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Identification of Peptide Substrate and Small Molecule Inhibitors of Testis-Specific Serine/Threonine Kinase1 (TSSK1) By the Developed Assays

Leilei Zhang, Yu Yan, Zijie Liu, Zeper Abliz, and Gang Liu*

Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

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In this paper, a peptide substrate (Pep8) of TSSK1 is identified. Using Pep8 as a substrate, two homogeneous and efficient assays for TSSK1 inhibitors screening have been developed, including luminescent kinase assay and LC−MS-based high-throughput assay. Two classes of compounds were identified that are able to efficiently inhibit phosphorylation catalyzed by TSSK1.

1. Introduction

Protein phosphorylation plays a critical role in the regulation of many cellular processes. Protein kinases are among the largest family of proteins known, with 518 putative protein kinase genes that were defined within the human genome.[1] Because abnormal kinase activities are associated with a wide range of diseases, including leukemias, tumors, vascular diseases, diabetes mellitus, and immune/inflammatory disorders, protein kinases have now become one of the most important groups of drug targets in addition to the G protein-coupled receptors.[2] The testis-specific serine/threonine kinase (TSSK) family is one with exclusive or predominant expression in the testis and consists of at least five members.[3−10] TSSKs were very recently validated as potential contraceptive targets.[11,12] The expression of TSSK1 was restricted to the last stages of spermatid maturation. Recent studies demonstrated that TSSK1 is localized in the cytoplasm of late spermatids and in structures resembling residual bodies, suggesting that TSSK1 probably participates in the reconstruction of cytoplasm during sperm tail maturation.[4] Therefore, inhibition of TSSK1 may provide male contraception. However, neither peptide substrate nor small molecule inhibitors of TSSK1 have been reported until recently, with TSKS (65 kDa protein) being shown to be phosphorylated at its Ser281 by TSSK2, which shared high sequence similarity with TSSK1.[4,13]

*To whom correspondence should be addressed. Phone/Fax: +86 10 63167165. E-mail:gangliu27@yahoo.com.

[a] Abbreviations: ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; BOP, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DIC, *N,N′*-diisopropylcarbodiimide; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ESI, electrospray ionization; HBTU, *O*-benzotriazole-*N,N,N′,N′*-tetramethyl-uronium-hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; HTS, high-throughput screening; IC_{50}, concentration for 50% inhibition; LC−MS, Liquid chromatography−mass spectrometry; Pep4, Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ser-Ser; Pep8, Ala-Ala-Leu-Val-Arg-Gln-Met-Ser-Val-Ala-Phe-Phe-Phe-Lys; Pep8-P, Ala-Ala-Leu-Val-Arg-Gln-Met-Ser(HPO_3)-Val-Ala-Phe-Phe-Phe-Lys; Pep8-P(O_2), Ala-Ala-Leu-Val-Arg-Gln-Met(O_2)-Ser(HPO_3)-Val-Ala-Phe-Phe-Phe-Lys; RP, reversed phase; Rt, retention time; RLU, relative light units; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol; TIC, total ion chromatography; TOF-MS, time-of-flight mass spectrometry; TFA, trifluoroacetic acid; UHP, urea−hydrogen peroxide.

2. Results and Discussion

2.1. Identification of TSSK1 Peptide Substrates. Identifying biologically relevant substances for protein kinases is a critical step to understanding the function of those new enzymes. In particular, an inexpensive substrate is necessary for high-throughput screening (HTS[a]) of kinase inhibitors. To search for a peptide substrate of TSSK1, two representative kinase substrate screening kits were selected and synthesized in this article, one from AnaSpec Corporate (10 peptides)[14] and the other from Cell Signaling Technology (76 peptides).[15] Accordingly, a total of 86 peptides were synthesized simultaneously, employing a "split−mix" combinatorial chemistry strategy and the standard Fmoc peptide chemistry by using Rf tag in a Microkan.[16] All synthesized crude peptides were subsequently analyzed using a fast liquid chromatographic−mass spectrometry (LC−MS) system to determine the purities and the correct molecular weights. The crude peptides were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mM and further diluted to 2 mM with 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)-buffer (Tris−HCl, pH = 7.4) as working solutions. The substrate screening was carried out at a final TSSK1 concentration of 1.0 μg/well with addition of 2 μM adenosine 5′-triphosphate (ATP). The phosphorylation of peptides was measured by detection of ATP remaining after certain reaction times using the Kinase-Glo reagent.[17] The substrate identification was subsequently performed as two runs. In the first, the synthetic crude peptides were used directly to obtain potential substrate peptides and eliminate the most inactive peptides. The active hits in the first run were then resynthesized and purified by HPLC to reproduce those results in the second run. Two peptides (Pep8 and Pep4) showed strikingly less luminescence, indicating that Pep4 and Pep8 were phosphorylated in the course of the TSSK1 reaction and the ATP was consumed. Dose curve studies demonstrated that Pep8 is more sensitive to phosphorylation than Pep4 (Figure 1a). Further studies demonstrated that these two peptides were unable to be phosphorylated experimentally by cyclin-dependent kinase 2 and 4 (data not shown). Pep4 contains multiple phosphorylation sites of threonine and serines, however, the specific one is unknown. It was reported that a mouse TSKS phosphopeptide

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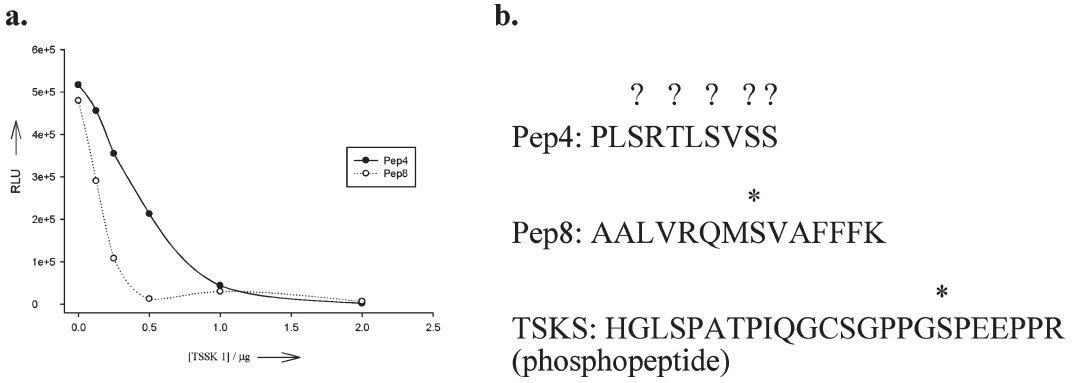


Figure 1. (a) Pep8 (AALVRQMSVAFFFK) was able to be phosphorylated at lower concentrations of TSSK1 in a dose-dependent manner than Pep4 (PLSRTLSVSS). (b) Sequence alignment of the TSKS phosphopeptide with Pep4 and Pep8. Asterisks indicate the phosphorylation positions. Question marks represent the plausible phosphorylation sites. RLU = relative light units.

(HGLSPATPIQGCSGPPGS*PEEPPR) by TSSK2 was identified from immunoprecipitated complexes of TSKS and its antibody by IMAC−LC−MS/MS method.[13] Analysis of sequence alignment mouse TSKS phosphopeptide with Pep4 and Pep8 did not reveal the conserved residues, indicating that Pep4 and Pep8 are novel substrates of TSSK1 (Figure 1b).

