation box large enough to fully contain the compound, centered on the center of mass of each compound conformation, was generated. The box was filled with a single molecule of the target compound, a single amino acid residue unit, and multiple water molecules (density 1 g/cm^3 , concentration 55 M) to create the initial configuration. The spatial positions of the amino acid residue unit and water molecules were randomized, and 100 such initial configurations were prepared. After structural relaxation for each configuration, MD simulations were performed at 300 K and 1 atm . Under periodic boundary conditions, the compound conformation was fixed while the amino acid residue unit and water molecules moved freely. The first 1 ns of simulation was used for thermal equilibration, and the subsequent 100 ns were used for analysis, recording the coordinates of the atoms in the amino acid residue unit at 0.01 ns intervals. Coordinate data from all 100 initial configurations were combined. This procedure was performed for 27 variants of naturally occurring amino acids (including hydroxyproline) to account for different protonation states.

The recorded coordinate data were divided into cubic cells of $1 \text{ \AA}$ spacing within the simulation box, and the occurrence frequency of each atom in each cell was calculated. Dividing the frequency in each cell by the average frequency of the boundary cells of the simulation box yielded the 3D spatial distribution of each atom in the amino acid residue unit. The resulting values are non-

negative; cells with values greater than 1 indicate attractive interactions between the compound and the amino acid residue unit atoms, whereas values less than 1 indicate repulsive interactions.

Step 2: To simplify and reduce the degrees of freedom, the 3D spatial distributions of atoms in the 27 amino acid residue units obtained in Step 1 were further consolidated. Among the 27 units, 24 amino acid residues whose backbone and side chain can be separated (Asp-side chain COOH, Asp-side chain COO⁻, Glu-side chain COOH, Glu-side chain COO⁻, Cys-side chain SH, Cys-side chain S⁻, Lys-side chain NH₂, Lys-side chain NH₃⁺, His δ -proton ± 0 , His ϵ -proton ± 0 , His fully protonated +1, Ala, Arg, Asn, Gln, Leu, Ile, Met, Phe, Ser, Thr, Trp, Tyr, Val) had their atoms divided into backbone and side chain groups. For the side chain atoms of 22 of these residues (excluding Leu and Ile), the geometric mean of the 3D spatial distributions across all side chain atoms of each residue was calculated to obtain the side chain 3D spatial distribution. For Leu and Ile, the geometric mean of the 3D spatial distribution of common side chain atoms was calculated to represent the side chain as a single type. For the backbone atoms of the 24 residues, the geometric mean across all residues was calculated to obtain the backbone 3D spatial distribution as a single type.

For the three residues whose backbone and side chain cannot be separated (Gly, Pro, hydroxyproline), the geometric mean of all atom distributions in each residue was calculated as their 3D spatial distribution. Thus, the final set of 3D spatial distributions comprises 23 side chain types, 1 backbone type, and 3 unseparated residues, totaling 27 voxel datasets. Each voxel dataset consists of 1 Å cubic cells, and the complete set represents the AAM descriptor (Fig. 2, Fig. S1).

4. Synthesis of 4*H*-pyrido[1,2-*a*]pyrimidin-4-one derivatives

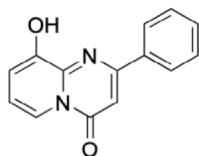
General

NMR spectra were recorded on a JEOL JNM-ECZ600R, with the solvent peak serving as the internal reference (DMSO- d_6 : δ H 2.50, δ C 39.5; CD₃CN: δ H 1.94, δ C 118.26 (CN); MeOH- d_4 : δ H 3.31, δ C 49.00). Multiplicities are denoted as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Coupling constants (J) are provided in Hertz (Hz). In the description of the chemical shift in ^{13}C NMR, multiline based on the C-F coupling were shown in parentheses. The purity of the synthesized compounds tested for biological evaluation was confirmed to be 90% or higher by ^1H NMR analysis. High resolution mass spectra were obtained using an Agilent Q-TOF G6520. Unless specified otherwise, materials were procured from commercial suppliers and used without further purification.

