



ARTICLE

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

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Abstract

Waldiomycin is a natural product originally isolated from actinomycete extracts. It inhibits histidine kinases (HKs), which are essential components of bacterial two-component signal transduction systems, by targeting the conserved H-box region and suppressing autophosphorylation. Here, we identified novel HK inhibitors using amino acid mapping (AAM)-based virtual screening. We compared the AAM descriptor of the naphthoquinone moiety of waldiomycin, which directly interacts with the H-box region, with those of approximately 12 million commercially available compounds and identified 714 candidates with similar descriptor values. From these candidates, we selected 9-hydroxy-4*H*-pyrido[1,2-*a*]pyrimidin-4-one as a promising and structurally distinct scaffold and synthesized 15 derivatives. The inhibitory activities of these derivatives were evaluated against the HKs WalK, VicK, and EnvZ. Three 4*H*-pyrido[1,2-*a*]pyrimidin-4-one derivatives (**FF-054**, **FF-055**, and **FF-060**) exhibited potent inhibitory activity, with IC₅₀ values ranging from 4.39 to 259 μM. Notably, the IC₅₀ values of these compounds increased three- to six-fold against the EnvZ S242A mutant, in which the conserved H-box residue is substituted. Furthermore, nuclear magnetic resonance (NMR) analysis using the EnvZ DHP domain (residues 223–289, encompassing the H-box region) confirmed direct interaction with this domain. Taken together, these results demonstrate that pyrido[1,2-*a*]pyrimidin-4-one derivatives **FF-054**, **FF-055**, and **FF-060** are novel HK inhibitors that target the H-box region. This study represents the first successful application of AAM-based screening to identify HK inhibitors targeting this highly conserved region.

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Introduction

Waldiomycin, a WalK (a histidine kinase, HK) inhibitor, was initially purified from a culture extract of the

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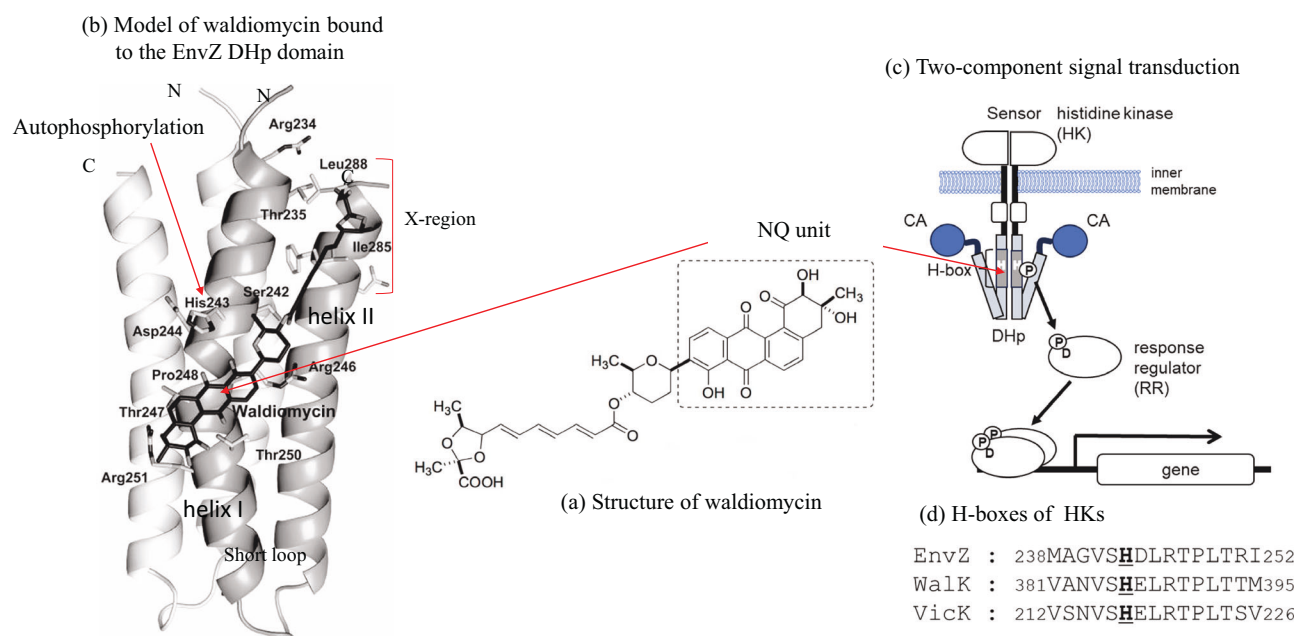


Fig. 1 Waldiomycin and two-component signal transduction. **a** The structure of Waldiomycin [1], **b** its mode of action [2], **c** two-component signal transduction [3, 4], and **d** sequence alignment of the H-boxes of EnvZ, WalK, and VicK. Autophosphorylated histidine residues are shown in bold

actinomycete *Streptomyces* sp. [1]. The molecule is composed of angucyclic rings with a naphthoquinone (NQ) scaffold connected to a dioxolan ring by a tetraene linker (Fig. 1a). Nuclear magnetic resonance (NMR) analysis of waldiomycin suggested that the angucyclic rings interact with the conserved amino acid residues within the H-box of the dimerization histidine phosphotransfer (DHp) domain in class I HKs. Based on this, we proposed a model in which waldiomycin acts on the H-box, thereby interfering with the kinase activity mediated by the catalytic adenosine triphosphate-binding (CA) domain of HKs (Fig. 1b) [2].

The amino acid sequence conserved in the HK H-box (Fig. 1d) is specific to HKs—it is not found in serine/threonine or tyrosine kinases in humans [3]. Genes involved in the multidrug resistance and virulence of pathogenic bacteria are regulated by two-component systems consisting of HKs and response regulators (Fig. 1c) [4]. Therefore, H-box inhibitors acting on such HKs are key targets as next-generation antimicrobial agents capable of modulating the drug resistance and pathogenicity of pathogens such as ESKAPE organisms [5, 6]. With this goal in mind, Ishikawa et al. [7] synthesized novel H-box inhibitors targeting HKs derived from the NQ moiety of the angucyclic ring in waldiomycin.

The HK inhibitors synthesized to date are classified as: trityles, benzoxazines, salicylanilides, triazoles, 6-oxa-isosteres, terpene derivatives, benzimidazoles, benzoxazoles, thiazolidiones, thiophenes, adenines, and other heterocycles [8]. None of these HK inhibitors have yet reached clinical

application, despite the highly diverse structures and wide range of properties exhibited [8]. It remains essential to generate novel HK inhibitor compounds suitable for clinical trials.

Tsumura et al. [9, 10] proposed a novel method—amino acid mapping (AAM)—that allows information on the ligand-binding site(s) for target proteins to be directly derived from a compound, bypassing the elucidation of the structure of the complex formed by the compound and the target protein (Fig. 2). The AAM descriptor is the spatial distribution of single amino acid residues surrounding the compound, calculated independently of the explicit protein structure (Fig. 2b; see Materials and Methods for computational details). Structure-independent virtual screening based on the AAM descriptor derived from a known ligand enables the efficient identification of compounds with distinct scaffolds that are likely to exhibit similar biological activities. Herein, we applied the AAM descriptor of the NQ moiety of waldiomycin to screen a library of 12 million compounds, leading to the identification of HK inhibitors with novel, non-NQ scaffolds.