2.2. Identification of Small Molecule Nonpeptide Inhibitors with a Luminescent Kinase Assay. To further identify the nonpeptide inhibitors of TSSK1, the kinase reaction conditions were optimized with respect to the amount of ATP, kinase, and final peptide substrate concentration. Accordingly, serial 2-fold dilutions of ATP resulted in a linear correlation with the amount of ATP depleted (Figure 2a), therefore, the middle concentration of 1.5 μM of ATP and excess Pep8 (20 μM) were used for selection of the optimal TSSK1 concentration (Figure 2b). Recordings during the optimization of the TSSK1 concentration resulted in the largest change in luminescence in wells that were missing Pep8 compared with the reaction wells in which the kinase reaction were completed in the presence of Pep8, where the concentration of TSSK1 was 0.5 μg/well. In addition, the amount of ATP depleted also increased as Pep8 increased (Figure 2c). Obvious luminescence changes were observed when Pep8 was at 10 μM. In all, the final conditions for HTS of TSSK1 inhibitor were defined as 1.5 μM of ATP, 0.5 μg/well of TSSK1, and 10 μM of Pep8.

Compounds with 14 different chemical skeletons (Figure 3a) were in stock in our laboratory as 50 mM solutions. These compounds were assembled through a scaffold-directed program, aimed at developing benzo-fused privileged structures with druglike properties.[18−29] A total of 640 single pure compounds were screened with initial concentration of 20 μM (see Supporting Information). Among them, eight compounds showed reproducible inhibitory activity (>40%) and two of them, 5-hydroxy-8-methyl-4-propyl-7,8-dihydropyrano[3,2-g]chromene-2,6-dione (**7**) and 1,1′-(biphenyl-4,4′-diyl)bis(2,2-dihydroxyethanone) (**8**), had measured IC_{50} values of 38.7 μM and 43.2 μM, respectively (Figure 3b).

2.3. Development and Validation of Liquid Chromatography−Mass Spectrometry (LC−MS) Based Assay. Conventional kinase assays include fluorescence analysis,[30−33] radioactive methods,[34,35] luminescence (bioluminescence) assays,[36,37] and colorimetric detection of a quantifiable substrate or product.[38,39] Because natural substrates or products are typically colorless, not fluorescent or luminescent, and not radioactive, traditional kinase assays usually require some modification of the substrates for compatibility with the means of detection, including fluorescent tagging, isotopic labeling, or enzyme coupling. Recent efforts have recognized that mass spectrometric techniques are useful for measuring enzyme activities and enzyme inhibitor screening as well as for studying enzyme kinetics.[40,41] LC−MS techniques can detect the reaction products directly and quantitatively, avoiding modification of substrates or secondary enzymatic reactions that are irrelevant to the target enzyme reaction. Thus, an LC−MS-based assay could be applied to any enzyme reaction system providing that the substrates and products are distinguishable by molecular weight and retention behavior on the basis of reasonable ionization efficiency.

2.3.1. Synthesis of Pep8-P and Pep8-P(O_2). To develop an LC−MS-based assay, phosphorylated product (Pep8-P) and an internal standard (Pep8-P(O_2)) were required, particularly to optimize the LC separation conditions for elimination of overlap between Pep8 and Pep8-P. There are currently two strategies for the synthesis of phosphopeptide. The building block approach uses protected phosphoamino acids and the global phosphorylation method involves post-synthetic phosphorylation of unprotected hydroxyl groups on the solid support. Owing to the commercial availability of suitably protected Fmoc-Ser(PO(OBzl)-OH)-OH, the Pep8-P was directly coupled to peptide on solid-phase using *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole (HOBt) as activator and additive, respectively. Deprotection of the side chain and cleavage of Pep8-P from the resin were achieved simultaneously by treatment of the resin with a cocktail solution of trifluoroacetic acid (TFA)/H_2O/thioanisole (v/v/v = 95:2.5:2.5). After purification of Pep8-P by semipreparative HPLC, direct confirmation was indicated by an increase in the molecular weight of Pep8 of 80 Da, corresponding to the addition of one phosphate group.

Because the Pep8-P contains a methionine in sequence, the sulfone analogue of Pep8-P at Met, with its physical and chemical properties close to Pep8-P, was chosen herein to be the internal standard. Many methods of preparing sulfones from sulfides have been reported. Among them, urea−hydrogen peroxide (UHP) oxidation is a simple and safe method.[21] UHP, an adduct of hydrogen peroxide and urea, is a mild, inexpensive, stable, and relatively safe oxidant.