General Procedure for the Synthesis of 2-aryl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones (Method A)

A mixture of 2-amino-3-hydroxypyridine **1** (1.00 mmol) and the appropriate β -keto ester (1.50 mmol) in PPA (2.00 g) was heated at 100 °C for 1 h. The mixture was slowly poured into crushed ice, and the resulting suspension was neutralized with 5% aqueous sodium hydroxide. The solid precipitate was collected by filtration and purified by silica gel column chromatography using ethanol-ethyl acetate mixed solvent to give 2-aryl-9-hydroxy-4*H*-pyrido[1,2-*a*]pyrimidin-4-one.

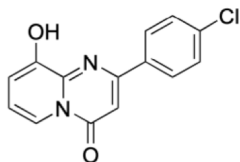
9-Hydroxy-2-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (FF-008)



(66 % yield as pale brown solid)

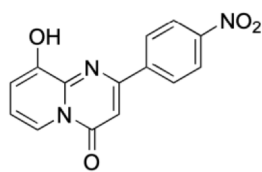
^1H NMR (600 MHz, DMSO- d_6) δ 7.03 (s, 1H), 7.23 (t, 1H, J = 7.2 Hz), 7.30 (d, 1H, J = 7.2 Hz), 7.48–7.58 (m, 3H), 8.40–8.47 (m, 2H), 8.52 (d, 1H, J = 7.2 Hz), 10.00–10.30 (br, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 98.4, 115.6, 116.2, 117.5, 127.7, 128.6, 130.7, 136.3, 144.5, 150.1, 157.9, 158.6; HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for C₁₄H₁₁N₂O₂: 239.0815; Found: 239.0810.

2-(4-Chlorophenyl)-9-hydroxy-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (FF-047)



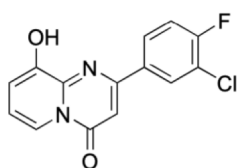
(62 % yield as pale-brown solid)

^1H NMR (600 MHz, DMSO- d_6) δ 7.06 (s, 1H), 7.24 (t, 1H, J = 6.6 Hz), 7.31 (d, 1H, J = 6.6 Hz), 7.58 (d, 2H, J = 8.4 Hz), 8.50 (d, 1H, J = 6.6 Hz), 8.50 (d, 2H, J = 8.4 Hz), 10.20–10.35 (br, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 98.4, 115.8, 116.4, 117.6, 128.6, 129.5, 135.2, 136.0, 144.5, 150.2, 157.3, 157.8; HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for C₁₄H₁₀ClN₂O₂: 273.0425; Found: 273.0421.

9-Hydroxy-2-(4-nitrophenyl)-4H-pyrido[1,2-a]pyrimidin-4-one (FF-048)

(45 % yield as pale-yellow solid)

^1H NMR (600 MHz, DMSO- d_6) δ 7.15 (s, 1H), 7.24 (t, 1H, J = 6.6 Hz), 7.30 (d, 1H, J = 6.6 Hz), 8.13 (d, 2H, J = 8.6 Hz), 8.50 (d, 1H, J = 6.6 Hz), 8.70 (d, 2H, J = 8.6 Hz), 10.70 (brs, 1H); ^{13}C NMR (150 MHz, CD_3CN) δ 98.7, 115.9, 116.7, 118.7, 128.2, 129.6, 135.4, 136.5, 145.5, 150.5, 157.6, 158.1; HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_{10}\text{N}_3\text{O}_4$: 284.0666; Found: 284.0662.

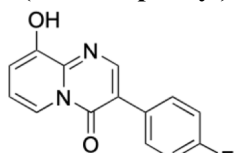
2-(3-Chloro-4-fluorophenyl)-9-hydroxy-4H-pyrido[1,2-a]pyrimidin-4-one (FF-053)

(51 % yield as pale-brown solid)

^1H NMR (600 MHz, DMSO- d_6) δ 7.11 (s, 1H), 7.25 (t, 1H, J = 7.2 Hz), 7.31 (dd, 1H, J = 1.8, 7.8 Hz), 7.55 (t, 1H, J = 7.8 Hz), 8.40–8.46 (m, 1H), 8.50 (dd, 1H, J = 1.2, 6.6 Hz), 8.89 (dd, 1H, J = 1.8, 7.8 Hz), 10.25–10.40 (br, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 98.6, 115.9, 116.5, (116.9, 117.1), 117.5, (128.3, 128.4), 130.3, (130.5, 130.6), 131.7, 134.2, 144.5, 150.2, (156.1, 157.8), 157.9; HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_9\text{ClFN}_2\text{O}_2$: 291.0331; Found: 291.0327.

