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Salpyran: A Cu(II) Selective Chelator with Therapeutic Potential

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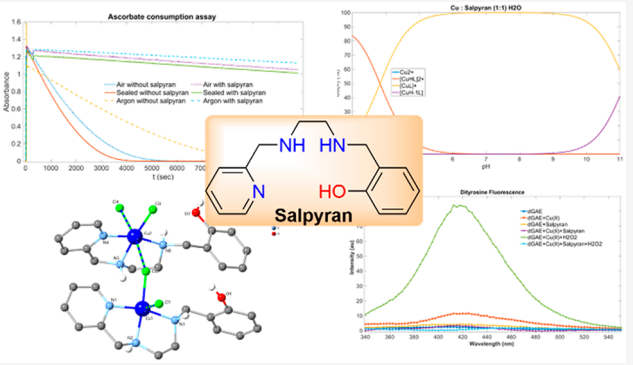
ABSTRACT: We report the rational design of a tunable Cu(II) chelating scaffold, 2-(((2-((pyridin-2-ylmethyl)amino)ethyl)-amino)methyl)phenol, **Salpyran**. This tetradentate (3N,1O) ligand is predicated to have suitable permeation, has an extremely high affinity for Cu compared to clioquniol ($pCu_{7.4}$ = 10.65 vs 5.91), and exhibits excellent selectivity for Cu(II) over Zn(II) in aqueous media. Solid and solution studies corroborate the formation of a stable $[Cu(II)(3N,1O)]^+$ monocationic species at physiological pH values (7.4). Its action as an antioxidant was tested in ascorbate, tau, and human prion protein assays, which reveal that **Salpyran** prevents the formation of reactive oxygen species from the binary Cu(II)/H_2O_2 system, demonstrating its potential use as a therapeutic small molecule metal chelator.



INTRODUCTION

The dysregulation and accumulation of biometals is a common pathological hallmark of many neurodegenerative disorders, such as Alzheimer's (AD), Parkinson's (PD) and prion diseases.[1−9] AD is the most prevalent adult neurogenerative disorder and the most significant cause of dementia.[10,11] Currently, 24 million people suffer globally, and, with an aging population, this figure may double by 2040.[12,13] AD is characterized by intracellular accumulation of neurofibrillary tangles formed of misfolded tau proteins and the extracellular deposition of fibrillar amyloid-β (Aβ) peptides. However, AD is a multiparameter disease, and other factors contribute to its etiology such as mitochondrial dysfunction, genetics, and age.[14] At present, a large body of research suggests that metal ion dyshomeostasis plays a role in AD's pathology; therefore, the restoration of biometal homeostasis offers a new clinical target when developing AD therapies.[6,15−22]

Recent trends show that drug development into disease-modifying therapies (DMTs) for AD is broadening its scope beyond the classical primary targets of Aβ and tau aggregation.[23,24] A paucity of new treatments for AD, for almost two decades, and the low success rate of drugs in clinical trials have furthered the need to widen the scope of both targets and approaches in curbing disease progression.[25,26] Recently, the first DMT (aducanumab) was approved by the Food and Drug Adminstration (FDA) for the treatment of AD patients. By targeting the production and aggregation of Aβ, this novel therapy was found to reduce senile plaques, although there still remains some uncertainty in its clinical benefits.

Metal ions can affect the self-assembly of amyloid proteins; for example, Aβ has a picomolar affinity for Cu(II) binding via histidine binding.[27,28] Cu(II) imbalances exist in AD affected brains, and Cu(II) can be found either upregulated or downregulated depending on the locality of the tissue.[6,29] Due to its redox potential when bound to Aβ, Cu(II) contributes to the generation of reactive oxygen species (ROS), leading to oxidative neuronal damage.[30−32]

In the past decade, there has been an increasing interest in designing Cu-specific small molecule metal chelators (SMMCs) aiming to reduce Cu(II)-Aβ induced oxidative stress and the resulting pathogenic consequences.[6,15,34−39,16−22,33] Chelation therapy aims to disrupt potential toxic interactions of metal ions and biomolecules by targeting specific metal ions and promoting redistribution or excretion. When designing a Cu-specific SMMC, both the thermodynamic properties of the metal chelate and the pharmacological properties of the ligand must be considered. The key criteria for Cu(II) targeting AD therapeutic are denticity, metal/ligand stoichiometry, and the coordination environment and geometry of the complex at physiological pH values. Ideally, the given ligand would coordinate to Cu(II) in a 1:1 stoichiometry, as ligands of this type exhibit a higher copper affinity than similar 1:2 complexes due to the

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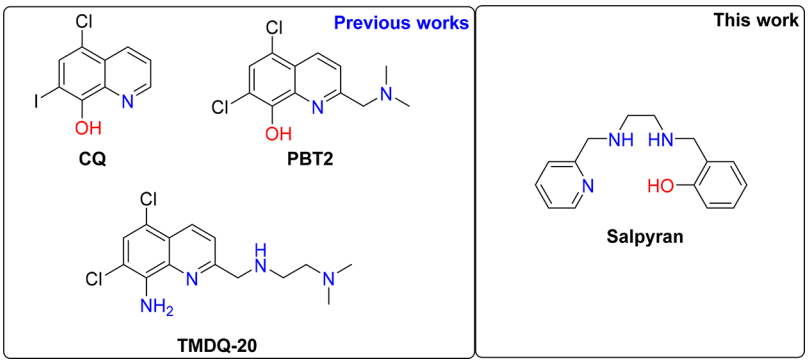

Figure 1. Previous and current SMMCs 2-(((2-((pyridin-2-ylmethyl)amino)ethyl)amino)methyl)phenol, **Salpyran**.

