Bivalent Inhibitors of Protein Tyrosine Kinases

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Abstract: The majority of protein kinase inhibitors described to date are ATP analogues. However, the selectivity of these species is highly suspect, given the enormous number of ATP-dependent processes that transpire in living cells. Inhibitors that target the protein binding site do not suffer from this disadvantage but exhibit comparatively low inhibitory activity. An alternative approach for the design of protein tyrosine kinase inhibitors is described herein. We have constructed species that simultaneously bind to the active site and the SH2 domain of the Src kinase. Since the region of the inhibitor that associates with the SH2 domain coordinates with relatively high affinity, the overall effect is a substantial enhancement in inhibitory potency (230-fold). This design element offers a strategy to overcome the otherwise poor efficacy of peptide-based protein tyrosine kinase inhibitors.

The Src family of protein tyrosine kinases (PTKs) are key participants in the signal transduction pathways that transmit extracellular signals across the cell membrane to distant locations in the cytoplasm and the nucleus. These enzymes share a strong primary sequence homology and utilize ATP as a common substrate. Enhanced protein kinase activity has been directly linked to cell transformation and carcinogenesis.[1] In addition, PTKs have also been shown to play a significant role in other proliferative diseases such as atherosclerosis, psoriasis, and restenosis.[2−4] Consequently, protein kinase inhibitors that can be precisely targeted to specific members of the kinase family could potentially be of great therapeutic value. Such inhibitors may also prove useful in deciphering the complexities of various signaling pathways.

The development of PTK inhibitors has been hampered by the broad overlapping substrate specificities exhibited by these enzymes. The fact that ATP serves as a common substrate for these enzymes raises serious questions about the utility of ATP-based analogues as specific kinase inhibitors. Indeed, any ATP-utilizing or forming process is a potential unintended target. For example, several PTK inhibitors have recently been shown to interfere with cellular respiration and fatty acid synthesis.[5] In addition, the high intracellular concentration of ATP (up to 8 mM) will significantly reduce the inhibitory potency of such compounds in vivo.[6] Although peptide-based active site directed inhibitors have been described, the performance of these compounds has been, in general, disappointing. K_i values typically lie in the 1−2 mM range.[7] These inhibitory species are routinely prepared by replacing the phosphorylatable tyrosine residue with a phenylalanine moiety. Much of the poor inhibitory activity of these peptides may be due to the missing phenolic hydroxyl moiety, which likely functions as a hydrogen-bond donor and/or acceptor to help facilitate productive active site interactions.[8]

The SH2 and SH3 domains present in the Src family of PTKs are protein modules that have evolved for the unique purpose of promoting protein−protein interactions.[9−11] SH2 domains are composed of approximately 100 amino acids and specifically recognize and bind to protein segments containing phosphotyrosine. In contrast, SH3 domains are approximately 60 amino acids in length and coordinate proline-rich regions that are present in a variety of signaling molecules. Peptides directed against the SH2 domain of a kinase can exhibit as much as 4 orders of magnitude enhanced affinity compared to active site directed peptides. Is it possible to exploit the SH2 and/or SH3 molecular recognition motifs to develop selective, high-affinity inhibitors for tyrosine kinases? Or, more simply stated, can SH2 and SH3 recognition sequences be utilized to "deliver" otherwise low-affinity peptides to the active site of PTKs?

In an effort to address these questions, we prepared a series of bivalent peptides that contain an SH2 recognition sequence linked, through a flexible tether, to an active site directed inhibitory peptide. The SH2 recognition sequence employed, acetyl-pTyr-Glu-Glu-Ile-Glu, is based on the SH2 recognition motif found in the hamster polyoma virus middle-T antigen. The X-ray crystal and NMR solution structures of this peptide bound to the SH2 domain of Src have been determined.[12,13] Acetyl-pTyr-Glu-Glu-Ile-Glu binds moderately well to the SH2 domain of Src with a K_d value of under 1 μM. The active site directed portion of the inhibitor, Glu-Glu-Leu-Leu-**(F_5)Phe-**

