Heterocyclic Bis-Cations as Starting Hits for Design of Inhibitors of the Bifunctional Enzyme Histidine-Containing Protein Kinase/Phosphatase from *Bacillus subtilis*

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The main mechanism of carbon catabolite repression/activation in low-guanine and low-cytosine Gram-positive bacteria seems to involve phosphorylation of HPr (histidine-containing protein) at Ser-46 by the ATP-dependent HPr kinase, which in *Bacillus subtilis*, *Lactobacillus casei*, and *Staphylococcus xylosus* also exhibits phosphatase activity and is thus a bifunctional enzyme (HPrK/P). Since deficiency of HPrK/P in *S. xylosus*, *L. casei*, and *B. subtilis* mutants leads to severe growth defects, inhibitors of the enzyme could form a new family of antibiotic drugs. The aim of the study was to screen an in-house chemical library for identification of hits as inhibitors of HPrK/P in *B. subtilis* and to further extract additional information of structural features from hit optimization using a radioactive in vitro assay. A symmetrical bis-cationic compound LPS 02-10-L-D09 (**2a**) with a 12-carbon alkyl linker bridging the two 2-aminobenzimidazole moieties was identified as a non-ATP mimetic compound exhibiting an EC_{50} value of 10 μM in a kinase assay with HPr as substrate. The substance also inhibited the phosphatase activity of HPrK/P triggered by the addition of inorganic phosphate. Similar results were obtained with **2a** and catabolite repression HPr, which, like HPr, can be phosphorylated at Ser-46 by HPrK/P and is involved in catabolite repression. Structure−activity relationship analysis indicated the importance in its structure of a substituted 2-aminobenzimidazole. This typical heterocycle is linked through a C12 alkyl chain to a second scaffold that can bear a cationic or a noncationic moiety but in all cases should present an aromatic ring in its vicinity.

Introduction

The major intracellular regulatory mechanism in many biological processes involves phosphorylation and dephosphorylation of proteins by protein kinases and protein phosphatases.[1−3] Since the discovery of protein phosphorylation in the mid-1950s in the case of rabbit skeletal muscle glycogen phosphorylase,[4] a large number of protein kinases and phosphatases have been characterized in a variety of systems.[1,2] For prokaryotes, the first conclusive evidence for the existence of protein kinase activity was presented in the late 1970s in *Salmonella typhimurium*[5] but is now well-known in a number of other bacterial species.[6]

In low-GC (guanine, cytosine) Gram-positive bacteria such as *Bacillus subtilis*, the main regulatory mechanism in the hierarchical use of carbon sources seems to involve phosphorylation of HPr (histidine-containing protein) at Ser-46 by the ATP-dependent HPr kinase.[7−15] HPr is a small phosphocarrier protein of the bacterial phosphoenolpyruvate (PEP)/sugar phosphotransferase system (PTS) that catalyzes phosphorylation and concomitant transport of PTS carbohydrates.[16] When HPr is phosphorylated by enzyme I (EI) at His-15, the phosphoryl group can then be transferred to the sugar-specific enzymes II (EIIAs) of the PTS system.[16] HPr of low-GC Gram-positive bacteria can also be phosphorylated at Ser-46 in the presence of ATP by HPr kinase in a mechanism that exclusively serves regulatory purposes.[7−11] In addition to its kinase activity, it has been shown for *Enterococcus faecalis*,[12] *B. subtilis*,[12] *Lactobacillus casei*,[13] and *Staphylococcus xylosus*[14] that the enzyme also possesses HPr(Ser-P) phosphatase activity, which becomes prevalent in the presence of inorganic phosphate. Thus, in these species it is a bifunctional enzyme (HPrK/P). Furthermore, HPrK/P of *B. subtilis* has been found to be a homo-oligomeric enzyme that shows strong positive cooperativity for fructose-1,6-bisphosphate (FBP), ATP, and other nucleotides.[17,18]

In a complex medium with different carbohydrates, the bacteria preferentially utilizes the one that allows the fastest growth, e.g., glucose, whereby the expression of secondary catabolic genes is repressed. The regulatory mechanism involves activation of the kinase of HPrK/P, which is typically stimulated by glycolytic intermediates such as FBP, although not in *E. faecalis*[12] and *Streptococcus salivarius*,[15] with concomitant phosphorylation of HPr at Ser-46.[7−15] HPr(Ser-P) then forms a

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complex with CcpA (catabolite control protein A),[19] a member of the GalR/LacI family of transcriptional repressors/activators,[20] and this complex interacts with cis-active operator sequences, cres (catabolite responsive elements).[21,22] Carbon catabolite repression (CCR)[23−26] and also carbon catabolite activation (CCA) of, for example, glycolysis and carbon overflow metabolism[27−29] are assumed to be the consequence of this complex binding to DNA.

Another protein, Crh (catabolite repression HPr), for which the gene was discovered within the *B. subtilis* genome sequencing program,[30] exhibits 45% sequence identity with HPr.[31] Crh can also be phosphorylated at Ser-46 by HPrK/P and has been shown to be implicated in CCR.[24,31,32] However, His-15 is replaced by a Gln residue that prevents phosphorylation by PEP and EI and is therefore probably not involved in the PTS transport of sugar.[31] Thus, Crh is presumed to carry out exclusively regulatory functions.[31]

Since HPrK/P-deficient *S. xylosus*,[14] *L. casei*,[13] and *B. subtilis* HPrK/P mutants[33] as well as mutants affecting in particular the phosphatase activity[34,35] lead to severe growth defects and since the main mechanism of CCR/CCA is suggested to involve the enzyme HPrK/P, inhibitors of HPrK/P could form a new family of antibiotic drugs. The need for new antibiotics can be seen in light of the increasing problem of pathogenic bacteria resistant to most known antibiotic drugs. For example, more than 95% of *Staphylococcus aureus* strains worldwide are resistant to penicillin and up to 60% are resistant to methicillin.[36] Although efforts are made to reduce the spread of antibiotic resistance, the problem still remains. The current situation strengthens the need for novel types of antibacterial agents with unique targets such that cross-resistance does not readily occur.

The rationale for building a focused library deals with small peptides able to mimic the residue Ser-46, taking into consideration both surrounding amino acids (Lys-Ser-Ile). However, the most potent compound exhibited moderate inhibitory capacity ($EC_{50} \approx 500\ \mu M$). Recently, a chemical library of synthetic compounds (see Experimental Section) originated from different teams and medicinal chemistry projects were established within the Laboratoire de Pharmacochimie de la Communication Cellulaire CNRS UMR 7081. The substances constituting the library were designed in the past as putative ligands of various central nervous system (CNS) and non-CNS targets including enzymes, receptors, and transport systems. Thus, a large chemical diversity (various heterocyclic compounds, synthetic amino acid derivatives, small peptides, etc.) could be found within the in-house library. In addition to storage in standard vials, the library is available for automated screening stored in 96-well plates with 80 substances per plate, allowing control samples in a total of 16 wells (first and last columns). The initial dilution was made with dimethyl sulfoxide (DMSO) to a final concentration of around 30 mM, and the plates were stored under argon at 4 °C. The structures of the compounds as well as characteristics and other data are stored in a relational database using the ISISBase software. The first version (about 1500 compounds) was screened in order to identify potential hits acting as HPrK/P inhibitors. Among them, an interesting compound was identified (**2a** in Figure 1A, $EC_{50} = 10\ \mu M$) and served as starting hit for further structural optimization. The first structure−activity relationship analysis is presented in this paper.