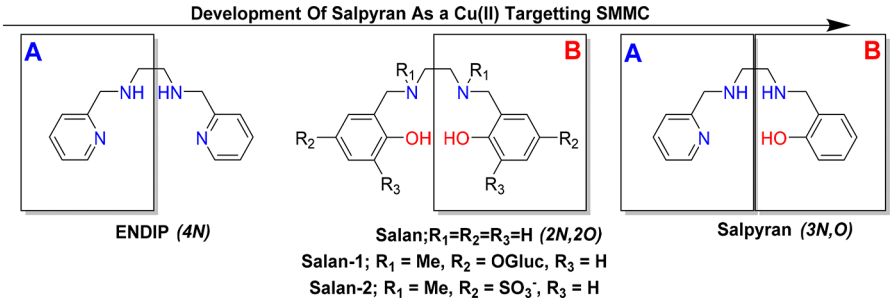

Figure 2. Development of **Salpyran** by combining structures of **ENDIP** and **Salan**.

chelation.[40] The increased shielding observed in 1:1 complexes protects the metal ion from the physiological environment, preventing further biological interactions such as the formation of [Aβ(Cu)L] ternary species.[39,41] Also, to be an effective therapeutic, both the ligand and the formed metal complex must be metabolically stable, nontoxic, and possess suitable aqueous solubility. Moreover, to be effective in AD, the SMMC should be able to pass through the blood−brain barrier (BBB) to reach the site of Cu(II) accumulation. For passive diffusion, this requires a SMMC that is suitably hydrophobic to passively pass through the membrane yet hydrophilic enough to stay soluble in physiological environments.[42,43]

Clioquinol (**CQ**, Figure 1) was investigated in phase II clinical trials for targeting metal homeostasis as an AD treatment. **CQ** is a bidentate ligand that forms a [Cu(II)L_2] complex with an 2N,2O coordination environment. By targeting both Cu(II) and Zn(II)binding, **CQ** showed some improvements in the cognition of the patients trialled.[44] However, due to neurotoxic side effects, the clinical progress of **CQ** was ultimately abandoned.[45] This led to the design of a second-generation tridentate 5,7-dichloro-2-((dimethylamino)methyl)quinolin-8-ol (**PBT2**, Figure 1), which completed Phase II clinical trials.[46,47] Introduction of a dimethylamino unit at the C2 position introduced a new binding site, but still [Cu(II)L_2] complexes are formed.[48] A lack of reduction in amyloid plaque levels in the brains of AD patients and only mild cognitive benefits mean that **PBT2** has not progressed into more extensive clinical studies. The poor metal selectivity is a possible reason for the clinical failure of **CQ**, as interactions with other biometals or metalloproteins/substrates *in vivo* are conceivable. The formation of the [Cu(II)L_2] species, in both **CQ** and **PBT2** cases, speculates the likely *in vivo* formation of ternary L(Cu)Aβ species that can contribute to increased ROS production.[49]

Due to the clinical potential demonstrated by **CQ** and **PBT2**, several tetradentate ligands based on similar scaffolds have been developed to increase Cu(II) selectivity and minimize unwanted biological interactions.[38,40,48,50,51] This incremental design led to the state-of-art Cu(II) chelator, **TDMQ-20** (Figure 1).[52] **TDMQ-20** is an 8-aminoquinoline derivative that offers a 4N coordination environment and shows exceptional selectivity for Cu(II) ions. Recently, **TMDQ20** has been studied as an AD therapeutic in early stage nontransgenic mouse models and late-stage transgenic models.[52] Oral treatment offered significant improvements in both the behavioral and cognitive impairments observed in each model, while also reducing oxidative stress in the mouse cortices. This efficacy paves the way for future pharmacological evaluation of SMMCs; thus, most research heavily focuses on chelators based around either 8-hydroxy/8-amino quinoline backbones. Having the chemical criteria and fall-outs from previous studies in mind,[39] and aiming to develop new chelators not derived from 8-hydroxy/8-amino quinoline cores, we hypothesized that the scaffold 2-(((2-((pyridin-2-ylmethyl)amino)ethyl)amino)methyl)phenol, **Salpyran** (Figure 1) would be an ideal therapeutic Cu(II) targeting SMMC. Herein, we report the criteria considered in designing **Salpyran**, its synthesis and characterization, solid-state and solution studies, and ROS inhibition.

RESULTS AND DISCUSSION

Scaffold Development. Several organic ligands, exclusive of hydroxy and aminoquinolines frameworks, have been investigated as potential Cu(II) SMMCs. A recent review by Hureau et al. highlights the pros and cons of these structures.[39] Among them, tetradentate bis(pyridine), **ENDIP**, competes for both copper and zinc in Aβ aggregates, preventing their formation and solubilizing amyloid precipitates.[53] Tetrahydrosalen (**Salan**) ligands are strong metal binders and offer

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Table 1. Thermodynamic and Calculated Pharmacological Relevant Properties of Chelators Targeting Cu(II) Homeostasis in Alzheimer's Disease*

	Thermodynamics					Drug Likeness[f]							
	pCu[a]	pZn[a]	Cu/Zn[d]	M/L ratio	Cu(II) Coordination Environment[e]	Mw (g/mol)	TPSA (Å)	cLog P	cLog S	HB D	BBB Perm.	GI Absorp.	Ref.
Cu(Aβ)	7.3-7.8	~6.0	~	-	$^{His}N_3^{Glu11/Asp1}O\ H_2O_{ap}$	-	-	-	-	-	-	-	39,65,75,76
CQ	5.91[b]	5.64[b]	0.27	1:2	N_2O_{2eq}	305.5	33.12	2.96	-4.37	1	Yes	High	77–79
PBT2	n.d	n.d	n.d	2:1	N_3O_2	271.14	36.36	2.85	-3.98	1	Yes	High	48
TMDQ-22	10.75[c]	5.06[c]	5.69	1:1	$N_{4eq}Cl_{ap}$	327.25	54.18	2.85	-4.43	3	Yes	High	40,51
ENDIP	10.35[b]	7.71[b]	2.64	1:1	n.d	242.32	49.84	1.27	-2.79	2	Yes	High	53
Salan	9.48[b]	5.35[b]	4.13	1:1	N_2O_{2eq}	272.34	64.52	2.26	-4.16	4	Yes	High	47
Salan-1	8.97[b]	5.33[b]	3.64	1:1	N_2O_{2eq}	656.68	245.7	-1.85	-1.72	10	No	Low	62
Salan-2	9.83[b]	5.97[b]	3.86	1:1	$N_2O_{2eq}\ OH_{2\ ap}$	458.51	195.82	-2.59	-0.86	2	No	Low	58,63
Salpyran	10.65	6.69	4.60	1:1	N_3Cl_{eq} or $N_{3eq}Cl_{ap}$	257.33	57.18	1.73	-3.47	3	Yes	High	**This work**

