Generation of Bis-Cationic Heterocyclic Inhibitors of *Bacillus subtilis* HPr Kinase/Phosphatase from a Ditopic Dynamic Combinatorial Library

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Ditopic dynamic combinatorial libraries were generated and screened toward inhibition of the bifunctional enzyme HPr kinase/phosphatase from *Bacillus subtilis*. The libraries were composed of all possible combinations resulting from the dynamic interconversion of 16 hydrazides and five monoaldehyde or dialdehyde building blocks, resulting in libraries containing up to 440 different constituents. Of all possible acyl hydrazones formed, active compounds containing two terminal cationic heterocyclic recognition groups separated by a spacer of appropriate structure could be rapidly identified using a dynamic deconvolution procedure. Thus, parallel testing of sublibraries where one specific component was excluded basically revealed all the essential components. A potent ditopic inhibitor, based on 2-amino-benzimidazole, was identified from the process.

Introduction

Dynamic combinatorial chemistry (DCC) is a recently introduced concept in drug discovery, based on the generation and implementation of adaptive libraries.[1−6] This supramolecular approach is based on the reversible connection between different basic components to produce continually interchanging library constituents representing all possible combinations of all components available (Figure 1). Each library constituent affects and is affected by all other surrounding constituents, allowing for target-driven and self-screening processes that lead to the preferential expression and retrieval from the library of the active species that present the strongest binding to the target entity.

Such libraries have been implemented in a number of cases concerning either the receptor-driven generation of a substrate/inhibitor or the reverse. In our laboratory, the anion-dependent generation of circular helicates led to the formulation of the DCC concept,[7,8] and the proof of principle has been further substantiated in studies including inorganic[9−12] and organic model systems,[13−18] as well as studies involving biological target molecules.[19−25] In the context of the present study, the induction of an inhibitor of carbonic anhydrase[20] and the discovery of a bis-cationic ligand toward acetylcholinesterase[24] have shown that DCC is well-adapted to the discovery of enzyme inhibitors.

Kinases and phosphatases are classes of enzymes that are becoming increasingly important as targets for drug discovery.[26−30] Protein phosphorylation (and dephosphorylation) is the key regulatory mechanism for most essential cellular functions, including gene transcription, cell growth, cell metabolism, and immune response.

Phosphorylation processes also underlie many severe disorders, such as diabetes, hypertension, infectious diseases, and cancer. Therefore, the direct inhibition of individual kinases or phosphatases could produce precise and efficient control over such cellular processes and diseases. In parallel with the intense mapping of such kinase-dependent regulatory processes, attempts have been made to discover kinase inhibitors. However, because of the complex nature of kinase regulation, it has been difficult to find highly potent—and at the same time highly specific—kinase/phosphatase inhibitors, and new approaches to inhibitor discovery are therefore warranted. Only recently has it become apparent that selective inhibitors can be obtained despite the great similarity of the active sites of different kinases.[31] Kinases are potentially very suitable targets for di- or oligotopic dynamic combinatorial libraries. These enzymes often have several binding sites for substrates (ATP, proteins), activators/attenuators, and protomer interface recognition. Thus, combinations of inhibitors of the respective sites could in principle lead to inhibitors presenting both high affinity and high selectivity.

One such target candidate is the HPr kinase (HPrK/P), a key enzyme in the bacterial carbon catabolite repression (CCR) pathway. In low-GC (guanine, cytosine) Gram-positive bacteria, the main mechanism for hierarchical control of carbohydrate utilization involves the reversible phosphorylation of HPr (histidine-containing protein) at Ser-46 by the ATP-dependent enzyme, which in addition to kinase activity also possesses phosphatase activity in *Bacillis subtilis*.[32−34] Metabolizing a preferred carbohydrate, e.g., glucose, generates high concentrations of ATP and FBP (fructose-1,6-diphosphate), whereby the expression of secondary catabolic genes becomes repressed in a cascade of events that is controlled by HPrK/P. On the contrary, during starvation with high concentrations of inorganic phosphate, the phosphatase activity of the enzyme becomes prevalent, leading to dephosphorylation of HPr(Ser-P).

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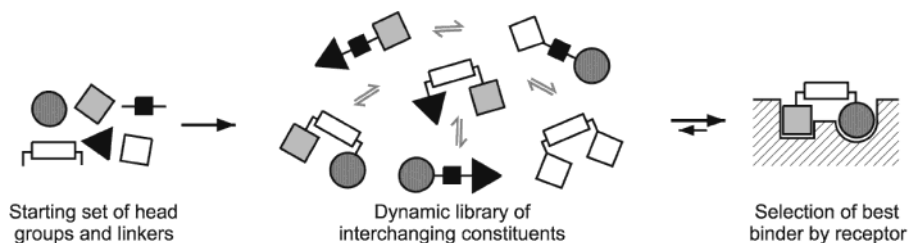
§ These authors contributed equally to this work.

Figure 1. Schematic representation of the DCC process. A collection of different building blocks is allowed to form an ensemble of assemblies/adducts, held together through reversible interconnections, and in continuous exchange with one another. Subsequent addition of a target species, e.g., an enzyme, leads to the selection of the best bound library constituent, allowing the identification of the active species.
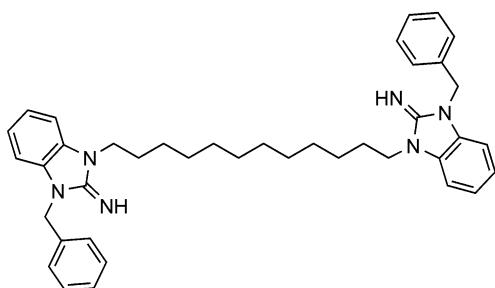

Figure 2. Structure of compound identified from an in-house patrimonial collection (University Louis Pasteur, Faculty of Pharmacy).

