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Remarkably Stereospecific Utilization of ATP α,β -Halomethylene Analogues by Protein Kinases

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Abstract

ATP analogues containing a CXY group in place of the α,β -bridging oxygen atom are powerful chemical probes for studying ATP-dependent enzymes. A limitation of such probes has been that conventional synthetic methods generate a mixture of diastereomers when the bridging carbon substitution is nonequivalent (X $\neq$ Y). We report here a novel method based on derivatization of a bisphosphonate precursor with a D-phenylglycine chiral auxiliary that enables preparation of the individual diastereomers of α,β -CHF-ATP and α,β -CHCl-ATP, which differ only in the configuration at the CHX carbon. When tested on a dozen divergent protein kinases, these individual diastereomers exhibit remarkable diastereospecificity (up to over 1000-fold) in utilization by the enzymes. This high selectivity can be exploited in an enzymatic approach to obtain the otherwise inaccessible diastereomers of α,β -CHBr-ATP. The crystal structure of a tyrosine kinase Src bound to α,β -CHX-ADP establishes the absolute configuration of the CHX carbon and helps clarify the origin of the remarkable diastereospecificity observed. We further

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Supporting Information

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Experimental details and characterization data (PDF)

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Notes

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synthesized the individual diastereomers of α,β -CHF- γ -thiol-ATP and demonstrated their utility in labeling a wide spectrum of kinase substrates. The novel ATP substrate analogues afforded by these two complementary strategies should have broad application in the study of the structure and function of ATP-dependent enzymes.

ATP is an essential nucleotide cofactor for numerous enzymes critical for life processes, ranging from protein kinases and ATPases to nitrogenases.^{1,2} Chemical modification of ATP has been a widely employed approach to probe the active-site structures, enzymatic mechanisms and cellular functions of ATP-dependent enzymes.^{3,4} Replacement of the α,β -bridging oxygen with a methylene linker (CH_2) facilitates protein crystallization⁵ and, in general, nucleotides with appropriately substituted bridging methylenes (CXY) are useful mechanistic probes⁶⁻⁹ or for other purposes such as *in vivo* expansion of the genetic alphabet.¹⁰ A limitation of such probes has been that conventional synthetic methods generate a mixture of diastereomers when the bridging carbon substitution is nonequivalent ($\text{X} \neq \text{Y}$).¹¹⁻¹³ Recently, the individual stereoisomers of β,γ -CXY-dGTP ($\text{X/Y} = \text{hydrogen}$ or halogen) were successfully prepared¹⁴ and exhibited 5–10-fold stereo-specificity in DNA polymerase β catalysis.¹⁵ Among other CXY nucleotide analogues, the α,β -CHN₃ dATP and β,γ -CHF ATP diastereomers, which are inhibitors for pol β and Src kinase, respectively, have been obtained as separate compounds.^{6,16} However, the synthesis of individual stereoisomers of α,β -CHF-ATP and its halo homologues, which are substrate mimics of ATP for kinases, has remained an unmet challenge.⁸

We report here a synthetic strategy to prepare the first examples of individual α,β -CHX-ATP diastereomers. The method involves three steps (Scheme 1): (i) conversion of the precursor α,β -CHX-ADP diastereomeric mixture **1** to a D-phenylglycine phosphoramidate derivative **2**; (ii) separation of the two diastereomers of **2** using preparative nonchiral reverse phase (RP) HPLC; and (iii) treatment of the isolated individual diastereomers of **2** with 85% H_3PO_4 to afford both individual triphosphate (**3**) and diphosphate (**4**) nucleotide analogues in a single reaction. In this synthesis, D-phenylglycine serves a dual role as both a chiral auxiliary ligand and to activate the bisphosphonate moiety for subsequent phosphorylation. H_3PO_4 also plays a dual role, providing an acidic medium to remove the protecting isopropylidene group and serving as the phosphate donor. The use of these reagents in dual roles enabled a concise and efficient chemical route to the α,β -CHX-ATP diastereomers that is 5 steps shorter than the route previously developed for the β,γ -CHX-dGTP stereoisomers.¹⁴ The two isomers of **3** synthesized by the present method are designated as **3x-1** and **3x-2** based on the elution order of their corresponding phosphoramidate precursors **2x-1/2x-2** ($\text{x} = \text{a}$ or b) under RP-HPLC (Figures S1 and S2). Their α -phosphorus nuclei show detectably different ³¹P NMR chemical shifts ($\delta \sim 0.13$ and 0.19 ppm for **3a-1/2** and **3b-1/2**, respectively) (Figures S3 and S4).¹⁷