Materials and methods

General

NMR spectra of the synthesized compounds were recorded on a JEOL JNM-ECZ600R. High-resolution mass

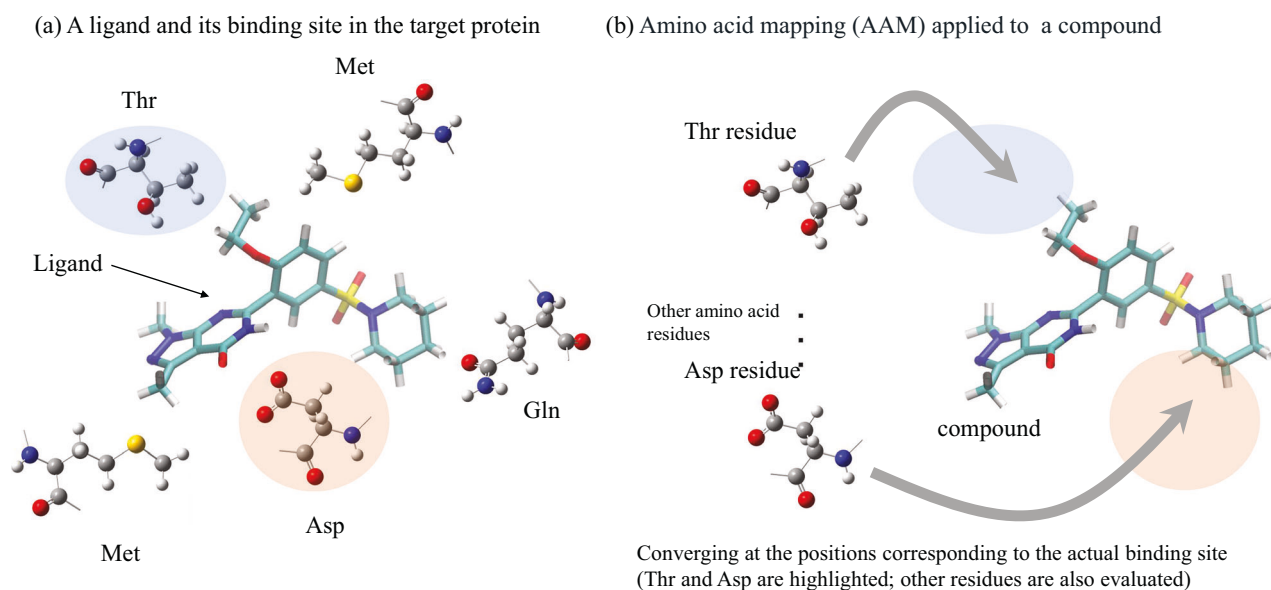


Fig. 2 Concept of the amino acid mapping (AAM) descriptor. **a** The binding site structure in which a ligand interacts with specific amino acid residues within the target protein. **b** The spatial distribution of

individual amino acid residues surrounding a compound calculated independently of protein structural information. This distribution constitutes the AAM descriptor

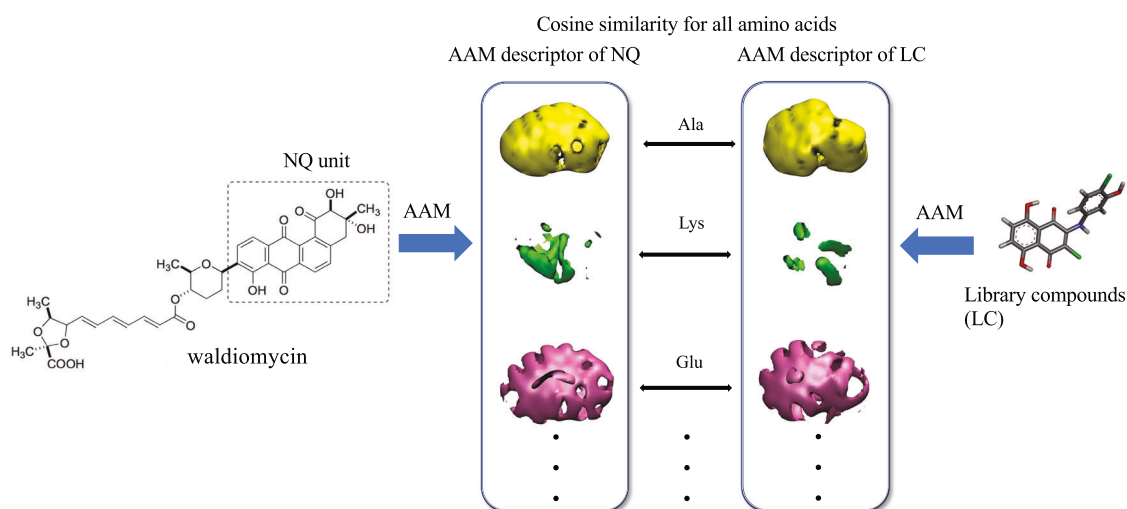


Fig. 3 Cosine similarity of amino acid mapping (AAM) descriptors for all amino acids interacting with naphthoquinone (NQ) and library compounds (LC), calculated as a 3D existence probability map of each

amino acid residue around the 3D conformation of NQ and LC through molecular dynamics simulations

spectra were obtained using an Agilent Q-TOF G6520. Materials were procured from commercial suppliers and used without further purification unless specified otherwise.

AAM descriptors

AAM descriptors were calculated (Figs. 3 and 4) for: (i) the angucyclic polyketide moiety containing the NQ scaffold (Fig. 1a), through which waldiomycin binds to the HK H-box; (ii) 11,973,825 compounds from a commercially

available library of compounds (LC: Namikishoji Co., Chemicup, <https://www.namiki-s.co.jp>); and (iii) H-box inhibitors (FF-054, FF-055, FF-060, 6a–f).

AAM descriptors are defined for individual target compound conformations; therefore, different conformations of the same compound produce distinct descriptors. The calculation of AAM descriptors for a specific conformation used a similar method (see Supplementary Method S1) to previously reported procedures [9, 10]. The three-dimensional (3D) conformations of atoms in the single amino acid residue units surrounding the compound in an

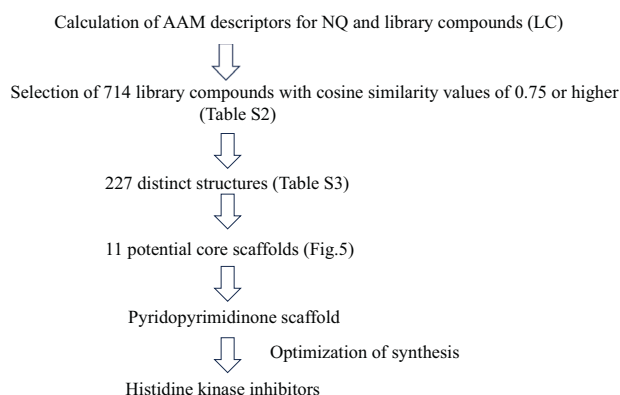


Fig. 4 Flow chart of amino acid mapping (AAM)-based selection of histidine kinase (HK) inhibitors with a novel scaffold from a library of compounds

aqueous solution were determined from molecular dynamics (MD) simulations. All MD simulations were performed using GROMACS version 5.1.5 [11]. The Generalized AMBER Force Field 2 [12] and Restrained Electrostatic Potential method charges [13] were applied to the compound. The TIP3P model [14] was used for water molecules, and the Amber-ff14SB force field [15] was used for the single amino acid residue units.