Thus, mechanistic control involves activation of either the kinase- or the phosphatase activity, depending on the energy state of the cell. Carbon catabolite repression and also carbon catabolite activation (CCA), including regulation of central metabolic pathways such as glycolysis, are assumed to be the consequence of this regulatory mechanism.[35-40] Since HPrK/P-deficient bacterial mutants show severe growth defects (*Lactobacillus casei*, *Staphylococcus xylosus*, and *B. subtilis*),[41-43] and the main mechanism of CCR/CCA is suggested to involve the enzyme HPrK/P, inhibitors of this key enzyme could form a new family of antibiotic drugs.

In search of potent inhibitors against kinases in general, and the kinase activity of HPrK/P in particular, an in-house patrimonial collection of molecules (University Louis Pasteur, Faculty of Pharmacy) was recently investigated.[44,45] Upon screening of this highly diverse 1440-membered library, at a concentration of each library member of approximately 30 μM, a heterocyclic bis-cation was found that inhibited HPrK/P (Figure 2), with an inhibitory binding efficiency in the micromolar range (IC$_{50}$ ~ 10 μM).

With this hit as a starting point, ditopic dynamic combinatorial libraries were designed where the recognition groups, as well as the structural linker component between them, were probed and evaluated. Thus, a set of different headgroups, resembling the original hit, was tested together with linkers of various type. Reversibility in the connections was based on acyl hydrazone formation and exchange, previously known to be easily controlled and also compatible with aqueous phase enzyme assays.[24,46] Because of the instability and limited availability of the enzyme, a preequilibration approach was also followed,[22,24] where a sensitive radioactive kinase assay was applied posterior to dynamic combinatorial library (DCL) generation. To identify active components, a dynamic deconvolution pro-

tocol[24] was employed for the efficient characterization of the active constituents through identification of their building blocks.
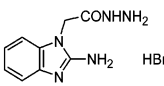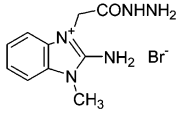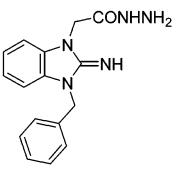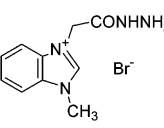
Results and Discussion
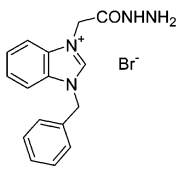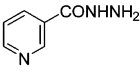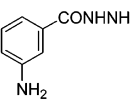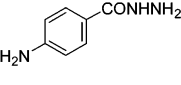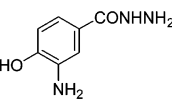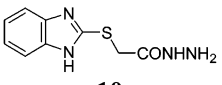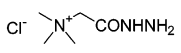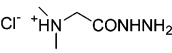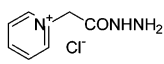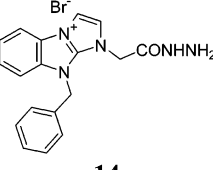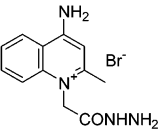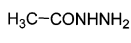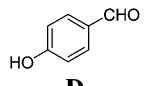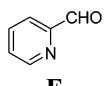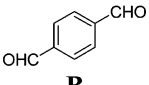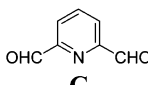
Generation of the Dynamic Combinatorial Library (DCL). Choice of Building Blocks. At the outset, a library composed of 16 different hydrazide headgroups and five different aldehydes were chosen as library components (Table 1). The headgroups selected were based on the previously known heterocyclic cationic structure emanating from the patrimonial library. Derivatives of this aminobenzimidazole structural motif were prepared and mixed with other cationic species, as well as uncharged, unrelated components for reference. As spacer units, three different dialdehydes were used, monitored in comparison with two different monoaldehydes. This ensemble of components amounts to a relatively focused library, in which several of the final constituents may lead to inhibition, potentially impeding their efficient identification.

DCL Generation. The complete library resulting from these 21 components would contain 800 different constituents (for a calculation, see ref 24), but on accounting for symmetry effects, this number is reduced to an ensemble of 440 different library members, all formed at the same time in the same solution under dynamic conditions. Generation of this library was easily accomplished at moderately acidic pH (acetate buffer, pH 4), upon agitation overnight. In general, generation and interconversion of acyl hydrazone libraries are completed within 15 min to a couple of hours, depending on the hydrazide/aldehyde combination and the pH of the solution, a lower pH resulting in a more rapid reaction.[24,46] In the present case, repeated studies with prolonged reaction times did not result in altered results, indicative of complete reaction progress. Since some of the components (notably compound **10**) resulted in acyl hydrazones of low solubility in the aqueous buffer; DMSO was also added to the samples. This modification was however of no concern, neither for the dynamic generation of the library nor for its interaction with the HPrK/P. The final concentration of the libraries amounted to 15.2 mM in total hydrazide and 9.5 mM in total aldehyde, respectively, resulting in a theoretical yield of 7.4−119 μM per library member (Table 2).

Screening of the DCL by Dynamic Deconvolution.

Sample Preparation. The protein substrate HPr and the enzyme HPrK/P were purified and tested as previously reported,[47] and a radioactive phosphorylation

Table 1. Library Elements

Hydrazides (*n* = 16)

Monoaldehydes (*m_1* = 2)

Dialdehydes (*m_2* = 3)

assay was used throughout in the selection process. All screening reactions were performed at pH 8.0 in Tris·HCl buffer, containing the phosphoryl donor, $[\gamma\text{-}^{32}P]$-ATP, together with $MgCl_2$ and FBP. The DCLs appropriately diluted 2−10 times were then added to the assay, to 20% (v/v), and in all samples a total concentration of 10% DMSO was maintained. The degree of dilution was probed in each case to ensure optimal observable effects, i.e., an overall inhibition by the complete libraries of approximately 80−90%. The reactions were then initiated by the addition of enzyme (1%

with respect to HPr), and after incubation for 10 min at 37 °C, they were stopped and the degree of phosphorylation determined.