The pure diastereomers of α,β -CHX-ATP (**3a-1**, **3a-2**, **3b-1** and **3b-2**) were first evaluated in comparison to ATP, α,β -CH₂-ATP and α,β -CF₂-ATP for their utilization by a prototypical tyrosine kinase Src, which is frequently overexpressed in cancers.¹⁸ The kinetic parameters (Table 1), obtained using the Omnia kinase assay, show that α,β -CF₂-ATP was utilized by Src with similar efficiency to ATP whereas α,β -CH₂-ATP was an extremely poor

substrate. An end-point assay based on ^1H NMR revealed the same trend (Figure S5). The specificity constants ($k_{\text{cat}}/K_{\text{M}}$) of these two nucleotides differ by >1000-fold suggesting that, for Src recognition, the CF_2 group is a much better mimic of the oxygen atom in ATP than CH_2 . **3a-2** has a comparable K_{M} to ATP whereas its k_{cat} is approximately 2-fold smaller. In contrast, **3a-1**'s K_{M} is over 30-fold greater than that of the active diastereomer whereas its k_{cat} is over 50-fold smaller. Interestingly, the two α , β -CHF-ATP diastereomers appear to mimic α , β - CH_2 -ATP and α , β - CF_2 -ATP rather than each other in terms of utilization by Src. Given that CHF phosphonates have long been considered to be isoacidic mimics of biological phosphates,¹⁹ our observation that the specificity constant of the inert diastereomer **3a-1** is at least 1000-fold lower than **3a-2**, in agreement with the high selectivity observed from NMR (^{31}P of ATP and ADP analogues, Figure S6) and Western-blot analysis (phosphotyrosine, Figure S7). The α , β -CHCl-ATP isomers (**3b-1** and **3b-2**) were utilized by the Src kinase with even higher diastereoselectivity than that of α , β -CHF-ATP, though their k_{cat} values are substantially smaller than those of α , β -CHF-ATP (Table 1 and Figure S8). The lower K_{M} value of **3b-2** than that of **3a-2** may reflect a higher binding affinity of the chlorine analog to the Src kinase than the fluorine one as the same trend was observed with K_{i} values of **4b-2** and **4a-2** (Table S1). This striking selectivity greatly exceeds that observed with DNA polymerase β ¹⁵ in utilizing substrates modified at β , γ -CXY bridge.

