Stabilization of Aliphatic Phosphines by Auxiliary Phosphine Sulfides Offers Zeptomolar Affinity and Unprecedented Selectivity for Probing Biological Cu[I]

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Abstract: Full elucidation of the functions and homeostatic pathways of biological copper requires tools that can selectively recognize and manipulate this trace nutrient within living cells and tissues, where it exists primarily as Cu[I]. Buffered at attomolar concentrations, intracellular Cu[I] is, however, not readily accessible to commonly employed amine and thioether-based chelators. Herein, we reveal a chelator design strategy in which phosphine sulfides aid in Cu[I] coordination while simultaneously stabilizing aliphatic phosphine donors, producing a charge-neutral ligand with low-zeptomolar dissociation constant and 10^{17}-fold selectivity for Cu[I] over Zn[II], Fe[II], and Mn[II]. As illustrated by reversing ATP7A trafficking in cells and blocking long-term potentiation of neurons in mouse hippocampal brain tissue, the ligand is capable of intercepting copper-dependent processes. The phosphine sulfide-stabilized phosphine (PSP) design approach, which confers resistance towards protonation, dioxygen, and disulfides, could be readily expanded towards ligands and probes with tailored properties for exploring Cu[I] in a broad range of biological systems.

As a cofactor of metalloproteins, copper plays a critical role in fundamental biological functions such as respiration or oxidative stress defense. While a major fraction of cellular copper is buried within the active sites of enzymes, cells also maintain a kinetically labile pool of Cu[I], serving not only as a reservoir for the metalation of cuproenzymes but also for rapidly adapting to changes in copper availability. This labile pool is regulated through a complex interplay between cytosolic metallochaperones, membrane-localized transporters, and storage proteins such as metallothionein.[1] Inferring from recent affinity measurements of endogenous Cu[I] ligands,[2] labile cellular Cu[I] is buffered at attomolar levels or below. Despite such exceptional binding affinities, the transfer of Cu[I] between chaperones and transport proteins occurs with rapid kinetics,[3] likely involving an associative exchange mediated through specific protein–protein interactions. However, the precise mechanisms governing the function and regulation of this labile copper pool remain poorly understood.

Membrane-permeant ligands that selectively bind to a metal ion of interest represent a critical tool for interrogating metal homeostasis;[4] however, the affinity of most currently available Cu[I] ligands does not match the biologically important attomolar buffer window. For example, mixed amine-thioether donors, which have been frequently employed for the construction of Cu[I]-selective fluorescent probes,[5] offer dissociation constants in the pico- to femtomolar range. While bathocuproine disulfonate (BCS)[6] has been successfully utilized to characterize cuproteins with attomolar dissociation constants,[2b] as a bidentate ligand it must be employed at significant excess over Cu[I] and in some cases ternary complex formation has been reported.[6,7] Synthetic ligands with sub-attomolar dissociation constants have been realized by mimicking the thiolate coordination environment of Cu[I] proteins;[8] however, thiolates may also bind to other biologically relevant metal cations such as Zn[II] or Fe[II] and are sensitive to pH and thiol–disulfide redox status, both of which may vary with the cellular microenvironment.

As soft Lewis bases, phosphine ligands form exceptionally stable complexes with Cu[I],[9] making them promising candidates for the development of alternative high-affinity Cu[I] chelators. While phosphorus is essential to life, it occurs almost exclusively in the +5 oxidation state as free phosphate or phosphoric acid derivatives. In contrast, P[III] compounds are not encountered in biology,[10] consistent with the susceptibility of aliphatic phosphines to rapid and irreversible oxidation by O_2 or disulfides. To address this challenge, we thought to explore the utility of phosphine sulfide substituents, which might stabilize nearby phosphines through an electron-withdrawing effect while also acting as auxiliary coordination donors toward Cu[I].[11] As indicated by the high aqueous solubility of Me₃PS (>1M), the P=S bond is substantially polarized, suggesting that the sulfur center may offer coordination properties intermediate between thioethers and thiolates. To test this design concept, we devised a phosphine sulfide-stabilized phosphine ligand, **PSP-1** (Figure 1), which features an identical donor topology and chelate ring size compared to **MCL-1**, our tightest-binding Cu[I] affinity standard built upon an amine-thioether framework.[6] Prepared in a single step from lithiated Me₃PS and