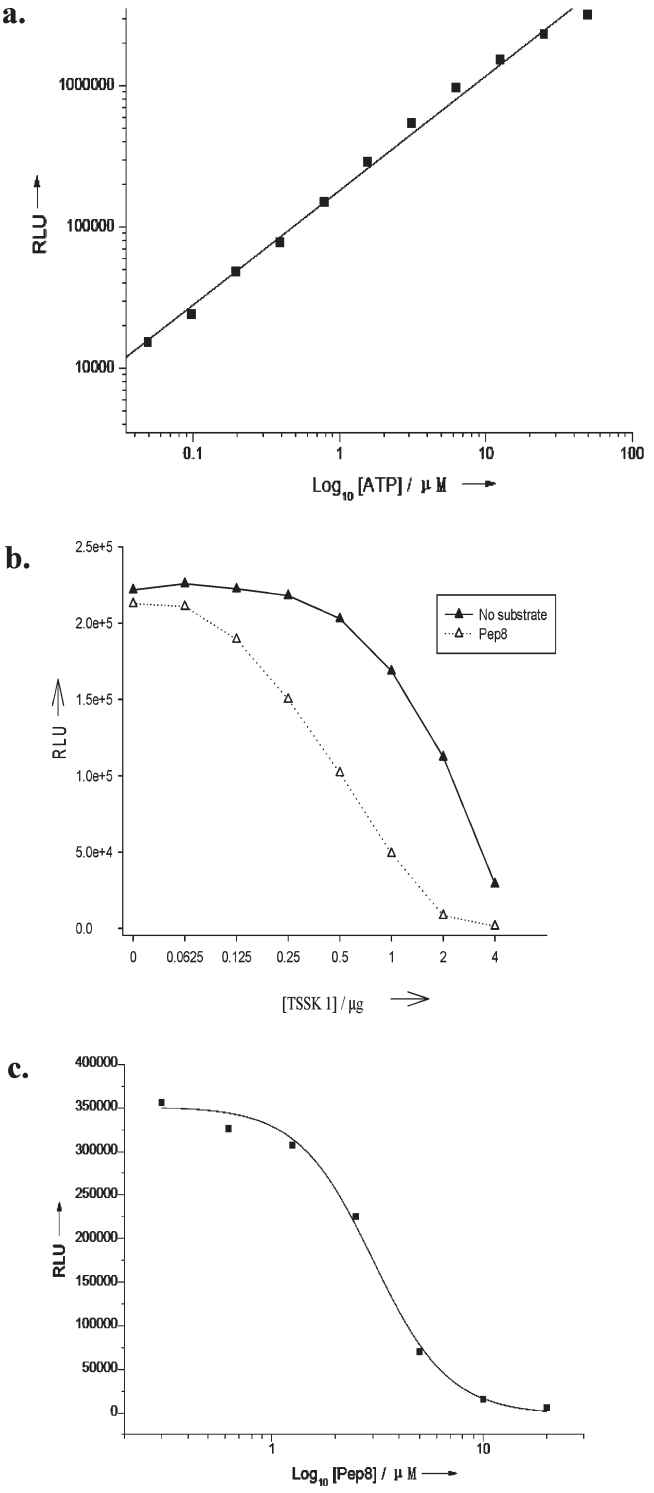


Figure 2. Optimization of the luminescent kinase assay for TSSK1. (a) Correlation of RLU readings and ATP concentrations (r^2 = 0.997). Serial 2-fold dilutions of ATP were made in a 96-well plate in 50 μL of kinase reaction buffer (40 mM Tris−HCl, 20 mM $MgCl_2$, and 0.1 mg/mL bovine serum albumin (BSA)). (b) Luminescence changes in the presence of TSSK1 after 2-fold serial dilutions in wells missing the Pep8 and in wells with completed kinase reactions. The amounts of ATP and Pep8 were 1.5 μM and 20 μM, respectively. (c) ATP depletion increased as Pep8 increased, in reactions where ATP and TSSK1 were 1.5 μM and 0.5 μg/well, respectively.

The byproducts of UHP oxidation are TFA and urea (Scheme 1), which are easily washed away with water. Therefore, UHP is particularly useful for peptide sulfonation. Here, UHP was used for the first time to oxidize peptide, and Pep8-P was conveniently and quantitatively converted into its sulfone analogue (Pep8-P(O_2)) (Scheme 1).

2.3.2. LC−MS-Based Assay Development. Theoretically, either peptide substrate or its phosphorylated product can be used for kinase activity evaluation by mass spectrometry analysis. However, primary experiments indicated that the signal from the Pep8-P was more facilitated to ionization than Pep8 (see Supporting Information). To efficiently separate Pep8-P from other reaction components, several commercially available columns were investigated. Among them, a Kromasil C18 (50 mm × 4.6 mm) column appeared to have the best efficacy for dealing with desalt procedures in a relatively short gradient running time. A formic acid/acetonitrile-based mobile phase at pH 2.9 was selected to achieve good HPLC performance as well as good volatility and appropriate pH for positive ion detection.

The sensitivity and accuracy of the mass spectrometry can be greatly compromised by the presence of a buffer. Therefore, a switching valve on the Agilent LC/MSD TOF was successfully combined with efficient HPLC separation to completely remove salt contaminants from the sample prior to analysis of the mass spectrometry. Basically, a proper HPLC gradient could elute all salts in a short time under polar conditions and these could be switched into the waste. The optimized HPLC conditions ensured that inorganic components were completely eluted from the column, but the substrate and phosphorylated substrate were still retained in the column. In fact, a total of 2.5 min of gradient flow was switched into waste in this study to guarantee at least 90 sample analyses without observing any contamination.

As discussed above, Pep8-P and Pep8 were separated efficiently under optimal HPLC conditions. For obtaining good repeatability as well as good chromatographic peak shape, an optimal fragmentor voltage was systemically investigated. Because peptides are easily ionized to multiply charged ions and show multiple charge distributions with different fragmentor voltages, the resulting mass spectra of Pep8-P showed prominent peaks at m/z 847.9 and m/z 565.3, which correspond to the +2 and +3 charge states of phosphorylated Pep8, respectively. Studies indicated that the lowest fragmentor voltage (150 V) resulted in a prominent +3 charged ion at m/z 565.3 ($[M + 3H]^{3+}$). In contrast, the highest fragmentor voltage (250 V) gave mainly +2 charged ions at m/z 847.9 ($[M + 2H]^{2+}$). Optimally, at 230 V, m/z 847.9 was prominent and both good repeatability and chromatographic peak shape were observed simultaneously.

LC−MS and a one-point normalization factor were used to determine the K_m values of two substrates.[42] In this study, Pep8-P(O_2), the sulfone analogue of Pep8-P, was the internal standard for K_m determination. Pep8-P and Pep8-P(O_2)'s TIC peaks were completely separated (see Supporting Information). Substrate time courses for ATP and Pep8 (Figure 4), respectively, were recorded in duplicate at varied substrate concentrations, while other substrate Pep8 or ATP was fixed at a saturated concentration. A kinetic plot was generated by plotting the initial reaction velocity against the different substrate concentrations. The calculated K_m values for ATP and Pep8 were 50.5 and 25.2 μM, respectively. In this article, therefore, we set ATP and Pep8 substrates at 25 μM and 10 μM for LC−MS-based

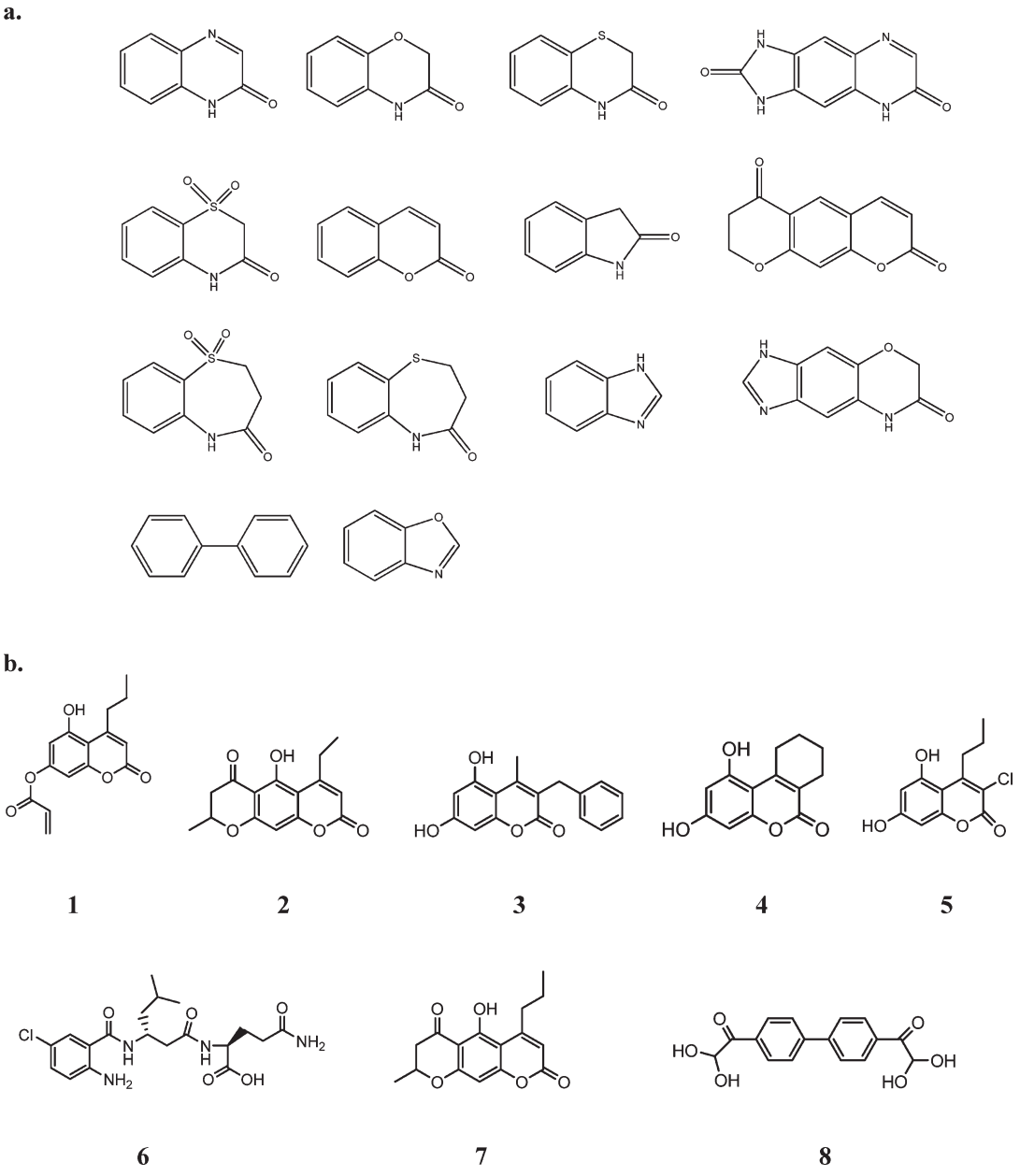
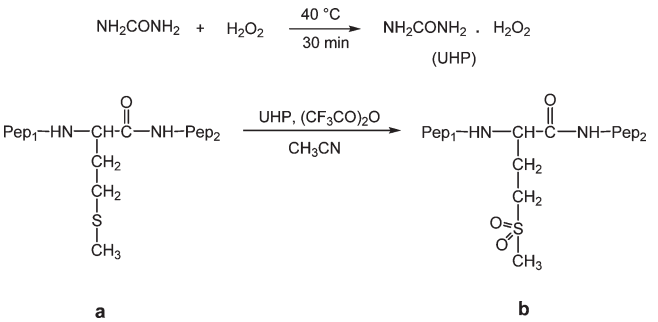