To assign absolute stereoconfiguration at the CHF carbon as a basis to understand the diastereoselective utilization of α , β -CHF-ATP by Src, we cocrystallized Src with **3a-2**, **3b-2**, **4a-2** and **4b-2**, the enzymatic substrates and products of the better substrate isomers (solved to 2.0–2.4 Å resolutions, Table S2). Given the low observable electron density for the γ -phosphoryl group in the **3b-2**/Src structure and the virtually identical complex structures for **3b-2** and **4b-2**, we suspect that **3b-2** underwent hydrolysis and lost its γ -phosphoryl group during the course of crystallization. Regardless, the well-defined electron density map allowed us to determine unambiguously the stereoconfiguration of the CHX carbon in **4a-2** and **4b-2** as *R* (Figure 1A,B). Therefore, the corresponding triphosphate **3a-2** and **3b-2** has the *S* configuration at the CHX carbon. Interestingly, the conformations of the kinase glycine-rich loop (P loop) and the nucleotide cofactor in the **4a-2**/Src structure resemble the previously described active state of Src whereas those in the **4b-2**/Src structure are close to the inactive state of Src (Figure 1C).²⁰ This may account for the lower k_{cat} for **3b-1** than that for **3a-1**. Moreover, the fluorine atom in **4a-2** is only 3.4 Å away from the ϵ -nitrogen of the catalytic lysine (K295) in Src, suggesting the presence of a favorable interaction through an electrostatic interaction with the polar F atom.¹² In contrast, the chlorine atom in the **4b-2** occupies a small hydrophobic pocket formed between the $\beta 1$ and $\beta 2$ strands, which may account for the lower K_{M} of **3b-2** than that of **3a-2**. When the opposite diastereomer (**4a-1** or **4b-1**) is modeled into the Src active site with the same conformation as **4a-2** or **4b-2**, the resulting complexes not only lose the halogen-mediated interactions but also have an unfavorable steric overlap between the halogen atom and the main chain amide of Q275/G276 within the glycine-rich loop (P loop) (Figure S9), highlighting the importance of the intimate interaction between ATP and the P loop in kinase catalysis. With its larger Cl atom, **4b-1** is predicted to have a greater steric clash than the

corresponding fluorine analog, thus accounting at least in part for the higher diastereoselectivity for **3b-1/2**.

We attempted to obtain the individual α , β -CHBr-ATP diastereomers, motivated by the Br atom's well-known utility in producing phase information for protein cocrystals²¹ and its ability to form stronger halogen bonds than F and Cl.²² Our auxiliary strategy failed to make available individual α , β -CHBr-ATP diastereomers because the CHBr phosphoramidate precursors could not be well resolved by RP-HPLC. However, the observed high stereoselectivity of Src toward **3a** and **3b** suggested an alternative method to produce individual α , β -CHX-ATP diastereomers. Incubation of a α , β -CHBr-ATP diastereomer mixture¹¹ with the Src kinase, Src-tide, and a tyrosine phosphatase YopH affords a catalytic cycle for phosphorylation of Src-tide (Figure 2). Using this enzymatic system, we succeeded in converting only one diastereomer of α , β -CHBr-ATP into the diphosphate form, which could be readily separated from the remaining triphosphate diastereomer by anion-exchange HPLC (Figure S10). The same method also succeeded in the enzymatic resolution of a mixture of α , β -CHCl-ATP diastereomers (Figure S11).

To test the generality of diastereoselective utilization of α , β -CHX-ATP by kinases, we measured the kinetic parameters of **3a-1** and **3a-2** along with ATP for a panel of 12 divergent kinases using an established microfluidic assay based on mobility shift of peptide substrates upon phosphorylation.²³ The K_M and k_{cat} values could be calculated for both **3a-1** and **3a-2** for nine out of the twelve kinases, which display stereospecificity constants ranging from 8 to 174 (Table S3). The different stereospecificity determined for Src kinase using the fluorescence-based and mobility shift-based assays may be due to the differences between the two assays and the Src proteins used. The kinetic parameters could be derived for **3a-2** but not **3a-1** (activity below detection limit) for three other kinases, which again show preference for the same diastereomer. These results suggest that high stereospecificity for α , β -CHF-ATP seems to be common to many protein kinases.

To examine further the scope of this selectivity, we tested the ability of α , β -CHF-ATPs to serve as the phosphoryl donor in the whole proteome in HEK293 cell lysates, taking advantage of a chemical-immunological method for detecting thiophosphorylation developed by Shokat.²⁴ The previously unavailable individual diastereomers of α , β -CHF- γ -thiol-ATP were synthesized by treating the separated diastereomers **2a** with thiophosphate followed by deprotection with HCl at $-20\text{ }^{\circ}\text{C}$ (Scheme 1). (*S*)- α , β -CHF- γ -S-ATP produced intense labeling of numerous proteins throughout the lane, whereas the *R* diastereomer caused no visible labeling of any bands (Figure 3), demonstrating that protein kinases generally exhibit high selectivity for (*S*)- α , β -CHF- γ -S-ATP in phosphorylating a wide spectrum of substrates. Although it produced a labeling pattern that is similar overall to that of γ -S-ATP, (*S*)- α , β -CHF- γ -S-ATP labeled a prominent band of $\sim 33\text{ kDa}$ not labeled by γ -S-ATP (Figure 3), suggesting that certain cellular kinases utilize (*S*)- α , β -CHF- γ -S-ATP more efficiently than γ -S-ATP. Identification of such kinases and their substrates using our probes will provide valuable new insight for understanding biological phosphorylation mechanisms.