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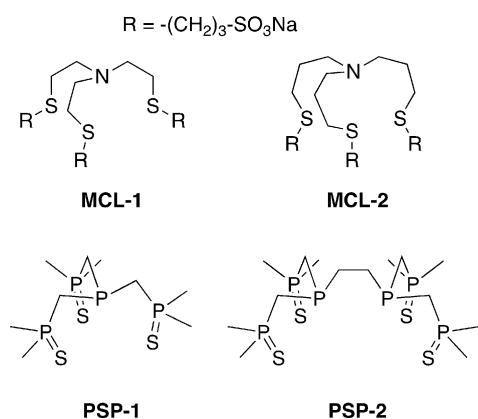
Figure 1. Structures of the amine-thioether-based Cu^I affinity standards **MCL-1** and **MCL-2** and the air-stable phosphine sulfide-stabilized phosphine ligands **PSP-1** and **PSP-2**.

(PhO)$_3$P, **PSP-1** was isolated as a colorless crystalline solid. It directly dissolved in water to a concentration of 1 mM at 37 °C, and ^{31}P NMR spectroscopy of the resulting solution revealed no detectable hydrolysis or oxidation after 15 days of continuous mixing with air (Supporting Information, Figure S1). Furthermore, in 1M HClO$_4$ only a slight peak broadening was observed, indicating that the ligand remains largely unprotonated. This represents a reduction in basicity of at least nine orders of magnitude compared to Me$_3$P (pK_a = 8.7) and three orders of magnitude compared to Ph$_3$P (pK_a = 2.7).[12] Given that tris(2-carboxyethyl)phosphine (TCEP) is frequently employed for the reduction of disulfides, we also tested the stability of **PSP-1** in the presence of 1 mM glutathione disulfide (GSSG). While TCEP is quantitatively oxidized within 5 min at 25 °C,[13] **PSP-1** suffered less than 1.5% oxidation after 23 h at 37 °C (Supporting Information, Figure S2).

Despite the low basicity of the phosphine center, Cu^I competition titrations with **PSP-1** (Supporting Information, Figure S4) revealed tight binding with an apparent K_d of 0.8 fM (10 mM PIPES, pH 7.0, 0.1M KCl, 25 °C), comparable to that of **MCL-1**.[6] Spectral deconvolution of the titration data indicated, however, an additional dimerization equilibrium with log K_{dimer} = 4.38 ± 0.02 [Eq. (1)].

$$2\ CuL \xrightleftharpoons[\quad]{K_{dimer}} Cu_2L_2 \qquad (1)$$
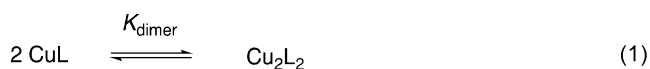
The predominant formation of coordination species with 1:1 stoichiometries was confirmed with a Job's plot, which revealed maxima at a 1:1 metal/ligand ratio regardless of the absorption wavelength (Supporting Information, Figure S9). Reaction of **PSP-1** with CuCl yielded the complex [(**PSP-1**)Cu^I]Cl as air-stable colorless crystals, which readily dissolved in water. The cyclic voltammogram of a 1 mM solution of [(**PSP-1**)Cu^I]Cl in 0.1M KCl revealed a quasi-reversible one-electron-redox process with a peak separation of 76 mV and half-wave potential of E^f = 0.73 V vs. SHE (25 °C; Supporting Information, Figure S11). The stabilization of Cu^I towards oxidation is thus comparable to that obtained with **MCL-1** (E^f = 0.72 V vs. SHE).[6]

While [(**PSP-1**)Cu^I]Cl crystallized as thin fibers, the corresponding hexafluorophosphate salt yielded prisms suitable for X-ray diffraction. The complex crystallized as a symmetrical Cu$_2$L$_2$ dimer (Figure 2) whose inversion center coincides with the crystallographic inversion center. The Cu^I coordination geometry is distorted tetrahedral with P-Cu-S angles of 96° and 131° (Table 1), likely imposed through geometrical constraints of the ligand backbone. The Cu-P bond length of 2.27 Å is comparable to that of homoleptic tetrahedral Cu^I-complexes with aliphatic phosphines, such as [Cu(PMe$_3$)$_4$]Cl·H$_2$O (2.27 Å average),[14] indicating a similar bond strength despite the dramatically reduced basicity of **PSP-1**. The longer Cu-S bond distances for S1 and S3 (2.37 and 2.40 Å) compared to S2 (2.31 Å) suggest significant strain within the 5-membered chelate rings, which likely provides the driving force for dimerization.
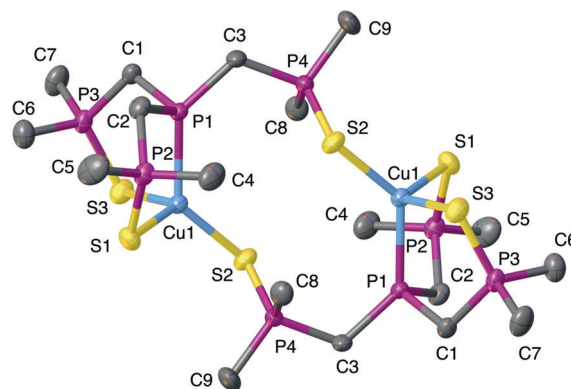