Figure 3. Identification of TSSK1 inhibitors. (a) The selected compound skeletons. (b) Eight compounds showed reproducible inhibitory activity (>40%).

Scheme 1. Oxidation of Pep8-P to Sulfonated Pep8-P(O2) at the Methionine Residue[a]



[a] Pep1 = NH_2-Ala-Ala-Leu-Val-Arg-Gln-CO, Pep2 = −Ser(HPO3)-Val-Ala-Phe-Phe-Phe-Lys-$CONH_2$.

high-throughput identification of TSSK1 small molecule inhibitors.

To determine the optimal TSSK1 concentration and incubation time, the kinase reaction was studied systematically at 150 ng/well, 75 ng/well, 37.5 ng/well, 18.75 ng/well, and 0 ng/well of TSSK1, respectively. The Pep8 phosphorylation level was determined within 180 min. A linear rate of product formation was observed within 120 min for 75 ng TSSK1 and within 60 min for 150 ng TSSK1 (Figure 5). Although phosphorylation of Pep8 also occurred linearly within the total testing time period for 18.75 and 37.5 ng of TSSK1, Pep8-P level was low enough that the results may be beyond the limitation of mass detection sensitivity. Hence, a kinase amount of 75 ng and 90 min incubation time were optimal for high-throughput screening (HTS) of chemical libraries.

To qualify the method, the linearity of the method was further investigated over a concentration range of 33.8−676.8 ng of Pep8-P. The product peak area versus Pep8-P concentration was plotted to generate a standard curve by

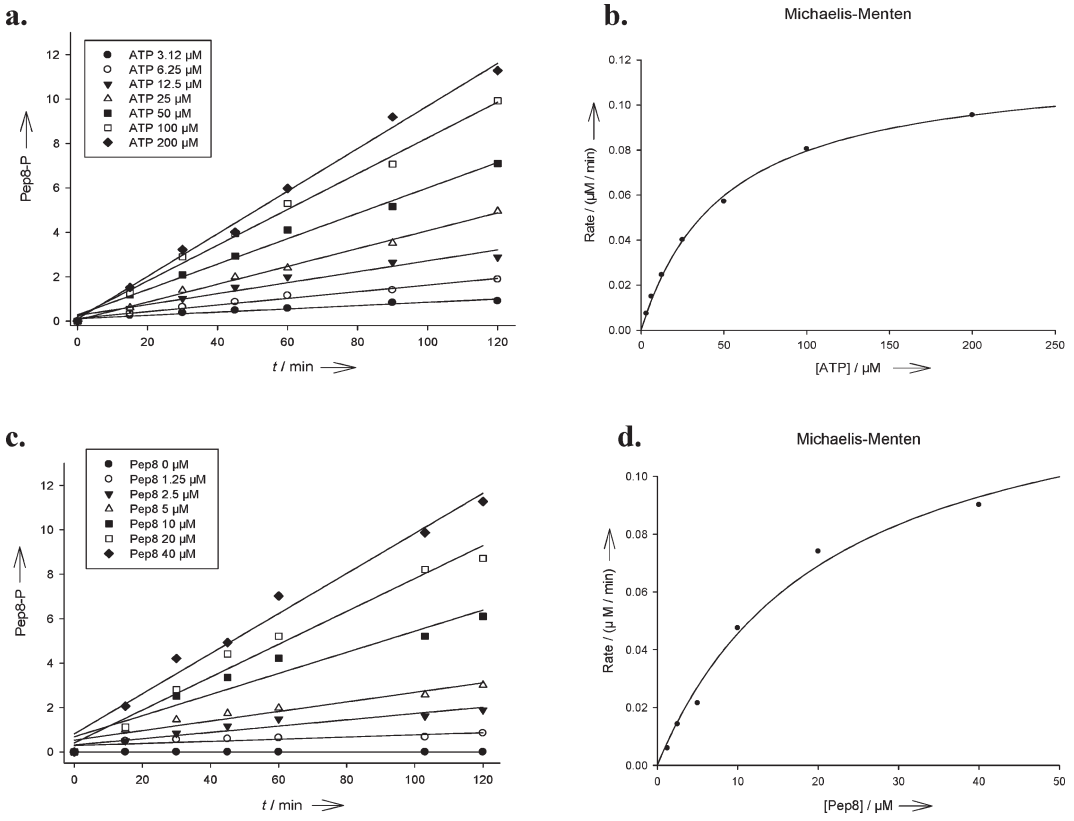


Figure 4. The K_ms of ATP and Pep8 were determined by LC−MS measurement. (a) Pep8 phosphorylation was time-dependent linear as the ATP concentration varied from 3.12 to 200 μM in the presence of 0.5 μg/well TSSK1 and 30 μM Pep8. (b) Michaelis−Menten plot for ATP. The K_m value of ATP was determined as 50.5 μM. (c) Pep8-P was linearly occurred in time-dependent manner as the Pep8 concentration varied from 0 to 40 μM in the presence of 0.5 μg/well TSSK1 and 200 μM ATP. (d) Michaelis−Menten plot for Pep8. The K_m value of Pep8 is obtained as 25.2 μM. The K_m values are calculated by Systat Sigmaplot 10.0.