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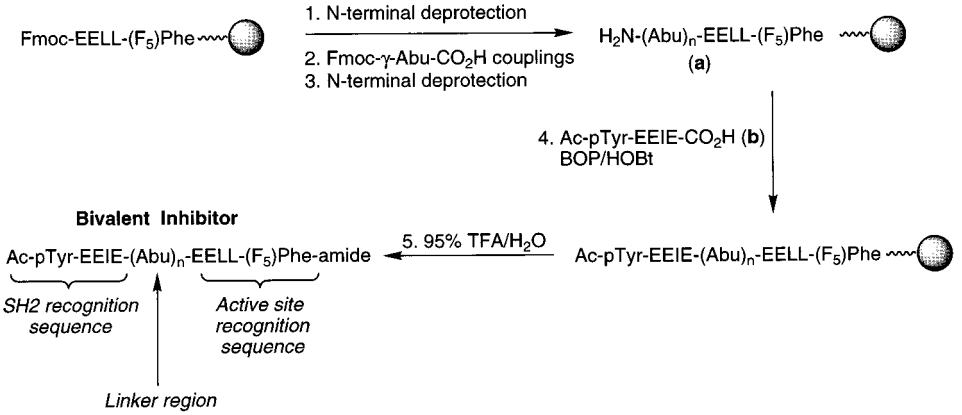
Scheme 1. Synthesis of Bivalent Src Inhibitors[a]



[a] Note: the glutamic acid residues (E) are side-chain-protected as *tert*-butyl esters in steps 1−4.

amide, is based on previous work from our laboratory which identified **pentafluorophenylalanine** as a nonphosphorylatable tyrosine surrogate.[14] Although the inhibitory potency of this peptide is, at best, only a modest improvement over its simpler Phe-containing counterpart (approximately a 4-fold decrease in K_i), we decided to incorporate this moiety into the active site directed sequence for our preliminary studies to assess the effect that a potent SH2-directed sequence linked to a poor active site targeted peptide has on inhibitory activity.

The X-ray crystal structures of two Src kinase family members, namely Hck (hematopoietic kinase) and c-Src, have been reported.[15−17] However, the structures are of the conformationally inactive form of these enzymes. Under these circumstances, the SH2 domain is intramolecularly associated with a phosphorylated tyrosine moiety, which in turn promotes the intramolecular association of the SH3 domain with a region linking the SH2 and kinase domains. Consequently, the inactive twisted conformational state is of little value in predicting the distance between the SH2 and kinase domains in the active enzyme form. Therefore, optimum tether length was empirically determined utilizing alkyl chains of varying length. We chose tethers that are structurally simple, reasonably flexible, and compatible with standard solid-phase peptide synthesis protocols. On the basis of these criteria, γ-aminobutyric acid (Abu) was employed as the monomeric building block for tether construction. Thus, a series of bivalent inhibitors of the general structure Ac-pTyr-Glu-Glu-Ile-Glu-$(Abu)_n$-Glu-Glu-Leu-Leu-(F_5)Phe-amide were prepared where *n* varied, in multiples of two, from 4 to 12. These inhibitors, along with the active site-directed control peptide, Ac-Glu-Glu-Leu-Leu-(F_5)Phe-amide (**1**), were evaluated for their ability to inhibit c-Src-catalyzed phosphoryl transfer.

Materials and Methods

Rink resin, Fmoc-protected amino acid derivatives, and all reagents for solid-phase peptide synthesis were obtained from Advanced Chem Tech. 2-Methoxy-4-alkoxybenzyl alcohol resin was obtained from Bachem. All of the other chemicals were purchased from Aldrich, except [γ-^{32}P]ATP (DuPont NEN), bovine serum albumin (Sigma), biotin succinimidyl ester (Pierce), and ScintiSafe (Fisher Scientific). Phosphocellulose P-81 filter paper was acquired from Whatman, and SAM^2 biotin capture membrane was obtained from Promega. Enzyme assay solutions were prepared with deionized/distilled H_2O.

Human Recombinant Src. Human Src was purchased from Upstate Biotechnology Inc. The enzyme was expressed by recombinant baculovirus containing the human *src* gene in SF9 insect cells and purified by the method of Bjorge et al.[18] The enzyme produced in this manner is not phosphorylated on its regulatory C-terminal tail (Tyr-530) and consequently exists in an activated state.