Similarity of the AAM descriptors

In this study, the AAM similarity between two specific conformations was defined as the cosine similarity between their AAM descriptors. For comparisons between compounds, AAM similarity was defined as the maximum similarity obtained through the optimization procedure described below. AAM similarities were calculated for two cases: (i) between the NQ reference compound and the 11,973,825 compounds from the LC (Fig. 3), and (ii) among a total of 10 compounds (NQ and nine H-box inhibitors: **FF-054**, **FF-055**, **FF-060**, and **6a–f**), for which all pairwise combinations were used to construct a 10×10 AAM similarity matrix. First, 100 low-precision conformations of NQ were generated using the open-source Open Babel software [16]. These conformations were then used as initial structures for geometry optimization with the Gaussian16 [17] quantum chemistry program—employing density functional theory with the B3LYP functional [18, 19], 6-31 G* basis set [20], and the polarizable continuum solvation model [21] with water as the solvent—to obtain 100 high-precision, locally stable conformations. The most stable conformation among the 100 was then selected. For the LC compounds, the geometries used in this study were not generated de novo during the present screening study. Rather, they had been prepared in advance, prior to this study, by large-scale DFT-based conformational

calculations, and these precomputed structures were used as input geometries for AAM-based virtual screening.

For each LC compound, the precomputed geometry was used as the initial conformation, and the centroid position, molecular orientation, and intramolecular dihedral angles were adjusted within an energy window of +10 kcal/mol to maximize the AAM similarity to the most stable NQ conformation. The final AAM similarity between NQ and each LC compound was defined as the maximum similarity obtained through this procedure (Figs. 3 and S1).

To reduce the computational cost of AAM similarity evaluation for the 11,973,825 LC compounds, a stepwise accelerated filtering procedure was applied. First, AAM similarity was calculated using only the descriptor for Phe, and compounds showing negative similarity values were excluded. Second, for the remaining compounds, AAM similarity was calculated using the descriptors for His fully protonated +1 and Cys-side chain S⁻, representing charged residue types, and compounds showing negative similarity values were excluded. Finally, full AAM similarity calculations using all 27 amino acid residue descriptors were performed only for compounds that passed both filtering steps. In this stepwise procedure, the final conformation obtained in the Phe-based calculation was used as the initial conformation for the second-step calculation, and the final conformation obtained in the second-step calculation was then used as the initial conformation for the full 27-residue calculation, thereby improving the efficiency of conformational optimization.

A threshold of 0.75 was used for hit selection in the LC screening. This cutoff was determined empirically based on previous AAM-based screening campaigns against several target protein systems, in which compounds showing activities comparable to those of reference ligands were identified with an approximate success rate of 10% among compounds exceeding this AAM similarity value. On this empirical basis, 0.75 was adopted as the hit-selection threshold in the present study. The conformations of the nine H-box inhibitors (**FF-054**, **FF-055**, **FF-060**, and **6a–f**) were determined using the same procedure as for the LC compounds.

The conformations of NQ and these nine H-box inhibitors were then used to compute a 10×10 AAM similarity matrix, in which each element represented the AAM similarity between a pair of compounds. Because AAM similarity is inherently asymmetric and depends on the order of comparison, the resulting matrix was symmetrized by adding it to its transpose and dividing each element by two.

Extraction of condensed ring scaffolds

Rigid substructures and their occurrence frequencies were analyzed for the 714 compounds selected by AAM-based

screening using an in-house Python script implemented with RDKit (Open-source cheminformatics, <https://www.rdkit.org>). In this study, a rigid substructure was defined as a condensed ring system together with adjacent atoms directly connected to that ring system through double or higher-order bonds. Ring information was obtained from each compound using the *GetSymmSSSR* function, and rings sharing atoms were merged to define ring systems. Non-ring atoms connected to ring-system atoms through multiple bonds were then added, and the resulting atom set was extracted as a fragment, sanitized, and converted to a canonical SMILES string. Duplicate occurrences of the same substructure within a molecule were counted only once, and frequencies were tabulated across all compounds. Exact molecular weights were calculated using *CalcExactMolWt*.

Multidimensional scaling analysis of AAM similarities

We converted the symmetrized 10×10 AAM similarity matrix into a distance matrix to visualize the relationships between H-box inhibitors. The distance between compounds i and j was defined as $d_{ij} = 1 - S_{ij}$, where S_{ij} is the AAM similarity between compounds i and j . We then performed classical multidimensional scaling (MDS) [22] using the scikit-learn module in Python [23] to create a two-dimensional map of the pairwise distances between the compounds from the distance matrix.

Protein expression and purification

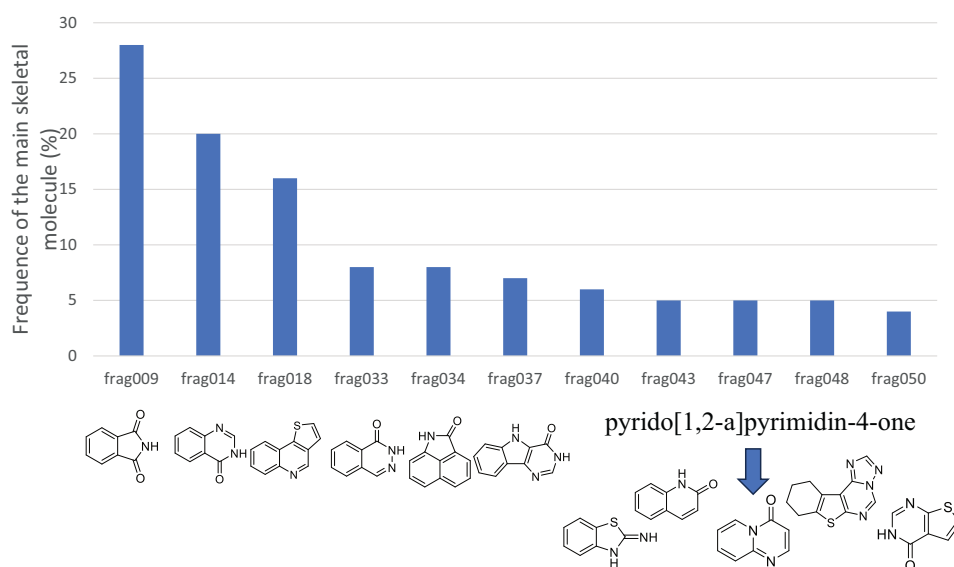
Cytoplasmic regions of *Bacillus subtilis* WalK(207-611) (amino acid residues 207-611), *Streptococcus mutans* VicK(31-450), and *Escherichia coli* EnvZ(223-450) were expressed from *E. coli* BL21 (DE3) cells transformed with the plasmids shown in Table S1. HKs for the autophosphorylation assays were expressed and purified as previously described [7]: 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to mid-exponential phase cells grown in 2 $\times$ YT medium, followed by induction for 5 hours at 30 °C; His-tagged HKs were purified from the induced cells by resuspension in lysis buffer (50 mM Tris-HCl (pH 8), 100 mM NaCl) with 1 mM phenylmethylsulfonyl fluoride (Nacalai tesque), cell lysis by sonication, and affinity purification using Ni (II)-NTA agarose (Qiagen). The eluted protein samples were dialyzed against lysis buffer and stored at -20 °C with the addition of 20% glycerol. 15 N-labeled EnvZ(223-289) was expressed from *E. coli* BL21 (DE3) cells transformed with pET-His-TEV-EnvZ(223-289) (Table S1). It was induced by adding 1 mM IPTG to cells grown in labeled synthetic