General Procedure for the Synthesis of 3-aryl-4H-pyrido[1,2-a]pyrimidin-4-ones (Method B)

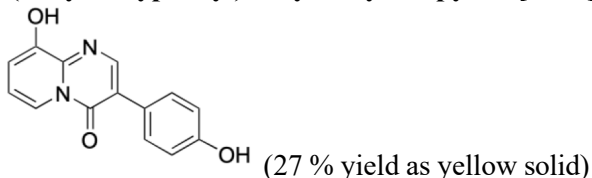
To a solution of an arylacetic acid ester (0.5 mmol) in acetonitrile (1 mL) was added dimethylformamide dimethyl acetal (1.5 mmol, 3 equiv.) and the mixture was heated at 90 °C for 20 h. The solvent was removed by evaporation to give a crude 3-(dimethylamino(-2-arylacrylate 3, which were added 2-amino-3-hydroxypyridine **1** (0.5 mmol), sodium acetate-3H $_2\text{O}$ (0.5 mmol), and acetic acid (1 mL). The mixture was heated at 90 °C for 20 h. The solvent was removed in vacuo and the crude product was recrystallized from ethanol-ethyl acetate mixed solvent to give 3-aryl-9-hydroxy-4H-pyrido[1,2-a]pyrimidin-4-one.

3-(4-Fluorophenyl)-9-hydroxy-4H-pyrido[1,2-a]pyrimidin-4-one (FF-058)

(70 % yield as yellow solid)

^1H NMR (600 MHz, DMSO- d_6) δ 7.22–7.35 (m, 4H), 7.85–7.93 (m, 2H), 8.57 (s, 1H), 8.63 (dd, 1H, J = 1.2, 6.0 Hz), 9.80–10.20 (br, 1H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 114.1, 115.1, (115.1, 115.2), 115.7, 117.9, (130.36, 130.40), 130.9, 131.3, 144.2, (150.49, 150.53), 156.1, (160.7, 162.3); HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_{10}\text{FN}_2\text{O}_2$: 257.0721; Found: 257.0727.

3-(4-Hydroxyphenyl)-9-hydroxy-4H-pyrido[1,2-a]pyrimidin-4-one (FF-061)



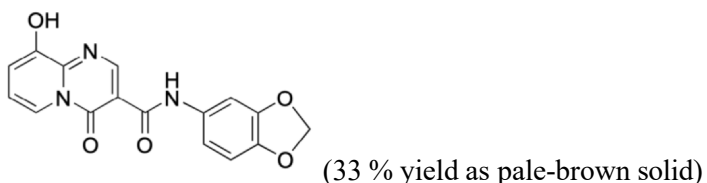
^1H NMR (600 MHz, DMSO- d_6) δ 7.27 (d, 2H, J = 9.0 Hz), 7.63 (d, 1H, J = 6.6 Hz), 7.67 (t, 1H, J = 6.6 Hz), 8.09 (d, 2H, J = 9.0 Hz), 8.92 (s, 1H), 9.02 (d, 1H, J = 6.6 Hz), 9.80–10.20 (br, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 114.8, 115.1, 115.4, 116.7, 117.7, 125.0, 129.6, 143.7, 149.6, 150.4, 156.2, 157.0; HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_{11}\text{N}_2\text{O}_3$: 255.0764; Found: 255.0769.

General Procedure for the Synthesis of 4H-pyrido[1,2-a]pyrimidin-4-one-3-carboxyamides (Method C)

A mixture of 2-amino-3-hydroxypyridine **1** (500 mg, 4.54 mmol) and triethyl orthoformate (5 mL) was heated at 140 °C for 7 h, then evaporated to give a crude oil, which was purified by silica gel column chromatography using hexane-ethyl acetate mixed solvent to afford oxazolo[4,5-*b*]pyridine **5** (390 mg, 72 %) as a colorless solid.