Figure 2. ORTEP and atom numbering scheme of the cationic unit in the crystal structure of [(**PSP-1**)$_2$Cu$^I_2$](PF$_6$)$_2$. Ellipsoids shown at 50% probability. Hydrogen atoms are omitted for clarity. CCDC 1853246.

Table 1: Selected bond distances [Å] and angles (deg) for the cationic unit in the crystal structure of [(**PSP-1**)$_2$Cu$^I_2$](PF$_6$)$_2$.

bond distance [Å]		bond angle (deg)	
Cu1-P1	2.272(1)	P1-Cu1-S1	96.70(4)
Cu1-S3	2.401(1)	P1-Cu1-S2	131.35(5)
Cu1-S1	2.370(1)	P1-Cu1-S3	96.41(4)
Cu1-S2	2.308(1)	S1-Cu1-S3	115.85(5)

In view of the excellent stability of **PSP-1** and its Cu^I complex, we hypothesized that a slightly more electron-rich phosphine donor might yet offer sufficient redox stability while further increasing the Cu^I affinity. To explore this idea, we devised the EDTA-like architecture of **PSP-2** in which each phosphine experiences the electron-withdrawing effect of only two pendant phosphine sulfides (Figure 1). Although Cu^I prefers a coordination number of four, the two extra donors might increase the affinity through a statistical effect.

Like **PSP-1**, **PSP-2** was accessible in a single step from Me$_3$PS and proved to be air-stable. Cu^I-competition titration against a 2000-fold excess of **MCL-1** (Figure 3 A) revealed a 1:1 binding equilibrium with a dramatically improved affinity of log K = 20.04 ± 0.06 (K_d = 10 zM), over two orders of magnitude higher than the Cu^I affinity of the metal-

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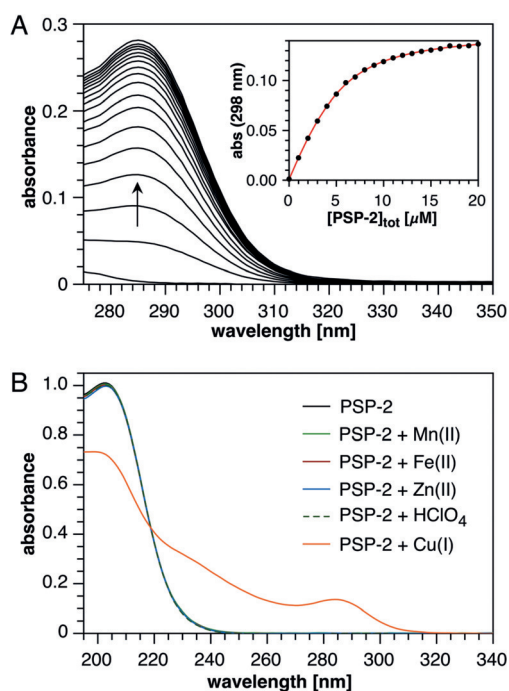
Figure 3. Metal-ion binding properties of **PSP-2**. A) Spectrophotometric determination of the Cu^I stability constant of [(**PSP-2**)Cu^I] in aqueous buffer at pH 8.0 (10 mM MCL-1, 1 mM HCl, 0.1 M KCl, 500 μM hydroxylamine, 25°C). A mixture of **MCL-1** (10 mM) and CuSO$_4$ (5 μM) was titrated with **PSP-2**. Inset: Nonlinear least-squares fit at 298 nm for a 1:1 complexation equilibrium model. B) Metal-ion binding selectivity of **PSP-2** (25 μM) probed by UV spectroscopy. The absorption traces in the presence of 0.1 M HClO$_4$ or 1 mM Mn^{II}, Fe^{II} or Zn^{II} are indistinguishable from that of the ligand alone.

lochaperone Atox1.[2a,b] Reaction of 0.3 mM **PSP-2** with equimolar GSSG in air-saturated buffer at 37°C yielded 8% of **PSP-2 oxide** after 4.5 h (Supporting Information, Figure S3), corresponding to a half-life of about 2 days. **PSP-2** is thus more reactive than **PSP-1**, consistent with more electron-rich phosphine donors. Nevertheless, taking into account the lower glutathione disulfide concentration under normal physiological conditions, **PSP-2** should be sufficiently resistant towards oxidation for biological studies.