medium (0.1% (W/V) 15 NH₄Cl, 0.7% (w/v) Na₂HPO₄, 0.3% (w/v) KH₂PO₄, 0.05% (w/v) NaCl, 3.3 mM FeCl₃, 1 mM MgSO₄, 50 μ M MnCl₂, 100 mM CaCl₂, 0.11% (w/v) glycerol, 0.4% (w/v) glucose, and 0.002% (w/v) each of adenosine, guanosine, cytidine, thymidine, thiamin, biotin, and 100 μ g/mL ampicillin) and culturing at 30 °C for 6 h. The labeled EnvZ(223-289) was purified from the induced cells as described above for His-tagged HKs. The eluted protein samples were dialyzed against lysis buffer, and the His-tag was cleaved by tobacco etch virus (TEV) protease (Nippon Gene). The reaction mixture was passed through a Ni(II)-NTA agarose column to remove the TEV proteases and cleaved His-tag, dialyzed against an NMR buffer solution (20 mM Na-phosphate buffer (pH 7.2), 50 mM KCl, 5 mM MgCl₂ and 5 mM dithiothreitol), and concentrated by ultrafiltration (Vivaspin 6, 3 kDa cut, Cytiva). 15 N-labeled EnvZ(223-289) samples were stored at -20 °C with the addition of 20% glycerol and dialyzed against the NMR buffer before NMR analyses.

Inhibition assay of HK autophosphorylation

The inhibition assay of HK autophosphorylation was carried out as previously described [7]. Autophosphorylations of WalK(207-611) and EnvZ(223-450) were performed at 1 and 2 μ M, respectively, in a reaction buffer (50 mM Tris-HCl (pH 8.5), 100 mM KCl, 5 mM MgCl₂, 100 mM NH₄Cl, 5% dimethyl sulfoxide (DMSO), and 40 μ M ATP for WalK(207-611); 400 μ M ATP for EnvZ(223-450)). For VicK(31-450), the reaction was conducted at 0.5 μ M in a buffer comprising 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 5% DMSO, and 20 μ M ATP. For all cases, the total reaction volume was 40 μ L. The autophosphorylation reactions were performed at 25 °C for 30 min (WalK(207-611), EnvZ(223-450)) or 10 min (VicK(31-450)) before being terminated by the addition of 40 μ L 2 $\times$ SDS-PAGE sample buffer. The reaction mixture was then subjected to SDS-PAGE (10% acrylamide) and Western blotting using a polyvinylidene difluoride membrane (Bio-Rad). Anti-N3-phosphohistidine antibody (clone SD56-2; EDM Millipore Corp.) was used as the primary antibody, goat anti-rabbit IgG-HRP (Abcam) as the secondary antibody, and 2% bovine serum albumin (Sigma-Aldrich) solution as the blocking agent. Signals originating from phosphorylated HKs were detected using a horseradish peroxidase (HRP) substrate (Immobilon Western Chemiluminescent HRP substrate, Millipore) and Multi-Imager II MultiBox (BioTools). The signal intensities were quantified using the CS Analyzer 4 (ATTO) image analysis software, and 50% inhibitory concentration (IC₅₀) values were determined using Prism 7 (GraphPad).

Fig. 5 The main skeletal fragments of the 714 selected library compounds



NMR spectroscopy

NMR measurements for protein-inhibitor binding were performed using a Bruker Avance III 600 MHz NMR spectrometer with a Z-axis gradient and triple resonance TXI probe (BrukerBioSpin Co.). The sample conditions were 20 mM Na-phosphate (pH = 7.2), 50 mM KCl, 5 mM MgCl₂, 5 mM DTT, and 5% (v/v) DMSO-*d*₆. Each sample contained 100 μM of ¹⁵N-labeled EnvZ(223-289) and either 200 μM or 1 mM of inhibitors. ¹H-¹⁵N hetero-nuclear single quantum coherence (HSQC) spectra were measured at 298 K. Spectral data were processed using nmrPipe [24] and analyzed using SPARKY (Goddard and Kneller, SPARKY3, University of California, San Francisco, <https://www.cgl.ucsf.edu/home/sparky/>). The signal intensity was evaluated as the peak height found by the “pc” function of SPARKY. The effects of the inhibitors for each amino acid residue of EnvZ(223-289) were evaluated as chemical shift perturbations (Δδ, ppm) defined by

$$\Delta\delta = \sqrt{\Delta\delta_{\text{H}}^2 + \left(\frac{\Delta\delta_{\text{N}}}{5}\right)^2} \quad (1)$$

where Δδ_H and Δδ_N are the differences between chemical shifts with and without the inhibitor for the ¹H and ¹⁵N axes, respectively.

Affinity selection/mass spectrometry

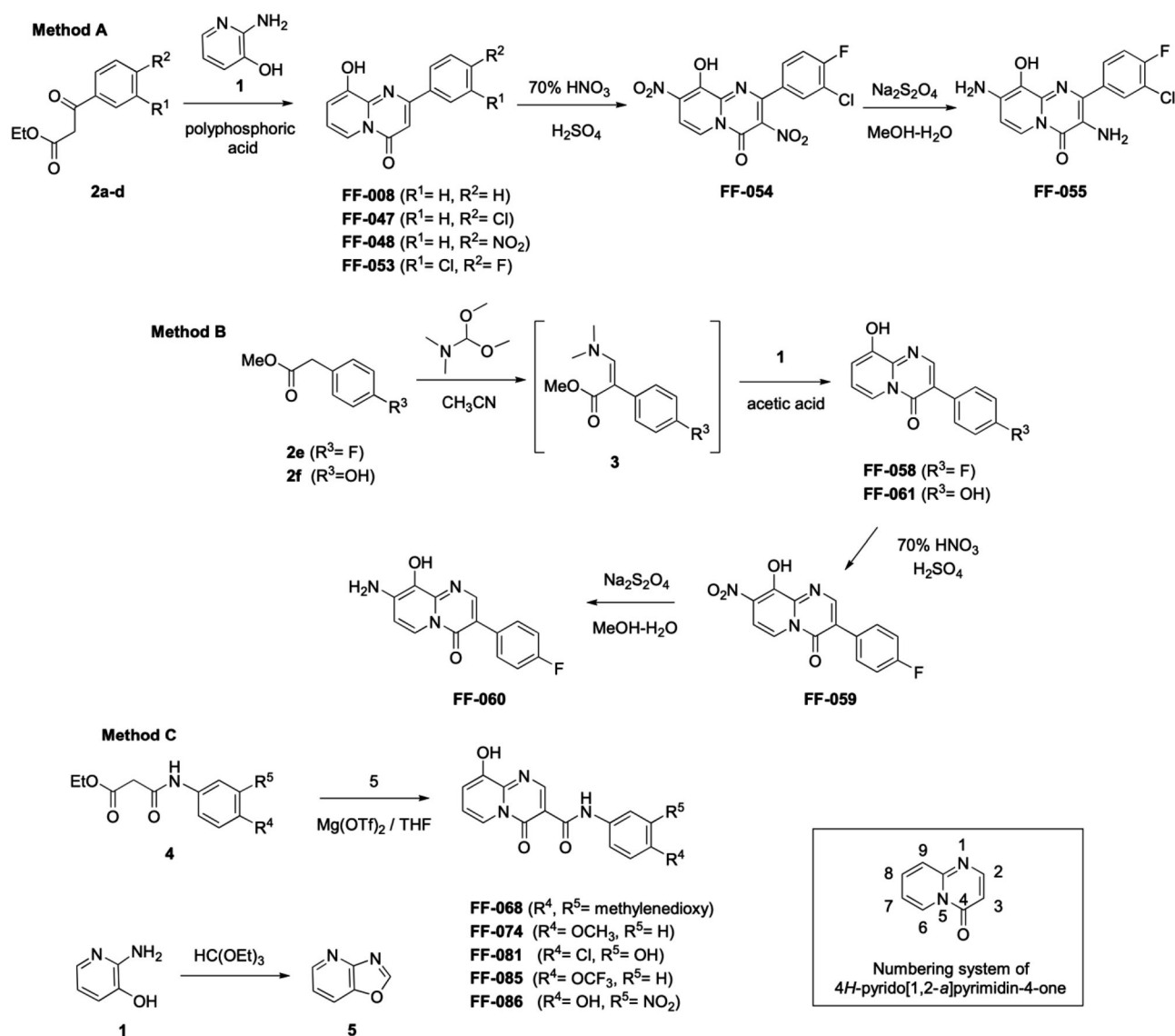
The affinity selection/mass spectrometry (AS/MS) method analyzes the competitive binding of small compounds to proteins against a reference ligand. Experiments followed a previously established protocol [7], wherein 1 μM of