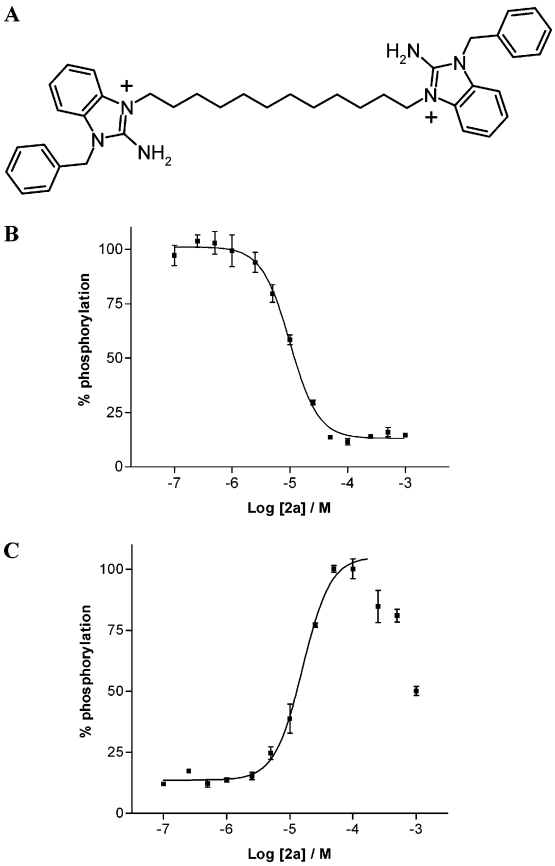
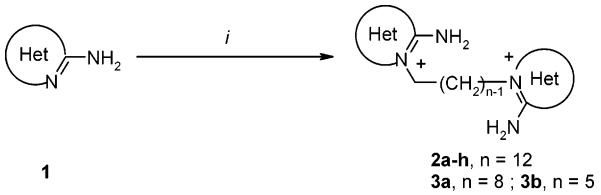


Figure 1. (A) Chemical structure of LPS 02-10-L-D09 (**2a**) (oxalate salt). (B) Phosphorylation assay with HPr (10 μM) after adding different amounts of **2a**. Data represent the mean ± SEM ($n = 3$). The EC_{50} value was determined to be 10 μM. (C) Dephosphorylation assay with HPr(Ser-P) (8 μM). After complete phosphorylation, inorganic phosphate (K_2HPO_4 and KH_2PO_4) was added to give a final concentration of 1 mM together with different concentrations of **2a**. The mixture was further incubated at 37 °C for 2 h. Addition of inorganic phosphate together with the three highest concentrations of **2a** to the assay mixtures led to precipitation, and these samples were excluded. The EC_{50} value was determined to be 14 μM. Data represent the mean ± SEM ($n = 3$).

Scheme 1[a]

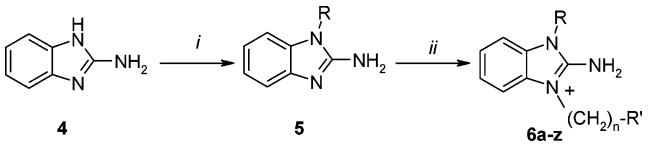


[a] Reagents and conditions: (i) 0.5 equiv of $Br(CH_2)_nBr$.

Chemistry

The symmetrical bis-cationic reference compound **2a** and other related compounds were prepared as described in Scheme 1. Depending on the nucleophilicity of the starting heterocyclic amidine **1**, the reaction was performed in refluxing butanone, acetone, or sulfolane in the presence of dibromoalkanes, affording the awaited

Scheme 2[a]



[a] Reagents and conditions: *i*: R−X, KOH, ethanol; *ii*: 2-butanone, 3 equiv. of $Br(CH_2)_nR'$ **7**, 80°C, 48 h, 55−95%.

bis-cations **2a**−**h** as dibromides in good yields (60−95%). When the crude salt was not pure enough, the free base could be chromatographed on silica gel and crystallized as an oxalate (**2a**−**d**). Because of the low reactivity for N-substituted 2-aminobenzimidazole **4** toward dibromoalkanes, the reaction needed to be performed in sulfolane in order to avoid significant amounts of the monosubstituted derivative. The progressive alkylation of the unsubsituted 2-aminobenzimidazole **4** using different functionalized halides $R'(CH_2)_nX'$ **7** was carried out as described in Scheme 2. The alcohol derivatives **6r** and **6s** (R′ = OH) served as intermediates because they were submitted to oxidation in the presence of chromium trioxide to afford the corresponding carboxylic acids **8a** and **8b**. Removal of the *N*-Boc protection in **6w**−**y** afforded the free amino derivatives **9a**−**c**. Finally, the treatment of the bromo derivatives with secondary amines yielded the unsymmetrical bis-cations **10a**−**i** (Scheme 3).

Enzymatic Kinase Assay

$HPr(His)_6$, $Crh(His)_6$, and HPrK/P(Trx-His_6-S-tag) from *B. subtilis* were purified by Ni-NTA affinity chromatography as described previously.[17] The degree of phosphorylation of $HPr(His)_6$/$Crh(His)_6$ (10 μM) by HPrK/P(Trx-His_6-S-tag) (100 nM) with and without the addition of test compound was determined in an in vitro kinase assay at pH 8.0 using ATP as the phosphoryl donor and with the addition of 2 mM FBP and 0.1% bovine serum albumine (BSA) (see the experimental part).

EC_{50} Curve. The EC_{50} (50% inhibition of phosphorylation/dephosphorylation) curves were based on 13 consecutively diluted concentrations of tested inhibitor included in the assay before initiating the reaction with the addition of enzyme. Typically, each concentration was tested in triplicate. The volume was 2 μL of the inhibitor dissolved in DMSO (10% v/v final DMSO concentration). Control reaction mixtures contained 10% v/v DMSO instead of the test compound.

ATP Competition Assay. Different concentrations of ATP (0.5, 0.75, 1, 2, and 3 mM) were included in the assay with the addition of tested inhibitor at a constant concentration equal to its EC_{50} value. This 6-fold increase in ATP concentration would correspond to a decrease in the inhibitor concentration to a similar extent if the inhibitor is directly competing with ATP. Since the nonspecific binding of ATP increased with increasing concentration of ATP, the values for the samples containing no enzyme (blank) were subtracted from the corresponding values for the samples containing enzyme.

Distinction of Inhibitor Type. HPr/Crh was used over a concentration range from 0.1 to 200 μM with and without three different fixed concentrations of tested inhibitor, which are the concentration equal to its EC_{50} value and the half and the double of this concentration. Initial velocities (pmol/min) were plotted against HPr/Crh concentrations.

Interdomain Interaction. The inhibitor was tested at three different constant concentrations: the concentration equal to its EC_{50} value, a lower concentration, which caused only a small inhibition, and a concentration that gave maximal inhibition at different concentrations of ATP ranging from 1 μM to 1 mM. A change in the Hill coefficient for ATP was used as an indication of an interaction between the subunits.