*Constants are for the form Aβ1-x. [a]pM = −log[M]$_{free}$; [M] = [L] = 10 μM, pH = 7.4. [b]Calculated from conditional affinity value. [c]Calculated from apparent affinity value at pH = 7.4. [d]Cu/Zn selectivity calculated by pCu − pZn. [e]Coordination environment in solid state; equatorial (eq) and apical (ap) sites. [f]Calculated using the SwissADME free to use webtool; both log *P* and log *S* and the consensus values.[72]

antioxidant properties.[54] Storr et al. designed multifunctional carbohydrate ligands based around an N-methylated salan core (**Salan-1**, Figure 2).[55] The pendant glucose arm facilitates access to the brain and passes through the BBB via glucose transporters. Both ligands were found to have significant antioxidant properties *in vitro*. The O-glycosylation of the **Salan** ligand was also investigated as a prochelator strategy, where the glucose moiety effectively masks the coordination pocket until hydrolysis occurs *in vivo*.[56,57] It was confirmed that the enzyme *Agrobacterium* sp. β-glucosidase could effectively cleave the C−O bond of the glucose moiety releasing the N-methylated **Salan** scaffold as the active chelator at the site. Other attempts to improve the pharmacological profile of the core **Salan** scaffold have involved the sulfonation of the phenolic groups (**Salan-2**), which significantly improves solubility (Table 1).[58] However, it is expected that the presence of an ionizable sulfonate group will result in poor BBB permeability, making it unsuitable for AD treatments.

Having all these in mind and building on our recent work in nonsymmetric salan ligands,[59] we envisaged that the combination of the **Salan** and **Endip** moieties should yield a nonsymmetric ligand, **Salpyran** (Figure 2). By breaking the C_2 symmetry, a new 3N,O coordination environment is formed that may partially fulfill the coordination environment of the Cu(II) center. **Salpyran** offers the same number of heteroatoms as **TDMQ-20**. Pearson's acid−base principle predicts that the addition of the pyridine will increase the Cu(II) affinity and selectivity versus the **Salan** scaffold; this is observed in the trend of pCu values observed for more nitrogen-rich coordination pockets (Table 1). Also, compared to the **Salan** (cLogP = 2.26, Table 1) scaffold replacement of a phenol with a pyridine entity improves the aqueous solubility by reducing the lipophilicity of the scaffold (cLogP = 1.73, Table 1). However, the phenolic moiety provides the scaffold with radical scavenging capabilities to act as an antioxidant during AD treatments.[54] Our approach introduces an entirely different scaffold for use in AD treatment, contrasting the more classical approach of modifying known metal coordinating scaffolds.[54,56−58,60−63]

Thermodynamic and Physiochemical Properties Compared to Other Cu Chelators. The selectivity of the ligand for Cu(II) over other metal ions is a critical factor in designing Cu(II) targeting SMMC. The chelator in question should have high selectivity toward copper to minimize competition with other essential metal ions and interactions with other metalloproteins. The stability constant (log β) of the metal complex (ML) is used to assess the affinity of a ligand for a specific metal (eq 1). Therefore, in designing AD therapeutics, it is beneficial to compare the stability constants for Cu(II) and Zn(II) due to the high concentration of Zn(II) in AD brains.[64,65] The variability in the method and conditions used to measure the metal/ligand affinities has led to the use of the pM (eq 2) value when comparing and assessing the chelation capability of copper targeting SMMCs. The pM value is calculated at physiological pH and micromolar metal and ligand concentrations. Consequently, this offers added benefit by comparing chelators regardless of denticity or metal/ligand stoichiometry.

$$mM + lL + hH \leftrightarrow [M_mL_lH_h]$$

$$\log\beta_{MLH} = \log\left(\frac{[M_mL_lH_h]}{[M]_m[L]_l[H]_h}\right) \quad (1)$$

$$pM = -\log[M]_{free} \quad (2)$$

Synthesis of Salpyran. **Salpyran** can be synthesized via a stepwise protecting group strategy in which consecutive reductive aminations using salicylaldehyde and 2-formylpyridine take place across an ethylenediamine backbone (Scheme 1, Figures S1−S10). First, the reductive amination of either

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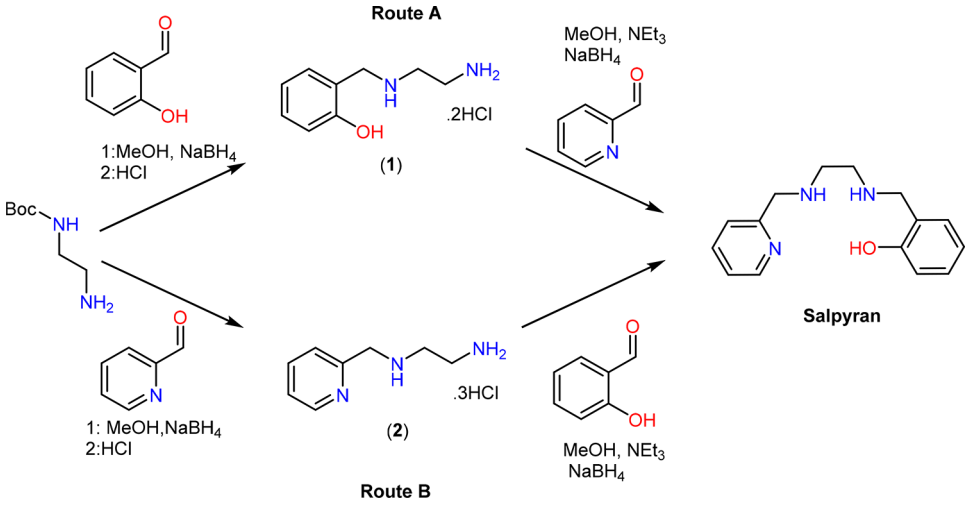
Scheme 1. Two Alternate Synthetic Routes Towards Salypyran Starting from N-Boc-ethylenediamine and Using Either (A) Salicylaldehyde or (B) 2-Formylpyridine