Screening/Dynamic Deconvolution. When testing the complete 440-membered library against HPrK/P, it became clear that it contained active species, potent inhibitors of the HPr phosphorylation. Thus, addition of the complete library to the kinase assay resulted in strong inhibition of the phosphorylation signal to a concentration of $^1/_{50}$ of the stock solutions, corresponding to approximately 0.15−2.4 μM of each library member.

Table 2. Concentrations of Library Constituents

Constituent		Concentration (μM)
monohydrazone	○—▭	119
homodihydrazone	○—▭—○	7.4
heterodihydrazone	◁—▭—○	14.9

To identify the active compounds of the libraries, a dynamic deconvolution protocol[24] was utilized. This method, which implements the dynamic features of the library, is based on the removal of a given building block, which leads to a redistribution of the remaining components, such that all constituents that contain this unit will automatically be suppressed from the equilibrating library. By removal of single building blocks from the pool library (Table 3),[24] for each component making up the DCL, a sublibrary is prepared from which all library constituents based on this element are deleted. Thus, when using this dynamic exclusive deconvolution (DED) strategy, a decrease in inhibitory effect reveals the importance of the removed component in the generation of active compounds in the dynamic library. This protocol leads to a limited number of samples (one for each building block + references), but is nevertheless highly efficient in targeting active species.

The complete pool library (**all**) was generated by adding all building blocks (**1**−**16**, **A**−**E**) simultaneously under preequilibrating conditions in acidic buffer at ambient temperature. At the same time, 21 sublibraries were formed by mixing all components, with the exception of one specific hydrazide or aldehyde building block, under the same conditions. The respective libraries obtained are composed of all possible condensation products in proportion to their relative thermodynamic

stability. Together with a reference sample (buffer), containing no building blocks, this series of 23 samples was sufficient for screening the entire 440-member library. Following equilibration, the libraries were subjected to the kinase assay, in which the inhibitory potency of HPrK/P for its natural substrate HPr was monitored.

The results obtained from this library generation/screening process are presented in Figure 3, where the sublibraries' effects have been related to the complete library's. The inhibition of the HPrK/P activity (low phosphorylation signal) by a library indicates the presence of one or several active adducts in a given equilibrated mixture. On sequential removal of each building block, one at a time, from the complete library, an increase in activity indicates that the omitted component contributed significantly to the inhibitory effect, and a decrease in activity that the component hampers the effect of the more active compounds. The data in Figure 3 show that several components proved active in the present study, the largest effect arising from removal of the dialdehyde **B** from the complete DCL. Clearly, **B** is necessary for inhibition to occur, whereas the other aldehyde building blocks are less important. Similarly, for the recognition headgroups, hydrazide **3** proved most active and smaller effects were observed for compounds **9** and **15**. Consequently, the most active constituent is likely to come from the assembly of fragments **3** and **B**. By testing the combinations of the headgroups **3** and **15** with linker **B**, it could furthermore be concluded that the homodimeric species **3**-**B**-**3** was more potent than the heterodimeric constituent **3**-**B**-**15**.

In the deconvolution protocol used, all samples can be prepared in advance and a readout can be obtained immediately after testing them. This is an advantage especially for larger libraries, where the deconvolution strategy can be directly programmed into the control software of a dispensing robot. Repetitive testing and intermittent analysis of sample pools are in this case

Table 3. Dynamic Exclusive Deconvolution (DED)

all	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	A	B	C	D	E
ref	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
E1	−	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	A	B	C	D	E
E2	1	−	3	4	5	6	7	8	9	10	11	12	13	14	15	16	A	B	C	D	E
E3	1	2	−	4	5	6	7	8	9	10	11	12	13	14	15	16	A	B	C	D	E
E4	1	2	3	−	5	6	7	8	9	10	11	12	13	14	15	16	A	B	C	D	E
E5	1	2	3	4	−	6	7	8	9	10	11	12	13	14	15	16	A	B	C	D	E
E6	1	2	3	4	5	−	7	8	9	10	11	12	13	14	15	16	A	B	C	D	E
E7	1	2	3	4	5	6	−	8	9	10	11	12	13	14	15	16	A	B	C	D	E
E8	1	2	3	4	5	6	7	−	9	10	11	12	13	14	15	16	A	B	C	D	E
E9	1	2	3	4	5	6	7	8	−	10	11	12	13	14	15	16	A	B	C	D	E
E10	1	2	3	4	5	6	7	8	9	−	11	12	13	14	15	16	A	B	C	D	E
E11	1	2	3	4	5	6	7	8	9	10	−	12	13	14	15	16	A	B	C	D	E
E12	1	2	3	4	5	6	7	8	9	10	11	−	13	14	15	16	A	B	C	D	E
E13	1	2	3	4	5	6	7	8	9	10	11	12	−	14	15	16	A	B	C	D	E
E14	1	2	3	4	5	6	7	8	9	10	11	12	13	−	15	16	A	B	C	D	E
E15	1	2	3	4	5	6	7	8	9	10	11	12	13	14	−	16	A	B	C	D	E
E16	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	−	A	B	C	D	E
EA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	−	B	C	D	E
EB	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	A	−	C	D	E
EC	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	A	B	−	D	E
ED	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	A	B	C	−	E
EE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	A	B	C	D	−