VicK(31-450) (Table S1) was combined and incubated with 5 μM of waldiomycin and **FF-060** (0, 10, 30, 100 μM) in a reaction buffer (25 mM Tris-HCl (pH = 8.0), 50 mM KCl, 50 mM NH₄Cl, 5 mM MgCl₂, 1 mM dithiothreitol, and 0.1% CHAPS(3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)). The mixture was subjected to size-exclusion via centrifugation to eliminate free compounds. The eluted protein was then denatured by adding an equal volume of methanol/0.1% formic acid. The compounds that were subsequently released from the protein were detected using a liquid chromatography–mass spectroscopy (LC–MS) system.

Results and discussion

Chemical library screening by AAM

714 compounds with sufficiently high AAM similarity values between NQ and the LC compounds (≥0.75) were identified (Fig. 4 and Table S2). Rigid substructures, defined as condensed ring systems together with adjacent atoms directly connected through double or higher-order bonds, were extracted from the 714 compound structures, yielding 227 distinct scaffolds along with their occurrence frequencies (Table S3). Scaffolds with a molecular weight of ≥140 were considered as potential core scaffolds in order to exclude overly small frameworks. This threshold was chosen as an approximate size criterion corresponding to a two-ring system, i.e., one ring fewer than the three-ring naphthoquinone-containing reference compound (NQ), and is roughly intermediate between the molecular weights of biphenyl and naphthalene. The 11 most frequently occurring structures



Scheme 1 Synthetic routes for 9-hydroxy-4H-pyrido[1,2-a]pyrimidin-4-one derivative

were then selected (Fig. 5). Synthesis of histidine kinase inhibitors featuring 4H-pyrido[1,2-a]pyrimidin-4-one as a core scaffold was subsequently conducted. Although this scaffold ranked ninth in terms of frequency of occurrence, we have selected it due to its novelty as an H-box inhibitor and the potential to synthesize a wide range of functional derivatives through more convergent and diverse synthetic methods compared to other scaffolds.

Synthesis

Synthetic methods for 9-hydroxy-4H-pyrido[1,2-a]pyrimidin-4-one derivatives (FF compounds) and the numbering system of these frameworks were outlined in Scheme 1. 2-Aryl-9-hydroxy derivatives were synthesized by the

condensation reactions of β -ketoesters (**2a–d**) and 2-amino-4-hydroxypyridine **1** under heating in polyphosphoric acid (Method A). Nitration of **FF-053** yielded **FF-054**, and the subsequent reduction process gave the 3,8-diamino-9-hydroxy derivative **FF-055**. 3-Aryl-9-hydroxy derivatives were synthesized by Method B, wherein arylacetic acid esters (**2e, f**) were converted to 2-aryl-3-(dimethylamino) acrylate intermediates **3**, followed by condensation reaction with **1** under heating in acetic acid to yield **FF-058** and **FF-061**, respectively. Nitration of **FF-058** gave **FF-059**, and subsequent reduction gave the 8-amino-9-hydroxy derivative **FF-060**. *N*-Aryl-9-hydroxy-3-carboxamide derivatives were synthesized by newly developed Mg (II) trifluoromethanesulfonate-mediated coupling reactions between the corresponding ethyl 3-oxo-3-(arylamino)

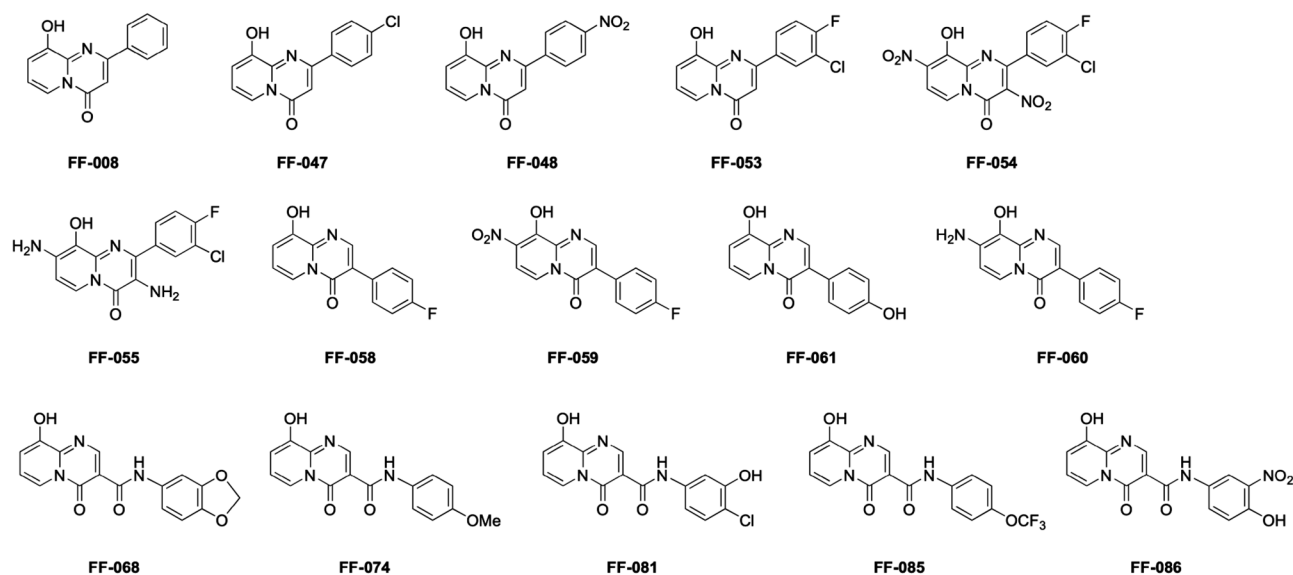


Fig. 6 Synthesized 9-hydroxy-4H-pyrido[1,2-a]pyrimidin-4-one derivatives

propanoate derivatives **4** and oxazolo[4,5-*b*]pyridine **5** derived from **1** (Method C). Experimental procedures and characterization data for synthetic FF compounds are provided in the Supplementary Information.