aldehyde with *N*-Boc-ethylenediamine and subsequent deprotection give the amine precursors (**1**) and (**2**) (Scheme 1). A second reductive amination, this time in the presence of stoichiometric base (NEt_3), yields **Salpyran**. It is possible to modify **Salpyran** via variation in the aromatic substitution of either aldehyde or by replacement of the diamine linker unit. Functionalization is also possible at either of the amine's groups, making **Salpyran** a highly tunable scaffold compared to similar symmetric structures. For this three-step synthesis, the total yield of **Salpyran** via route A is 49% and significantly drops to 19% for route B. The fact that there are two simple synthetic routes demonstrates the synthetic accessibility toward **Salpyran**, which offers flexibility in analogue design in further medicinal chemistry pursuits. In the development of drugs targeting neurodegenerative disorders, there has been a trend in the design of multifunctional drugs that contain structural moieties aiming to target multiple pathological features at once or the addition of bioisosteres or isosteres to modify the pharmacokinetic properties.[66] This has led to an interest in multifunctional drugs containing a metal-binding unit;[67−69] therefore, the high synthetic accessibility and tunability of **Salpyran** may offer future opportunities for use in multifunctional drugs.

Complexation Behavior with Cu(II) and Zn(II). The protonation constants (Table S1) of **Salpyran** were determined by pH-metric titrations. Using these data, the stability constants of the Cu(II) and Zn(II) complexes were calculated (Table 2). At low pH (<4) values, the dicationic $[CuLH]^{2+}$ is the dominant species. At the same time, the phenolic hydroxyl group remains protonated and uncoordinated. Across the physiological pH values (7.4), the monocationic $[CuL]^{+}$ is the dominant species. In contrast, at high pH values (>11), a further deprotonation process occurs, forming a neutral $[CuLH_{-1}]$ species likely via the deprotonation of a coordinated water molecule (Figure 3A,B). The aqueous solution behavior is alike for Zn(II); however, no protonated complex is formed. At pH 5, 50% of Zn(II) is found unbound (Figure 3C). In all, the stability of the formed Zn(II) species is lower than that of the corresponding Cu(II) species, demonstrating the Cu(II) selectivity of **Salpyran** (Table 1). The species distribution plots of the Cu(II) complexes formed in equimolar metal to ligand solutions are shown in Figure 3. Further solution studies with Cu(II) were performed in a mixture of DMSO:H_2O (70:30), as it is a common practice for biological studies. Notably, the ligand behavior changes drastically, corroborated by UV−vis studies (Figure 3D,E), showcasing the formation of other species and indicating that speciation is highly dependent on the solvent system (Table S2). In the less polar DMSO-containing solvent mixture, the positively charged $[CuL]^{+}$ species is dominant in both systems in the physiological pH range (Figure 3) but is present in a narrower pH range, and the formation of the neutral $[CuLH_{-1}]$ species is favorable. Based on this evidence, we considered that solution studies in DMSO solution would add no value to our conclusion.

The complex formation of **Salpyran** with Cu(II) and Zn(II) ions was studied at 1:2 and 1:1 ligand to metal ion ratios in the pH range 3−11 (Figure S11). Comparison of the UV−vis spectra of the Cu(II)-**Salpyran** system at 1:2 and 1:1 metal to ligand ratios (Figure S12) shows that similar spectra are obtained. These studies indicate that irrespectively of the metal to ligand ratio, only the 1:1 complex forms at pH values ranging from 3 to 11. Therefore, we assume that during *in vivo* studies, the 1:1 species is dominant, reducing the possibility of interactions with endogenous metalloproteins

The thermodynamic properties and drug-likeness of **Salpyran** and other chelators discussed in this work are summarized in Table 1. The affinity of the ligands for Cu(II) and Zn(II) is measured using pCu and pZn values calculated from the reported conditional ($\log \beta_{con}$) or apparent ($\log \beta_{app}$) stability constants using [M] = [L] = 10 μM, p.H = 7.4. This was achieved using the Hyperquad simulation and speciation (HySS) software.[71] Copper/zinc selectivity is given as pCu/

Table 2. Stability Constants ($\log \beta$) for Salpyran complexes with Cu(II) and Zn(II) in Aqueous Solution Calculated Using the SUPERQUAD software (ref 70)*

logβ	Cu(II)	Zn(II)
MLH	23.93 (8)	—
ML	20.10 (8)	11.98 (5)
MLH_{-1}	8.94 (10)	2.38 (13)

*I = 0.2 mol × dm^{-3} KCl, T = 298 K, standard deviations are in parentheses.