[a] Sublibraries (S1−16 and SA−SE) were prepared, where one specific component (**1**−**16**, **A**−**E**) is excluded in each library. The response (negative or positive) in the analytical assay is indicative of the importance of this component. In addition, the complete library (**all**), composed of all components, and reference samples (ref) containing no components were made for comparison. In the present example, 23 different samples needed to be prepared.
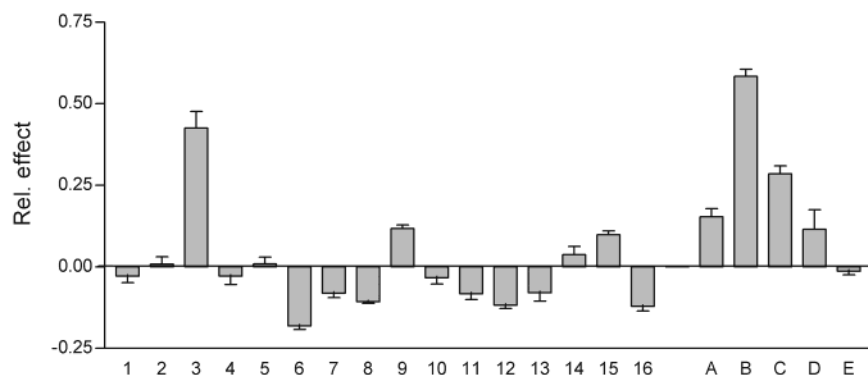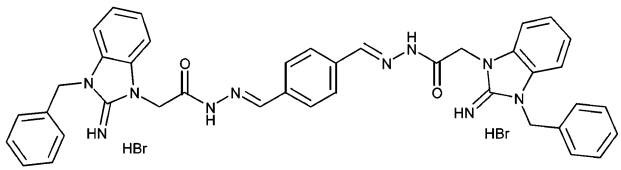
Figure 3. Dynamic exclusive deconvolution: relative activity (kinase assay) of each sublibrary (**E1−EE**), compared to complete library. Exclusion of hydrazides **3**, **9**, and **15**, and especially dialdehyde **B**, leads to reduction in inhibitory effect (SEM, $n = 3$).

Table 4. Inhibitory Activities (IC$_{50}$ Values) of Selected Ditopic Inhibitor

Compound		pH	Hill coeff.	IC$_{50}$ (μM)
3-B-3		7.0	1.4	17
		8.0	2.9	18

not needed, and the entire process can be performed in one single operation.

Further Evaluation of Observed Species. Once the active building blocks had been identified with the deconvolution procedure, a more detailed study was undertaken. The most potent library component **3-B-3** was synthesized separately and its inhibitory effects further characterized. The results, shown in Table 4, indicate that **3-B-3** is indeed a relatively potent HPrK/P inhibitor, displaying a 50% inhibitory concentration (IC$_{50}$) in the micromolar range, comparable to the compound identified from the patrimonial library.

The screening results also reveal some structural features required for efficient inhibition. From the exclusive deconvolution protocol, it is seen that a ditopic structure leads to higher inhibition than a monotopic adduct. Also, the orientation and/or distance between the headgroups are important factors, where a 1,4-phenyl scaffold (**B**) leads to a higher effect. When a longer spacer unit than **B** based on pentaethylene glycol (26-atom linker) was tested, no improvement could be detected (data not shown), indicating that the linker structure was sensitive to change. The structural determinants of the headgroup hydrazides can also be deduced to some extent, a charged 1,3-disubstituted 2-amino-benzimidazole unit (compound **3**) being very efficient for inhibition in combination with linker **B**. With an uncharged headgroup (**1**), no inhibition was detected. Likewise, removal of the 2-amino group (**5**) yielded a considerably reduced effect. In addition, substituting the *N*-benzyl group for a methyl resulted in loss of activity, indicating a substantial contribution of an aromatic/lipophilic moiety in that position. Other structural motifs proved less efficient, including the 4-ami-

noquinaldine group (**15**) previously known to display potent inhibitory effect against protein kinase C.[48]

The mechanism of inhibition for HPr kinase has been found to be of complex nature, similar to many other kinases.[45] Although, a detailed mechanistic study of compound **3-B-3** has not been the focus in the present study, studies with the compound found from the patrimonial library have shown that this structure is not an ATP competitor, but rather acts on the protein substrate binding site. **3-B-3** is likely to present the same type of mechanism.

Conclusions

It has been shown that potent kinase inhibitors can be generated by acyl hydrazone formation and exchange that allow the efficient generation of dynamic combinatorial libraries in aqueous media. A set of 21 initial building blocks yielded a library containing 440 different species in a single step in a short time. Among all possible acyl hydrazones formed, active compounds of appropriate length containing potent recognition groups could be rapidly identified using a dynamic deconvolution process. In addition to its straightforward nature, this strategy is also amenable to modern high-throughput robotics, the deconvolution process facilitating the screening operation. The concept can in principle be extended to encompass much larger combinatorial libraries, allowing swifter library generation and more expeditious screening than when using individual testing.

Experimental Section

General. All reagents were purchased from commercial sources and used after appropriate purification. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker AC200 spectrom-

Scheme 1. General Procedure for the Preparation of the Hydrazide Elements[a]

1: R^1 = H, R^2 = NH

2: R^1 = Me, R^2 = NH$_2$Br

3: R^1 = CH$_2$Ph, R^2 = NH$_2$Br

4: R = Me

5: R = CH$_2$Ph
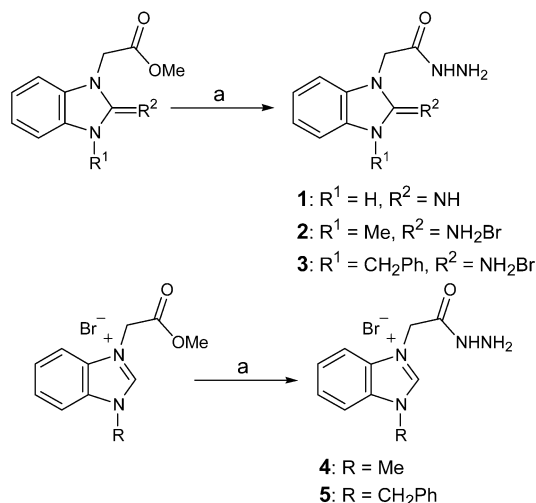
[a] (a) Hydrazine hydrate, MeOH, rt.

eter at 298 K. Mass spectra were determined by the Service de spectrométrie de masse at the Institut de Chimie, Université Louis Pasteur. Microanalyses were performed at Service de microanalyse at the Institut de Chimie, Université Louis Pasteur, or at the Institut Universitaire de Technologie, Strasbourg-sud, France.