Multiple Drug Effect/Combination Index. For multiple drug effects, the combination index (CI) method described by Chou-Talalay was used.[37] Briefly, two inhibitors were included in the assay at equipotent doses; i.e., the concentrations of the individual substances were varied, but the ratio between them was kept constant, equal to the ratio at their EC_{50} values. At least 13 different conditions, tested in triplicate, were included. The CI was calculated at EC_{50} according to the equation

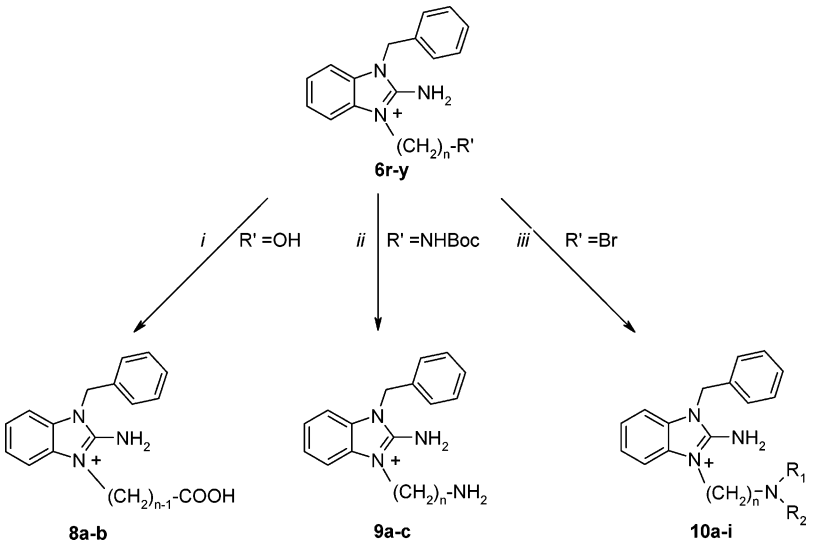
$$\mathrm{CI} = \frac{D_1}{D_{x,1}} + \frac{D_2}{D_{x,2}} + \frac{\alpha D_1 D_2}{D_{x,1} D_{x,2}}$$

where D_1 and D_2 refer to the concentrations of each individual substance when mixed together. The terms $D_{x,1}$ and $D_{x,2}$ are the concentrations of the two different substances when each compound is tested alone. The value of α is 0 if the two compounds are mutually exclusive and is 1 if they are mutually nonexclusive. The tool to classify the effects of two compounds is the median-effect plot, where log(dose) was plotted against log(fraction phosphorylated/fraction nonphosphorylated). If the median-effect plots of each individual compound, and when mixed together, are parallel or if the compounds have similar modes of action, then α is 0. However, if the plots of the individual compounds are still parallel but the plot of the mixture of the two compounds is upwardly concave, or if the two compounds have independent modes of action, then α is 1. A CI value less than 1 indicates synergism, a value equal to 1 indicates summation, and a value greater than 1 indicates antagonism.

Results

Phosphorylation Assay. To study the phosphorylation reaction of $HPr(His_6)$/$Crh(His_6)$, a radioactive assay for HPrK/P(Trx-His_6-S-tag) was developed. The phosphoryl donor [γ-^{32}P]ATP was used together with an excess of about 5 mM $MgCl_2$. The final concentration of FBP was 2 mM, since higher concentrations did not increase the degree of phosphorylation. The reaction was performed at pH 8 using Tris as the buffer and initiated by a 100 times lower concentration of the enzyme compared to protein substrates. Except for the necessary ingredients for the reaction, BSA was included in the assay to avoid nonspecific binding. A final concentration of 0.1% BSA was used, and an increase in concentration did not result in further improvement. Parvalbumin was also evaluated but was less efficient than BSA. After incubation, it was furthermore necessary to immediately place the P81 papers containing the samples in phosphoric acid solution. If the papers were

Scheme 3[a]



[a] Reagents and conditions: (i) CrO_3, H_2SO_4, acetone/water, 30 min, 60−75%; (ii) HCl_g, AcOEt; (iii) acetonitrile, 3 equiv of HNR_1R_2, 85 °C, 48 h, 40−70%.

Table 1. Characteristics of **2a** Alone, ADP Alone, and When Mixed Together[a]

	EC_{50} (μM) mean (95% confidence interval)		Hill slope mean (95% confidence interval)		CI ($\alpha = 0$)	
inhibitor	HPr	Crh	HPr	Crh	HPr	Crh
		Kinase Assay				
2a	10 (8.6−12)	21 (17−25)	1.8 (1.3−2.2)	2.4 (1.5−3.3)		
ADP	4300 (2400−7800)	7400 (6400−8700)	0.90 (0.5−1.3)	1.4 (1.2−1.7)		
2a + ADP	5.2 (4.8−5.6) + 2100 (1900−2200)	12 (11−13) + 4200 (3800−4600)	2.2 (1.9−2.5)	2.3 (1.9−2.7)	1.0	1.1
		Phosphatase Assay				
2a	16 (14−19)	14 (8.5 to 23)	2.1 (0.8−3.5)	1.7 (0.6−2.8)		

[a] Mean (=3) and 95% confidence intervals for the EC_{50} values and the Hill slope values were estimated using the program GraphPad Prism. The following equations were used: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/\{1 + 10^{[X - \log(EC_{50})]\text{Hill slope}}\}$ for phosphorylation of HPr/Crh; $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/\{1 + 10^{[\log(EC_{50}) - X]\text{Hill slope}}\}$ for dephosphorylation of HPr/Crh. X is the logarithm of the concentration of inhibitor, and Y is the response.

allowed to dry at room temperature before being washed with phosphoric acid, a lower degree of phosphorylation was obtained possibly because of the phosphate content of the papers, which triggered the enzyme to act as a phosphatase. Since the samples were prepared consecutively, the time spent in the first wash with phosphoric acid for the P81 papers was investigated. A longer time spent in the first wash, up to 1 h, did not influence the results significantly.

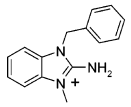
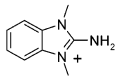
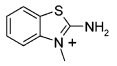
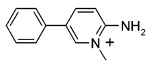
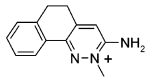
To obtain a fast, simple, and convenient screening procedure, a somewhat modified kinase assay was used in an automated 96-well format. Because the substances in the library were diluted with DMSO, this solvent needed to be included in the assay. Control experiment revealed that 10% v/v of DMSO did not affect the degree of phosphorylation. With use of a dispensing robot, the phosphorylation reaction was terminated by adding 75 mM H_3PO_4 to the samples in the wells in the same order as the reactions were initiated before spotting the samples on P81 paper 96-well filter plates. This procedure was performed to ensure that all enzymatic activity was prevented on the P81 papers because the papers contain phosphate and the robot worked column-by-column. The washing procedure to remove unreacted ATP from the P81 papers was subsequently carried out with the plates mounted on a vacuum manifold.

Parent Inhibitor. The screening campaign was performed with the different compounds from the in-house library at a final concentration of approximately 30 μM. From this first screening, a single substance, compound **2a** (Figure 1A), was identified and submitted to further investigations. It is a symmetrical bis-cationic heterocyclic compound that was the only active structure identified among the substances in the library at the chosen activity level (30 μM), which otherwise contained at least 18 other different bis-cationic compounds. In the phosphorylation assay with HPr, this substance resulted in an EC_{50} value of 10 μM (Figure 1B) and a value in the same range (21 μM) was obtained in a kinase assay with Crh (Table 1). Since BSA has been reported to significantly increase the EC_{50} value for inhibitors of tyrosine kinase c-Src,[38] an experiment was performed with an assay containing HPr/Crh and the bis-cationic compound but without BSA. The doses required to produce the EC_{50} were found to be comparable (10 and 11 μM for HPr, 21 and 19 μM for Crh) for the assays with and without BSA. The enzyme was also preincubated with the inhibitor for 1 h at 37 °C, and then the reaction was initiated by adding HPr. The obtained EC_{50} value was the same as that received without preincubation, consistent with a rapid equilibrium enzyme system.