Peptide Synthesis. Preparation of H_2N-$(Abu)_n$-Glu(*O-tert*-butyl)-Glu(*O-tert*-butyl)-Leu-Leu-(F_5)Phe-resin (a). The overall synthetic strategy employed for the synthesis of the inhibitors is illustrated in Scheme 1. The protected active site directed peptide Fmoc-Glu(O-*tert*-butyl)-Glu(O-*tert*-butyl)-Leu-Leu-(F_5)Phe was synthesized on Rink resin (substitution level = 0.18 mmol/g), utilizing a standard Fmoc solid-phase synthesis protocol on an Advanced Chem Tech 90 peptide synthesizer. The N-terminus was deprotected, and tether construction and elongation were accomplished through multiple couplings of FmocAbu. Aliquots of peptidyl resin were removed at the appropriate times in the synthetic sequence to provide tethers containing the desired number of monomeric units. The N-terminal Fmoc group was removed from each aliquot just prior to segment coupling.

Preparation of Ac-Tyr(PO_3H_2)-Glu(*O-tert*-butyl)-Glu(*O-tert*-butyl)-Ile-Glu(*O-tert*-butyl)-OH (b). Peptide fragment **b** was synthesized on 2-methoxy-4-alkoxybenzyl alcohol resin (substitution level = 0.50 mmol/g). The protected peptide was cleaved from the resin using 1% TFA in DCM. Typically, 1 g of peptide resin was incubated with 15 mL of 1% TFA in DCM for 15 min. The resin was isolated by filtration and the filtrate cooled to 0 °C and neutralized by the addition of *N*-methylmorpholine. The process was repeated several times to ensure complete removal of the peptide from the resin. The filtrate was evaporated to dryness and the residue dissolved in a small amount of methanol. The peptide fragment was precipitated with Et_2O and isolated by filtration. Typically, 1 g of peptide−resin gave approximately 325 to 400 mg of the desired peptide (51−63% yield).

General Protocol for the Preparation of Ac-Tyr(PO_3H_2)-Glu-Glu-Ile-Glu-$(Abu)_n$-Glu-Glu-Leu-Leu-(F_5)Phe-amide (2−6). A segment condensation approach was employed for the construction of the bivalent inhibitors. Typically, 1.2 equiv of **b** was condensed with 500 mg of amino terminal deprotected peptidyl resin **a** in the presence of 1.8 equiv of BOP and HOBT utilizing 2 to 3 mL of DCM/DMF (1:1) as solvent. Couplings were allowed to proceed at room temperature for 1.5 to 2 h. The completeness of the segment condensations was monitored by the Kaiser test. Double couplings were employed in those cases where couplings were deemed to be incomplete. Cleavage of the peptides from the resin with concomitant removal of all side-chain protecting groups was accomplished with 95% TFA/H_2O. The mixture was filtered and the TFA/H_2O removed under reduced pressure. The residue was dissolved in H_2O and the pH adjusted to between 7 and 8. Crude peptides were purified by preparative reverse phase HPLC on a C_{18} column utilizing a linear gradient of CH_3CN/H_2O containing 0.1%

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Table 1. IC_{50} Values of Bivalent Inhibitors of Src[a]

peptide	structure	IC_{50} (μM)
1	Ac-EELL-(F_5)Phe-amide	1590 ± 170
2	Ac-pYEEIE-$(Abu)_4$-EELL-(F_5)Phe-amide	195 ± 26
3	Ac-pYEEIE-$(Abu)_6$-EELL-(F_5)Phe-amide	35 ± 5
4	Ac-pYEEIE-$(Abu)_8$-EELL-(F_5)Phe-amide	18.5 ± 1
5	Ac-pYEEIE-$(Abu)_{10}$-EELL-(F_5)Phe-amide	250 ± 25
6	Ac-pYEEIE-$(Abu)_{12}$-EELL-(F_5)Phe-amide	400 ± 50
7	Ac-$(Abu)_8$-EELL-(F_5)Phe-amide	860 ± 90
8	coumarin-pYEEIE-$(Abu)_8$-EELL-(F_5)Phe-amide	6.9 ± 0.4

[a] Inhibitors were assayed using 30 μM Arg-Arg-Arg-Arg-Arg-Ala-Glu-Glu-Glu-Glu-Tyr-phenethylamine as peptide substrate.