General Procedure for the Synthesis of Hydrazide Derivatives. Library hydrazides **1−5** were synthesized from the corresponding methyl esters,[49−52] by addition of hydrazine hydrate in methanol at ambient temperature (Scheme 1) analogous to the protocol for compound **2**.

Compound 2. Hydrazine hydrate (96 µL, 2.0 mmol) was added to a suspension of 2-amino-1-methyl-3-carbomethoxy-methylbenzimidazolium bromide (300 mg, 1.0 mmol) in 15 mL of MeOH. The mixture was stirred at ambient temperature overnight. The resulting precipitate was filtered off, washed with ethanol, and dried. Recrystallization from ethanol/diethyl ether yielded 244 mg of pure product (82%). ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C): δ = 9.49, 8.97 (s, 1H, NH-amide), 8.86 (br, 2H, NH$_2$), 7.60−7.56 (m, 1H, H−Ar), 7.43−7.27 (m, 3H, H−Ar), 5.20, 4.87 (s, 2H, CH$_2$), 4.56, 4.39 (br, 2H, NH$_2$-hydrazide), 3.67 (s, 3H, Me). ^{13}C NMR (50 MHz, DMSO-d_6, 25 °C): δ = 168.29, 164.07, 150.53, 130.16, 130.01, 129.67, 123.56, 123.41, 110.22, 110.02, 44.04, 29.59. FAB-MS (positive mode): m/z 220.1 [M − Br$^-$], calcd 220.12. Anal. Calcd for C$_{10}$H$_{14}$BrN$_5$O (%): C 40.01, H 4.70, N 23.33. Found: C 39.88, H 4.55, N 23.45.

Compound 1. Yield: 67%. ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C): δ = 9.45, 8.78 (s, 1H, NH-amide), 7.15 (d, J = 7.4 Hz, 1H, H−Ar), 7.03 (d, J = 7.4 Hz, 1H, H−Ar), 6.95 (t, J = 7.4 Hz, 1H, H−Ar), 6.86 (t, J = 7.4 Hz, 1H, H−Ar), 6.47 (br, 2H, NH$_2$), 4.96, 4.60 (2H, CH$_2$), 4.35 (br, 2H, NH$_2$-hydrazide). ^{13}C NMR (50 MHz, DMSO-d_6, 25 °C): δ = 170.37, 166.15, 155.33, 142.62, 134.96, 134.42, 120.40, 120.06, 117.98, 117.78, 114.63, 114.43, 107.40, 107.26, 43.36. FAB-MS (positive mode): m/z 206.0 [M − H$^+$], calcd 206.10. Anal. Calcd for C$_9$H$_{11}$N$_5$O (%): C 52.67, H 5.40, N 34.13. Found: C 52.90, H 5.20, N 34.33.

Compound 3. Yield: 80%. ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C): δ = 9.60, 8.96 (br, 1H, NH-amide), 9.07 (br, 2H, NH$_2$), 7.48−7.23 (m, 9H, H−Ar and H-phenyl), 5.55 (s, 2H, CH$_2$-benzyl), 5.31, 4.98 (s, 2H, CH$_2$), 4.59, 4.44 (br, 2H, NH$_2$-hydrazide). ^{13}C NMR (50 MHz, DMSO-d_6, 25 °C): δ = 168.29, 164.16, 150.82, 150.73, 134.38, 129.33, 128.70, 127.92, 126.91, 123.70, 110.65, 110.31, 45.50, 44.19; FAB-MS (positive mode): m/z 296.1 [M − Br$^-$], calcd 296.15. Anal. Calcd for C$_{16}$H$_{18}$BrN$_5$O (%): C 51.08, H 4.82, N 18.61. Found: C 50.90, H 4.86, N 18.49.

Compound 4. Yield: 100%. ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C): δ = 9.77, 9.71 (s, 1H, H−Ar), 9.69, 9.07 (br, 1H, H-amide), 8.09−8.00 (m, 1H, H−Ar), 7.98−7.88 (m, 1H, H−Ar),

7.75−7.67 (m, 2H, H−Ar), 5.60, 5.28 (s, 2H, CH$_2$), 4.68, 4.44 (br, 2H, NH$_2$-hydrazide), 4.15 (s, 3H, Me). ^{13}C NMR (50 MHz, DMSO-d_6, 25 °C): δ = 168.34, 163.82, 143.74, 143.59, 143.50, 131.66, 131.47, 131.32, 131.22, 126.61, 126.52, 126.42, 126.23, 113.61, 113.52, 113.32, 47.29, 47.10, 33.32. FAB-MS (positive mode): m/z 205.1 [M − Br$^-$], calcd 205.11. Anal. Calcd for C$_{10}$H$_{13}$BrN$_4$O (%): C 42.12, H 4.60, N 19.65. Found: C 41.88, H 4.50, N 19.56.