HK inhibitory activity of the synthesized compounds

The 15 synthesized candidate compounds (Fig. 6) were first evaluated at 100 $\mu\text{g/mL}$ for their inhibition of WalK(207-611) and VicK(31-450) autophosphorylation. Compounds showing >30% inhibition (i.e., <70% residual activity compared to the DMSO control) were subjected to IC_{50} determination: IC_{50} values against WalK(207-611) ranged from 105–305 μM and those against VicK(31-450) from 4.39–1817 μM (Table 1). No compounds had stronger inhibitory activity against WalK(207-611) than waldiomycin (13.1 μM), although **FF-008**, **054**, **060**, and **081** showed relatively strong inhibition. However, the IC_{50} values against VicK for **FF-055** (6.92 μM) and **FF-060** (4.39 μM) were lower than that of waldiomycin (25.8 μM) (Fig. S2 and Table 1). The amino acid sequences constituting the H-box regions of WalK and VicK are highly similar (Fig. 1d). However, the structural differences between the dimeric forms of WalK and VicK have not yet been fully elucidated. The conformation of the H-box regions is affected by the prior and subsequent regions and the CA domain. In addition, according to the binding model shown in Fig. 10, **FF-060** and **FF-055** are predicted to bind to a site that is close to but distinct from the waldiomycin-binding site and inhibit the histidine kinase activity of the CA domain. Our results provide no evidence that **FF-060** and **FF-055** bind to the binding site through the identical interactions between WalK and VicK. Therefore, it is

Table 1 IC_{50} of the synthesized compounds for WalK and VicK

Compounds	IC_{50} (μM)	
	WalK ₂₀₇₋₆₁₁	VicK ₃₁₋₄₅₀
FF-008	105	1817
FF-047	>367*	>367
FF-048	>353	>353
FF-053	>344	>344
FF-054	119	167
FF-055	354	6.92
FF-058	>390	>390
FF-059	>322	>322
FF-060	160	4.39
FF-061	>393	>393
FF-068	305	354
FF-074	229	276
FF-081	176	312
FF-085	>274	>274
FF-086	>292	665
Waldiomycin	13.1	25.8

*Values with > indicate that the compounds showed less than 30% inhibition of HK activity at 100 $\mu\text{g/mL}$ in the first screening

possible that structural differences between the dimeric forms of WalK and VicK contribute to the enhanced affinity of **FF-060** and **FF-055** for VicK. The potent inhibitory activities of **FF-055** and **FF-060** against VicK(31-450), and **FF-054** against both WalK(207-611) and VicK(31-450), lead us to further examine how these compounds inhibit the activity.

Table 2 Effects of **FF-054**, **FF-055** and **FF-060** on the histidine kinase activity of EnvZ mutated at S242 and T247

Compounds	IC ₅₀ (μM)		
	EnvZ	EnvZ S242A	EnvZ T247A
FF-054	259	618	237
FF-055	20.2	139	15.5
FF-060	22.4	139	26.5
Waldiomycin	71.1	>712*	>712

*Values with > indicate that the compound showed less than 50% inhibition of HK activity at the maximum concentration (500 μg/mL)

We previously showed that mutations within the H-box region rendered the site-directed mutant proteins of EnvZ(223–450) mutants highly resistant to waldiomycin [2]. These showed autophosphorylation activities comparable with that of the wild-type protein in the absence of the inhibitors and had enough stability under the measurement conditions [2]. We compared the inhibitory potential of the FF compounds against EnvZ(223–450) and its S242A and T247A mutants (Table 2): **FF-054** inhibited EnvZ(223–450) and EnvZ(223–450)T247A at the same level, but not EnvZ(223–450)S242A, which became modestly resistant. **FF-055** and **FF-060** also showed similar levels of inhibition against EnvZ(223–450) and EnvZ(223–450)T247A, but weaker inhibition against EnvZ(223–450)S242A. These results suggest that S242 (which is adjacent to the site of autophosphorylation H243 (Fig. 1b,d)) is related to the binding of **FF-055** and **FF-060**, while T247 (which is within the H-box and has a side chain protruding in a similar direction to S242) is not. Our results suggest that **FF-054**, **FF-055**, and **FF-060** may bind to the S242 side of H243, in contrast to our model of the binding of waldiomycin to EnvZ(223–289) where the NQ moiety interacts with the T247 side of H243 [2].

Binding of 4H-pyrido[1,2-a]pyrimidin-4-one derivatives to the DHp domain

We performed NMR analyses of EnvZ(223–289) in the presence of **FF-054**, **055**, and **060** to further clarify the binding. EnvZ(223–289) is composed of two helices, helix I (R234–M258) and helix II (G264–L288), connected with a five-residue short loop (Fig. 1b) [2]. Helix I contains the H243 autophosphorylation site and the H-box region (M238–I252) (Fig. 1b, d). EnvZ(223–289) exists as a four-helix bundle dimer in solution.

¹H–¹⁵N HSQC spectra of ¹⁵N-labeled EnvZ(223–289) were obtained and the chemical shifts assigned (Fig. S2(a)) as previously [25]. All signals except for P248 and S260 were assigned. The signal intensity of S260 was too weak

for assignment. **FF-054**, **FF-055**, and **FF-060** at a concentration of 200 μM were added to 100 μM of ¹⁵N-labeled EnvZ(223–289) to determine whether these compounds bind directly to EnvZ(223–289). The chemical shift perturbations in the ¹H–¹⁵N HSQC spectra (Fig. S3) between treated (200 μM or 1 mM compounds) and untreated (DMSO) samples were calculated (Y axis of Fig. 7). The addition of **FF-054** to EnvZ(223–289) affected the chemical shifts ($\Delta\delta > 0.02$ ppm) of the V241, D244, and R246 residues in the H-box region (Fig. 7a). The chemical shifts of S242, R246, and T247 showed $\Delta\delta > 0.02$ ppm only when the concentration of **FF-060** was 1 mM, suggesting that the addition of **FF-060** to EnvZ(223–289) has a dose-dependent effect on the H-box region. Similar analysis was performed with **FF-055**, but it resulted in a weaker influence (Fig. S3(c)). Higher **FF-055** concentrations could not be analyzed due to the limited amount of synthesized **FF-055**. We previously showed [2] that the addition of waldiomycin to EnvZ(223–289) results in a reduction in the residue signal intensities in the H-box and C-terminal X-regions (Fig. 1b, 270–289; a region composed of central hydrophobic and flanking charged residues). The X-region may be important for hydrophobic interactions between the helices [26]. A similar reduction by waldiomycin was observed herein (Fig. S4(c)). Signal intensity reduction was also observed for the addition of **FF-054** and **060** (Fig. S4a, b, d), although the decrease was not as significant for waldiomycin. This may be due to the smaller sizes of the FF compounds compared to waldiomycin. $\Delta\delta$ for the addition of waldiomycin could not be calculated in our previous study [2] due to the overly reduced signal intensities. However, we were able to detect the reduced signals and to determine $\Delta\delta$ for the addition of 200 μM waldiomycin in the present study (Fig. 7c). The difference between the two studies may have been caused by the DMSO concentration in the NMR samples (5% herein and 10% previously), or the difference in the NMR probe used. Waldiomycin affected the amino acid residues G240 and L249 within the H-box and N271, N278, A279, I280, E282, Y287, and R289 within the X-region, reconfirming that waldiomycin binds to the H-box and X-region.