ATP Competition Assay and Synergism/Antagonism Analysis. 2a was further investigated for its ability to compete directly with ATP. Increasing concentrations of ATP, up to 6-fold, were included in a

Table 2. Importance of the Heterocyclic Moiety for HPrK/P Inhibitory Activity in a Kinase Assay with HPr

Het-$(CH_2)_{12}$-Het

	Heterocycle[a]		Formula[b]	m.p. °C	% inhibition at 100 μM
2a		B	$C_{44}H_{52}N_6O_8$	191	88
2b		B	$C_{32}H_{44}N_6O_8$	182	64
2c		B	$C_{30}H_{38}N_4O_8S_2$	172	<5
2d		B	$C_{22}H_{34}N_4O_8$	126	<5
2e		A	$C_{22}H_{36}Br_2N_4$	231	<5
2f		A	$C_{34}H_{44}Br_2N_4$	183	<5
2g		A	$C_{36}H_{46}Br_2N_6$	266	<5
2h		A	$C_{30}H_{40}Br_2N_4$	172	<5

[a] A, bromide; B, oxalate. [b] CHN analysis.

phosphorylation assay with **2a** at a concentration equal to its EC_{50} value. Since the nonspecific binding of the phosphate donor increased with increasing concentrations of ATP, the values for the samples containing no enzyme (blank samples) were subtracted from the samples containing enzyme. The degree of phosphorylation did not change with increasing concentrations of ATP, suggesting that the substance was not competing with the binding of ATP substrate under these assay conditions. To further support the nonrecognition of the ATP site by the bis-cationic compound, ADP was used as an ATP competitor and synergism/antagonism analysis was performed. The EC_{50} values for ADP alone in an assay with HPr was determined to be 4 mM (Table 2). A competition assay with the bis-cationic compound in combination with ADP was performed at a constant ratio of the concentrations equal to the ratio of the compounds at their EC_{50} values when tested alone. The concentrations obtained for each compound at EC_{50} when combined were 5 μM **2a** and 2 mM ADP (Table 1). The data were graphed with the median-effect plot of the dose−effect relationship, where log(dose) was plotted against log(fraction phosphorylated/fraction nonphosphorylated) for **2a** alone, ADP alone, and **2a** with ADP (Figure 2). Parallel plots of the mixture with respect to the parent substances indicate that the inhibitory effect of **2a** and ADP is mutually exclusive; thus, $\alpha = 0$. The CI value for the combination when $\alpha = 0$ was determined to 1.0, which reveals an additive effect of the two substances in a mixture. Under the same conditions, using Crh as a protein substrate instead of HPr, a similar CI value, 1.1, was obtained (Table 1). These experiments support an action on two different sites for ADP and the bis-cationic compound.

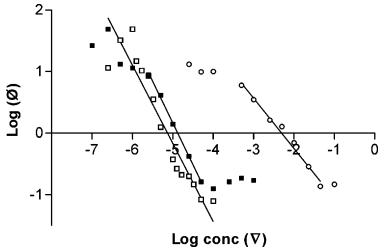


Figure 2. Median-effect plot of the dose−effect relationship of phosphorylation of HPr. The log(ϕ) function (which is log(fraction phosphorylated/fraction nonphosphorylated)) was plotted against log conc (∇) (which are the concentrations of individual compounds (when mixed together the concentration of the bis-cationic compound is shown in the graph)): **2a** alone (■); ADP alone (○); **2a** plus ADP (□). Parallel plots of the mixtures with respect to the parent compounds indicate that the inhibition of the phosphorylation of **2a** and ADP is mutually exclusive.

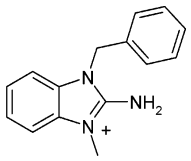
Distinction of Inhibitor Type and Interdomain Interaction. To distinguish between different types of inhibition, **2a** was included at three different fixed concentrations (equal to its EC_{50} value and the half and the double of this concentration) in an assay with HPr/Crh at protein concentrations ranging from 0.1 to 200 μM. Nonlinear regression analysis indicated that the substance is a competitive inhibitor, since the values for V_{max} were constant and those for $K_{0.5}$ were increased. To further evaluate the inhibition, the substance was also tested for potential interface properties because HPrK/P of *B. subtilis* exists as a homo-oligomeric enzyme with a strong positive cooperativity for FBP, ATP, and other nucleotides.[17,18] In an assay with HPr present at different concentrations of ATP, ranging from 1 μM to 1 mM, the inhibitor was tested at three different constant concentrations, i.e., the concentration equal to its EC_{50} value, a lower concentration, which caused only a small inhibition, and a concentration that gave maximal inhibition. The Hill coefficient did not change (around 4), consistent with no change in cooperativity, and thus suggests that **2a** does not seem to participate in interface interactions between the different subunits.

Phosphatase Assay. Since HPrK/P is a bifunctional enzyme that in addition to its kinase activity also possesses phosphatase activity, the inhibitor was added at different concentrations together with a final concentration of 1 mM inorganic phosphate to trigger the phosphatase activity in an assay with HPr/Crh after complete phosphorylation. The three highest concentrations of **2a** together with inorganic phosphate, prepared in advance, were excluded when the EC_{50} value was determined because precipitation was observed after adding the solutions to the assay mix. Compound **2a** inhibited dephosphorylation of HPr(Ser-P) and Crh(Ser-P) with an estimated EC_{50} value of 16 and 14 μM, respectively (Figure 1C, Table 1).

The inhibition of both phosphorylation and dephosphorylation by the bis-cationic compound was cooperative with a Hill coefficient around 2 (Table 1). In contrast, ADP did not show such cooperativity under the experimental conditions used.

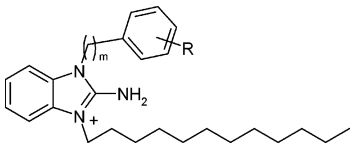
Hit Optimization. A series of derivatives of **2a** and structurally related compounds were prepared and tested at final concentrations of 10 and 100 μM in a kinase assay using HPr as the protein substrate. Data

Table 3. Correlation between the Linker Length and the EC_{50} Values for HPrK/P Inhibition by **2a** and Its Homologues

Q = 

	compound	EC_{50} (μM) mean (95% confidence interval)		Hill slope mean (95% confidence interval)	
		HPr	Crh	HPr	Crh
2a	**Q**$(CH_2)_{12}$**Q**	10 (8.6−12)	21 (17−25)	1.8 (1.3−2.2)	2.4 (1.5−3.3)
3a	**Q**$(CH_2)_8$**Q**	20 (16−25)	71 (64−78)	1.1 (0.9−1.3)	2.2 (1.8−2.6)
3b	**Q**$(CH_2)_5$**Q**	93 (72−120)	210 (170−250)	1.3 (0.9−1.6)	1.3 (1.1−1.6)
6a	**Q**$(CH_2)_{11}CH_3$	29 (27−32)	32 (28−36)	5.3 (2.8−7.8)	6.4 (3.5−9.3)

Table 4. Effects of N1 Substitution on HPrK Inhibition of **6a** and Its Derivatives



	m	R	% inhibition at 100 μM (SEM), $n = 3$	% inhibition at 10 μM (SEM), $n = 3$
6a	1	H	90(±1)	18(±2)
6b	2	H	98(±1)	0(±2)
6c	3	H	96(±1)	38(±4)
6d	1	4-CH_3	23(±4)	0(±2)
6e	1	4-Cl	21(±4)	17(±4)
6f	1	3,4-Cl	51(±8)	9(±4)
6g	1	4-OCH_3	95(±1)	34(±2)

are listed in Tables 2−5. Replacing the benzimidazole ring in **2a** by other heterocycles, including monocycles (pyridines **2e** and **2f**, pyridazine **2g**, imidazole **2d**) and bicycles (isoquinoline **2h**, benzothiazole **2c**), led to inactive compounds (Table 2). These results emphasized the critical role played by the benzimidazole ring for HPrK inhibition. In addition the *N*-benzyl moiety seemed to significantly increase the potency (compare **2a** with the corresponding *N*-methyl derivative **2b**).

Table 3 highlights the effect of the length of the linker between the cationic heads and both their potency and selectivity toward phosphorylation inhibition of HPr and Crh. Shortening the length progressively led to decreased potencies. In particular, the pentylene derivative **3b** was found to be about 10 times less potent than the reference compound **2a**. It is noteworthy that suppressing the second cationic head, but with the same lipophilic side chain, led to a compound (**6a**) with 3 times less potency when compared to the reference compound in a kinase assay with HPr. However, the mode of action of this monocation **6a** is probably different as shown by the strong difference in their Hill slope coefficients.

