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Article

Hydrophilic CO-releasing material of PEGlyated Ruthenium Carbonyl Complex

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Abstract: The poor water-solubility and instability of Ru(II) carbonyl complex hamper the therapeutic application as CO releasing materials (CO-RMs). To enhance the hydrophilicity and bio-utility of CO, a robust Ru(I) carbonyl sawhorse skeleton were grafted with water-soluble PEGlyated sidearms. Twelve PEGlyted sawhorse $Ru_2(CO)_4$ complexes were prepared with satisfactory yields and characterized by IR and ^{1}H- and ^{13}C- NMR. X-ray diffraction analysis of CO-RM **8**, **13** and **14** revealed the featured diruthenium sawhorse skeleton and PEGylated axial ligands. The flask-shaking method measures the hydrophilicity of CO-RMs, indicating that both bridging carboxylate ligand and PEGlyated axial ligands regulate the hydrophilicity of these CO-RMs. Under photolysis conditions, CO-RM **4-13** sustainable released therapeutic amounts of CO in myoglobin assay. The correlation of the CO release kinetics and hydrophilicity of CO-RMs demonstrated that the more hydrophilic CO-RM released CO faster. The biological test found the low cytotoxic CO-RM **4** showed a specific anticancer activity toward HT-29 tumour cells.

Keywords: Ruthenium complex; Carbon monoxide releasing molecule; Hydrophilicity, PEGylation.

1. Introduction

Recently, biologic experiments using transition metal carbonyl complexes as CO-Releasing molecule (CO-RM) [1] revealed the therapeutic effects of endogenous CO. These effects include anti-inflammatory function, vasodilatation, anti-apoptotic, anti-proliferative, and anti-hypoxia [2]. The organ protection of CO is desirable attractive because controlling a low concentration of CO indeed protects donor tissues from ischaemia-reperfusion injury [3]. The ruthenium carbonyl complex, $[Ru(CO)_3Cl_2]_2$, CO-RM-02, mainly were fasinating because it attenuated acute hepatic ischemia-reperfusion injury in rats by reducing serum AST/ALT levels and improves the liver histology score [4]. However, the poor solubility of CO-RM-02 and its unregulated CO releasing property in aqueous systems hinder the therapeutic application under physiological conditions [5]. To solve the water-solubility issue, CO-RM-02 was solubilized in DMSO, which readily reacts with the CO-RM dimer to generate DMSO-ligated monomeric ruthenium carbonyl species [6]. Motterlini and Mann found that glycinate ligands chelate to Ru (II) carbonyl moiety, and the corresponding Ru(II) complex (CO-RM-03) were water- soluble. Unfortunately, CO-RM-03 degrades rapidly in the human plasma, and the half-life of CO release was only 3.6 minutes [7]. In fact, due to the intrinsic hydrophobic nature of CO, most metal carbonyl complexes have minimal solubility in an aqueous solution. Although a few ionic transition metal carbonyl complexes are water-soluble, the CO release tests indicated that the M-CO bonds of simple ionic CO-RM degraded quickly under complicated physiological conditions and produced unpredictable side effects, such as blocking blood vessels and causing cytotoxic effects. To increase the water-solubility and finely control the CO release kinetics of transition metal complexes are challenges in the design of therapeutic CO-RMs [8-12].