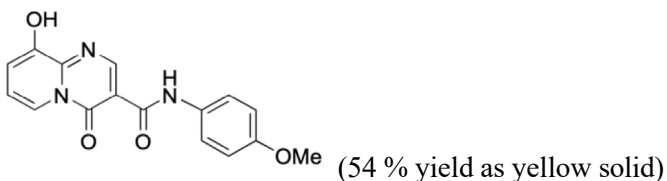
To a solution of ethyl 3-oxo-3-(arylamino)propanoate **4** (0.20 mmol) in THF (1 mL) were added **5** (0.26 mmol) and magnesium trifluoromethanesulfonate (0.26 mmol). The suspension was stirred at 100 °C for 24 h in a sealed tube. The mixture was concentrated and purified by silica gel column chromatography using ethanol-ethyl acetate mixed solvent to afford 4H-pyrido[1,2-*a*]pyrimidin-4-one-3-carboxyamide.

9-Hydroxy-4-oxo-N-(3,4-methylenedioxyphenyl)-4H-pyrido[1,2-a]pyrimidine-3-carboxamide (FF-068)



^1H NMR (600 MHz, DMSO- d_6) δ 5.96 (s, 2H), 6.86 (d, 1H, J = 8.8 Hz), 7.02 (s, 1H), 7.02 (d, 1H, J = 8.8 Hz), 7.45 (t, 1H, J = 7.7 Hz), 7.48 (d, 1H, J = 7.7 Hz), 8.64 (d, 1H, J = 7.7 Hz), 9.01 (s, 1H), 10.95–11.03 (br, 1H), 11.10 (brs, 1H); ^{13}C NMR (150 MHz, MeOH- d_4) δ 101.1, 105.6, 112.0, 114.7, 116.3, 116.8, 120.7, 129.3, 129.2, 144.3, 147.5, 147.8, 150.4, 155.0, 155.2, 163.1; HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{16}\text{H}_{12}\text{N}_3\text{O}_5$: 326.0772; Found: 326.0768.

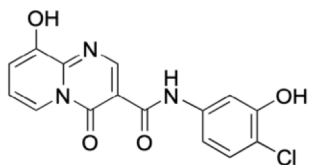
9-Hydroxy-4-oxo-N-(4-methoxyphenyl)-4H-pyrido[1,2-a]pyrimidine-3-carboxamide (FF-074)



^1H NMR (600 MHz, DMSO- d_6) δ 3.71 (s, 3H), 6.90 (d, 2H, J = 9.0 Hz), 7.49 (t, 1H, J = 7.8 Hz), 7.60 (d, 1H, J = 7.8 Hz), 7.61 (d, 2H, J = 9.0 Hz), 8.73 (d, 1H, J = 7.8 Hz), 9.04 (s, 1H), 10.95–11.02 (br, 2H); ^{13}C NMR (150 MHz,

DMSO- d_6) δ 55.8, 106.2, 114.8, 119.1, 119.6, 120.3, 121.8, 132.0, 145.8, 151.8, 155.1, 157.1, 158.2, 161.8; HRMS (ESI-TOF) m/z : $[M+H]^+$ Calcd for $C_{16}H_{14}N_3O_4$: 312.0979; Found: 312.0973.

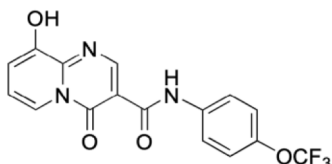
9-Hydroxy-4-oxo-N-(4-chloro-3-hydroxyphenyl)-4H-pyrido[1,2-a]pyrimidine-3-carboxamide (FF-081)



(41 % yield as pale brown solid)

^1H NMR (600 MHz, DMSO- d_6) δ 6.97 (d, 1H, J = 6.6 Hz), 7.08 (d, 1H, J = 8.4 Hz), 7.24 (d, 1H, J = 8.4 Hz), 7.45 (t, 1H, J = 6.6 Hz), 7.57 (s, 1H), 8.22 (d, 1H, J = 6.6 Hz), 8.63 (s, 1H), 11.07 (brs, 1H), 11.07–11.12 (br, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 103.8, 107.7, 109.7, 111.3, 114.2, 118.9, 122.0, 129.9, 138.0, 147.9, 151.8, 153.2, 158.2, 161.5, 161.7; HRMS (ESI-TOF) m/z : $[M+H]^+$ Calcd for $C_{15}H_{11}ClN_3O_4$: 332.0433; Found: 332.0428.