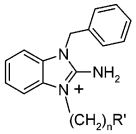
Table 4 shows the effect of the *N*-benzyl moiety (compounds **6b** and **6c**) and the ring substituted derivatives (compounds **6d**−**g**). When compared to the reference monocation **6a**, the superior homologue **6c** was found to be more potent. In addition, the presence of a *p*-methoxy group on the aromatic ring in compound **6g** may have some beneficial effect, whereas a similar effect was not found with the methyl group in **6d**. This may reflect some additional H bond interaction with the oxygen of the methoxy group in **6g**.

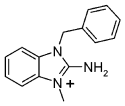
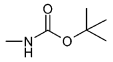
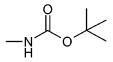
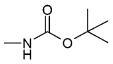
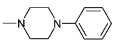
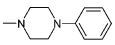
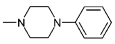
Table 5 deals with the topological exploration along the alkyl side chain. In particular, the second identical cationic head was replaced by other various cations (**9** and **10**), anions (**6m**, **8**), hydrophobic groups (**6k** and **6l**), or various H bond acceptor−donor systems (**6n**−**s**,**w**−**z**). For the optimal fit of a novel putative interaction, various homologues were considered with the length *n* varying from 1 to 12.

Data taken together allowed for the following comments: (i) Among the lipophilic derivatives with short or intermediate lengths (**6h**−**l**), only the compound with the largest length **6a** ($n = 12$) retained some significant potency. (ii) Introducing anions (carboxylic acids **6m**, **8ab**) and H bond acceptor−donor systems (amides **6n**−**p**,**w**−**y**, alcohols **6q**−**s**) led to inactive compounds. Only the phthalimide **6z** and the aniline **10a** showed an EC_{50} of about 10 μM, as found for the starting bis-cation hit **2a**. (iii) Introduction of a second, nonheterocyclic cationic head within the side chain seemed more promising.

First, it is noteworthy that other bis-cations resulting from primary (**9a**−**c**) or secondary (**10b**) or tertiary amines (**10e**) with relatively short lengths ($n < 6$) were inactive. However, within the series of increased length of the spacer ($n = 11$ or 12), a significant increase in potency was observed when introducing an aromatic ring at the NH_2 nitrogen (compare **9c** with **10a** and **10c)**. Thus, the second symmetrical aminobenzimidazole ring could be replaced by other cationic systems such as benzylamine (**10b**), morpholine (**10d**), and *N*-methyl- and *N*-phenylpiperazines (**10h** and **10g**, respectively). However, the presence of an aromatic ring in the vicinity of the cationic head may produce some beneficial effects (better activities for the set of compounds **10c** and **10g** when compared to **9c**, **10d**, **10h**, and **10i**). However, the aniline derivative **10a** is probably not protonated at pH 7.4 and may participate in a typical H bond interaction, since it could be hypothesized for the active noncationic phthalimide **6z**. Taken together, biological data highlighted the compounds **6z**, **10a**, **10c**, and **10g** as the most potent inhibitors within this series. Finally, the good activity of the monocationic compound **6z** allowed us to hypothesize that the second cationic moiety present in **2a** is not always needed for HPrK inhibition. However, the presence of a phenyl ring in the vicinity of the chemical moiety R′ seems to be favorable for activity. On the other hand, R′ (amine or amide) served as an anchor point for introducing a phenyl ring, which might establish an additional hydrophobic interaction.

Table 5. Inhibitory Activity of **2a** and Related Compounds in a Kinase Assay with HPr



Cpd	Linker length n	Functional group (R')	% inhibition at 100 μM (SEM), n=3	% inhibition at 10 μM (SEM), n=3
2a	12		88 (± 1)	50 (± 1)
6h	2	Me	14 (± 2)	13 (± 5)
6i	4	Me	32 (± 2)	12 (± 3)
6j	7	Me	55 (± 22)	9 (± 7)
6a	11	Me	90 (± 1)	18 (± 2)
6k	1	Ph	33 (± 3)	13 (± 4)
6l	3	Ph	20 (± 7)	11 (± 2)
6m	1	COOH	3 (± 9)	2 (± 3)
8a	5	COOH	10 (± 2)	3 (± 4)
8b	10	COOH	39 (± 3)	2 (± 2)
6n	5	$CONH_2$	11 (± 3)	3 (± 5)
6o	1	CONHMe	7 (± 5)	7 (± 4)
6p	1	CONHPh	16 (± 4)	13 (± 2)
6q	3	OH	6 (± 6)	4 (± 7)
6r	6	OH	5 (± 4)	13 (± 2)
6s	11	OH	37 (± 2)	9 (± 3)
6t[a]	5	Br	nt	nt
6u[a]	8	Br	nt	nt
6v[a]	12	Br	nt	nt
6w	3		12 (± 5)	18 (± 6)
6x	6		14 (± 1)	9 (± 5)
6y	11		96 (± 1)	14 (± 2)
6z	12		97 (± 1)	40 (± 2)
9a	3	NH_2	24 (± 10)	18 (± 6)
9b	6	NH_2	33 (± 3)	12 (± 5)
9c	11	NH_2	62 (± 2)	24 (± 5)
10a	12	NHPh	96 (± 1)	46 (± 6)
10b	5	$NHCH_2Ph$	53 (± 1)	12 (± 2)
10c	12	$NHCH_2Ph$	96 (± 1)	50 (± 1)
10d	12		90 (± 1)	31 (± 4)
10e	5		34 (± 2)	13 (± 4)
10f	8		86 (± 1)	33 (± 8)
10g	12		96 (± 1)	41 (± 1)
10h	12		93 (± 6)	35 (± 5)
10i	12		93 (± 1)	11 (± 4)

[a] Not tested, intermediates.

Discussion

From the screening campaign of the in-house chemical library, a single inhibitor of HPrK/P in *B. subtilis* was identified. In contrast to most kinase inhibitors, which are competitive with ATP,[39−41] this substance inhibited protein kinase activity independently of the ATP concentration, consistent with an alternative mechanism of inhibition. Non-ATP mimetic kinase inhibitors can be considered more favorably when compared to ATP-competitive inhibitors because the ATP-binding site is highly conserved among a large class of protein kinases. Consequently, many ATP inhibitors may be described as general rather than specific protein kinase inhibitors,[40] and in order to obtain highly selective ATP site-directed inhibitors, optimization by use of the structural information in the vicinity of the ATP site of the specific kinase needs to be further undertaken.[40,42,43] Moreover, ATP-competitive inhibitors and their application to intact eukaryotic cells may be limited because of numerous factors such as that high intracellular concentrations of inhibitor must overcome physiological millimolar levels of ATP.[41,44,45] The consequence of the requirement of high intracellular levels of inhibitors may compromise the selectivity and increase the likelihood of nonspecific effects.[44] For prokaryotes, intracellular concentrations of glycolytic intermediates, e.g., ATP, have been measured in *Streptococcus lactis*.[46] Following the addition of glucose to starved cells, the intracellular levels of ATP increased to around 2 mM. Thus, a non-ATP inhibitor binding to its site more weakly than an ATP-competitive inhibitor binding to the ATP site could be equally potent intracellularly because the inhibitor in the first case may compete with micromolar concentrations of protein substrates compared to millimolar levels of ATP in the second case.