In conclusion, we describe the first preparation of the individual diastereomers of α , β -CHX-ATP where X = F or Cl. Remarkable stereospecificity (>1000-fold) was observed in the utilization of these α , β -CHX-ATP diastereomers by Src kinase. Crystal structure analysis established the absolute configuration of the CHF and CHCl nucleotide analogues and reveals a potential electrostatic interaction between the fluorine atom in (*R*)- α , β -CHF-ADP and the ϵ -amino group of the catalytic lysine. Finally, we demonstrate that the observed selectivity is general for a wide range of eukaryotic protein kinases. Our results raise the interesting possibility that an engineered kinase capable of accepting (*R*)- α , β -CHX-ATP (the inert isomer) with high efficiency can be used for kinase substrate identification as in the bump–hole approach.²⁵ Beyond providing new mechanistic insights on one of the most centrally important enzymes utilizing ATP, individual α , β -CHX-ATP stereoisomers should have wide application in the study of numerous additional ATP-dependent enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Co-crystal structures of Src and ATP analogs. (A) Co-crystal structure of Src/**4a-2** establishes the absolute configuration of the nucleotide and reveals its interaction with the K295 of Src. Stick model of α , β -CHF-ADP overlaid with $|2F_o - F_c|$ electron density (blue mesh, contoured at 1σ). (B) Co-crystal structure of Src/**4b-2**. (C) Structural overlay reveals that the bisphosphonate moiety of (R)- α , β -CHF-ADP (green) assumes a similar conformation to that of γ -thiol-ATP (cyan) bound to Src (3DQW). (D) Overlay of the α , β -CHX nucleotide bound Src structures with the previously solved active (3DQW) and inactive (2SRC) Src structures reveal different conformations of the glycine-rich loop.

Figure 2.
Enzymatic approach to resolve a mixture of α, β -CHX-ATP diastereomers.

Figure 3.

Utilization of γ -S-ATP, (*R*)- α , β -CHF- γ -S-ATP and (*S*)- α , β -CHF- γ -S-ATP by various kinases to phosphorylate substrate proteins in cell lysates. All γ -S nucleotides were used at 0.25 mM, and thiolphosphorylation was detected using a monoclonal antibody.

Scheme 1.

Synthesis of Individual α , β -CHX (X = F, Cl) ATP Diastereomers

Table 1

Kinetic Parameters of ATP Analogues as Src Substrates

ATP analogue		K_M (mM)	k_{cat} (min ⁻¹)	Stereospecificity (k_{cat}/K_M) _S /(k_{cat}/K_M) _R
ATP		0.085 ± 0.010	167 ± 56	n.a.
α , β -CH ₂ -ATP		1.6 ± 0.47	0.17 ± 0.025	
α , β -CF ₂ -ATP		0.034 ± 0.004	84 ± 1.3	
3a-1	(<i>R</i>)-CHF	1.6 ± 0.3	1.5 ± 0.6	1.5 × 10 ³
3a-2	(<i>S</i>)-CHF	0.042 ± 0.0053	59 ± 21	
3b-1	(<i>R</i>)-CHCl	5.1 ± 0.52	0.22 ± 0.0018	1.8 × 10 ³
3b-2	(<i>S</i>)-CHCl	0.0087 ± 0.0020	0.69 ± 0.10	