Compound 5. Yield: 79%. ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C): δ = 10.07, 10.01 (s, 1H, H−Ar), 9.79, 9.11 (br, 1H, H-amide), 8.04−7.95 (m, 2H, H−Ar), 7.72−7.61 (m, 2H, H−Ar), 7.56−7.37 (m, 5H, H-phenyl), 5.89 (s, 2H, CH$_2$-benzyl), 5.66, 5.36 (s, 2H, CH$_2$), 4.72, 4.47 (br, 2H, NH$_2$-hydrazide). ^{13}C NMR (50 MHz, DMSO-d_6, 25 °C): δ = 168.29, 163.82, 143.69, 143.45, 133.94, 133.84, 132.00, 131.61, 130.45, 128.89, 128.65, 128.17, 126.76, 126.66, 113.85, 113.66, 49.77, 47.63, 47.54. FAB-MS (positive mode): m/z 281.1 [M − Br$^-$], calcd 281.14. Anal. Calcd for C$_{16}$H$_{17}$BrN$_4$O (%): C 53.20, H 4.74, N 15.51. Found: C 53.44, H 4.68, N 15.70.

Compound 14 was prepared in four steps from 2-amino-1-benzylbenzimidazole according to Scheme 2. Thus, bromoacetaldehyde dimethyl acetal was initially coupled at position 3 to give compound **I**, and upon hydrolysis of the acetal in dilute HBr, cyclization occurred readily, yielding compound **II**. Subsequent coupling of methyl bromoacetate at the 1-position gave **III**, which by reaction with hydrazine, following a procedure analogous to that used for the preparation of **2**, yielded **14**.

Compound I. To a suspension of 2-amino-1-benzylbenz-imidazole (5.0 g, 22.4 mmol) in butanone (150 mL) was added bromoacetaldehyde dimethyl acetal (8 mL, 67.2 mmol). The reaction mixture was refluxed for 72 h (after heating for 5 min, the suspension became soluble), then the resulting precipitate was filtered off, washed with acetone, and dried to yield the white product (1.93 g, 22%). ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C): δ = 9.01 (br, 2H, NH$_2$), 7.66−7.61 (m, 1H, H−Ar), 7.51−7.25 (m, 8H, H−Ar), 5.53, 5.51 (s, 2H, CH$_2$-benzyl), 4.75 (t, J = 5.1 Hz, 1H, CH), 4.37 (d, J = 5.1 Hz, 2H, CH$_2$-acetal), 3.66 (s, 3H, OCH$_3$). ^{13}C NMR (50 MHz, DMSO-d_6, 25 °C): δ = 150.14, 134.42, 129.96, 129.14, 128.75, 127.92, 126.81, 123.61, 111.28, 110.51, 101.39, 55.35, 45.50, 44.96. FAB-MS (positive mode): m/z 312.4 [M − Br$^-$], calcd 312.17. Anal. Calcd for C$_{18}$H$_{22}$BrN$_3$O$_2$ (%): C 55.11, H 5.65, N 10.71. Found: C 55.17, H 5.43, N 10.85.

Compound II. A suspension of acetal **I** (1.5 g, 3.8 mmol) in hydrobromic acid (10 wt % in water, mL) was stirred at 60 °C for 24 h. After cooling the reaction mixture, the new precipitate was filtered off, washed with water, and dried to yield the white product (1.14 g, 91%). ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C): δ = 8.25 (d, J = 2.4 Hz, 1H), 8.15−8.11 (m, 1H), 7.83−7.75 (m, 1H), 7.73 (d, J = 2.4 Hz, 1H), 7.57−7.29 (m, 7H), 5.66 (s, 2H, CH$_2$). ^{13}C NMR (50 MHz, DMSO-d_6, 25 °C): δ = 142.82, 134.62, 134.47, 128.80, 128.17, 127.39, 125.55, 123.75, 122.93, 120.74, 113.08, 122.30, 109.78, 47.24; FAB-MS (positive mode): m/z 248.1 [M − Br$^-$], calcd 248.12. Anal. Calcd for C$_{16}$H$_{14}$BrN$_3$ (%): C 58.55, H 4.30, N 12.80. Found: C 58.38, H 4.31, N 12.80.

Compound III. To a solution of ester **II** (1.0 g, 3.1 mmol) in methanol (40 mL) was added methyl bromoacetate (0.6 mL, 6.2 mmol) and sodium methoxide (0.2 g, 3.7 mmol). The reaction mixture was refluxed for 48 h. Then the solvent was evaporated under reduced pressure and the residual reaction was purified by flash chromatography (silica gel, step gradient from 5% CH$_2$Cl$_2$ in MeOH to 20% CH$_2$Cl$_2$ in MeOH) to yield the product (0.49 g, 38% yield). When using ethanol as solvent in the reaction, the yield was 71%, but a mixture of methyl ester and ethyl ester was obtained. ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C): δ = 8.42 (d, J = 2.4 Hz, 1H, H−Ar), 8.28−8.19 (m, 1H, H−Ar), 7.93−7.84 (m, 1H, H−Ar), 7.69 (d, J = 2.4 Hz, 1H, H−Ar), 7.67−7.53 (m, 2H, H−Ar), 7.42−7.22 (m, 5H, H−Ar), 5.86 (s, 2H, CH$_2$-benzyl), 5.36 (s, 2H, CH$_2$), 3.44 (s, 3H, OCH$_3$). ^{13}C NMR (50 MHz, DMSO-d_6, 25 °C): δ = 166.98, 140.68, 135.40, 134.76, 128.85, 128.07, 126.37, 126.27, 125.01, 123.80, 123.27, 113.27, 112.50, 109.78, 52.53, 47.68, 46.66.