Compound **2a** seems to have a similar effect in vitro when considering the degree of inhibition of phosphorylation and dephosphorylation regarding HPr and Crh, respectively, even though a tendency for higher concentrations of the inhibitor was needed in a kinase assay with Crh compared to HPr (Table 1). The tendency for higher concentrations of inhibitor in a phosphorylation assay with Crh than with HPr was further supported when the refinement compounds **3a**, **3b**, and **6a** (Table 3) were analyzed. The background may be due to the different behavior of the proteins in solution, as was

envisaged by NMR analyses indicating that Crh may exist as a homodimer, whereas HPr is known to be monomeric in solution.[47,48] However, the concentration was rather high, 0.5 mM, and yielded a distribution of 70−80% monomer and 20−30% dimer.[47,48] When electrospray ionization mass measurements at 20 μM were performed, no dimer of Crh could be detected.[17] Nevertheless, if Crh behaves as a dimer under certain conditions, the consequence may be the need for higher concentrations of inhibitor. The fact that both the kinase and the phosphatase activity was affected suggests a close association between these two opposing activities. A close association between the kinase and phosphatase activity indicating the same catalytic site was reported for HPrK/P of *B. subtilis*[34,49] as well as for HPrK/P of *L. casei*.[35,50] Furthermore, the bis-cationic inhibitor suggests a bivalent contact between the compound and the enzyme, and the length of the linker proved to be a critical parameter (the distance between the two guanidines in the extended state of the molecule with a 12-carbon spacer was estimated to be around 17 Å as judged by standard bond lengths). When the alkyl linker was reduced from 12 to 5 carbon atoms (see **2a** and **3b** in Table 3), the EC_{50} value increased 10 times. The two-site binding model is further supported by the increasing EC_{50} value observed for monomer **6a** and may contribute to a higher selectivity of the inhibitor for HPrK/P than for other kinases.

The type of receptor−ligand interaction for the bis-cationic compound **2a** remains unknown, but the structure of the substance indicates that at least one of the binding sites may be negatively charged and surrounded by lipophilic substituents. It can also be envisaged that a π-cation-type interaction might take place between cations and aromatic systems in HPrK/P, as observed in other systems.[51] The active compounds emphasized the importance of the *N*-benzyl-2-aminobenzimidazole moiety containing at least an eight-carbon alkyl linker. Moreover, as shown in Tables 4 and 5, the most active compounds (percent inhibition of more than 40% at 10 μM) present various scaffolds bearing an aromatic ring. As supported by the activity of monocations **6a** and **6z**, at least one binding site involving a typical lipophilic benzimidazole ring is the minimum required for binding to HPr K/P.

The type of inhibition was examined, suggesting that **2a** is a competitive inhibitor because the values for V_{max} were constant and the values for $K_{0.5}$ were increased when tested at three different concentrations of the substance. Furthermore, since HPrK/P of *B. subtilis* is an oligomeric enzyme with strong cooperativity for ATP[17,18] the possibility of the bis-cationic compound acting also as an interface inhibitor was investigated in an assay with different concentrations of ATP and at three constant concentrations of inhibitor. However, the Hill coefficient for ATP did not change, indicating that the bis-cationic compound does not participate in interface interactions between the different subunits.

2a was also added to a mixture with ADP. For ADP alone, the Hill coefficient was determined to be 0.9, indicating that the nucleotide probably does not induce any cooperativity under the experimental conditions in an assay with HPr. A positive cooperativity was reported for the *N*-methylanthraniloyl (Mant) derivative of ADP for its binding to HPrK/P of *B. subtilis* using HPr as the protein substrate.[18] However, this is not in contradiction to each other because different substances may behave differently. For the bis-cationic compound, the inhibition is cooperative with a Hill coefficient around 2 in both the phosphorylation and the dephosphorylation assays. The cooperativity decreased when the alkyl linker length decreased (**3a**,**b**) but increased approximately 3 times when one of the benzyl benzimidazole moieties was removed, the monomer (**6a**), compared to the parent compound.

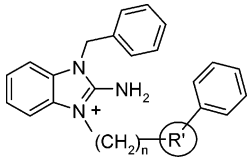
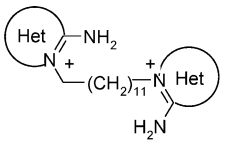
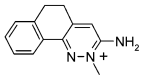
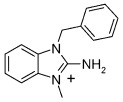


Figure 3. Pharmacophoric model of HPrK/P inhibitors: n = 11, 12; R′ = basic amine (**10c**, **10g**), fairly amine (**10a**), amide (**6z**).

Table 6. Influence of the Structure of the Heterocyclic Amidines (Cationic Head) onto the Selective Inhibition of Specific Kinases[a]



Cpd	Het	Kinase inhibition			
		PKC	CaMK	HPrK	MLCK
2g		na	a	na	nt
2a		nt	a	a	na

[a] nt: not tested. a: active. na: non active.

The 1-substituted 2-aminobenzimidazoles represented here by the prototype shown in Figure 3 belong to the general class of symmetrical heterocyclic amidines linked through their endocyclic nitrogen. These bis-cations are fully protonated at pH 7.4 and are known to present various pharmacological effects (antimicrobials,[52] antifungals[53]). Moreover, they act at different targets, including receptors (GPCR muscarinic receptors,[54] channel receptors[55]) and enzymes (protein kinase C[56]). It is interesting to note that earlier screening of the same chemical library against another specific kinase, calmodulin regulated protein kinase (CaMK),[57] highlighted another bis-cationic entity (compound **2g**) as shown in Table 6. Moreover, depending on the nature of the chemical entity yielding the cationic species (pyridazine or benzimidazole), some selectivity was observed. The bis-pyridazine **2g** and bis-quinoline **2h** did not inhibit the HPr kinase (see Table 2). It is noteworthy that hit optimization starting from the bispyridazine **2g** for designing more potent CaMK inhibitors emphasized the possible replacement of the second pyridazine nucleus in **2g** by a noncationic moiety bearing an aromatic ring.[58]

In conclusion, starting from a specific bis-cationic hit derived from 3-benzyl-2-aminobenzimidazole, a first SAR analysis clearly identified this heterocyclic system as a specific feature needed for HPrK inhibition. The second cationic head could be replaced by a noncationic chemical moiety bearing an aromatic ring. Further studies will optimize the nature of both the connecting group R′ and the aromatic system including substituent effects at the aromatic ring. When compared with recent studies dealing with CaMK starting from another bis-cationic hit compound, they may support the existence of a similar mode of interaction of the heterocyclic amidines presented here. Because they do not compete with the ATP binding site, they may provide interesting challenges for designing potent protein kinase inhibitors with significant increased selectivity profiles.

Antimicrobial activity associated with aromatic bis-cationic compounds is well established. An example is pentamidine, which is clinically used against *Pneumocystis carinii*. Compounds related to pentamidine and other bis-cationic compounds have also been reported to possess antimicrobial effects.[53,59] However, the mechanism of action, even though DNA binding has been hypothesized,[59] and the potential of these latter compounds for clinical use seem to be unknown. The first preliminary results indicated that the parent compound, as well as some of the compounds related to **2a**, i.e., **2b**, **3a**, **6g**, **6y**, **10c**, **10d**, and **10g**, inhibited growth of *B. subtilis*. However, no inhibition was observed when samples were tested in cultures of *E. coli*. Because significant cytotoxicity was observed for the most interesting compounds ($ED_{50} \approx 1\ \mu M$), the series was abandoned. Since the HPrK/P is involved in the regulatory mechanism in the hierarchical use of carbon sources for growth of bacteria, a better strategy could be to evaluate the effect of compounds on growth retardation. Further studies will need to evaluate whether inhibitors of HPrK/P are valuable therapeutical agents as antimicrobials.

Experimental Section

1. Chemistry. The structure of all the compounds were checked by NMR on a Bruker AC-200 or DPX-300 Avance spectrometer. Shifts are given in ppm (δ) with respect to the TMS signal, and coupling constants (J) are given in Hz. Column chromatography was performed on Merck silica gel 60 (230−400 mesh). Melting points were measured with a Mettler FP62 apparatus. Mass spectra were recorded on a Perseptive-Biosystem Mariner electrospray (ES) spectrometer. Elemental analyses (C, H, N) were carried out at a microanalytical center (Lyon).