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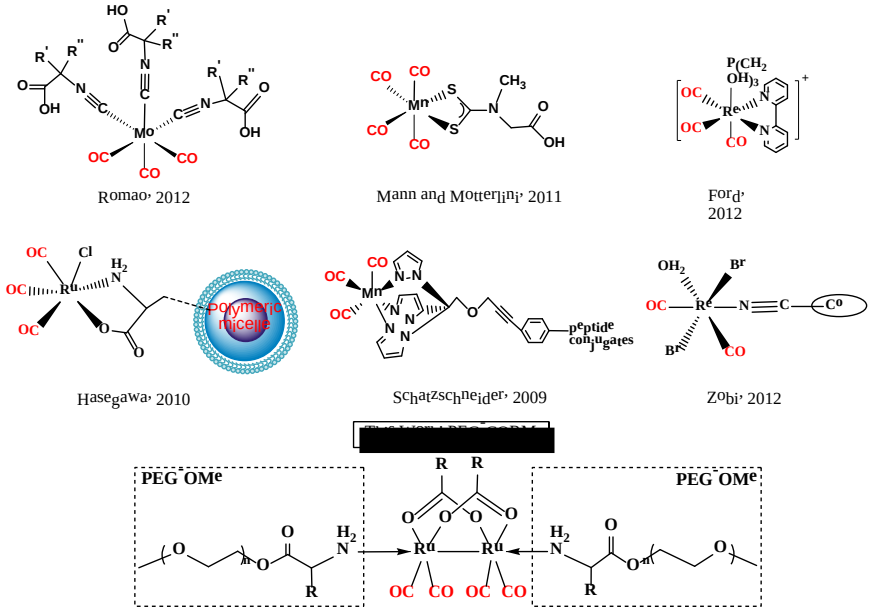
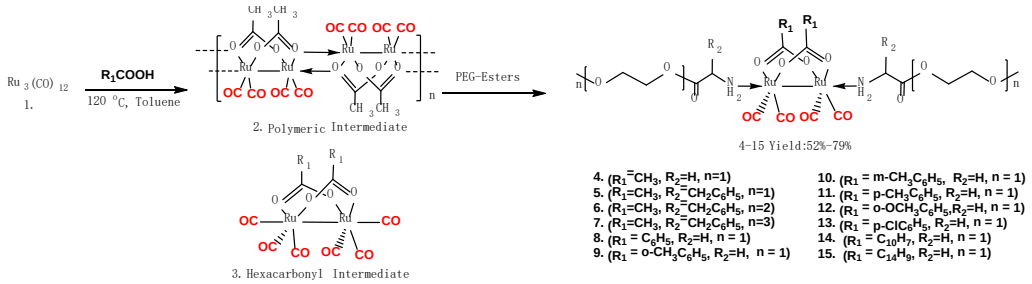


Scheme 1. Selected Water-soluble CO-RMs

Introducing the hydrophilic functional groups into the coordination sphere of transition metal complexes is an efficient way to enhance the water-solubility of the leading CO-RM structure (Scheme **1**.). The hydrophilic auxiliary ligands bearing carboxylic acid groups improve the hydrophilicity of $(Mo(CO)_3(CNCR'R''CO_2R''')_3$ [13] and $[Mn(CO)_4\{S_2CNMe(CH_2CO_2H)\}]$ [14], respectively. The classic water-soluble phosphine ligand, $P(CH_2OH)_3$ coordinates to Ru(I) center and significantly enhance the solubility of Photo-CO-RM, 2, 2′-bipyridine tricarbonyl rhenium(I) in PBS solution [15]. Conjugation of transition metal carbonyl moiety with the hydrophilic biomacromolecules also improves the water solubility and biocompatibility of CO-RMs. The micellar bearing [RuCl(glycinate)$(CO)_3$] [16], peptide conjugates of $[Mn(CO)_3(tmp)]^+$ [17], and a photo-CO-RM based on vitaminB12, namely, B12-ReCO-RM2 [18] were fabricated to delivery CO in aqueous system. However, the synthesis and purification of these hydrophilic CO-RMs are sophisticated. More importantly, the CO release kinetics of most of the water-soluble CO-RMs are unpredictable and thus cannot satisfy the basic requirements of ADME properties for CO pre-drugs [19]. Polyethylene glycol (PEG) is a non-ionic polymer that improves water solubility and selective drug absorption. Inspired by the idea of PEG-polymer linking drug system proposed by H. Ringsdorf [20], and the subsequent precedents of PEGlyated therapeutic agents by A. Abuchowski [21]. Herein, CO-RMs, which are PEGlylated Sawhorse Ru carbonyl complexes, were synthesized by incorporating a robust CO-RM lead structure $[Ru_2(CO)_4(COOR)_2]$ with functionalized PEG chains. X-ray single-crystal analysis revealed that the designed PEG esters of amino acids coordinate to Ru(I) of the sawhorse CO-RM lead structure. From the results of the logP measurements and myoglobin assay experiments, it was found that these hydrophilic CO-RMs show a well-controlled CO release property with broad kinetics under physiologic conditions.