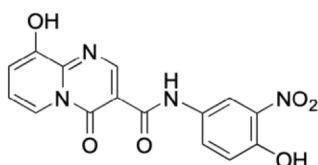
9-Hydroxy-4-oxo-N-(4-trifluoromethoxyphenyl)-4H-pyrido[1,2-a]pyrimidine-3-carboxamide (FF-085)



(45 % yield as pale brown solid)

^1H NMR (600 MHz, DMSO- d_6) δ 6.92 (d, 2H, J = 8.8 Hz), 7.51 (t, 1H, J = 7.8 Hz), 7.62 (d, 1H, J = 7.8 Hz), 7.64 (d, 2H, J = 8.8 Hz), 8.75 (d, 1H, J = 7.8 Hz), 9.06 (s, 1H), 11.02–11.12 (br, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ 106.4, 114.9, 119.3, 119.6, 120.6, 121.8, 129.0 (q), 132.2, 145.9, 151.7, 155.3, 157.3, 158.4, 161.9; HRMS (ESI-TOF) m/z : $[M+H]^+$ Calcd for $C_{16}H_{11}F_3N_3O_4$: 366.0696; Found: 366.0691.

9-Hydroxy-4-oxo-N-(4-hydroxy-3-nitrophenyl)-4H-pyrido[1,2-a]pyrimidine-3-carboxamide (FF-086)



(21 % yield as pale brown solid)

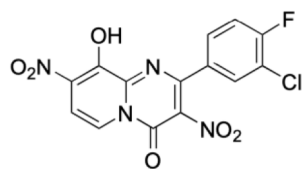
^1H NMR (600 MHz, CD_3CN) δ 7.07 (d, 1H, J = 8.8 Hz), 7.51 (t, 1H, J = 7.6 Hz), 7.62 (d, 1H, J = 7.6 Hz), 7.63 (d, 1H, J = 8.8 Hz), 8.32 (s, 1H), 8.77 (d, 1H, J = 7.6 Hz), 9.04 (s, 1H), 11.10 (brs, 1H), 11.10–11.14 (br, 2H); ^{13}C NMR (150 MHz, CD_3CN) δ 115.0, 116.5, 115.8, 119.7, 120.7, 129.0, 129.3, 129.5, 135.3, 147.1, 148.7, 150.4, 155.1, 155.4, 162.0; HRMS (ESI-TOF) m/z : $[M+H]^+$ Calcd for $C_{15}H_{11}N_4O_6$: 343.0673; Found: 343.0669.

General Procedure for nitration reaction of 4H-pyrido[1,2-a]pyrimidin-4-ones

To a suspension of a 4H-pyrido[1,2-a]pyrimidin-4-ones (0.50 mmol) was dissolved in concentrated sulfuric acid (0.5 ml) and cooled to 0 °C. 70% solution of nitric acid (0.04 ml, 0.6 mmol) was added dropwise to the solution causing a yellow color change. The reaction was stirred at 0 °C for 1 h, then at room temperature for

1.5 h. Ice was added and the mixture was stirred for 1 h, then filtered to afford a yellow solid, which was washed with water (x2) and purified by silica gel column chromatography using ethanol-ethyl acetate mixed solvent to give nitro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one.

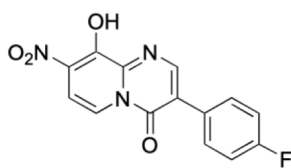
2-(3-Chloro-4-fluorophenyl)-9-hydroxy-3,8-dinitro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (FF-054)



(31 % yield as pale brown gum)

¹H NMR (600 MHz, CD₃CN) δ 7.42 (t, 1H, *J* = 8.8 Hz), 7.66–7.71 (m, 1H), 7.82 (d, 1H, *J* = 8.0 Hz), 8.03 (dd, 1H, *J* = 2.4, 7.0 Hz), 8.57 (d, 1H, *J* = 8.0 Hz), 10.50–10.65 (br, 1H); ¹³C NMR (150 MHz, CD₃CN) δ 116.5, (117.1, 117.3), 117.5, 120.1, (128.7, 128.8), 130.5, (130.6, 130.7), 131.4, 131.7, 134.3, 144.8, 150.6, (156.3, 157.9), 157.6; HRMS (ESI-TOF) *m/z*: [M+H]⁺ Calcd for C₁₄H₇ClFN₄O₆: 381.0033; Found: 381.0039.