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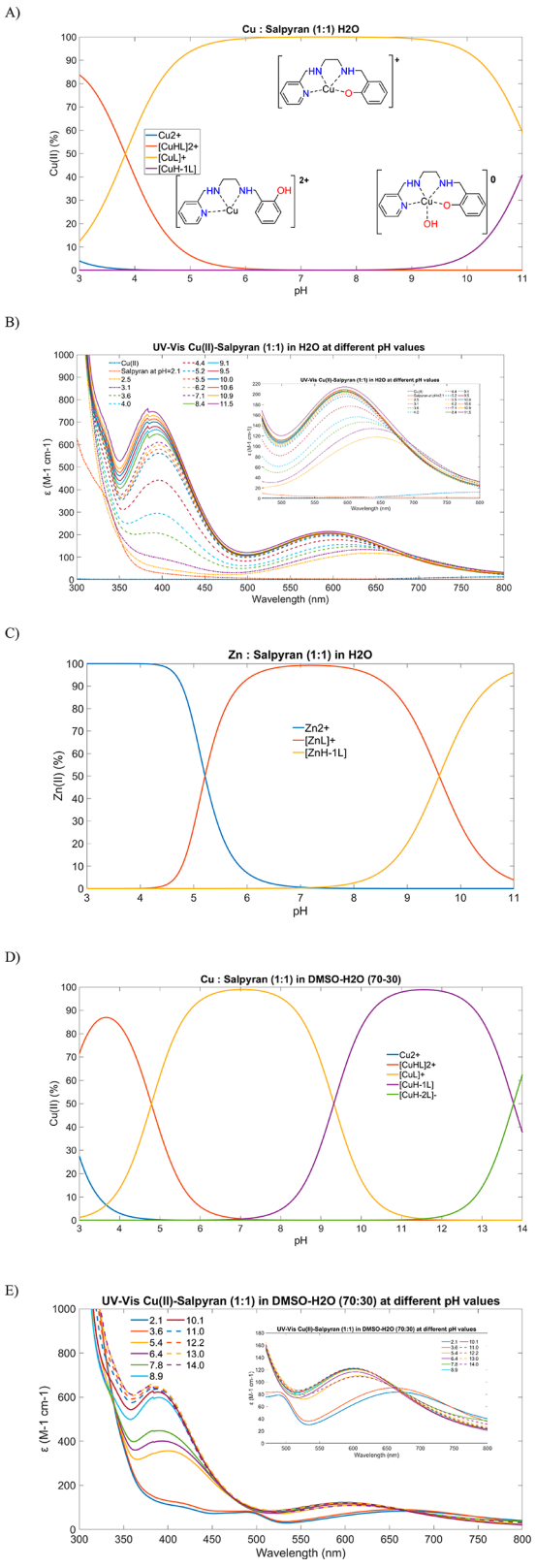

Figure 3. (A, B) Species distribution and UV−vis data of the Cu(II)-**Salpyran** complexes formed in the equimolar solutions as a function of pH in H_2O. (C) Species distribution of the Zn(II)-**Salpyran** complexes formed in the equimolar solutions as a function of pH. (D, E) Species distribution and UV−vis data of the Cu(II)-**Salpyran** complexes formed in the equimolar solutions as a function of pH in mixture DMSO:H_2O (70:30).

pZn; the larger the value, the greater selectivity toward Cu(II) over Zn(II). Also included in Table 1 is the stoichiometry and coordination environment of the copper complexes according to the reported solid-state structures. The drug-likeness of the ligands has been predicted using the SwissADME web tool, and the calculated physicochemical properties and predicted BBB permeation and gastrointestinal absorption are also given.[72] Ideally, any SMMC would follow the ' Lipinski rule of 5'[73] and have a topological polar surface not exceeding 140 Å^2 (Veber rule).[74] In all, the complexation behavior of **Salpyran** supports its potential use as a Cu(II) targeting SMMC. It has an exceptional affinity for Cu(II) (pCu = 10.65) and good selectivity for Cu(II) over Zn(II) (Cu/Zn = 4.60) (Table 1). **Salpyran** acts as a tridentate or tetradentate, dependent on the pH and only forms the 1:1 complex with Cu(II). (The characteristic bands of the Cu(II) complexes are summarized in Table S3.) **Salpyran** has a higher affinity and selectivity for copper when compared to its C_2 symmetric analogues, **ENDIP** and **Salan** and comparable affinity but with lower selectivity when compared to **TMDQ-20** (Table 1).

From Table 1, it is evident that **Salpyran** offers both high affinity and selectivity for Cu(II) (pCu = 10.65, Cu/Zn = 4.6). Compared to both "parent" ligands, **EDNIP** and **Salan**, **Salpyran** outperforms, and its values are close to the state of the art **TDMQ-20** (pCu = 10.75, Cu/Zn = 5.06). **Salpyran** has good solubility, and its calculated log *P* value suggests that good BBB permeation could be expected, although the number of hydrogen bond donors (HBD = 3) may be deleterious to BBB influx and may need to be factored into future drug design (e.g., masked HBDs, rigidification).[80,81]

Salpyran Copper Crystal Structure. To better understand the complexation behavior of **Salpyran** with Cu(II), we carried out several complexation reactions in protic or aprotic solvents. The reflux of an equimolar solution of **Salpyran**, $CuCl_2$, and NEt_3 for 1 h in methanol yielded a viscous, green oil, which upon dissolving in DMF, followed by vapor diffusion of diethyl ether over 1 week, yielded blue crystals suitable for single X-ray diffraction in low yield (13%, Tables S4 and S5). The solid-state structure is shown in Figure 4. Upon complexation with $CuCl_2$, **Salpyran** yields an asymmetric Cu(II)-dimer consisting of two different ($CuCl_2HL$) units, and Cl_2 serves as a bridge of these two entities. The coordination geometry of the two Cu centers varies; Cu1 adopts a 3N,2Cl coordination environment (square pyramidal), while Cu2 adopts a 3N,3Cl environment (distorted octahedron) (Figure 4); notably, both phenol moieties remain protonated. This observation is in line with the potentiometric studies, which suggest that at low pH values (pH < 4), the $[CuHL]^{2+}$ species is dominant. The crystal structure confirms that the ligands exhibit two five-membered chelated rings via coordination of the three nitrogen donor atoms (NH, NH, N_{py}), which may account for the high stability of $[CuHL]^{2+}$ species (Table 1). Moreover, a close inspection of bond lengths and angles (Table S3) reveals three different Cu−Cl bond types: Cl2 and Cl3 strongly bind to Cu1 and Cu2, respectively, [2.2780(14) Å and 2.2649(15) Å], the Cu1−Cl1 [2.6466(15) Å] and Cu2−Cl4 [2.7294(15) Å] are weakened bonds, while the value of the Cu2−Cl2 bond is 3.0454(15) Å, which is indicative of a secondary, very weak interaction.[82]

Further attempts to isolate crystals of the complex with the deprotonated ligand were unsuccessful. HRMS of the isolated crystals and viscous green oil is provided in the Supporting Information (Figures S13 and S14) and is in line with the