A Job's plot for the Cu^I-**PSP-2** system showed maxima at either 1:1 or 2:1 metal/ligand ratios (Supporting Information, Figure S9), suggesting formation of an additional Cu$_2$L species. Spectrophotometric titrations against **MCL-2** indeed revealed coordination of a second Cu^I to **PSP-2**, albeit with a far lower affinity of log$K_2 = 12.25 \pm 0.01$ ($K_d = 0.6$ pM) (Supporting Information, Figure S6). Under Cu^I-limiting conditions; however, exclusive 1:1 binding of Cu^I to **PSP-2** was observed (Supporting Information, Figure S10). Analogous to **PSP-1**, we also observed dimerization of [(**PSP-2**)Cu^I] with a logK_{dimer} of 3.70 ± 0.08 (Supporting Information, Figure S7), corresponding to a dimer dissociation constant of 200 μM. This result was independently confirmed by ^{31}P NMR spectroscopy in D$_2$O (Supporting Information, Figure S8). While the cyclic voltammogram of [(**PSP-2**)Cu^I]Cl in aqueous solution indicated even greater stabilization of the Cu^I oxidation state compared to the **PSP-**

1 complex, with an anodic shift of the oxidation wave by 50 mV, the one-electron oxidation process proved to be irreversible (Supporting Information, Figure S11).

As a small-molecule ligand, **PSP-2** offers a Cu^I affinity that is unprecedented for a neutral donor set and matched only by ligands with anionic sulfur donors. As the latter are pH-sensitive and may also coordinate to other metal ions, we evaluated the basicity of **PSP-2** and its selectivity for Cu^I over other biologically relevant first-row transition metal ions. As shown in Figure 3 B, the UV spectrum of **PSP-2** is unaffected by acidification or by the presence of Mn^{II}, Fe^{II}, or Zn^{II}, even at 1 mM metal ion concentrations, thus demonstrating a binding selectivity for Cu^I of greater than 17 orders of magnitude.

The unprecedented Cu^I-selectivity and affinity of **PSP-2** harbors the potential to probe the role of biological Cu^I in new ways, for example by selectively intercepting intracellular copper trafficking pathways without affecting other metal-dependent biological processes. Preliminary experiments with live mouse fibroblasts showed that **PSP-2** is readily taken up by cells, as confirmed by LC-MS analysis of the cell lysate (Supporting Information, Figure S13). While the ionization efficiency of neutral **PSP-2** proved to be very low, the ligand was readily detected in its oxidized form and as Cu^I complex (Supporting Information, Figure S14). An MTT cell viability assay additionally revealed low acute toxicity up to a concentration of 100 μM over 24 h at 37°C (Supporting Information, Figure S15). By comparison, the membrane-permeant Zn^{II}-selective chelator TPEN[15] resulted in a dramatically reduced viability at concentrations as low as 5–10 μM under otherwise identical conditions. Consistent with previous observations that selective removal of copper from the growth medium had little effect on the viability of cultured cells,[16] the low cytotoxicity of **PSP-2** suggests that the ligand cannot sequester copper from metalloproteins whose function is essential for cellular survival.