Our NMR analyses indicated that both S242 and T247 were affected by the addition of **FF-060** and waldiomycin. Accordingly, the EnvZ S242A and T247A mutants conferred resistance to waldiomycin (Table 2, ref. [2]). However, **FF-060** induced resistance only in the EnvZ S242A mutant and not in the T247A mutant (Table 2). This suggests that T247 is not involved in **FF-060** binding and that the chemical shift perturbation observed for T247 (Fig. 7d) might be induced indirectly. Indeed, AS/MS analysis of the binding of waldiomycin and **FF-060** to the H-box of VicK (HK) suggested that **FF-060** and waldiomycin bind to distinct sites within the H-box region.

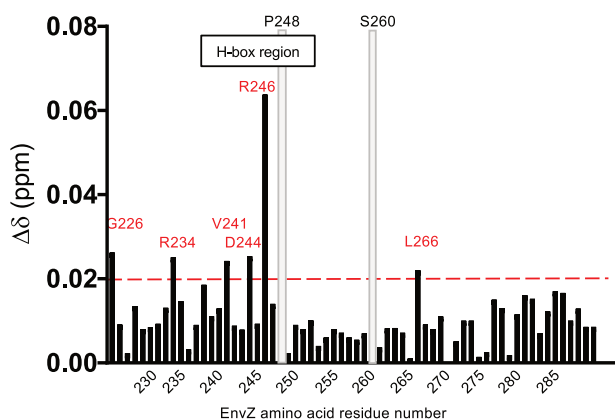
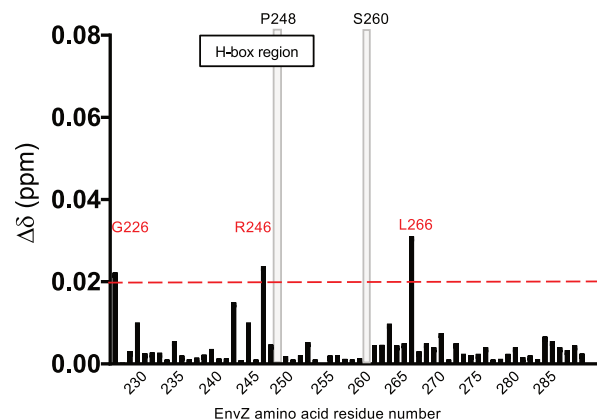
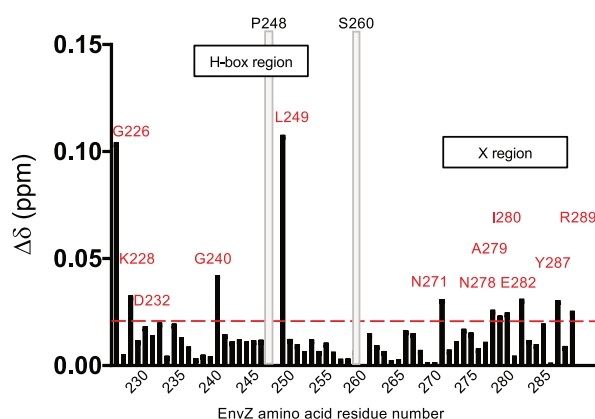
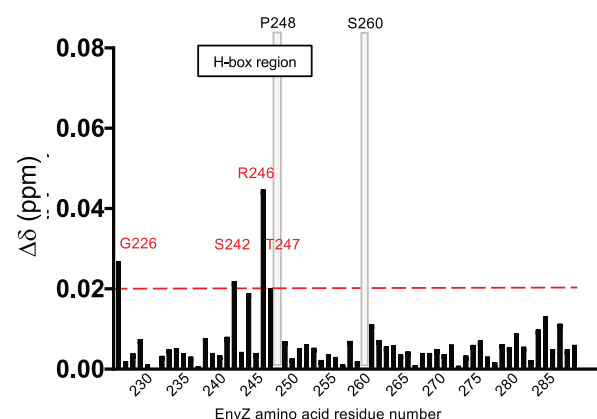
(a) 100 μ M EnvZ₂₂₃₋₂₈₉ vs 200 μ M FF-054(b) 100 μ M EnvZ₂₂₃₋₂₈₉ vs 200 μ M FF-060(c) 100 μ M EnvZ₂₂₃₋₂₈₉ vs 200 μ M waldiomycin(d) 100 μ M EnvZ₂₂₃₋₂₈₉ vs 1 mM FF-060

Fig. 7 Effects of **FF-054**, **FF-060**, and waldiomycin on the chemical shifts of amino acid residues within the EnvZ DHp domain in ^1H - ^{15}N HSQC spectra. **a** 100 μ M ^{15}N -labeled EnvZ(223-289) with 200 μ M **FF-054**; **b** 100 μ M ^{15}N -labeled EnvZ(223-289) with 200 μ M **FF-060**; **c** 100 μ M ^{15}N -labeled EnvZ(223-289) with 200 μ M waldiomycin (note

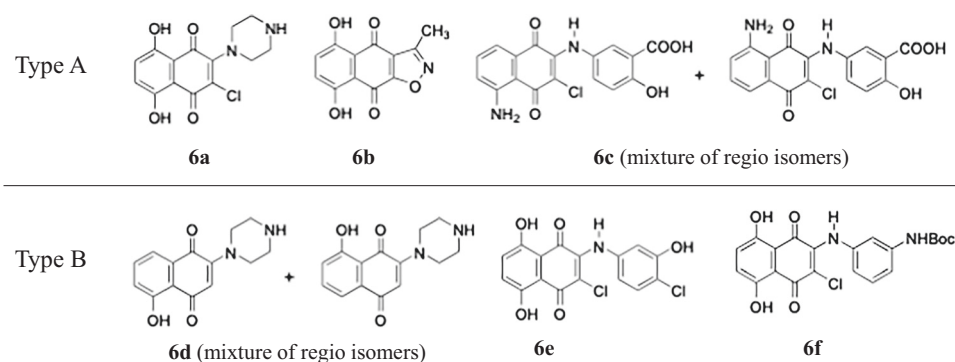
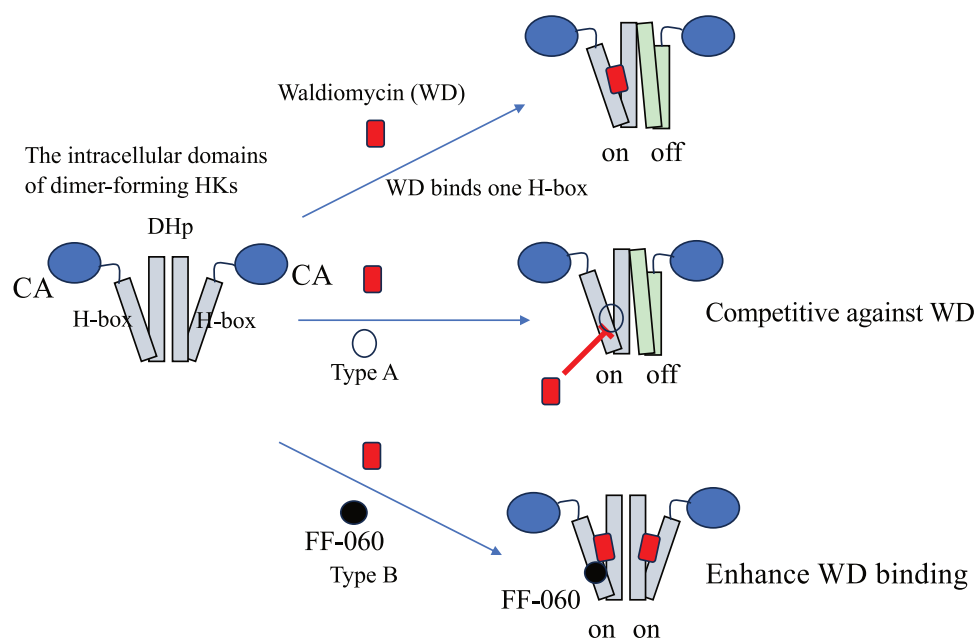
that the y-axis range is different to the other panels); **d** 100 μ M ^{15}N -labeled EnvZ(223-289) with 1 mM **FF-060**. Red dashed lines correspond to changes of 0.02, and residues showing perturbation of more than 0.02 are labeled. The “H-box region” box indicates the area of the H-box