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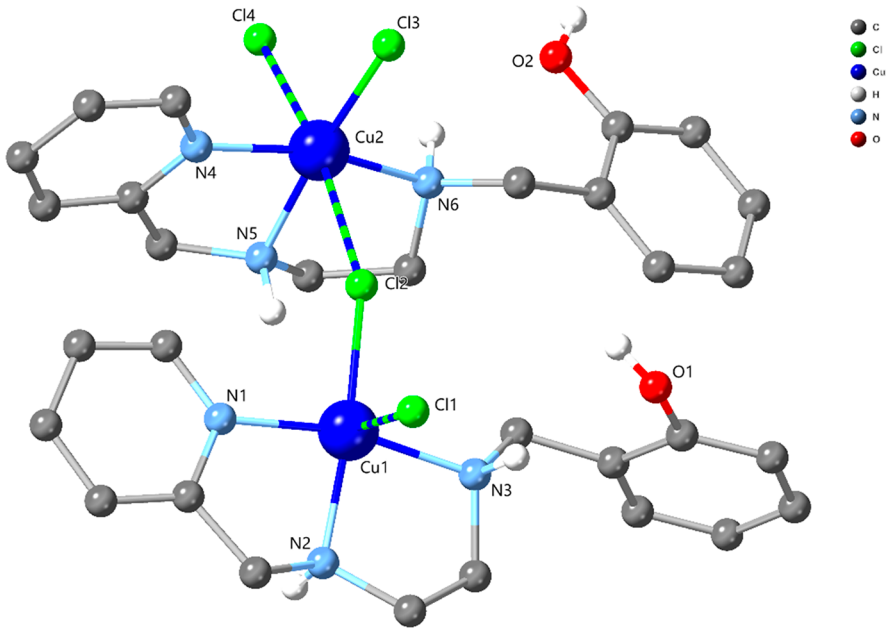

Figure 4. Solid-state structure of protonated **Salpyran**−copper complex.

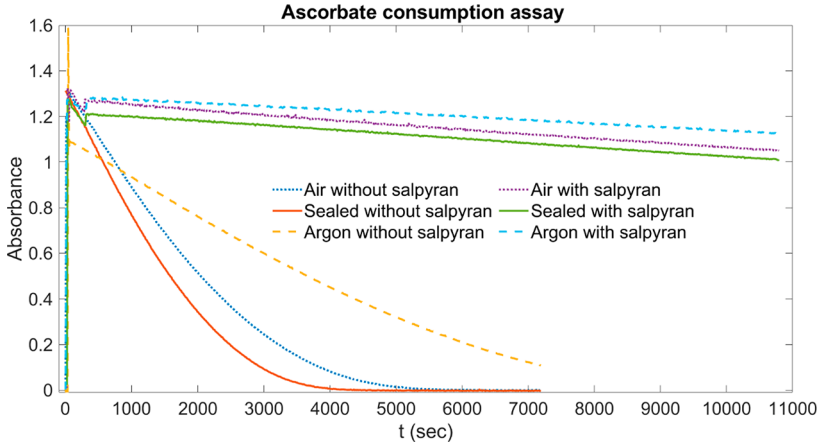

Figure 5. Kinetics of ascorbate consumption with(out) **Salpyran** in different conditions (open air, Ar, and sealed cuvette). The reactants **Salpyran** (if any)/$CuCl_2$/ascorbate (12 μM/10 μM/100 μM) ratio.

$[CuL]^+$ and $[CuHL]^{2+}$ structures, respectively. In all, taking into account that (a) differentiation in Cu−Cl bonding is due to the weakly binding character of the Cl anion, (b) solution studies were carried out using $CuCl_2$ stock solutions, (c) UV−vis studies suggest the existence of a Cu,3N (low pH value) and Cu,3N,O (physiological pH values) chromophores, and (d) ESI-MS studies corroborate the existence of monomeric, not dimeric species, in methanolic or aqueous solution, we can correlate the solid and solution phases and confirm the dominance of the $[CuL]^+$ species at physiological pH values.

Antioxidant Properties. Redox-active Cu(II) is known to induce ROS formation and oxidative stress accumulation.[83] Therefore, potential therapeutic SMMCs must be capable of effectively inhibiting Cu(II)-induced ROS formation. As a starting point, we adopted a recently reported protocol[83] and investigated the ability of **Salpyran** to arrest the ROS production by monitoring ascorbate consumption under three different conditions (open air, Ar, and sealed cuvette). The ascorbate consumption is plotted as a function of time in seconds (Figure 5 and Figures S15−S20). The ascorbate consumption without **Salpyran** was followed for 2 h, while in the presence of **Salpyran**, the samples were monitored for 3 h. Samples were prepared in situ from stock solutions in 100 mM HEPES buffer at pH 7.1, and the pH was adjusted with 0.2 M HCl. The components were added in the following order: HEPES, HCl, water, ascorbate, $CuCl_2$, and **Salpyran** (if any). The assay was carried out under anaerobic and aerobic conditions. In the anaerobic studies, the ascorbate consumption was not completed even after 2 h, while under aerobic conditions, the ascorbate is fully consumed in 1.5 h. The calculated rate constants (from 5000 to 10,000 s, Table 3) for the samples containing **Salpyran** are under argon, 1.07 × 10^{-9} Ms^{-1} (=1.07 nMs^{-1}), in open air, 1.37 × 10^{-9} Ms^{-1} (1.37 nMs^{-1}), and in a sealed cuvette, 1.36 × 10^{-9} Ms^{-1} (=1.36 nMs^{-1}). The rate constants were calculated by dividing the slope by the extinction coefficient of ascorbate, ε = 14,500 M^{-1} cm^{-1}. Any difference in rates with the reported protocol[83] may be attributed to the stirring rate (300 rpm over 800 rpm) and ligand framework. These studies clearly show **Salpyran** slows

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Table 3. Calculated Rate Constants* for Kinetics of Ascorbate Consumption in Different Conditions with Ratio Salpyran/$CuCl_2$/Ascorbate (12 μM/10 μM/100 μM)

Condition	Without Salpyran	With Salpyran
Air	Not calculated	1.37
Sealed	Not calculated	1.36
Argon	Not calculated	1.07

*In nMs^{-1}.

the ascorbate consumption, thus demonstrating its capability to prevent ROS production.