TFA. The appropriate fractions were combined and lyophilized. All peptides gave satisfactory MALDI-TOF mass spectral analysis.

Preparation of Biotin-ϵ-aminocaproyl-Tyr(PO_3H_2)-Glu-Glu-Ile-Glu-$(Abu)_6$-Glu-Glu-Leu-Leu-Tyr-amide (9). This Src substrate **9** was synthesized on rink resin (substitution level = 0.6 mmol/g) using a standard Fmoc synthesis protocol. The N-terminus was biotinylated utilizing *N*-hydroxysuccinimidobiotin (3 equiv) and NMM (4 equiv) in DMF. The side-chain protecting groups were removed, and the peptide was simultaneously cleaved from the resin with 95% TFA/H_2O. The mixture was filtered and the filtrate evaporated under reduced pressure. The residue was dissolved in H_2O and adjusted to pH 8 with NH_4OH. The crude peptide was purified by reverse phase HPLC, using the following protocol with solvent A (0.1% TFA in H_2O) and solvent B (0.1% TFA in CH_3CN): 0 to 3 min (100% A), a linear gradient from 3 to 5 min (100−85% A), 5 to 65 min (85−63% A). Fractions containing the desired peptide were pooled and lyophilized.

Kinase Assays. Assays were performed in triplicate at pH 7.5 in a thermostated water bath maintained at 30 °C. For the determination of IC_{50} values, the following protocol was typically employed. Phosphorylation reactions were initiated by the addition of 10 μL of Src from a concentrated stock solution to produce a final 40 μL solution containing peptide inhibitor concentrations that varied about their respective IC_{50} values, 100 μM [γ-^{32}P] (500−1000 cpm/pmol), 30 μM Arg-Arg-Arg-Arg-Arg-Ala-Glu-Glu-Glu-Glu-Tyr-NH$(CH_2)_2C_6H_5$ substrate (K_m of 18 ± 2 μM and V_{max} of 337 ± 56 nmol min^{-1} mg^{-1}), 50 mM Tris, 10 mM $MgCl_2$, 0.2 mg/mL bovine serum albumin and 1.10 nM Src. Reactions were terminated after 20 min by spotting 25 μL aliquots onto 2.1-cm diameter phosphocellulose paper disks. After 10 s, the disks were immersed in 10% glacial acetic acid and allowed to soak with occasional stirring for 1 h. The acetic acid was decanted, and the disks were collectively washed with 4 volumes of 0.5% H_3PO_4 and a single volume of water, and followed by a final acetone rinse. The disks were air-dried and placed in plastic scintillation vials containing 3 mL of ScintiSafe and subjected to scintillation counting for radioactivity.

The above protocol was also employed for the determination of IC_{50} values in the presence of 300 μM phosphopeptide (Ac-pTyr-Glu-Glu-Ile). It should be noted that at high concentrations, the phosphopeptide itself was found to serve as an inhibitor of Src-catalyzed phosphoryl transfer. However, at 300 μM, no significant change in catalytic activity was observed.

The following conditions were employed to determine the K_i value of compound **8**. [γ-^{32}P]ATP (2000 cpm/pmol) was fixed at 100 μM with compound **9** being utilized as the variable substrate. The concentration of **8** was varied around its K_i value. Reactions were initiated by the addition of 10 μL of Src from a concentrated stock solution as described above to give a final enzyme concentration of 1.74 nM. Reactions were terminated by spotting 20 μL aliquots onto 1.25 × 1.15 cm SAM^2 biotin capture membranes. The membranes were collectively washed with 2 M NaCl, 2 M NaCl in 1% H_3PO_4, and deionized water, followed by a final ethanol wash as per the manufacturers instructions. The membranes were allowed to air-dry and were subjected to scintillation counting as described above.

Results and Discussion

The results obtained with the bivalent inhibitors are summarized in Table 1. An obvious trend emerges from the data. The active site directed control peptide **1** is clearly a poor inhibitor with an IC_{50} of nearly 1.6 mM. However, as can be seen from the results obtained with peptide **2**, inhibitory potency is enhanced roughly 8-fold by tethering the active site directed peptide to an SH2 recognition sequence. The inhibitory efficacy of the bivalent inhibitors improves as a function of increasing chain length until a maximum is reached at eight aminobutyric acid residues (18.5 μM). This represents a nearly 86-fold enhancement in inhibitory activity relative to peptide **1**. At a tether length of 10 Abu residues, inhibitory potency sharply decreases and continues to decline with peptide **6** being the poorest inhibitor of those sampled.