2. Results and Discussion



Scheme 2. PEGlyation of Sawhorse $Ru_2(CO)_4$ complex of **4**-**15.**

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Since 1969 when J. Lewis first reported $Ru_2(CO)_4(O(C=O)R)_2L_2$, features a sawhorse structure [22], a considerable number of the ruthenium(I) carbonyl complexes have been prepared and characterized. However, the inherited poor water-solubility and biological incompatibility of sawhorse ruthenium complexes are obstacles to its application in the biological system [23]. To increase the hydrophilicity of these ruthenium carbonyl complexes, the water-soluble amino acid glycol and PEG esters were tethered at the axial position of the sawhorse structure, respectively (Scheme **2**). The thermolysis of $Ru_3(CO)_{12}$ **1** in acetic acids affords polymeric sawhorse ruthenium carbonyl **2**, and the sequential ligand substitution reaction using glycine methyl esters flourishes the PEGylated sawhorse complexes **4-7** with 61-74% yield. The light yellow products **4-7** are moisture and air-stable and easily manipulated without proof from oxygen or light. The thermolysis of $Ru_3(CO)_{12}$ **1** and aromatic carboxylic acid at 120 °C in toluene generates hexacarbonyl Ru(I) intermediates **2** and has the characteristic carbonyl bands at 2103w, 2079vs, 2035vs, 2004vs and 1938w cm^{-1}. The axial carbonyl ligands of **2** are labile and can readily be substituted by glycine esters of ethylene glycol monomethyl ester. Thermolysis using various bridged aromatic carboxylic acids afforded **8-15**, and FT-IR, ^{1}H-NMR, mass spectrometry and elemental analysis were used to fully characterize complexes **4-15**. The IR spectra of the Ru(I) complexes identified the four characteristic carbonyl bands at 2028-1938 cm^{-1} of sawhorse $Ru_2(CO)_4$ complexes whilst the bridged-carboxylato ligand showed C=O band at 1743 cm^{-1}. The NMR spectra revealed more detailed information about the molecular structures of these ruthenium complexes with more details. In the ^{1}H-NMR spectrum of **4**, the bridged-acetate was observed as a singlet at δ 1.94 ppm, and the protons of NH_2 appeared as a singlet at δ 2.90 ppm. CH_2 and OCH_3 of axial ethylene glycol monomethyl ester glycine ester appeared as triplets at δ 4.35, 3.75, 3.62ppm, and a singlet at 3.39 ppm, respectively. The ^{13}C{^{1}H} NMR spectrum of **4** shows three types of CO resonances, and the Ru bonded carbonyl groups are at δ 204 ppm, the bridging carboxlyate is at δ 172 ppm, and the ester group is at δ 184 ppm, respectively. **4-15** showed similar resonances for both bridged and axial ligands. Notably, the amino protons of μ_2-acetato complex **4** appear at δ 2.90 ppm, lower than the corresponding chemical shift of μ_2-arylcarboxylato complexes **8-15**. The shielded amino proton of **4** reflects less electron donation to the sawhorse unite, indicating the corresponding axial ligand may be more liable.

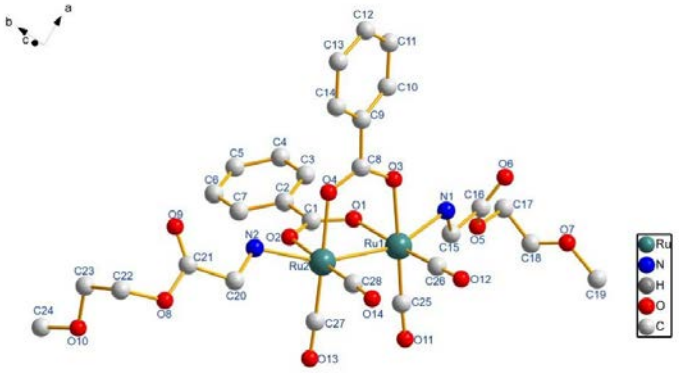

Fig. 1. Molecular structures of **8** (H atoms are omitted for clarity).