Also, we investigated **Salpyran**'s oxidation ability in the presence of H_2O_2 (Figure 6 and Figures S21 and S22). The reaction mixtures containing 1.0 mM **Salpyran** at metal to ligand molar ratio 1:1 were incubated at 25 °C for different time periods in the presence of H_2O_2 at ligand to H_2O_2 molar ratio 1:4. The pH was adjusted to 7.4. The reaction was initiated by the addition of a freshly prepared 1% H_2O_2 solution. The reaction was stopped by the addition of Na_2EDTA at ligand to Na_2EDTA ratio 1:5. The reaction process was monitored by analytical RP-HPLC using a Jasco instrument, equipped with a Jasco MD-2010 plus a multi-wavelength detector. From these data, it is evident that oxidation does not occur in the sample containing equivalent amount of Cu(II) and **Salpyran** even after 2 days (Figure S21, upper). While in the sample containing 4-fold excess 1% H_2O_2, some oxidation occurs in the first 4 h (Figure S21, lower).

Then, we assessed the ability of **Salpyran** in preventing Cu(II)-catalyzed oxidation in two different protein fragment assays at physiological pH values. It has previously been shown that a fragment of the human prion protein (HuPrP(103−112), dMKHM) (Figure S23) undergoes oxidation in the presence of radicals formed from the Cu(II)/H_2O_2 system.[84] The oxidation occurs only at the methionine residues, yielding three main products: two singly oxidized products (dMKHM + O, orange) and a doubly oxidized product (dMKHM + 2O, yellow). Both methionine residues at position 7 (Met109) or/ and at position 10 (Met112) can be oxidized. However, only methionine sulfoxides are produced and not the corresponding sulfones. The oxidation was initiated by adding H_2O_2 to an equimolar Cu(II)-dMKHM-**Salpyran** solution, and the reaction was monitored by HPLC for 1 day (Figure 7). After 1 h, almost 60% of HuPrP(103−112) remains intact, and no oxidation occurs in any methionine group, three times higher than the blank experiment. In contrast, after 2 h, the

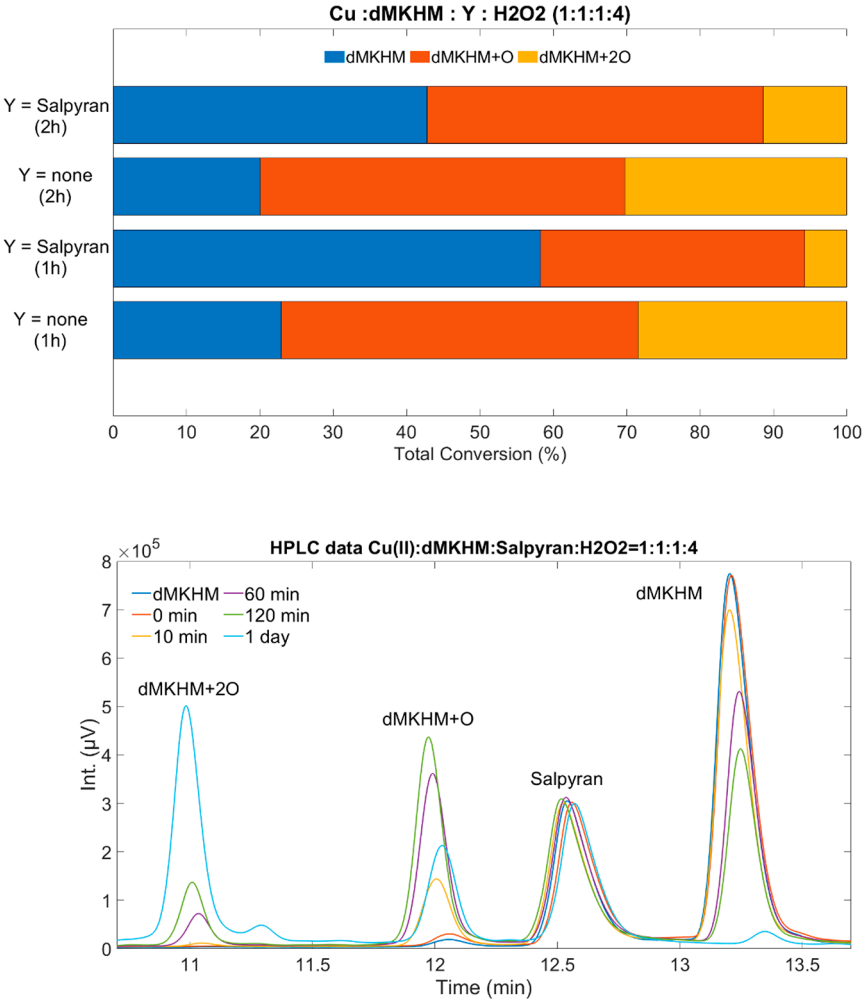

Figure 6. (upper) Ratio of the Cu(II)/H_2O_2 oxidized prion protein fragment, HuPr(103−112) (dMKHM), formed products with and without **Salpyran**. (lower) An HPLC chromatograph of the oxidation process 0 min, 10 min, 60 min, 120 min, and 1 day. Teknokroma Europa Protein C18 (250 × 4.6 mm, 300 Å, 5 μm) column at a flow rate of 1 $mL{\cdot}min^{-1}$, monitoring the absorbance at 222 nm. Mobile phases were water (A) and acetonitrile (B) containing 0.1% TFA.