Is the dramatic difference in IC_{50} values between peptides **1** and **4** due to the simultaneous occupancy of the SH2 and active site regions of Src by peptide **4**? We performed two experiments to address this question. First, we prepared peptide **7**, which is identical to **4** except that it lacks the SH2 recognition sequence. If the SH2 recognition motif is responsible for the enhanced potency exhibited by **4**, then **7** should be a much poorer inhibitor than **4**. As can be seen in Table 1, this is indeed the case. In addition, we also assessed the inhibitory activity of **4** in the presence of 300 μM Ac-pTyr-Glu-Glu-Ile-amide, which at this concentration should saturate the SH2 domain of Src. Under these conditions, the ability of peptide **4** to serve as an inhibitor of Src kinase activity should be significantly impaired since the SH2 targeting motif contained within **4** must compete with the overwhelming presence of Ac-pTyr-Glu-Glu-Ile-amide. Indeed, **4** exhibits an IC_{50} of 240 ± 70 μM under these circumstances. Thus, both experiments provide strong evidence that peptide **4** coordinates to Src in a bivalent fashion.

Recent work from our laboratory has demonstrated that 7-hydroxy-coumarin-4-acetic acid linked to the N-terminus of pTyr-Glu-Glu-Ile-amide significantly enhances the affinity of this peptide for the SH2 domain of Lck (lymphoid T cell kinase).[19] Although this effect appears to be quite specific for Lck, we do observe a modest enhancement for the SH2 domain of Fyn (Fgr and Yes-related kinase) as well. Since both Lck and Fyn are members of the Src kinase family, we prepared the coumarin derivative **8** and found that it serves as an approximately 2.5-fold better inhibitor than **4** (Table 1). The coumarin-derivatized peptide **8** is a 230-fold more potent inhibitor of Src than the monovalent active site directed peptide **1**.

Since **8** is the best inhibitory agent in this study, we decided to examine its mode of action in greater detail. We previously demonstrated that the simple active site directed peptide, Glu-Glu-Leu-Leu-(pentafluoro)Phe-Gly-Glu-Ile, is a competitive inhibitor with respect to peptide substrate.[14] In contrast, peptide **8** exhibits a complex inhibition pattern versus the variable substrate [$(Arg)_5$-Ala-$(Glu)_4$-Try-phenethylamine] (data not shown). Since binding to the higher affinity SH2 site is the primary driving force for the complexation of the bivalent inhibitors with Src, one might envision the formation of several discrete intermediates. For example, it is possible that some fraction of the peptide **8**/Src complex contains the SH2 recognition sequence coordinated to the SH2 domain with the active site portion free in solution. Competition with a substrate that has only one binding modality available to it (i.e., only active site directed) may lead to mechanistic complexities that preclude the observation of a clean competitive inhibition pattern. In an effort to address this matter, we prepared the bivalent substrate (biotin-aminohexanoic acid)−pTyr-Glu-Glu-Ile-Glu-$(Abu)_6$-Glu-Glu-Leu-Leu-Tyr-amide (**9**). An avidin-impregnated membrane was employed for the assays utilizing

(19) Lee, T. R.; Lawrence, D. S., unpublished results.

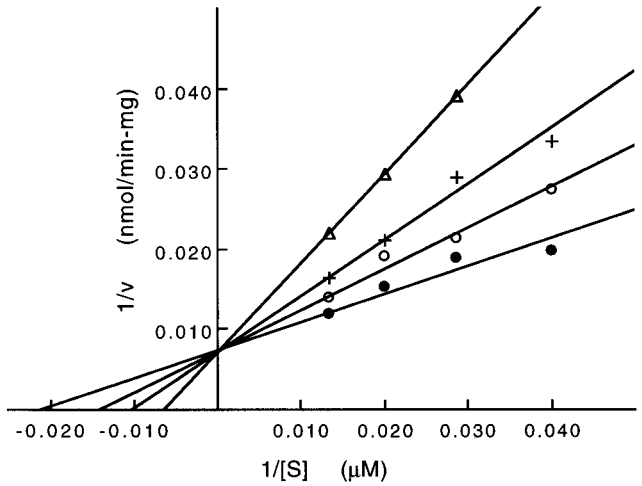

Figure 1. Inhibition pattern of peptide **8** [0 μM (●), 5 μM (○), 10 μM (+), and 20 μM (△)] versus variable bivalent substrate **9** (25 μM, 35 μM, 50 μM, 75 μM) at a fixed ATP concentration of 100 μM.