2-Substituted-1-benzylbenzimidazole **5** was obtained using the established alkylation of 2-aminobenzimidazole **4** with KOH and arylalkyl bromide. The alkyl bromide **7** was commercially available or was made by standard procedures from commercially available starting materials and precursors described in the literature.

General Procedure for the Synthesis of the Bis-Cationic Compounds 2a−g and 3. Method A: Procedure for Compounds 2a and 2b. Dibromododecane (1.5 mmol) was added to a solution of N-substituted 2-aminobenzimidazole (3 mmol) in sulfolane (100 mL), and the resulting solution was heated at 80 °C for 70 h with stirring. After the mixture was cooled to 0 °C, the precipitate was filtered, washed with acetonitrile (30 mL) and then with diethyl ether (50 mL), and recrystallized from isopropyl alcohol. The resulting solid was poured into a 20% K_2CO_3 solution (80 mL) and extracted with ethyl acetate (3 × 50 mL). The combined extracts were washed with water and brine and dried (Na_2SO_4). After removal of the solvent under reduced pressure, the residue was chromatographed on silica gel (ethyl acetate/triethylamine, 95/5) to provide the free bases of **2a** and **2b**.

Method B: Procedure for Compounds 2c,e−h and 3a,b. Dibromoalkane (1.5 mmol) was added to a solution of heterocyclic amidine (3 mmol) in 2-butanone (100 mL), and the resulting solution was heated at 80 °C for 4 days. After the mixture was cooled, the precipitate was filtered and washed twice with 2-butanone (2 × 30 mL). Recrystallization of the product in isopropyl alcohol yielded the dibromides **2c**,**e**−**h** and **3a**,**b**.

Method C: Procedure for Compound 2d. A solution of imidazole (18.5 mmol) in acetone (50 mL) and KOH (55.5 mmol, 3 equiv) was stirred for 30 min at room temperature. Dibromododecane (6.25 mmol) was then added, and the mixture was stirred for 4 h. The solution was then concentrated in vacuo and partitioned between EtOAc (50 mL) and water. The organic layer was washed with brine and extracted with 2 N HCl (50 mL). The aqueous layer was made basic with a 20% K_2CO_3 solution (60 mL) and extracted with AcOEt (60 mL). The organic layer was dried over Na_2SO_4 and was evaporated in vacuo to give the free base as an oil, which was purified by flash chromatography on silica gel (ethyl acetate/triethylamine, 95/5).

Procedure for Oxalate Salts. The free base was dissolved in a small amount of isopropyl alcohol previously heated to 80 °C, and then 2 equiv of oxalic acid dissolved in isopropyl alcohol was added. The product recrystallized at room temperature.

***N,N′*-Bis(2-amino-1-benzylbenzimidazol-3-yl)-1,12-dodecane Oxalate (2a).** Compound **2a** was prepared from *N*-benzyl-2-aminobenzimidazole by method A and was obtained as a white solid (95% yield). $R_f = 0.26$ (AcOEt/TEA, 95:5); mp 190−192°C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.17−1.24 (m, 16H, 4 −CH_2−), 1.60 (m, 4H, 2 −CH_2−), 3.82 (t, 4H, $J = 6.7$, 2 −CH_2N), 5.05 (s, 4H, 2 −CH_2Ph), 6.80−6.93 (m, 8H, Ar−H), 7.19−7.29 (m, 10H, Ar−H). Anal. ($C_{44}H_{52}N_6O_8$) C, H, N.

***N,N′*-Bis(2-amino-1-methylbenzimidazol-3-yl)-1,12-dodecane Oxalate (2b).** Compound **2b** was prepared from *N*-methyl-2-aminobenzimidazole by method A (85% yield). $R_f = 0.23$ (AcOEt/TEA, 95:5); mp 181−183 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.23−1.31 (m, 16H, 4 −CH_2−), 1.63−1.74 (m, 4H, 2 −CH_2−), 3.34 (s, 6H, −N1CH_3), 3.76 (t, 4H, $J = 6.5$, 2 −CH_2-N3), 4.55 (br s, 4H, 2 NH_2 exchange with D_2O), 6.78−6.85 (m, 4H, H5 and H6), 6.92−6.98 (m, 4H, H4 and H7). Anal. ($C_{32}H_{44}N_6O_8$) C, H, N.

***N,N′*-Bis(2-aminobenzothiazol-3-yl)-1,12-dodecane Oxalate (2c).** Compound **2c** was prepared from 2-aminobenzothiazole by method B (85% yield). $R_f = 0.5$ (AcOEt/TEA, 95:5); mp 171−173 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.25−1.37 (m, 16H, 4 −CH_2−), 1.69−1.79 (m, 4H, 2 −CH_2−), 3.90 (t, 4H, $J = 6.5$, 2 −CH_2N3), 6.74−6.85 (m, 4H, H7 and NH), 6.92−7.04 (m, 2H, H6), 7.08−7.26 (m, 4H, H4 and H5). Anal. ($C_{30}H_{38}N_4O_8S_2$) C, H, N.

***N,N′*-Bis(imidazol-1-yl)-1,12-dodecane Oxalate (2d).** 80% yield; $R_f = 0.24$ (AcOEt/TEA, 95:5); mp 158−160 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.14−1.23 (m, 16H, 4 −CH_2−), 1.75 (m, 4H, 2 −CH_2−), 3.91 (t, 4H, $J = 7.1$, 2 −CH_2N1), 6.89 (br s, 2H, H4), 7.04 (br s, 2H, H5), 7.45 (br s, 2H, H2). Anal. ($C_{22}H_{34}N_4O_8$) C, H, N.

***N,N′*-Bis(2-aminopyridin-1-yl)-1,12-dodecane Dihydrobromide (2e).** Compound **2e** was prepared from 2-aminopyridine by method B (95% yield). $R_f = 0.4$ (AcOEt/TEA, 95:5); mp 230−232 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.14−1.23 (m, 16H), 1.65 (m, 4H), 4.16 (t, 4H, $J = 7.0$, −CH_2N1), 6.90 (t, 4H, $J = 6.4$, H4), 7.00 (d, 2H, $J = 8,7$, H3), 7.87 (t, 2H, $J = 7.1$, H5), 8.10 (d, 2H, $J = 5.8$, H6), 8.51 (br s, 4H, NH_2 exchangeable with D_2O). Anal. ($C_{22}H_{36}Br_2N_4$) C, H, N.

***N,N′*-Bis(2-amino-5-phenylpyridin-1-yl)-1,12-dodecane Dihydrobromide (2f).** Compound **2f** was prepared from 2-aminobenzothiazole by method B (60% yield). $R_f = 0.37$ (AcOEt/TEA, 95:5); mp 182−184 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.14−1.25 (m, 16H, 4 −CH_2−), 1.67 (m, 4H, 2

−CH_2−), 4.19 (m, 4H, 2 −CH_2N1), 7.32 (m, 6H, Ar−H), 7.53 (m, 4H, Ar−H), 7.70 (B/AB, 2H, J_{AB} = 7.6, H4), 7.90 (A/AB, 2H, J_{AB} = 7.6, H3), 8.24 (s, 2H, H6), 8.35 (br s, 4H, NH_2 exchangeable with D_2O). Anal. ($C_{34}H_{44}Br_2N_4$) C, H, N.