Table 1. Selected Bond Distances (Å) and Angles (deg) for **8**, **13** and **14**

Entry	Ru(1)-Ru(2)	Ru(1)-N(1)	Ru(2)-N(2)	Ru(1)-Ru(2)-N(2)	Ru(2)-Ru(1)-N(1)
8	2.6694(10)	2.239(5)	2.210(5)	157.86(15)	158.27(15)
13	2.6634(7)	2.249(4)	2.249(4)	159.09(11)	160.28(11)
14	2.6727(5)	2.233(4)	2.240(4)	158.27(11)	159.84(10)

Yellow crystals of three diruthenium (I) complexes **8**, **13** and **14** were obtained via the diffusion of petroleum ether to CH_2Cl_2 solution of complexes at 0 °C. Single crystal X-ray diffraction was used to characterize these complexes' molecular structures, and the results are shown in Table 1. The molecular structures feature a typical sawhorse structure that consists of a diruthenium tetracarbonyl core surrounded by two ethylene glycol monome-

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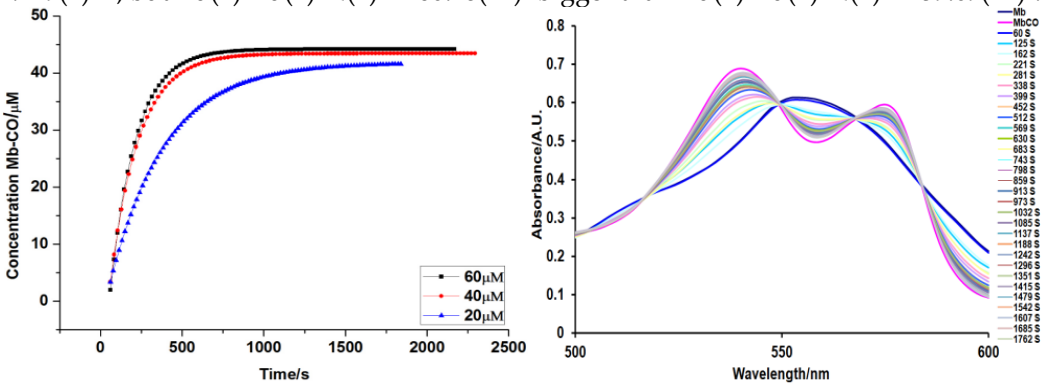
thyl ester glycine esters as axial ligands and two arylcarboxylato ligands at equatorial positions. Three crystals belong to the monoclinic system with the C2/c space group. The Ru-Ru bond distances in these sawhorse skeletons are 2.6694(10) Å (**8**), 2.6634(7) Å (**13**) and 2.6727(5) Å (**14**), respectively; these values are with a metal-metal single bond[24]. The Ru-CO bond length of each terminal carbonyl is slightly different. For instance, the average Ru-CO bond length of these complexes is about 1.83 Å, which is shorter than Ru-CO (1.943(3) Å and 1.903(3) Å) of CO-RM-3 [7], but longer than Ru-CO(1.76 Å) in those of axial triphenylphosphine analogues [24]. In complex **8**, Ru(1)-N(1) 2.239(5) Å is slightly more than Ru(2)-N(2) 2.210(5) Å, and the average Ru-Ru-N angle about 158º, indicating the former axial ligand might is more labile and readily dissociate during the CO releasing process. Interesting, in complex 13, Ru(1)-N(1) and Ru(2)-N(2) have same distance at 2.249(4) Å, but Ru(2)-Ru(1)-N(1) = 160.28(11)º bigger than Ru(1)-Ru(2)-N(2) = 159.09(11)º.



(a) (b)

Fig. 2 Photo-activated CO release profile for **4** (a) UV-vis spectrum showing the Q-bands during the conversion of deoxy-Mb to Mb-CO with time while the concentration of CO-RMs is 60; (b) The CO-releasing kinetics of **4** in which [Mb-CO] was plotted with CO-RM at 60, 40, 20μM against time.

The CO release activity of each CO-RM in vivo was measured with the "golden standard" of myoglobin assay. Firstly, sodium dithionite was added to reduce myoglobin to deoxy-myoglobin (deoxyMb) in PBS (pH=7.4) at 37.8 ºC. A stock solution of CO-RM was added and then activated by LED-UV radiation at 365nm, releasing CO in vivo. The change of deoxyMb to carbonmonoxy-myogblobin (MbCO) were monitored by UV-vis spectroscopy. A typical series of electronic absorption spectra (**Fig. 3**) showed the conversion of deoxyMb to MbCO in the presence of CO released from **4**. The half-lives of CO release rate of **4** at 60 μM is 166 s, 40 μM is 172 s, and 20 μM is 267 s. Four isosbetic points demonstrated the biocompatibility of this CO-RM. The controlled CO release experiment in the dark showed that all CO-RMs are stable and do not spontaneously degrade at the physiological condition. Tuning the time and dense of UV radiation also control the kinetic of CO releasing from CO-RM whilst the molecular structures of CO-RMs determined their photo-sensitivity and CO release activity.