To test whether **PSP-2** can be used to selectively target copper-dependent pathways within a complex biological environment, we further explored its activity both in cell culture and in mouse hippocampal brain tissue. At the cellular level, we employed HeLa cells to explore the effect of **PSP-2** on trafficking of the Menkes protein (ATP7A), a copper-transporting P-type ATPase involved in metalation of cuproenzymes in the Golgi apparatus.[17] In the presence of excess copper, the Menkes protein relocates from the trans-Golgi network to the plasma membrane to mediate copper efflux.[18] Consistent with previous reports,[8a,18] under basal growth conditions the Menkes ATPase is mostly localized to the Golgi network near the nucleus but relocates to the cytoplasm upon supplementation of the medium with CuCl$_2$ (Figure 4 A). After replacement of the copper-supplemented incubation buffer with 100 μM **PSP-2**, the cytoplasmic localization of the Menkes protein is reversed back to the Golgi (Figure 4 A, right panel). In contrast, a control experiment with buffer lacking **PSP-2** showed no significant changes (Supporting Information, Figure S12). Likewise, electrophysiological studies with hippocampal brain tissue slices revealed that **PSP-2** suppresses long-term potentiation (LTP). The importance of copper in the central nervous system is well established,[19] and increasing evidence suggests
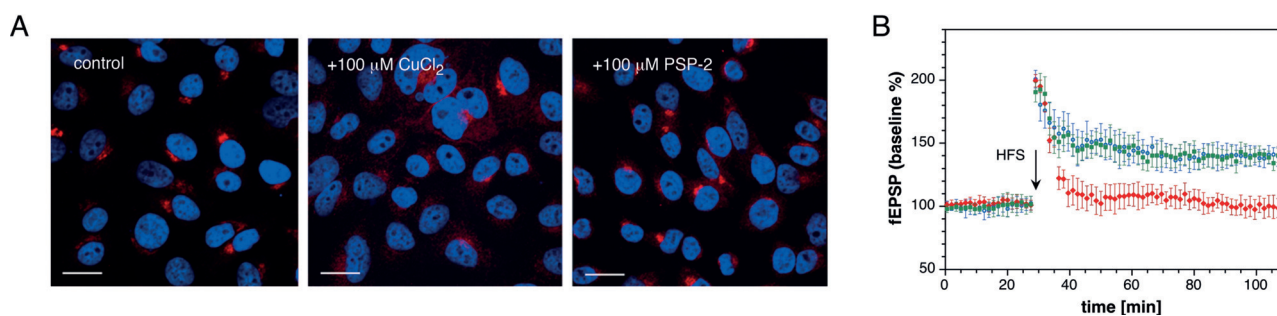
Figure 4. Targeting Cu^I-dependent trafficking pathways with **PSP-2**. A) Copper-dependent relocalization of ATP7A (red) in HeLa cells visualized by immunohistochemistry under basal growth conditions (left), upon supplementation with 100 μM CuCl$_2$ for 3 h (middle), followed by incubation with 100 μM **PSP-2** for 1 h after removal of CuCl$_2$ (right). Cell nuclei were stained with Hoechst 33258 (blue). Scale bars = 20 μm. B) Inhibition of long-term potentiation (LTP) by **PSP-2** in mouse hippocampal neurons. Induction of LTP upon high-frequency stimulation (HFS, 100 Hz) for 1 s was significantly suppressed in the presence of 20 μM **PSP-2** (red squares) compared to control (blue diamonds), but remained unaffected by 20 μM [(**PSP-2**)Cu^I]Cl (green circles) ($P < 0.05$, $n = 6$).

that copper may be directly involved in modulating neuronal activity.[20] Induced by high-frequency stimulation of synapse inputs, LTP results in an increase of synaptic strength and is thought to play a critical role in learning and memory. As illustrated with Figure 4 B, we induced LTP with two trains of 100 Hz stimulation in hippocampal neurons of brain slices from 4–5 week-old C57BL/6 mice and recorded field potentials (fEPSPs) in CA1 neurons. Upon application of 20 μM **PSP-2** for 20 min, LTP was reproducibly blocked ($P < 0.05$, $n = 6$), as indicated by return to baseline at 100%. Compared to control, LTP was thus significantly suppressed in **PSP-2**-treated slices while a paired-pulse test did not show significant difference between the two conditions. In contrast, [(**PSP-2**)Cu^I]Cl had no effect on LTP induction under identical conditions. Altogether, these data demonstrate that **PSP-2** can affect copper-dependent activities within complex biological systems, likely through competitive chelation of the metal ion from endogenous cuproproteins and ligands.

In summary, the presented ligand design approach using phosphine sulfide-stabilized phosphine (PSP) donors yielded a membrane-permeant Cu^I-chelator, **PSP-2**, with an unprecedented 10 zeptomolar dissociation constant. The ligand is remarkably resistant towards oxidation by disulfides and air under physiologically relevant conditions. Given its negligible affinity towards Zn^{II} and Fe^{II}, **PSP-2** is expected to make a valuable addition to the bioinorganic toolbox for elucidating biological copper trafficking pathways. The PSP design concept may also serve as a versatile approach towards developing chemically stable Cu^I-selective chelators for other applications, including fluorescent probes with sub-attomolar dissociation constants for visualizing the cellular dynamics of labile copper pools by fluorescence microscopy.

Conflict of interest

The authors declare no conflict of interest.

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