***N,N′*-Bis(2-amino-benzocyclohexa[4,5-*c*]pyridazin-1-yl)-1,12-dodecane Dihydrobromide (2g)**. Compound **2g** was prepared from 2-aminobenzocyclohexa[4,5-*c*]pyridazine by method B (65% yield). R_f = 0.45 (AcOEt/TEA, 95:5); mp 265−267 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.32−1.50 (m, 16H, 4 −CH_2−), 1.99 (m, 4H, 2 −CH_2−), 3.18−3.30 (m, 8H, 4 −CH_2), 4.49 (m, 4H, 2 −CH_2N1), 7.52−7.67 (m, 10H, Ar−H and H4), 9.10 (br s, 4H, NH_2 exchangeable with D_2O). Anal. ($C_{36}H_{46}Br_2N_6$) C, H, N.

***N,N′*-Bis(1-aminoisoquinolin-2-yl)-1,12-dodecane Dihydrobromide (2h)**. Compound **2h** was prepared from aminoisoquinoline by method B (80% yield). R_f = 0.5 (AcOEt/TEA, 95:5); mp 283−285 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.14−1.23 (m, 16H, 4 −CH_2−), 1.73 (m, 4H, 2 −CH_2−), 4.27 (m, 4H, 2 −CH_2N2), 7.32 (d, 2H, J = 6.9, H4), 7.83−7.96 (m, 8H, Ar−H), 9.66 (d, 2H, J = 7.0, H3), 9.21 (br s, 4H, NH_2 exchangeable with D_2O). Anal. ($C_{30}H_{40}Br_2N_4$) C, H, N.

***N,N′*-Bis(2-amino-1-benzylbenzimidazol-3-yl)-1,5-pentane Dihydrobromide (3a).** Compound **3a** was prepared from 2-amino-1-benzylbenzimidazole with 1,5-dibromopentane by method B (80% yield). R_f = 0.2 (AcOEt/TEA, 95:5); mp 299−301 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.51 (m, 2H, −CH_2−), 1.80 (m, 4H, −CH_2−CH$_2$−CH_2−), 4.23 (t, 4H, J = 7.1, 2 −CH_2N), 5.51 (s, 4H, 2 −CH_2Ph), 7.52 (m, 18H, Ar−H), 9.03 (br s, 4H, 2 NH_2 exchange with D_2O). Anal. ($C_{33}H_{36}Br_2N_6$) C, H, N.

***N,N′*-Bis(2-amino-1-benzylbenzimidazol-3-yl)-1,8-octane Dihydrobromide (3b).** Compound **3b** was prepared from 2-amino-1-benzylbenzimidazole with 1,8-dibromooctane by method B (80% yield). R_f = 0.2 (AcOEt/TEA, 95:5); mp 302 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.28 (m, 9H, 3 −CH_2−), 1.69 (m, 4H, 2 −CH_2−), 4.16 (t, 4H, J = 7.2, 2 −CH_2N), 5.51 (s, 4H, 2 −CH_2Ph), 7.33 (m, 18H, Ar−H), 8.92 (br s, 4H, 2 NH_2 exchange with D_2O). Anal. ($C_{36}H_{42}Br_2N_6$) C, H, N.

2. Pharmacology. Purification of HPr(His)$_6$, Crh(His)$_6$, and HPrK/P(Trx-His$_6$-S-tag) from *B. subtilis*. HPr(His)$_6$, Crh(His)$_6$, and HPrK/P(Trx-His$_6$-S-tag) from *B. subtilis* were purified by Ni-NTA affinity chromatography as described previously.[17] The concentration of HPrK/P(Trx-His$_6$-S-tag) was determined spectrophotometrically using the Bio-Rad protein assay (Bio-Rad Laboratories, München, Germany) with bovine γ globulin as standard, and the concentrations of Crh(His)$_6$ and HPr(His)$_6$ were determined by UV spectrophotometry using the extinction coefficient for one and two tyrosine residues, respectively (1500 and 2900 M^{-1} cm^{-1}). Protein solutions were stored at −20 °C.

In-House Chemical Library. The compounds were stored in 96-well plates with the first and last column empty of substances for control samples, i.e., 80 compounds per plate. The initial concentration of the compounds, in DMSO, was 10 g/L. The mean molecular weight of the compounds is close to 300 g/mol, which gives a mean molar concentration of approximately 30 mM. The solutions were further diluted with DMSO using a dispensing robot, Biomek 2000 (Beckman Coulter, Roissy CDG, France), to a final concentration of 0.1 g/L (mean molar concentration of approximately 300 μM) in 96-well flat bottom plates (Costar, Corning, NY). All dilutions were stored under argon at 4 °C.

Radioactive Protein Phosphorylation Assay. In vitro phosphorylation of HPr(His)$_6$/Crh(His)$_6$ by HPrK/P(Trx-His$_6$-S-tag) was performed as described previously.[17] Briefly, the standard assay with a final volume of 20 μL contained 50 mM Tris, pH 8, 5 mM $MgCl_2$, 0.5 mM ATP, 3.3−5 Bq (leading to 200−300 cpm [γ-^{32}P]ATP/pmol ATP) (Amersham Biosciences, Uppsala, Sweden), 2 mM FBP, 0.1% BSA, 10 μM HPr(His)$_6$/Crh(His)$_6$, and to initiate the reaction 100 nM HPrK/P(Trx-His$_6$-S-tag). After incubation at 37 °C for 10 min, the reaction was terminated by spotting samples on P81 phosphocellulose paper (Whatman, Maidstone, England), which were then washed three times with 75 mM H_3PO_4 to remove unreacted ATP and, finally, once with ethanol. The papers were dried and transferred to scintillation vials, and the radioactivity was determined in a scintillation counter.

Automated Protein Phosphorylation Assay. The radioactive phosphorylation assay with HPr(His)$_6$, described above, with the addition of different compounds from the in-house library, mean final concentration of 0.01 g/L (approximately 30 μM), was carried out in 96-well round-bottom plates (Greiner, Poitiers, France) one column at a time using a dispensing robot, Biomek 2000. The final concentration of DMSO used for the dilutions of the compounds in the in-house library was 10% v/v. The reactions were initiated by the addition of HPrK/P(Trx-His$_6$-S-tag), and after incubation at 37 °C for 10 min, the reactions were stopped by adding 100 μL of 75 mM H_3PO_4 to the samples in the wells in the same order as the reactions were initiated. Subsequently, 90 μL from each sample was transferred to 96-well P81 filter plates (Whatman, Maidstone, England) mounted on a manifold and vacuum was applied for 1.5 min. The papers were washed three times with 200 μL of 75 mM H_3PO_4 and the last time with 200 μL of ethanol. The wells were incubated with the washing solutions for 3 min, and the solutions were removed by applying the vacuum for 20 s except for ethanol where the vacuum was applied for 5 min. The P81 papers were left to dry overnight. The next day, 25 μL of scintillation solution was added to each well and the radioactivity was measured with a Microbeta 1450 (PerkinElmer, Courtaboeuf, France).

Radioactive Phosphatase Assay. The phosphorylation assay described above was carried out to first receive phosphorylation of HPr(His)$_6$/Crh(His)$_6$. Then 5 μL of inorganic phosphate (K_2HPO_4 and KH_2PO_4, pH 8.0) was added to give a final concentration of 1 mM, and the reaction mixture was further incubated at 37 °C for 2 h. Termination of the reaction and the rest of the steps was performed as described above. The final concentration of HPr(His)$_6$/Crh(His)$_6$ was 8 μM.

EC_{50} Curve. Thirteen different consecutively diluted concentrations of tested inhibitor were mixed in advance with inorganic phosphate. After complete phosphorylation, the mixtures were added to the assay.

Estimation of EC_{50} Values and Kinetic Parameters. The program GraphPad Prism was adapted for nonlinear regression analysis to determine EC_{50} values, the Hill coefficient, $K_{0.5}$ (which is the half-saturation constant in the Hill equation), and V_{max}.

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Supporting Information Available: Representative experimental procedures and spectral data for the preparation and characterization of compounds **6** and **8**−**10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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