Table 2. The Correlation of Hydrophilicityand CO releasing kinetics of CO-RM

CO-RM	log P [a].	$t_{1/2}$, 60 μM [b].
4	0.39	166
5	1.41	276
6	1.17	249
7	1.05	189
8	1.71	1209
9	1.67	632
10	1.03	962
11	1.06	1096
12	1.78	1450
13	1.26	966
14	N. D.	2699
15	N. D.	2472

Note: [a] Oil-water partition coefficient by UV-vis.

[b] CO releasing Kinetics measured with myoglobin assay as t1/2, s.

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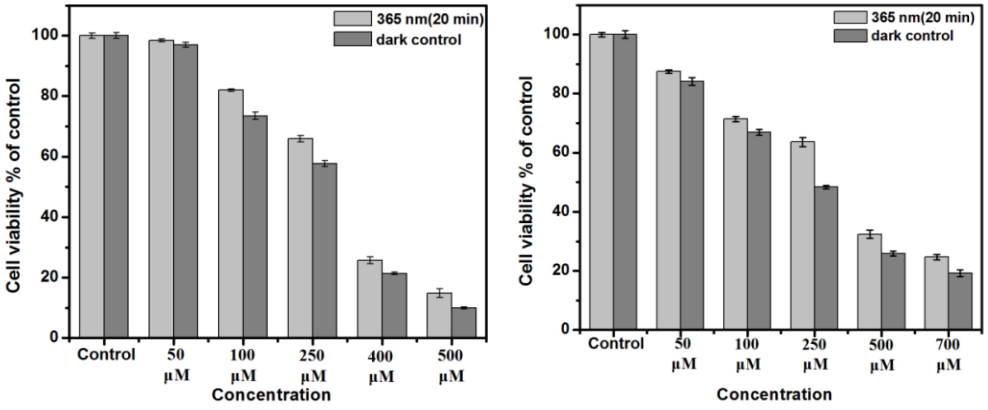
To identify the structural features of CO-RMs that govern the CO releasing behaviour, the CO release kinetics of **4-15** were correlated to the corresponding M-CO band and lipophilicity in Table **3**. Firstly, the oil-water partition coefficient log P values of complexes were measured by the "flask-shaking" method with n-octanol and water, respectively [25]. The log P value of **4-13** ranges from 0.39 to 1.78. The water-solubility of these sawhorse ruthenium copmlex mainly depends on both the axial glycol amino esters and bridging carboxylate, respectively. **4** exhibited the lowest logP value of 0.39, which release CO fastest and convert 30um Mb to MbCO for just 163s using 60um CO-RM. The benzyl substitutes of axial glycol glycinate ester significantly reduce the hydrophilicity of **5**. The LogP value of **5** increased to 1.41 and the CO release half-life of **5** $t_{1/2}$, 60um decreased to 276s. The longer PEG chain of **6** and **7** enhanced the hydrophilicity of acetate bridging CO-RM. The higher LogP and faster CO release rate were observed (Table **2**, CO-RM **6** and **7**). The aromatic bridging ligands were utilized to finely tune the sawhorse structures. The substitutes on the arene are related to the hydrophilicity of aromatic carboxylate bridging CO-RM. **8**, **9** and **12** showed higher LogP values as 1.71, 1.67 and 1.78, respectively, which released CO much slower with $t_{1/2}$,60um around 1000s. Interestingly, The para-substituted methyl-, chloro- **10**, **11** and meta methoxy- **13** groups increase the hydrophilicity, and the corresponding CO rate of each aromatic CO-RM. **14** and **15** are too hydrophobic to be evaluated via the "flask-shaking" method, which $t_{1/2}$, 60um is over 2000s. These experiments demonstrated that hydrophilicity is another factor controlling CO release in the aqueous system.



(a) (b)

Fig. 4. Cell viability of RAW264.7 cell (a) and HT29 (b) in presence of **4**. Cells were grown in the presence of **4** (50-500 μM) and right in the dark (b) or Irradiated at 365 nm for 20 min.