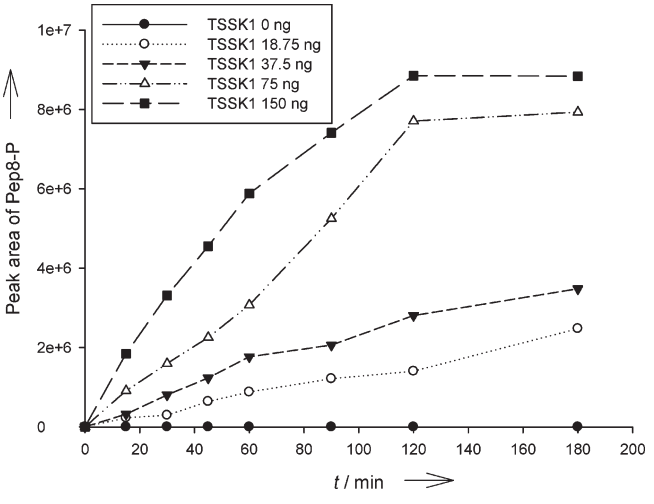


Figure 5. Phosphorylation level of Pep8 as time changes of incubation and variable TSSK1 doses.

linear regression. The obtained high correlation coefficient (0.99) demonstrates that this method was highly suitable (Figure 6a).

The major advantage of the LC−MS-based assay was that it could be automated. Because many factors influence MS analysis, the reproducibility and stability of this method were determined in the following manner. When Pep8-P was dissolved in a buffer system (acetonitrile:kinase buffer = 50:50 (v/v)), the obtained RSD was 7.34% for 91 continuous injections (Figure 6b). In another experiment, 89 TSSK1 reactions were carried out simultaneously under optimal conditions and analyzed by LC−MS. The precision evaluated by repeatedly measuring the peak areas of Pep8-P was 10.6% (Figure 6c). All of these results indicate that this LC−MS-based assay of TSSK1 reaction developed was highly reproducible.

To assess the high-throughput screening, we also estimated the effect of added DMSO on the TSSK1 activity prior to the evaluation of inhibitors. The results indicated that DMSO less than 2% did not affect both the analysis and enzymic reaction. To valuate the Z'-factor, a total of 48 mass spectra were acquired, of which 24 spectra were collected in the presence of TSSK1 (75 ng in the assay buffer) and 24 were not (see Supporting Information). Calculations based on these data provided a Z' score of 0.836, indicating that the developed mass-based assay was excellent.

2.4. High-Throughput Screening of Single Compound or Mixture Compound Libraries by the Developed LC−MS-Based Assay. 2.4.1. Screening Biphenyl Compounds. The LC−MS-based assay was then used to screen small molecule inhibitors of TSSK1. In above luminescent kinase assay, compound **8** was identified, which belongs to biphenyl compound scaffold. Twenty analogues in our stock library were further screened as TSSK1 potential inhibitors seeking more potent compound. Fortunately, one of these, sodium 2,2′-(biphenyl-4,4′-diyl)bis(1-hydroxy-2-oxoethanesulfonate (**9**), which is the adduct of **8** and sodium hydrogen sulfite, was found to be more potent.[43] Its IC_{50} value

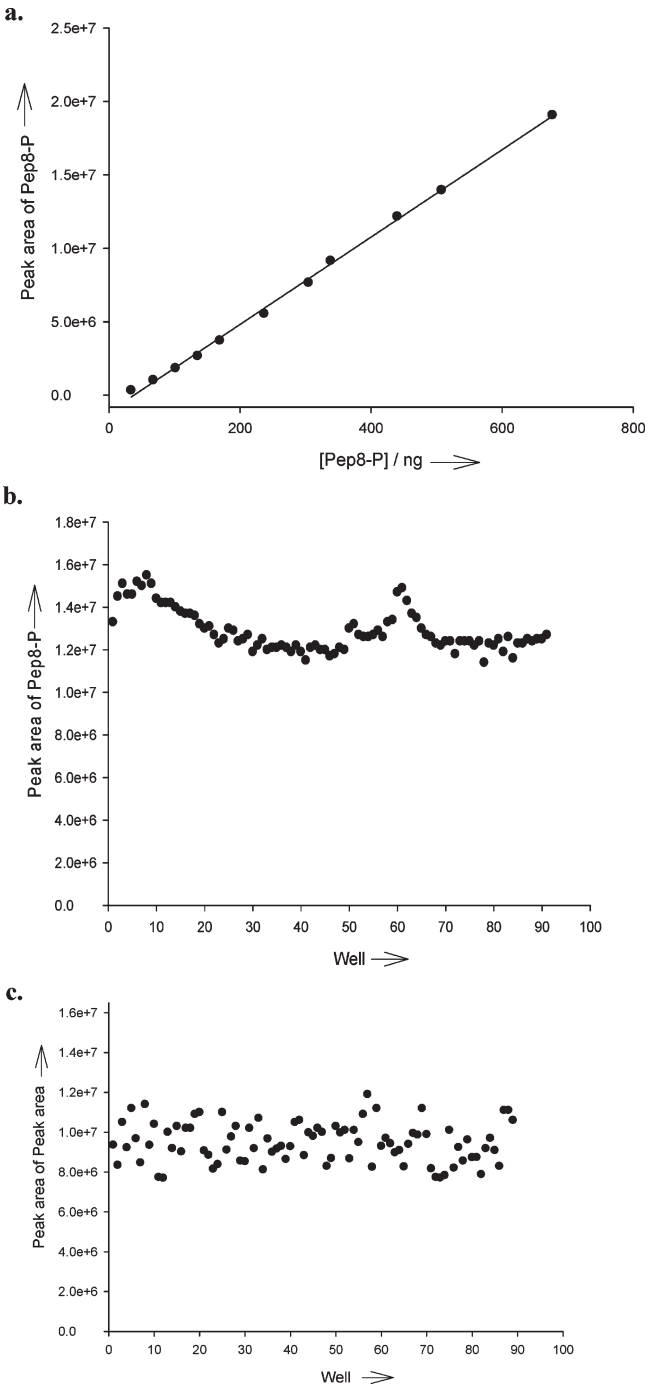


Figure 6. (a) LC−MS peak area counts for Pep8-P was correlatively linear with Pep8-P concentration (r^2 > 0.99). (b) Repeatability of Pep8-P in 50% ACN−kinase buffer for 91 continuous injections. (c) Repeatability of Pep8-P peak area in 89 TSSK1 catalyzed reactions. The optimal reaction conditions were: 40 mM Tris−HCl, pH 7.4, 20 mM $MgCl_2$, 2 mM Dithiothreitol (DTT), 0.1 mg/mL BSA, 25 μM ATP, 75 ng TSSK1, and 10 μM Pep8. These results were obtained by LC−MS analysis system (the analysis conditions are included in [4]Experimental Section).

was calculated by three different methods developed in this study to give 19.1 μM for LC−MS-based assay with internal standard (Pep8(O_2)-P), 14.4 μM for LC−MS-based assay without internal standard, and 24.8 μM for a luminescent kinase assay, respectively (Figure 7a,b,c). The smaller range in the results confirms the validity of