Effect of FF-060 on waldiomycin–DHp domain binding

Ishikawa et al. [7] used the AS/MS method to classify H-box inhibitors, synthesized as derivatives based on the NQ skeleton, into types A and B (Fig. 8) according to differences in their sites of action (Fig. 9). Type A inhibitors (**6a–c**) competitively inhibit the action of waldiomycin on the DHp domain, whereas Type B inhibitors (**6d–f**) enhance the binding of waldiomycin to the DHp domain. A possible model for the action of such H-box inhibitors is illustrated in Fig. 9. Waldiomycin has been reported to bind to only one of the two H-boxes present in the dimeric DHp domain of HKs [2]. Type A H-box inhibitors share the binding site with waldiomycin, inhibiting its action. In contrast, Type B inhibitors bind to the other site to waldiomycin, facilitating waldiomycin binding to both H-boxes through allosteric effects. Herein, the

effect of **FF-060** on waldiomycin–DHp domain binding was investigated using the AS/MS method (Fig. 10). **FF-060** has been suggested to bind to the H-box region located within the DHp domain. The concentration of waldiomycin that was bound to VicK and released was approximately 50 nM in the absence of **FF-060** and at low (10 μ M and 30 μ M) **FF-060** concentrations (Fig. 10, lanes 5–7). However, the concentration increased to 120 nM for 100 μ M **FF-060**. These results indicate that **FF-060** exhibits an inhibitory mechanism similar to that of the previously reported Type B H-box inhibitors [7].

We calculated AAM descriptors for 10 compounds (waldiomycin NQ, **FF-054**, **FF-055**, **FF-060**, and **6a–f**) to further investigate binding site differences between H-box inhibitors. The calculated 10×10 pairwise AAM similarity matrix contains cosine similarity values, ranging from 1 to -1 . The similarity matrix was converted into a distance matrix by calculating “ $1 - \text{AAM}$

Fig. 8 Chemical structures of naphthoquinone derivatives (**6a–f**)**Fig. 9** Model of inhibition and enhancement of waldiomycin binding to the H-box by Type A (○) and Type B (●) H-box inhibitors

similarity” for each pair. MDS was applied to generate a two-dimensional plot (Fig. 11), where compounds with similar AAM descriptors (and which are thus likely to share similar binding sites) are positioned closer together. The map revealed a clear separation between the two classes of compounds. Type A inhibitors (**6a–c**) and waldiomycin NQ clustered together in the left ($X < -0.08$) region of the plot, whereas Type B inhibitors (**6d–f**, **FF-054**, **FF-055**, and **FF-060**) are grouped on the right ($X > -0.08$) side. The boundaries between these clusters are indicated by a double-headed arrow in Fig. 11. Notably, **FF-054**, **FF-055**, and **FF-060** are positioned with the Type B group, whereas **6a–c** are grouped with waldiomycin NQ (the representative Type A inhibitor). The observed clustering supports the hypothesis that Type A and Type B H-box inhibitors bind to distinct sites on the target protein.

Conclusion

AAM-based screening combined with structural simplification of waldiomycin identified 4*H*-pyrido[1,2-*a*]pyrimidin-4-one derivatives as novel histidine kinase inhibitors that affect the H-box region. Our approach provides a practical strategy for identifying novel inhibitor scaffolds based on amino acid interaction patterns, without requiring detailed structural information of the target protein. Furthermore, visualization of the positional relationships between nine synthesized H-box inhibitors, 4*H*-pyrido[1,2-*a*]pyrimidin-4-ones, in the present study and previously reported naphthoquinones (Fig. 11), based on their AAM descriptors, provided mechanistic insights that correlated with differences in their modes of action. Collectively, our findings suggest that AAM/MDS provides a framework for investigating compounds with diverse modes of action and for generating

Fig. 10 AS/MS analysis of the effect of **FF-060** on waldiomycin binding to the VicK H-box. Variable concentrations of **FF-060** and 5 μ M waldiomycin were incubated in the absence (lanes 1–4) or presence (lanes 5–8) of VicK (1 μ M). Data points represent the mean of triplicate measurements, with error bars showing the standard deviations

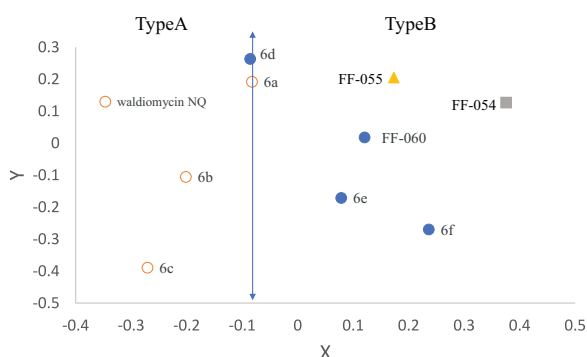
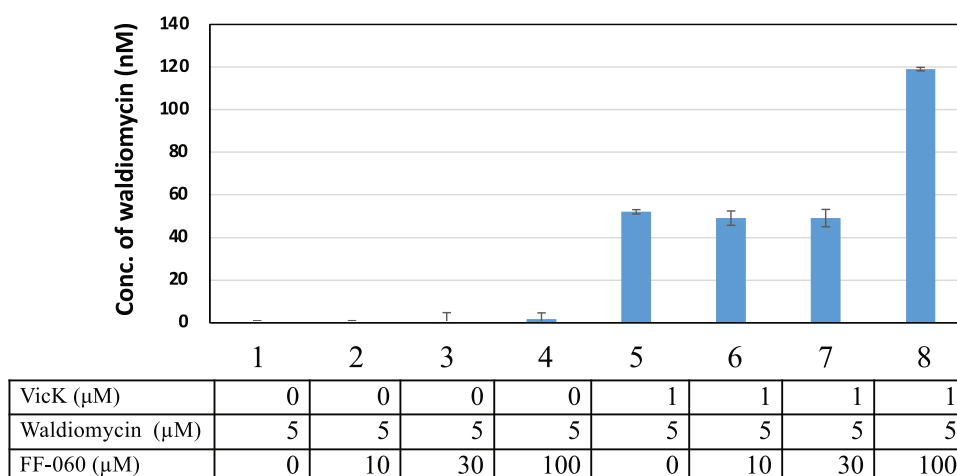


Fig. 11 A two-dimensional map of the correlations between AAM descriptor similarities for naphthoquinone (NQ) and nine H-box inhibitors (**FF-054**, **FF-055**, **FF-060**, and **6a–f**). Type A H-box inhibitors (NQ, **6a–c**) are represented by open circles, and Type B H-box inhibitors (**FF-060**, **6d–f**) by filled circles. **FF-054** and **FF-055** are represented by a square and a triangle, respectively

hypotheses regarding their interaction mechanisms. Future studies will be required to determine whether such information can be leveraged to guide the rational identification of inhibitors with specific functional properties.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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