3-(4-Fluorophenyl)-9-hydroxy-8-nitro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (FF-059)



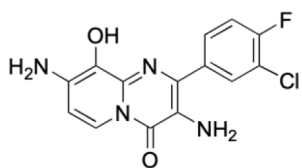
(25 % yield as pale-yellow solid)

¹H NMR (600 MHz, MeOH-*d*₄) δ 7.24 (t, 2H, *J* = 8.6 Hz), 7.34 (d, 1H, *J* = 5.4 Hz), 7.82 (dd, 2H, *J* = 4.1, 8.6 Hz), 8.70 (s, 1H), 8.92 (d, 1H, *J* = 5.4 Hz); ¹³C NMR (150 MHz, MeOH-*d*₄) δ (115.2, 115.3), 115.6, 117.8, 120.9, (130.4, 130.5), 130.9, 131.2, 132.1, 144.3, (150.5, 150.6), 156.4, (160.9, 162.5); HRMS (ESI-TOF) *m/z*: [M+H]⁺ Calcd for C₁₄H₉FN₃O₄: 302.0572; Found: 302.0576.

General Procedure for nitro group reduction of 4*H*-pyrido[1,2-*a*]pyrimidin-4-one compounds

To a stirred suspension of a nitro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (0.30 mmol) in a 1:1 mixture of methanol and water (1.5 mL each) was added sodium dithionite (1.5 mmol) and the mixture was stirred at room temperature for 20 h. The solvent was removed under reduced pressure and the resulting solid was washed with water (x3) and purified by silica gel column chromatography using ethanol-ethyl acetate mixed solvent to give 4*H*-pyrido[1,2-*a*]pyrimidin-4-one bearing amino group.

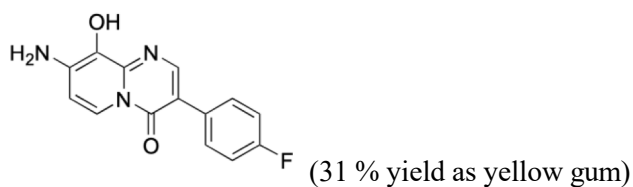
3,8-Diamino-2-(3-chloro-4-fluorophenyl)-9-hydroxy-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (FF-055)



(25 % yield as pale brown gum)

^1H NMR (600 MHz, MeOH-d_4) δ 6.10 (d, 1H, J = 7.8 Hz), 7.30 (t, 1H, J = 8.8 Hz), 7.63–7.69 (m, 1H), 8.01–8.05 (m, 1H), 8.80 (d, 1H, J = 7.8 Hz); ^{13}C NMR (150 MHz, MeOH-d_4) δ 112.3, 116.5, (117.1, 117.3), 117.7, 120.2, (128.4, 128.5), 130.5, (130.4, 130.5), 131.7, 134.3, 144.8, 150.6, (156.2, 157.8), 159.8; HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_{11}\text{ClFN}_4\text{O}_2$: 321.0549; Found: 321.0553.

3-(4-Fluorophenyl)-9-hydroxy-8-amino-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (FF-060)



^1H NMR (600 MHz, MeOH-d_4) δ 7.21 (t, 2H, J = 8.6 Hz), 7.45 (d, 1H, J = 5.4 Hz), 7.80 (dd, 2H, J = 4.1, 8.6 Hz), 8.44 (s, 1H), 8.82 (d, 1H, J = 5.4 Hz); ^{13}C NMR (150 MHz, MeOH-d_4) δ 110.9, (115.0, 115.1), 115.7, 117.9, (130.36, 130.40), 120.9, 130.9, 131.3, 144.2, (150.4, 150.5), 156.3, (160.8, 162.4); HRMS (ESI-TOF) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_{11}\text{FN}_3\text{O}_2$: 272.0830; Found: 272.0835.