Each component of CO-RM, such as Ru, carboxylic acid and amino acid esters, are known as less harmless than most of the other CO-RM, the cytotoxicity of CO-RM is still unknown. To predict the potential effect of CO-RM in vivo or on primary cells, the cytotoxicity of **4** were investigated with the popular murine macrophage cell line, RAW 264.7 and the human colon adenocarcinoma cell line HT29. Generally, IC50 of **4** over two cell lines showed less cytotoxicity in the dark, in constant with the cellular protection effect of endogenous CO. The MMT experiments showed that 100μM to 500 μM of **4** significantly impacted the cell viability of RAW264.7 **(Fig. 4a)**. 100 μM of **4** started to reduce the cell survival rate. As the concentration of CO-RM increased, the survival rate of the cell dropped sharply. In the presence of 400uM and 500uM of **4**, RAW264.7's survival rate was 22.3 % and 10%, respectively. The IC50 value is 253.3 μmol/L, which showed that **4** was less toxic to RAW264.7 cells. The human colon adenocarcinoma cell line HT29, is not only used to study the biology of human colon cancers, but it is receiving special interest in studies focused on food digestion and bioavailability due to the ability to express characteristics of mature intestinal cells. To evaluate the potential of CO-RM for CO therapy as anti-cancer agents, the cytotoxicity of **4** over HT-29 were measured with the IC50 value of 300.3 μmol/L. The further experiments with HT29 cells using the concentration of **4** in the range from 50 μM to 700 μM (**Fig. 4b**). A concentration of **4** in 50 μM, 12.5 % of the cells

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lose activity in light stimulation. When the concentration increased to 700 μM, HT29 cells survived just 24.7 %. The IC50 value is 342.4 μmol/L. Interestingly, **4** showed the similar anticancer activity in the dark, indicating the anticancer activity of **4** might result from CO-RM as a whole rather than its' released CO.

3. Materials and Methods

All manipulations were accomplished with standard Schlenk techniques. Decacarbonyl-ruthenium ($Ru_3(CO)_{12}$) and mPEG amino acid esters were prepared according to literature procedures [24].CO releasing test were performed using myogblin assay. The cytotoxicity and anticancer activity were measured with RAW264.7 and HTC-29 cells, respectively. The details of experiments were listed in ESI.

4. Conclusions

In conclusion, the robust sawhorse skeletons of the diruthenium carbonyl complex were devised with PEGlyated ligands to tune the CO releasing and bioavailability of CO-RMs. The myoglobin assay test on the CO releasing rate showed well-controlled release kinetics of CO-RMs **4-13** with $t_{1/2}$, 60uM from 166s to 2699s. The Log P values of CO-RMs were correlated with CO release rates, revealing the intrinsic relationship between the water-solubility and CO releasing activity of CO-RM. The CO-RMs with smaller LogP released CO faster, which might prove the concept of enhancing water-solubility to improve the release CO properties. The hydrophilicity of CO-RM was finely tuned via selecting carboxylate bridging ligands and glycol amino acid esters. MTT assay confirmed that CO-RM 4 consisted of acetate and glycol glycine ester as ligands were less cytotoxic to RAW264.7, but specificly toxic to HT29 cancer cells. These CO releasing and bioactivity experiments demonstrated the PEGylated Sawhorse ruthenium carbonyl complex's drug-like properties and the promising therapeutic potentials.

Supplementary Materials: Figure S1, S2: Molecular structure of complex **13**, **14**, Table S1: Data Collection and Structural Refinements Details for Single-Crystal X-ray Diffraction Stud-ies of Complexes **8**, **13** and **14**.

Author Contributions: Conceptualization, W. Z. (Weiqiang Zhang); Methodology, X. Z. (Xiao Zhang), S. Y. (Shuhong Yang), N. G. (Nan Gao) and W.Z.; Writing —Original Draft Preparation, X. Z. (Xiao Zhang), N. G. (Nan Gao), and S. Y. (Shuhong Yang) ; Writing — Review and Editing, X. Z. (Xiao Zhang), N. G. (Nan Gao), S. Y. (Shuhong Yang) , H. K. (Huma Khan) and W.Z. (Weiqiang Zhang) ; Supervision, W.Z. (Weiqiang Zhang); Project Administration, W.Z. (Weiqiang Zhang).

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Conflicts of Interest: The authors declare that there is no conflict of interests regarding the publication of the paper.

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