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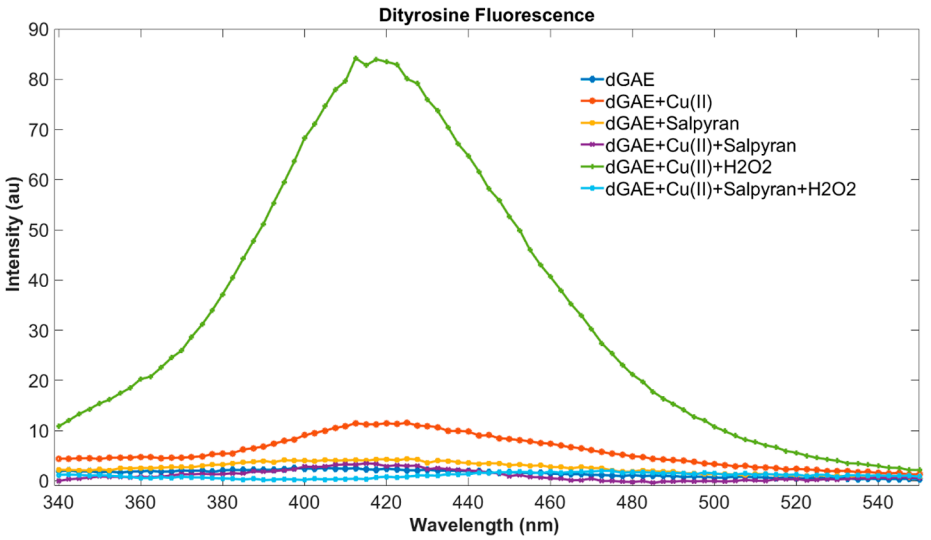

Figure 7. Fluorescence monitoring of the formation of dityrosine bridges from Cu(II)/H_2O_2 oxidation of the tau dGAE fragment. Reactions were prepared using μM dGAE mixed with Cu(II) at a 1:10 ratio or in combination with 2.5 mM H_2O_2 to induce oxidation and dityrosine cross-linking. A separate dGAE reaction was prepared with **Salpyran** at a 1:10 ratio or **Salpyran** in combination with Cu(II) at a 1:1 ratio alone and in combination with 2.5 mM H_2O_2. The reactions were quenched after 1 h with the addition of 2 mM EDTA.

percentage of dMKHM is still high (40%, doubled compared to that of the blank), while unreacted dMKHM parts are still evident after 1 day (HPLC, Figure 6). These data demonstrate **Salpyran's** efficiency in hindering the oxidation of the peptide, possibly by protecting the Cu(II) ions and inhibiting the ROS formation from the binary Cu(II)/H_2O_2 system. The lack of total inhibition of peptide oxidation was not observed, potentially due to an excess of peroxide used in the experiment. These results demonstrate the potential of **Salpyran** in targeting Cu(II) dyshomeostasis and reducing the oxidative stress associated with neuronal death.

One known product of oxidation induced by Cu(II) is dityrosine cross-links on proteins, such as Aβ.[85] Dityrosine (DiY) formation, whereby closely spaced tyrosines covalently cross-link by ortho−ortho coupling at C3 of their benzene rings, has been used as a marker of oxidative stress, and DiY has been shown to form under Cu(I/II)/H_2O_2 oxidative conditions for Aβ and tau *in vitro*[86−89] and within AD amyloid plaques *in vivo*.[87] In the presence of H_2O_2, Cu(II) induces dityrosine cross-linking more efficiently, serving as an excellent marker of oxidation.[89] Also, Cu(II) is known to bind tau and induce tau oxidation, dimerization, and aggregation.[90,91] Recently, it was demonstrated that Cu(II) alone or in the presence of H_2O_2 induces oxidation and dityrosine cross-linking of a tau297−391 fragment which contains one tyrosine at position 310.[89,92] To further demonstrate the antioxidant ability of **Salpyran**, we performed a series of reactions using tau297−391 and Cu(II) (1:10 ratio) in combination with H_2O_2 to induce oxidation and dityrosine formation, which were quenched after 1 h with the addition of EDTA. The appearance of the dityrosine species was observed by monitoring the intensity of the peak at 410 nm (Figure 7). Unlike the reactions with just Cu(II) or more so in combination with H_2O_2, which showed robust induction of dityrosine to approximately 1% and 7% dityrosine levels (Figure S24), similar reactions mixed with **Salpyran** showed no dityrosine cross-linking alongside the controls (below 0.5%) (Figure 7). This suggests that **Salpyran** effectively prevents dityrosine formation and thus oxidation of dGAE via binding to Cu(II). Combined with the aforementioned antioxidant studies, these results indicate that **Salpyran** can reduce ROS production in both Cu(II)/H_2O_2 and Cu(II)/O_2/reductant systems.

CONCLUSION

We rationally designed and synthesized a highly modifiable copper chelating scaffold, **Salpyran**. This tetradentate ligand offers a 3N,O coordination environment and possesses good drug-likeness. **Salpyran** exhibits an extremely high affinity for Cu and excellent Cu(II) selectivity over Zn(II), comparable to the state of the art components. Solid and solution studies corroborate variation in coordination behavior at different pH values, but confirm the existence of only one dominant species at physiological pH values in aqueous solutions. Under physiological pH values and unaerobic conditions, the $[Cu(II)(3N,1O)]^+$ complex remains intact for at least 2 days, while in the presence of H_2O_2, an oxidation procedure occurs. Further studies showcase that **Salpyran** slows the ascorbate consumption, thus preventing ROS production. Finally, two different protein fragment assays that investigate antioxidant properties revealed **Salpyran's** excellent efficacy to prevent the formation of ROS from Cu(II)/H_2O_2. Due to its drug-likeness, desirable coordination behavior, antioxidant properties, and tunability, **Salpyran** is an alternative scaffold to 8-hydroxy/aminoquinolines for further pharmaceutical development of Cu(II) targeting drugs in neurodegenerative disorders such as AD.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.inorgchem.1c01912.

Copies of ^1H,^{13}C NMR, HRMS, and LCMS data for the ligand (PDF)

Accession Codes

CCDC 2090343 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge

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via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Author Contributions

All authors contributed to writing the manuscript and approved its final version. J.D. devised the project with critical input and comments from G.E.K, J.S., and C.K. J.D. designed, synthesized, and characterized the ligand and performed and evaluated, with G.E.K., the crystallographic data. C.K. and N.B. performed and evaluated the potentiometry, UV−vis, and human prion fragment studies. L.S. and M.B.M. performed and evaluated the dityrosine studies. A.M. provided valuable feedback and comments.

Notes

The authors declare no competing financial interest.

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