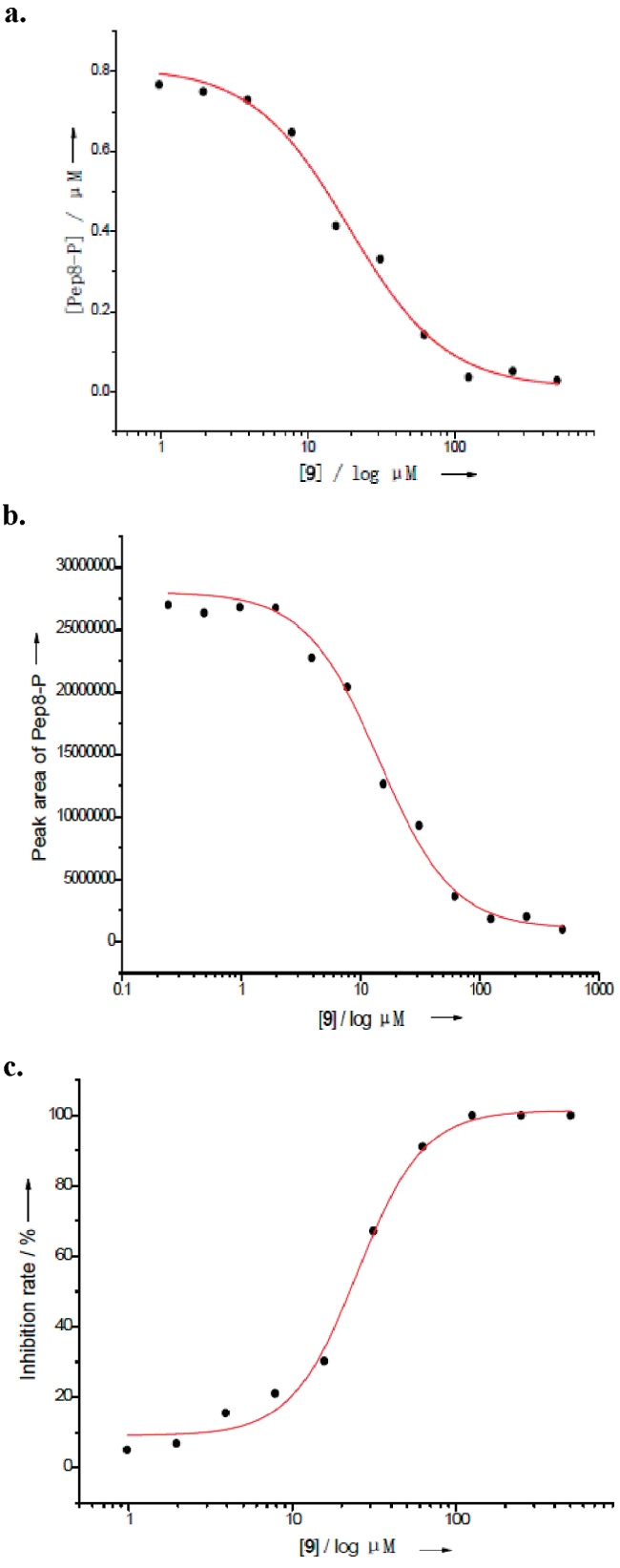
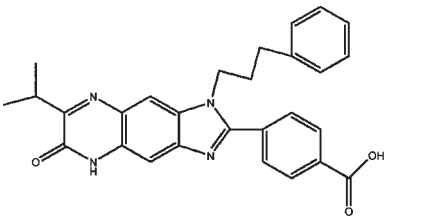
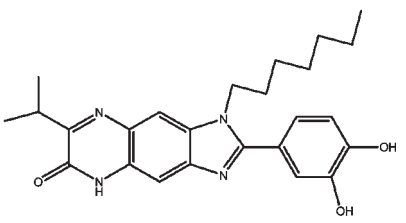


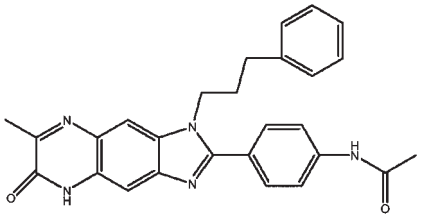
Figure 7. Inhibitory curves of compound **9** to TSSK1 were illustrated by developed LC−MS-based assay with or without internal standard (Pep8-(O2)) (a,b) and a luminescent kinase assay (c). X axis represents the log 10 value of the compound **9** concentration, while *Y* axis is the peak area of Pep8-p for (a), the concentration of Pep8-p for (b), and the inhibitory rate for (c). All of the IC_{50} values were plotted and calculated by the software Origin 7.5.



14 (IC_{50}=38 μM)



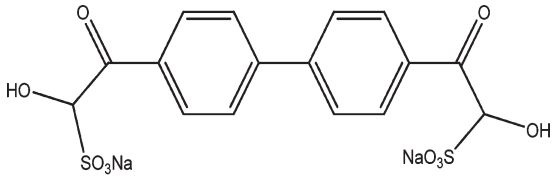
15 (IC_{50}=72 μM)



16 (IC_{50}=98 μM)

Figure 8. Novel 1,2,7-trialky-1*H*-imidazo[4,5-*g*]quinoxalin-6-ones that inhibited TSSK1 activity.

the developed assays.



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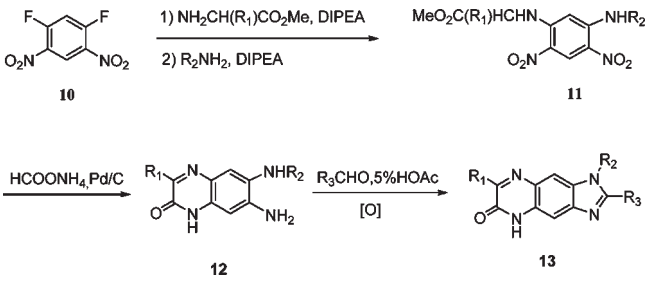
2.4.2. Screening a chemical library of 1,2,7-trialky-1*H*-imidazo[4,5-*g*]quinoxalin-6-one. Privileged structures have been successfully exploited across and within different target families and promises to be an effective approach to the discovery and optimization of novel bioactive molecules. Both benzimidazole and quinoxalin-2-one are important pharmacophores and privileged structures in medicinal chemistry encompassing a diverse range of biological activities. The incorporation of two privileged pharmacophores may provide additional opportunities to discover novel lead compounds using one synthetic process. A chemical library of 1,2,7-trialky-1*H*-imidazo[4,5-*g*]quinoxalin-6-one was designed and synthesized by our laboratory to integrate benzimidazole and quinoxalin-2-one privileged structures into one molecule.[26] This library was composed of five mixed analogues per well, and the total purity of five compounds/well is > 80% by HPLC analysis (UV wavelength at 254 nm).