Scheme 2[a]
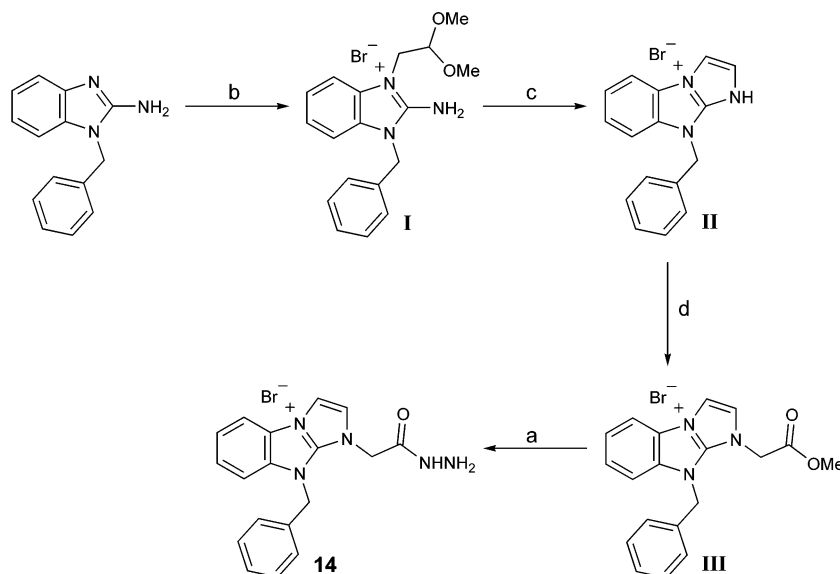
[a] (a) Hydrazine hydrate, MeOH, rt; (b) BrCH₂CH(OMe)₂, butanone, reflux; (c) 10% HBr, 60 °C; (d) BrCH₂CO₂Me, NaOMe, MeOH, reflux.

Scheme 3
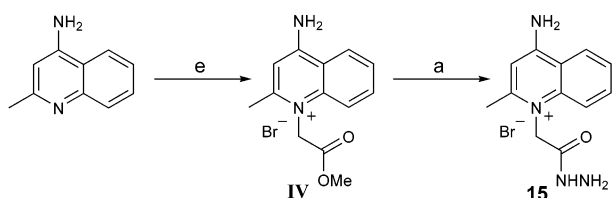
[a] (a) Hydrazine hydrate, MeOH, rt; (e) BrCH₂CO₂Me, butanone, reflux.

FAB-MS (positive mode): m/z 320.1 [M − Br⁻], calcd 320.14. Anal. Calcd for $C_{19}H_{18}BrN_3O_2$ (%): C 57.01, H 4.53, N 10.50. Found: C 57.28, H 4.51, N 10.52.

Compound 14. Yield: 75%. ¹H NMR (200 MHz, DMSO-d_6, 25 °C): $\delta = 9.60, 9.00$ (s, 1H, H-amide), 8.51, 8.48 (d, $J = 2.4$ Hz, 1H, H−Ar), 8.30−8.24 (m, 1H, H−Ar), 7.80−7.65 (m, 2H, H−Ar), 7.58−7.47 (m, 2H, H−Ar), 7.41−7.27 (m, 5H, H-phenyl), 5.78, 5.67 (s, 2H, CH₂-benzyl), 5.37, 5.05 (s, 2H, CH₂), 4.57, 4.29 (br, 2H, NH₂-hydrazide). ¹³C NMR (50 MHz, DMSO-d_6, 25 °C): $\delta = 168.87, 164.60, 140.97, 135.01, 134.86, 128.80, 128.21, 128.07, 126.95, 126.71, 126.03, 124.96, 123.61, 123.36, 113.32, 112.64, 109.73, 109.39, 47.92, 47.20$. FAB-MS (positive mode): m/z 320.0 [M − Br⁻], calcd 320.15. Anal. Calcd for $C_{18}H_{18}BrN_5O\cdot EtOH$ (%): C 53.82, H 5.42, N 15.69. Found: C 53.72, H 5.39, N 15.79.

Compound 15. Compound **15** was prepared from 4-aminoquinaldine according to Scheme 3. The 1-methoxycarbonylmethyl derivative (**IV**) was prepared by condensation with methyl bromoacetate, and **15** was subsequently acquired following addition of hydrazine in a manner analogous to that used for the preparation of **2**.

Compound IV. To a suspension of 4-aminoquinaldine (5.0 g, 31.6 mmol) in butanone (130 mL) was added methyl bromoacetate (3.6 mL, 37.9 mmol). The reaction mixture was refluxed for 24 h, then the resulting precipitate was filtered off and washed with acetone. The resulting precipitate (mixture of product and starting material) was purified by flash chromatography (neutral alumina, step gradient from 10% CHCl₃ in MeOH to 20% CHCl₃ in MeOH) to yield the product (1.87 g, 19% yield). ¹H NMR (200 MHz, DMSO-d_6, 25 °C): $\delta = 9.58, 9.52$ (br, 2H, NH₂), 8.69 (d, $J = 8.2$ Hz, 1H, H−Ar), 8.02−7.91 (m, 2H, H−Ar), 7.71−7.63 (m, 1H, H−Ar), 6.88 (s, 1H, H−Ar), 5.53 (s, 2H, CH₂), 3.75 (s, 3H, OMe), 2.64 (s, 3H, Me). ¹³C NMR (50 MHz, DMSO-d_6, 25 °C): $\delta = 167.79, 157.65, 155.32, 139.75, 134.51, 125.78, 124.90, 117.63, 116.17, 103.66,$

52.86, 49.22, 21.52. FAB-MS (positive mode): m/z 231.1 [M − Br⁻], calcd 231.11. Anal. Calcd for $0.45C_{13}H_{15}BrN_2O_2\cdot 0.55C_{13}H_{14}N_2O_2$ (%): C 58.55, H 5.46, N 10.50. Found: C 58.52, H 5.66, N 10.37.