this substrate. The bivalent nature of substrate **9** suggests that it should form the same discrete intermediates upon binding to Src as **8**. Furthermore, as with all the bivalent inhibitors in this study, binding of the substrate peptide to the SH2 domain should be the dominant driving force for complexation.

Peptide **9** is a reasonably effective substrate for Src, exhibiting a K_m of 47 ± 9 μM and a V_{max} of 130 ± 20 nmol/min-mg.[20] Peptide **8** displays an IC_{50} of 19 ± 3 μM with this substrate. Most importantly, **8** serves as a competitive inhibitor versus variable peptide substrate **9** and exhibits a K_i of 9 ± 2 μM (Figure 1).

The most potent peptide-based PTK inhibitors reported to date are those in which the phosphorylatable tyrosine moiety has been replaced by a nonphosphorylatable phenolic analogue of tyrosine. For example, Yuan et al. prepared an undecapeptide analogue of gastrin containing a tetrafluorinated Tyr residue in place of the phosphorylatable tyrosine.[21] This peptide serves as a potent competitive inhibitor with respect to peptide substrate (K_i = 4 μM). Likewise, Fry et al. incorporated (D)F_4-Tyr into an EGF receptor active site directed peptide, which was based on the primary sequence that envelops Tyr-472 of phospholipase C-γ1.[22] The inhibitor exhibits a K_i of 18 μM. More recently, L-dopa has been utilized as a potent nonphosphorylatable tyrosine mimetic in a Src-targeted peptide (K_i = 16 μM).[23] Finally, Alfaro-Lopez and co-workers developed a series of conformationally and topographically constrained inhibitors of Src that utilize 3-iodotyrosine and 3,5-diiodotyrosine as nonphosphorylatable tyrosine mimetics.[24] In the present study, we have taken an active site directed peptide with poor inhibitory efficacy and tethered it to an SH2 recognition sequence. The resultant bivalent peptide **8** exhibits 2 orders of magnitude greater inhibitory efficacy against Src than the corresponding monovalent active site directed peptide **1**. Clearly, several additional issues need to be addressed in order to optimize the potency of this new family of PTK inhibitors. The incorporation of more potent active site-directed peptides (e.g., those containing L-dopa) into these compounds should markedly improve their inhibitory efficacy. More ridged tethers, with fewer degrees of rotational freedom, could likewise enhance the effectiveness of these species. Finally, the relative orientation of the active site directed and SH2-targeted segments in **8** may not be optimal. By varying the N- to C-terminal orientation of each subligand, it may ultimately be possible to dramatically minimize the current $(Abu)_8$ tether length. We are currently addressing these issues.

There are many possible applications and variations of the bivalent approach. Cowburn et al. prepared a series of "consolidated" ligands containing SH2- and SH3-binding subligands.[25] These species bind to the regulatory apparatus (SH2 and SH3 domains) of the Abelson protein tyrosine kinase with enhanced affinity. Likewise, Pluskey and co-workers tethered two phosphotyrosyl peptides together via aminohexanoic acid linkages to produce bivalent ligands that bind to the two SH2 domains of the phosphatase SH-PTP2, which results in enzyme activation.[26,27] The bivalent ligands were found to stimulate catalytic activity in a fashion more pronounced than their monovalent phosphopeptide counterparts. As in the SH-PTP2 example, the interaction of an SH2 or SH3 ligand with members of the Src family of tyrosine kinases often results in enhanced enzymatic activity. In contrast, the bivalent inhibitors described in this study behave in a dramatically different fashion. These species are both bivalent and bifunctional. Not only do these compounds coordinate to the SH2 domain of Src, which should block the assembly of Src-based signaling complexes, but they shut down the catalytic activity of the enzyme as well.

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