In the primary screening, 370 wells consisting of 1850 compounds were assayed at a total estimated concentration of 20 μM of five compounds in each well. Every mixture well was measured twice in parallel. A total of eight wells with greater than 50% repetitive inhibition of TSSK1 activity were selected for secondary screening. Each individual compound in these eight positive wells was then resynthesized as outlined in Scheme 2. Two fluorine groups on the starting material 1,5-difluoro-2,4-dinitrobenzene (DFDNB)

Scheme 2. Synthesis of 1,2,7-trialky-1*H*-imidazo[4,5-*g*]quinoxalin-6-one



(**10**) were subsequently and quantitatively displaced by alkyl primary amines and amino acid methyl or ethyl esters in the presence of *N*-ethyldiisopropylamine (DIPEA).[30] Subsequently, the quantitative reduction of two aromatic nitro groups of **11** to produce **12** was achieved in high yield using $HCOONH_4$ and Pd/C. In the presence of 5% acetic acid in dioxane, treatment of **12** with aldehydes at 77 °C gave high yields of **13**. All of 40 compounds were finally characterized by ^{1}H NMR and high-resolution MS after recrystallization or purification by semipreparative HPLC until the purity was greater than 95% (see Supporting Information).

These 40 individual pure compounds were further tested for inhibitory activity against TSSK1. Three compounds showed positive activity (Figure 8). Herein, false-positive wells were thought to arise from impurities in the combinatorial library. Nevertheless, it was shown that mixture library synthesis and mixture screening could greatly improve the efficiency of lead compound discovery by a LC−MS-based assay.

3. Conclusion

In this study, a peptide substrate of TSSK1 was identified for the first time. Furthermore, a luminescent kinase assay using Kinase-Glo reagent and a highly efficient LC−MS-based assay were developed for high-throughput identification of novel TSSK1 inhibitors. Two classes of compounds

(biphenyl compounds and 1,2,7-trialky-1*H*-imidazo[4,5-*g*] quinoxalin-6-ones) were identified as being able to efficiently inhibit TSSK1-catalyzed phosphorylation. The LC−MS-based assay proved to be more sensitive than the luminescent method. For instance, the amount of kinase required for the LC−MS-based assay was 75 ng, but 500 ng of TSSK1 was required for the luminescent method when optimal kinase reaction conditions were reached for TSSK1-catalyzed phosphorylation. In addition, the LC−MS-based assay directly detects the native substrate, whereas the luminescent method indirectly detects ATP consumption. The former is compatible with mixture analysis and significantly enhances the screening throughput.

4. Experimental Section

4.1. Materials. ATP disodium salt, BSA, DTT, ($MgCl_2 \cdot 6H_2O$), and Tris were purchased from Amresco (Solon, OH). All Kinase-Glo reagents were obtained from Promega (Milano, Italy). Purified TSSK1 was a gift from Shanghai Genomics (Shanghai, China). Acetonitrile and water were HPLC gradient grade. Formic acid was obtained from Fluka (Vienna, Austria). All amino acids used for peptide synthesis and coupling reagents (HBTU, BOP, and DIC) and HOBt were purchased from GL Biochem Ltd. (Shanghai, China). Rink amide resin (loading 0.44 mmol/g, 1% DVB, 100−200 mesh) was purchased from Hecheng, Co. (Tianjin, China). All amino acid methyl or ethyl esters were purchased from Chem-Impex International, Inc. (Wood Dale, IL). All alkyl primary amines and aldehydes were purchased from Acros Organics (Geel, Belgium). All organic solvents were redistilled after a proper drying program.

4.2. LC−MS Analysis System. Liquid chromatography/electrospray/time-of-flight mass spectrometry in positive ionization mode was used to separate and identify Pep8 and Pep8-P. The analytes were separated using an HPLC (series 1100, Agilent Technologies, Palo Alto, CA) equipped with a 50 mm × 4.6 mm reversed-phase C18 analytical Kromasil column (Beijing Analytical Instrument Institute, China) with 5 μm particle diameter. Mobile phase A was acetonitrile with 0.1% formic acid, and mobile phase B consisted of water with 0.1% formic acid. The HPLC conditions were as follows: 5% A for 1.5 min, followed by a gradient to 25% A in 0.1 min, then a linear gradient progressing from 25% A to 60% A in 4.9 min, after which a gradient increased A to 95% in 1 min, followed by a stop time of 7.5 min and a post time of 1.5 min 5% A. The flow rate was 0.8 mL/min, and 60 μL of sample was injected. The HPLC system was connected to a time-of-flight mass spectrometer (MSD-TOF, Agilent Technologies) equipped with an electrospray interface under the following operating parameters: capillary 2500 V, nebulizer 30 psig, drying gas 10 L/min, gas temperature 300 °C, fragmentor 230 V, skimmer 50 V. The mass axis was calibrated using the mixture provided by the manufacturer over the m/z 50−3200 range. A second orthogonal sprayer with a reference solution was used as a continuous calibration using the reference masses 121.0509 and 922.0098 m/z. Spectra were acquired over the m/z 50−3000 range at a scan rate of 1 scan/s.

4.3. Peptide Library Synthesis. The peptide library containing 86 peptides was synthesized using a Fmoc-based synthesis protocol and mix-and-split approach using the IRORI sorting system. Rink amide resin (28 mg) was put into every MicroKan using a resin-packing device. At the same time, an Rf tag was put in and MicroKans were covered by a parallel capping device. The tag was coded before the first coupling and decoded before the next coupling step. The resin-bound Fmoc-protected peptide was treated twice with 20% piperidine in DMF for 15 min for deprotection. The coupling procedure involved the appropriate Fmoc-amino acids (3 equiv), HOBt (3 equiv), and coupling reagent (BOP or DIC, 3 equiv) dissolved in DMF. Coupling reactions were allowed to proceed for different times, depending on the particular sequence. In cases of insufficient coupling, repeated coupling was performed until the reaction was complete. The N-terminus was acetylated using 15% acetic anhydride in DCM for 30 min. Several difficult sequences were selected as controls during the course of the coupling reaction and were monitored using the Kaiser test. Crude peptides were cleaved using a one-step TFA cleavage method with different cleavage conditions (according to peptide synthesis manuals, such as Nova Biochem). The TFA cleavage mixture was added to a tube containing a peptide resin, and the mixture was shaken for 2 h at room temperature. Upon completion of the cleavage reaction, the "peptide + resin" mixture was filtered and the tube was rinsed with 2−3 mL of cleavage mixture. The solution was treated with ice-cooled methyl *tert*-butyl ether/petroleum ether (3:1) to precipitate the peptide. The crude peptides were washed six times with ice-cooled methyl *tert*-butyl ether/petroleum ether (3:1). Crude peptides were redissolved in 30−60% CH_3CN−H_2O (v/v) and analyzed by LC/MS, indicating that the purities were from 30% to 95% under UV 214 nm wavelength.