Compound 15. Yield 43%. ¹H NMR (200 MHz, DMSO-d_6, 25 °C): $\delta = 9.89, 9.04$ (br, 1H, H-amide), 9.18 (br, 2H, NH₂), 8.57 (d, $J = 8.2$ Hz, 1H, H−Ar), 7.97−7.92 (m, 2H, H−Ar), 7.72−7.64 (m, 1H, H−Ar), 6.82 (s, 1H, H−Ar), 5.54, 5.26 (s, 2H, CH₂-hydrazide), 4.83, 4.40 (s, 2H, NH₂-hydrazide), 2.66, 2.60 (s, 3H, Me). ¹³C NMR (50 MHz, DMSO-d_6, 25 °C): $\delta = 168.92, 164.89, 157.27, 157.18, 155.97, 155.82, 139.86, 134.33, 125.69, 125.60, 124.53, 124.38, 117.88, 117.64, 116.28, 116.18, 103.57, 103.42, 49.14, 21.77, 21.63$. FAB-MS (positive mode): m/z 231.0 [M − Br⁻], calcd 231.12. Anal. Calcd for $0.5C_{12}H_{15}BrN_4O\cdot 0.5C_{12}H_{14}N_4O$ (%): C 53.24, H 5.40, N 20.70. Found: C 53.32, H 5.67, N 20.58.

Compound 3-B-3. Terephthalaldehyde (47.7 mg, 0.36 mmol) was suspended in a solution of hydrazide **3** (211 mg, 0.56 mmol) in hydrobromic acid (3 wt % in water, 15 mL). After stirring at ambient temperature for 2 h, the resulting precipitate was filtered off, washed with water and acetonitrile, and dried to yield the desired compound as a pale yellow precipitate (229 mg, 92%). ¹H NMR (200 MHz, DMSO-d_6, 25 °C): $\delta = 12.28, 12.08$ (2H, H-amide), 9.24, 9.19 (br, 4H, amine), 8.41, 8.20 (2H, H-imine), 8.02−7.80 (m, 4H, H−Ar), 7.75−7.58 (m, 2H, H−Ar), 7.58−7.46 (m, 2H, H−Ar), 7.46−7.25 (m, 14H, H−Ar and H−Ph), 5.60, 5.24 (4H, CH₂). ¹³C NMR (50 MHz, DMSO-d_6, 25 °C): $\delta = 166.56, 150.84, 143.71, 135.51, 135.31, 134.39, 130.41, 130.12, 129.25, 128.76, 127.98, 127.40, 126.87, 123.76, 110.71, 45.50, 44.97$. FAB-MS (positive mode): m/z 345.2 [M − 2Br⁻], calcd 345.16. Anal. Calcd for $C_{40}H_{38}Br_2N_{10}O_2\cdot 2H_2O$ (%): C 54.19, H 4.77, N 15.80. Found: C 54.35, H 4.51, N 15.84.

Generation of the Dynamic Combinatorial Libraries (DCLs). DCLs Used in DED. Solutions of individual hydrazides (20 mM) and aldehydes (40 mM) were prepared in NaOAc buffer pH 4.0, 50% DMSO, v/v. The libraries were subsequently generated by combining the solutions (15 μL, each) and allowing the resulting mixture to equilibrate at ambient temperature for 3 days to ensure full reaction. Aliquots of the equilibrated solution were subsequently tested in the kinase assay. Sublibraries were prepared in the same way, using NaOAc buffer pH 4.0, 50% DMSO, v/v, solution instead of the excluded building blocks.

Purification of HPrK/P and HPr from *B. subtilis.* HPrK/P and HPr from *B. subtilis* were purified as previously reported, expressed as HPrK/P(Trx-His₆-S-tag) and HPr(His)₆

in *E. coli*.[47] The concentration of HPrK/P was determined spectrophotometrically using the Bio-Rad protein assay with bovine gammaglobulin as standard, and the concentrations of HPr were determined by UV spectrophotometry ($\epsilon = 2900$ M^{-1} cm^{-1}). Protein solutions were stored at -20 °C.

Radioactive Kinase Assay. The general kinase assay for phosphorylation of HPr(His)$_6$ was composed of the DCL or inhibitor sample (4 μL, 50 mM NaOAc, pH 4.0, 50% DMSO, v/v), 50 mM Tris·HCl, pH 7.0 or 8.0, 5 mM MgCl$_2$, 0.5 mM ATP (3.3–5 Bq, leading to 200–300 cpm [γ-^{32}P]ATP/pmol ATP), 2 mM FBP, 0.1% BSA, 10 μM HPr, and, to initiate the reaction, 100 nM HPrK/P, in a final volume of 20 μL. The mixture was incubated at 37 °C for 10 min and the phosphorylation reaction was then terminated by spotting samples onto 1 × 1-cm P81 phosphocellulose paper (Whatman) and dropped immediately into a beaker containing 75 mM H$_3$PO$_4$. The total volume of phosphoric acid solution used was approximately 10 mL for each paper. Unreacted ATP was removed by washing three times with 75 mM H$_3$PO$_4$, 15 min each, and once with ethanol, just covering the papers, for 5 min. The papers were dried and transferred to scintillation vials containing 6 mL of scintillation solution for water samples (Rotiszint ecoplus, Carl Roth), and the radioactivity was determined in a scintillation counter (LKB 1211, Rackbeta, Perkin-Elmer).

Estimation of IC$_{50}$ Values. The IC$_{50}$ (50% inhibition of phosphorylation) curves were based on 13 consecutively diluted concentrations of tested inhibitor included in the assay before initiating the reaction by the addition of enzyme. Typically, each condition 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 test compound. The program GraphPad Prism (GraphPad Software) was adopted for nonlinear regression analysis determining IC$_{50}$ values and Hill coefficient using the following equation: $P = P_{min} + (P_{max} - P_{max})/(1 + 10^{(C-\log IC_{50})H})$, where P is the degree of phosphorylation, C is the inhibitor concentration, and H is the Hill coefficient.

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