4.4. Synthesis of Pep8 and Pep8-P. Pep8 and Pep8-P, which correspond with the sequences of Ala-Ala-Leu-Val-Arg-Gln-Met-Ser-Val-Ala-Phe-Phe-Phe-Lys and Ala-Ala-Leu-Val-Arg-Gln-Met-Ser(HPO_3)-Val-Ala-Phe-Phe-Phe-Lys, respectively, were manually synthesized according to standard Fmoc solid-phase strategy.[44] Noticeably, the coupling procedure of Fmoc-Ser(PO(OBzl)-OH)-OH for synthesis of Pep8-P involved the appropriate Fmoc-amino acids (5 equiv), HOBt (5 equiv), and coupling reagent (HBTU, 5 equiv) dissolved in DMF containing 15 equiv of DIPEA. Double coupling was performed until the reaction was complete. The N-terminus was acetylated using 15% acetic anhydride in DCM for 30 min. The side chain benzyl group was removed using TFA/TIS/water (95:2.5:2.5) for 3 h. Peptides were lyophilized and purified by HPLC until >97% under UV 214 nm wavelength.

4.5. Synthesis of Internal Standard (Pep8-P(O_2)). Freshly prepared UHP (0.07 mmol) was dissolved in acetonitrile (300 μL), and trifluoroacetic anhydride (50 μL) was added slowly with stirring to achieve full dissolution. UHP−trifluoroacetic anhydride oxidate was then prepared. Pep8-P (12 mg) was dissolved in acetonitrile (2 mL), and UHP−trifluoroacetic anhydride oxidate (70 μL) was added with stirring at room temperature. After completion of oxidation, the reaction mixture was subjected to HPLC analysis, and the mixture was filtered with a 0.45 μM membrane and subjected to desalting with RP-HPLC until the purity was >97% under UV 214 nm wavelength.

4.6. K_m Determination. Stock solutions of Pep8-P (1 mM) and Pep8-P(O_2) (0.7 mM) were prepared in kinase buffer. The mixed solution of Pep8-P(O_2) (7 μM) and Pep8-P (10 μM) was then prepared in 50% acetonitrile−kinase buffer (v/v). This mixture was analyzed by the developed LC−MS analysis system. Thirty samples were run sequentially with an injection volume of 15 μL. The normalization factor was determined from eq 1, shown below. $A_{Pep8\text{-}P}$ was the peak area of Pep8-P and A_{IS} was the peak area of the internal standard. This ratio was multiplied by the respective concentrations of internal standard and Pep8-P in order to obtain R, the normalization factor. The average R value was used for subsequent calculations of product concentration.

$$R = \frac{(A_{Pep8\text{-}P})[\text{internal std}]}{(A_{IS})[\text{Pep8-P}]} \quad \text{(eq1)}$$

Determination of K_m for two substrates (ATP concentrations ranged from 3.13 to 200 μM and Pep 8 concentrations ranged from 0 to 40 μM) was performed using the LC−MS assay under conditions of saturation of the other substrates as follows: [ATP] = 200 μM, [Pep8] = 30 μM. Data were collected in duplicate and averaged. Other reaction components were 75 ng TSSK1, 40 mM Tris−HCl, pH 7.4, 20 mM $MgCl_2$, 0.1 mg/mL BSA, and 2 mM DTT. Kinase reactions with 50 μL volume were incubated at 37 °C. After incubation for different periods, the

reactions were quenched with 50 μL of acetonitrile containing 7 μM internal standard (Pep8-P(O_2)), then centrifuged (15000g × 10 min) and subjected to HPLC−MS analysis. The K_m value was determined by performing a nonlinear least-squares best fit to the Michaelis−Menten equation using Systat Sigmaplot 10.0.

4.7. Luminescent Kinase Assay. All TSSK1 reactions were performed in a kinase buffer (40 mM Tris−HCl buffer, 20 mM $MgCl_2$, 0.1 mg/mL BSA, 2 mM DTT). To obtain the best performance when using Kinase-Glo reagent, optimal kinase conditions were established. TSSK1 was resuspended in 25 μL of kinase buffer. The kinase reaction was performed by adding 25 μL of a mixture containing ATP and peptide substrate (Pep8) in kinase buffer. The final reaction conditions were 0.5 μg/well TSSK1, 10 μM Pep8, and 1.5 μM ATP. The reaction was incubated for 2 h at 37 °C, and then an equal volume of Kinase-Glo reagent (50 μL) was added. As a control, the kinase reaction was performed with the same samples in the absence of peptide substrate (no pep8) and without TSSK1. Meanwhile, staurosporine was chosen as a positive control at the concentration of 100 μM. Samples were then incubated for 10 min at room temperature, and the luminescence developed was recorded by a luminometer and expressed as RLU.

4.8. LC−MS-Based Assay. The LC−MS-based TSSK1 assay, an end-point assay that measures Pep8-P production, was performed in a 0.5 mL Eppendorf tube with a reaction volume of 50 μL. All TSSK1 reactions were performed in a kinase buffer (40 mM Tris−HCl buffer, 20 mM $MgCl_2$, 0.1 mg/mL BSA, 2 mM DTT). TSSK1 was resuspended in 25 μL of kinase buffer. The kinase reaction was performed by adding 25 μL of a mixture containing ATP and peptide substrate (Pep8) in kinase buffer. The final reaction conditions were 75 ng/well TSSK1, 10 μM Pep8, and 25 μM ATP. The reaction was incubated for 90 min at 37 °C. Enzyme reactions were quenched by addition of acetonitrile (50 μL) and centrifuged (15000g × 10 min). A 60 μL volume of quenched reaction mixture was subjected to HPLC−MS analysis.

4.9. Preparation of Individual 1,2,7-Trialkyl-1*H*-imidazo[4,5-*g*]quinoxalin-6-one Compounds. To a stirred solution of DFDNB (204 mg, 1.0 mmol) in THF (20 mL), DIPEA (4.0 mmol), and $NH_2CH(R^1)COOMe \cdot HCl$ (1.0 mmol) were added sequentially. The reactions were then continued for 3 h at room temperature with gentle stirring. Continuously, exactly 1.0 mmol of alkyl primary amine was added and allowed to stand for an additional 18 h. The solvent was removed under reduced pressure to give compound **11**, which was dissolved in dichloromethane, washed with water, and dried. Compound **11** was then dissolved in a mixed solvent of THF (10 mL) and EtOH (10 mL), and 10% Pd/C was added, followed by immediate addition of ammonium formate (1.26 g). The suspensions were stirred continuously at room temperature. When hydrogenation was complete, the Pd/C was filtered and the solvent removed to give crude compound **12**. After purification, parallel synthesis was applied to obtain compound **13**. To each reaction tube in a 96-well H + P parallel synthesizer, 0.16 mmol of compound **12** and 0.32 mmol of aldehyde were added and dissolved in 6 mL dioxane, to which 300 μL of acetic acid was added. The suspensions were stirred at 77 °C for 8 h, after which the solvent was removed to give the crude product. All reactions were monitored by LC−MS. All the products were recrystallized or purified by preparative RP-HPLC and were further characterized by LC/MS and ^{1}H NMR.

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Supporting Information Available: Protein sequence and purification methods of TSSK1, screening results of 640 single pure compounds, LC−MS analysis of the kinase reaction mixture and the mixture solution including internal standard and Pep8-P, HPLC and MS analysis of Pep8, Pep8-P, and Pep8-P(O_2), table listing peptide library for TSSK1 peptide substrate screening, Z' factor determination, the structure, and their physical data of single compound with 1,2,7-trialky-1*H*-imidazo[4,5-*g*]quinoxalin-6-one. This material is available free of charge via the Internet at http://pubs.acs.org.

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